Microenvironment and Immunology

Cancer Research

Identification of a Natural Killer Cell Receptor Allele That Prolongs Survival of Cytomegalovirus-Positive Glioblastoma Patients

Mev Dominguez–Valentin[1], Andrea Gras Navarro[1], Aminur Mohummad Rahman[1], Surendra Kumar[2,3], Christèle Retière[4], Elling Ulvestad[5,6], Vessela Kristensen[2], Morten Lund-Johansen[6,7], Benedicte Alexandra Lie[8], Per Øyvind Enger[1,7], Gro Njølstad[5], Einar Kristoffersen[6,9], Stein Atle Lie[10], and Martha Chekenya[1]

Abstract

By affecting immunological presentation, the presence of cytomegalovirus in some glioblastomas may impact progression. In this study, we examined a hypothesized role for natural killer (NK) cells in impacting disease progression in this setting. We characterized 108 glioblastoma patients and 454 healthy controls for *HLA-A,-B,-C*, NK-cell KIR receptors, and CMV-specific antibodies and correlated these metrics with clinical parameters. Exome sequences from a large validation set of glioblastoma patients and control individuals were examined from *in silico* databases. We demonstrated that the KIR allele *KIR2DS4*00101* was independently prognostic of prolonged survival. *KIR2DS4*00101* displayed 100% concordance with cognate *HLA-C1* ligands in glioblastoma patients, but not controls. In the context of both *HLA-C1/C2* ligands for the KIR2DS4 receptor, patient survival was further extended. Notably, all patients carrying *KIR2DS4*00101* alleles were CMV seropositive, but not control individuals, and exhibited increased NK-cell subpopulations, which expressed the cytotoxicity receptors CD16, NKG2D, and CD94/NKG2C. Finally, healthy controls exhibited a reduced risk for developing glioblastoma if they carried two *KIR2DS4*00101* alleles, where protection was greatest among Caucasian individuals. Our findings suggest that *KIR2DS4*00101* may offer a molecular biomarker to identify intrinsically milder forms of glioblastoma. *Cancer Res; 76(18); 5326–36. ©2016 AACR.*

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

Introduction

One of the more devious strategies utilized by cancer is evasion of immune surveillance, our primary defense against pathogens and rogue mutated cells (1). The molecular mechanisms underlying this property of cancer cells have been the topic of intense investigation. Specifically, as a treatment strategy of human cancers, new generation immunotherapy has shown unprecedented success in some solid tumor types (2, 3). These approaches, including checkpoint-inhibiting immunotherapy, have unfortunately proven largely unsuccessful in the treatment of human glioblastoma, a highly aggressive malignant brain tumor in adults, where the standard, multimodal treatment extends survival to only 14.6 months (4, 5). The approach in most ongoing immunotherapy trials is to use genetically modified autologous T lymphocytes and dendritic cells. None propose to investigate natural killer (NK) cells as therapeutic effectors despite their ability to spontaneously detect and destroy malignant and virus-infected cells without prior sensitization or antigen specificity (6, 7). NK cells' activity is regulated by the balance of activating and inhibitory signals that are transmitted through their receptors, including killer immunoglobulin-like receptors (KIRs) upon ligation to their cognate HLA ligands (8, 9). The KIR receptors are encoded by a set of 15 genes for inhibitory and activating receptors on chromosome 19q13.4. Ligation of inhibitory KIRs (iKIRs, comprising two or three immunoglobulin domains and long cytoplasmic tails, KIR2DL or KIR3DL) to their cognate HLA ligands leads to inhibitory signaling, rendering the NK-cell tolerant to self cells (10). The KIRs are categorized into phylogenetic lineages II and III (11), where the latter is denoted by a polymorphism at position 44 in the D1 domain. Amino acid substitutions at this position confer specificity for particular HLA-C epitopes.

Activating KIRs, (aKIRs, comprising two or three immunoglobulin domains and short cytoplasmic tails, KIR2DS or KIR3DS) have high sequence homology to the corresponding iKIR and

[1]Department of Biomedicine, University of Bergen, Bergen, Norway. [2]Department of Cancer Genetics, Oslo University Norwegian Radium Hospital, Institute for Cancer Research, Oslo, Norway. [3]Division of Medicine, Department of Clinical Molecular Biology (EpiGen), Akershus University Hospital, Lørenskog, Norway. [4]Blood Transfusion Center Pays de la Loire, Nantes, France. [5]Department of Microbiology, Haukeland University Hospital, Bergen, Norway. [6]Department of Clinical Medicine, University of Bergen, Bergen, Norway. [7]Department of Neurosurgery, Haukeland University Hospital, Bergen, Norway. [8]Department of Immunology, Oslo University Hospital, Oslo, Norway. [9]Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway. [10]Department of Clinical Dentistry, University of Bergen, Bergen, Norway.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Dominguez–Valentin, A. Gras Navarro, and A.M. Rahman contributed equally to this article.

S.A. Lie and M. Chekenya are co-senior authors of this article.

Corresponding Authors: Martha Chekenya, University of Bergen, Jonas Lies Vei 91, Bergen N-5020, Norway. Phone: 47-55586380; Fax: 47-55586360; E-mail: martha.enger@uib.no; and Stein Atle Lie, Stein.Lie@uib.no

doi: 10.1158/0008-5472.CAN-16-1162

©2016 American Association for Cancer Research.

share HLA-ligand specificity, albeit with reduced binding affinity (12). It has been suggested that ligation of aKIRs to cognate HLA ligands results in NK-cell licensing (13). Despite epidemiological studies implicating aKIR–HLA–ligand combinations with disease entities, the physiological role of aKIR receptors in cancer has been elusive. *KIR2DS1* and *KIR2DL1* both bind class I HLA-C2 group ligands, whereas *KIR2DS4*00101* was shown to bind some HLA-$C1^{Asp80}$, HLA-$C2^{Lys80}$ epitopes that are also recognized by *KIR2DL2/3* (14), and *HLA-A*1101*, *HLA-A*1102* epitopes recognized by KIR3DL2 (15, 16). However, careful analysis indicated that KIR2DS4 does have selectivity for HLA-C epitopes that is distinct from *KIR2DL1* and *KIR2DL2/3*, possibly implying an important and unique role for *KIR2DS4*. *KIR2DS4* is the most prevalent and evolutionary conserved lineage III KIR (11) distinguished from other lineage III KIRs by a Pro71-Val72 motif in the D1 domain, that it shares with lineage II *KIR3DL2* as a result of gene conversion before separation of humans from chimpanzee ancestry 6.5 to 10 million years ago (15, 17). *KIR2DS4* gene also exists as deletion mutants (*KIR2DS4*00030101-2; KIR2DS4*004-9*), resulting in nonfunctional protein lacking intracellular and transmembrane anchoring (18). Moreover, *KIR2DS4*00101* specifically recognizes an as yet classified, non-class I HLA ligand expressed on melanoma, leading to potent killing of these targets (19). Cytokines and viral infection may break tolerance induced by ligation of iKIRs to self-HLA, invoking potent cytolysis via the activating receptors (20, 21). Hence, some activating KIRs have been associated with protection from viral infections (20, 22, 23) and induction of autoimmunity (24, 25). Intriguingly, some autoimmune diseases are associated with reduced risk of glioma (26). The common denominator being increased inflammation due to pronounced IFNγ release and augmented cytolytic responses.

However, virus infections, such as with cytomegalovirus (CMV), may render infected cells highly resistant to immunosurveillance. CMV proteins contribute to immune evasion in part by targeting NK cells (27). In addition, viral proteins downregulate class I HLA to evade antigen presentation and detection by $CD8^{+}$ T cells (28). CMV infection alters the distribution of NK-cell receptors (28) to generate a greater abundance of terminally differentiated NK-cell subsets characterized by elevated CD57, KIRs, activating receptor CD94/NKG2C, and prolonged persistence in peripheral blood (29). Although CD94/$NKG2C^{+}$ NK cells have been shown to be remobilized upon subsequent CMV reactivation (30, 31) and to potently kill antibody-coated cells via antibody-dependent cellular cytotoxicity (ADCC; ref. 32), these findings have largely been demonstrated only in healthy individuals.

Interestingly, greater than 90% of glioblastomas express CMV gene products (33–35), and high-grade CMV infection is associated with poor overall survival (36). These results suggest that CMV could contribute to the development of glioblastoma through evasion of immune surveillance, and in fact, treatment of CMV-positive glioblastoma patients with anti-retroviral drugs prolonged survival (37). Although most studies have focused on tumor molecular changes that impact disease progression, few have investigated immune molecular profiles associated with glioblastoma. Here, we hypothesized that CMV infection interferes with immune responsiveness and contributes to glioblastoma progression by altering the distribution of activating KIR–HLA ligand interactions, which regulate NK-cell functionality and are important for target recognition (38). Our results reveal for the first time the association of a specific *KIR2DS4*00101* gene allele with protection from glioblastoma development and enhanced survival in patients who do contract glioblastoma. This novel finding is of direct clinical relevance for the identification of patients who may exhibit a less aggressive clinical course and who may potentially respond to immunotherapy.

Patients and Methods

Norwegian glioblastoma patients

Hundred and eight (108) glioblastoma biopsies and paired blood samples were obtained during surgical resections at the Haukeland University Hospital, Norway, between 1998 and 2015. There were $n = 62$ males and $n = 46$ females of median 61 years (range 10–81 years). Material was collected with the informed consent of patients and healthy controls. The regional ethical committee approved the study (REK vest 013.09/20879; 2014/588). Neuropathologists confirmed glioblastoma diagnosis and eligibility criteria included availability of follow-up data, less than 50% necrosis, and only biopsies obtained at primary glioblastoma diagnosis. Survival was determined as time elapsed from the date of surgery to death or last follow-up until death or August 2015. The six living patients at this last follow-up had been followed up for 10.7 months.

Glioblastoma patients included in The Cancer Genome Atlas database

Tumor-derived DNA exome sequences from mixed American glioblastoma patients ($n = 300$; 191 males and 108 females; wherein white Caucasian patients accounting for $n = 265$, 96 males and 168 females that could be substratified in analyses; Supplementary Tables S1 and S2) collected into the TCGA database between 1993 and 2013 were analyzed. Frequency of *KIR2DS4*00101* that codes full-length membrane anchored activating receptor (FUNC) or (*KIR2DS4*0030101-2; KIR2DS4*004-9*) 22 bp deletion variants (DEL) that encode nonfunctional protein was determined (detailed bioinformatics description in Supplementary Methods and in Supplementary Figs. S1, S2, and S3). Ethical approval and access to the database was obtained from the TCGA Network and The NCBI dbGaP. Survival time was determined as the time elapsed to the date of death and/or last follow-up.

Norwegian and 1000 Genome Healthy controls

Plasma and peripheral blood mononuclear cells (PBMC) were isolated from age-matched controls ($n = 454$ Caucasian donors; 301 females and 153 males) using standard procedures. Median age was 49 years (range 20–73 years). Blood-derived DNA exome sequences from controls ($n = 2504$) in the 1000 Genome (OKG) public domain database were also analyzed. Information on sex, ethnic origin, and *KIR2DS4* status was obtained (Supplementary Table S3), and bioinformatics analysis is described in supplementary methods.

KIR genotyping and HLA genotyping

HLA class I (*A, B,* and *C*) locus was genotyped in glioblastoma patients ($n = 108$) and controls ($n = 76$) using sequence-specific oligonucleotide probe hybridization (ProImmune) within four-digit resolution and KIR genotyping utilized the KIR Typing Kit (Miltenyi Biotec), according to the manufacturer's protocol. The

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

remaining controls ($n = 363$) were KIR and class I HLA genotyped as previously reported (39).

MGMT methylation analysis

Tumor DNA was sodium bisulfite converted using the DNA Methylation-Gold Kit (Zymo Research) and methylation-specific PCR performed using standard protocols.

Flow cytometry

PBMCs were harvested by density gradient centrifugation in Lymphoprep using standard procedures. Thereafter, PBMCs from healthy controls ($n = 56$) and glioblastoma patients ($n = 21$) were fixed and phenotyped as listed in Supplementary Table S4. For good separation of KIR3DL2 and KIR3DL1, as well as KIR2DL2 and KIR2DL3, two-step staining was performed. PBMCs were preincubated for 20 minutes with anti-KIR2DL1 and anti-KIR3DL1 Abs before incubation with anti-KIR3DL2/1 and anti-KIR2DL2/3 Abs. Fluorescence minus one (FMO) for each channel was used and lymphocytes gated as indicated in Fig. 1B. Data acquired on LSR Fortessa (BD Biosciences) was analyzed using FlowJo, version 10 (Tree Star Inc.).

CMV serology and qPCR

Plasma from patients ($n = 27$) and healthy controls ($n = 91$) was serotyped for CMV-specific IgG and IgM antibodies using ARCHITECT CMV ELISA and PCR-based assays (Abbott). A clinical diagnostic cut-off for seropositivity was designated as IgG (Au/mL) ≥ 6 and IgM (index) ≥ 1. The CMV antigen UL83 (pp65) was detected by qPCR on genomic DNA from paired blood and glioblastoma biopsy samples ($n = 108$) as previously described (40, 41). A CMV-infected patient sample was used as positive control and pp65 considered positive when cycle threshold (C_t) values were ≤ 34.

Statistical analysis

Patient survival was presented using the Kaplan–Meier method (42), and Cox-proportional hazards regression was used to assess significant differences adjusted for age, postoperative treatment, sex, and MGMT as possible confounders. Fisher exact test was used to compare gene frequencies between patient and healthy control cohorts. Two-way ANOVA with Bonferoni correction for multiple testing was used. Descriptive statistics were reported as mean ± SEM unless otherwise stated and two-sided *P*-values less than 0.05 were considered significant. Statistical analyses were performed in Stata version 13.1 (Texas) or the Graphpad PRISM 6.0 software (La Jolla, CA). Figures were made in *R* version 3, The R foundation.

Results

*KIR2DS4**00101 preferentially licensed by *HLA-C1* in glioblastoma patients

We previously demonstrated that NK cells infiltrating patient glioblastoma tumors highly express NKG2D (43) and that subsets derived from *KIR2DS4* immunogenotype donors were more potent against glioblastoma stem-like cells (44). Therefore, we hypothesized that altered distribution of NK cells' KIR–HLA ligand interactions as a result of CMV infections may influence malignant progression of glioblastoma. Specifically, we postulated that reduced frequency of the potent *KIR2DS4**00101 allele may underlie this progression. Genomic analysis of all individuals demonstrated that iKIR and aKIR gene frequencies were as previously reported (39), as were frequencies of the haplotypes within glioblastoma and healthy Norwegian control groups when analyzed independently (Fig. 1A). The frequency of *KIR2DS4**00101 did not differ between glioblastoma patients and controls (41.12% vs. 41.46% respectively; χ^2, $P = 0.94$; Fig. 1A). Stochastic expression of KIR genes on NK cells means they may not necessarily be expressed even if the gene is present. Thus, we confirmed surface expression of the KIR receptors on $CD56^+CD3^-$ NK cells (Fig. 1B), where KIR3DL2 and KIR2DL2/3 were highly expressed in controls than glioblastoma NK cells (two-way ANOVA, $df = 1$, $P < 0.0001$ and $P < 0.01$, respectively, Fig. 1C). KIR2DL3 expression appeared in negative linkage disequilibrium with KIR2DL2.

Engagement of the inhibitory KIR receptors that are licenced by self-HLA ligands during development potentiates NK-cell responses against target cells lacking these self-HLA ligands (45). Therefore, we asked whether the presence of KIRs in the context of their cognate HLA ligands differed in glioblastoma patients compared to controls. The *KIR2DS4**00101 allele was present at 100% concordance with *HLA-C1* ligands in glioblastoma patients but not in controls (Fisher exact test, $P = 0.004$; Fig. 1D; and Table 1), whereas it was reduced in the presence of *HLA-C2* ligands in patients compared to controls (43.18% and 53.59%, respectively; Fisher exact test, $P = 0.027$; Fig. 1E; and Table 1). Finally, there was a trend for *KIR3DS1* association with *HLA-Bw4* in controls but not in glioblastoma patients (Fisher exact text, $P = 0.079$; Table 1). Taken together, these results indicated a potentially functional and specific interaction of the *KIR2DS4**00101 allele with both of its ligands in glioblastoma patients but not in controls.

*KIR2DS4**00101 is independently prognostic for improved overall survival

Next, we asked whether the strong association of *KIR2DS4**00101 with *HLA-C1* had impact on patient outcomes. Carrying *KIR2DS4**00101 was significantly associated with improved patients' survival by 2.1 months (median 11.8 months vs. 9.7 months, HR 0.6, $P = 0.034$), or restricted mean survival by 3.3 months (14.9 months vs. 11.6 months; Fig. 1F and Table 2). Importantly, the survival benefit of carrying *KIR2DS4**00101 was independent of other strong prognostic factors, including age (HR 1.34; $P = 0.002$; Fig. 1G), *MGMT* promoter methylation (HR 2.0, $P = 0.008$; Fig. 1H), and postoperative treatment ($P = 0.049$; Fig. 1I; Table 2).

*KIR2DS4**00101 in context of *HLA-C1/C2* ligands enhances overall survival

To rule out the possibility that survival benefit observed with *KIR2DS4**00101 was simply due to *HLA-C1* in the absence of *HLA-C2* ligation of *KIR2DL1* that render autologous NK cells strongly hyporesponsive (46), we performed Cox regression survival analyses comparing associations of *HLA-C1* versus *HLA-C2* corrected for *KIR2DS4**00101 on glioblastoma patient survival. Patients bearing *HLA-C1/C1* in absence of *KIR2DS4**00101 had median survival of 8.2 months, which was the poorest survival time for all groups (Fig. 1J), whereas the median survival times of glioblastoma patients *HLA-C2/C2* and *HLA-C1/C2*

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

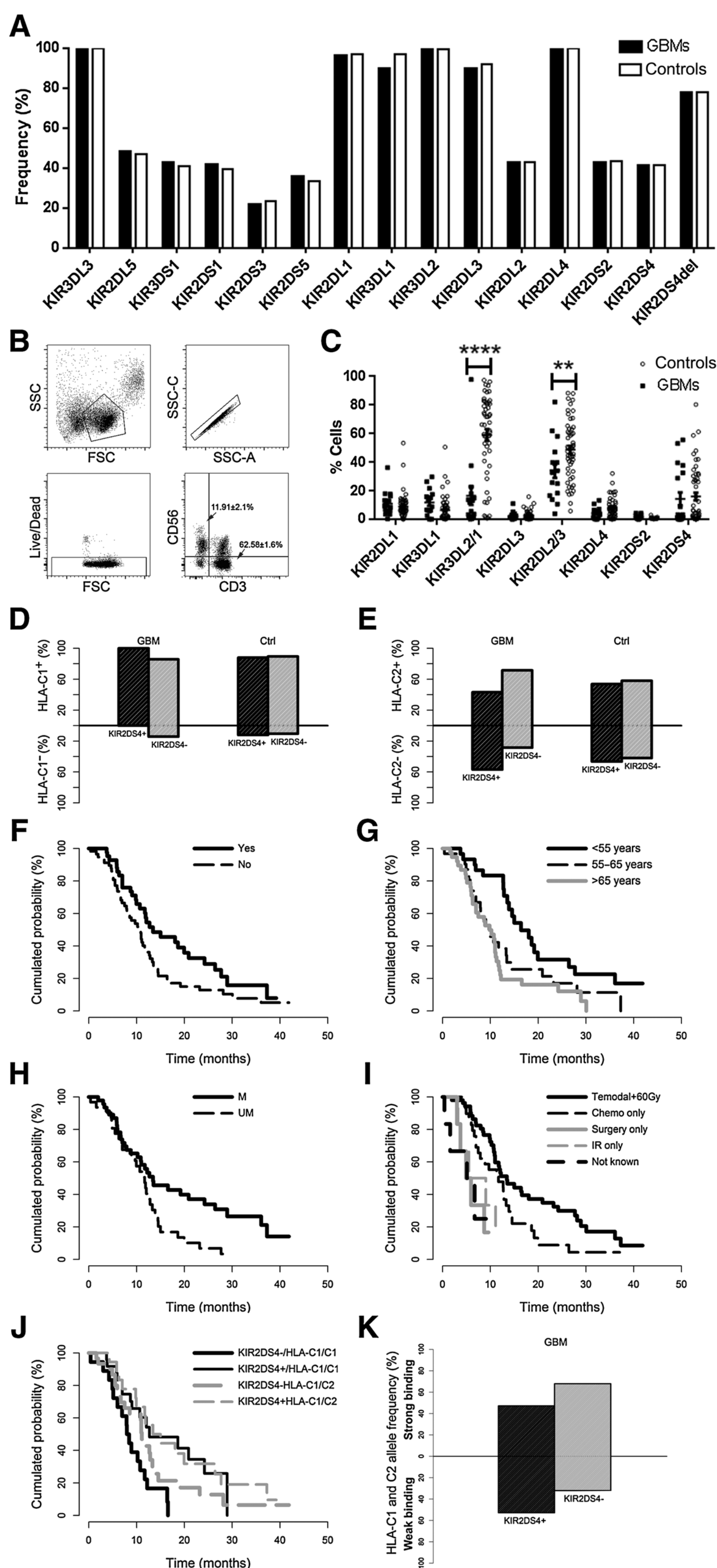

Figure 1.

KIR expression and association of *KIR2DS4*00101* with *HLA-C1* and *HLA-C2* ligands with patient survival. **A,** percentage of KIR gene frequency in glioblastoma patients ($n = 107$) and healthy controls ($n = 436$). **B,** gating strategy for NK cells showing lymphocytes on SSC vs. FSC, singlets on SSC-H vs. SSC-A, live cells on live/dead vs. FSC, and dot plots showing NK-cell gate CD56 vs. CD3. **C,** percentage of cells expressing KIRs on their surface by flow cytometry (two-way ANOVA with Bonferoni correction for multiple testing, $df = 1$; **, $P < 0.01$; ****, $P < 0.0001$). **D** and **E,** percent frequency of *KIR2DS4*00101* alleles in the presence or absence of *HLA-C1* ligands (**D**) or *HLA-C2* ligands (**E**) in glioblastoma patients ($n = 107$) and healthy controls ($n = 436$). **F,** cumulative survival for glioblastoma patients possessing *KIR2DS4*00101* alleles (Yes) or *KIR2DS4*-negative patients (No); Cox regression, $P = 0.034$. Kaplan–Meier cumulative survival curves for glioblastoma patients by age (**G**); MGMT promoter methylation (M, methylated; UM, unmethylated; **H**); and postoperative treatment (**I**). **J,** Kaplan–Meier cumulative survival curves for glioblastoma patients based on *KIR2DS4*00101* in presence or absence of *HLA-C1* and *HLA-C2* ligands. **K,** percent frequency of patients carrying *KIR2DS4*00101* alleles or not in the presence of strongly binding *HLA-C1* (*C*1601, C*0102, C*1402*) or *HLA-C2* (*C*0501, C*0201, C*0401*) ligands compared to weakly binding ligands (*C*0304* and *C*0702*) in glioblastoma patients ($n = 92$).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

Table 1. KIR–HLA frequencies in glioblastoma patients compared to healthy controls

KIR-ligand	Glioblastoma (N = 107) N (%)	χ^2 Fisher exact test
KIR2DL1*C2	61 (59.22)	0.53
KIR2DL2*C2	24 (52.18)	0.16
KIR2DL2*C1	43 (93.48)	0.54
KIR2DL3*C1	87 (90.63)	0.29
KIR2DS1*C2	31 (68.89)	0.10
KIR2DS2*C1	43 (93.48)	0.54
KIR2DS2*C2	24 (52.17)	0.16
KIR2DS2*A11	6 (13.04)	0.80
KIR2DS3*C1	20 (86.96)	0.37
KIR2DS4*C1	44 (100)	**0.009/0.010**
KIR2DS4*C2	19 (43.18)	**0.003/0.005**
KIR2DS4*A11	6 (13.64)	0.92
KIR3DL1*Bw4	50 (52.08)	0.06/0.12
KIR3DL2*A03	30 (28.04)	N/A
KIR3DL2*A11	15 (14.02)	N/A
KIR3DS1*Bw4	26 (56.52)	0.80

KIR-ligand	Controls (N = 436) N (%)	χ^2 Fisher exact test
KIR2DL1*C2	239 (56.66)	0.31
KIR2DL2*C2	111 (59.36)	0.25
KIR2DL2*C1	165 (88.24)	0.76
KIR2DL3*C1	355 (88.53)	0.60
KIR2DS1*C2	239 (56.64)	0.31
KIR2DS2*C1	169 (88.48)	0.87
KIR2DS2*C2	113 (59.16)	0.27
KIR2DS2*A11	22 (11.46)	0.86
KIR2DS3*C1	92 (89.32)	0.84
KIR2DS4*C1	159 (87.85)	0.61
KIR2DS4*C2	97 (53.59)	0.36
KIR2DS4*A11	21 (11.54)	0.83
KIR3DL1*Bw4	221 (52.24)	0.87
KIR3DL2*A11	49 (11.19)	0.72
KIR3DS1*Bw4	82 (46.33)	**0.044/0.051**

KIR-HLA GBM vs. controls	Logistic regression P value[a]
KIR2DS4ins*C1/C1	**0.004**
KIR2DS4ins*C1/C2	**0.027**
HLA-Bw4	0.112
KIR3DS1*Bw4	0.079

NOTE: Numbers in bold text are significant P values.
Abbreviations: N/A, not applicable; %, of KIR positive.
[a]Logistic regression analysis.

without KIR2DS4*00101 were better at 10.5 months (data not shown) and 10.9 months (HR = 0.537; 95% CI, 0.28–1.02; P = 0.056; Fig. 1J). Carrying KIR2DS4*00101 in the presence of HLA-C1/C1, however, significantly extended patient median and restricted mean survival outcomes to 13.8 and 17.4 months, respectively (HR 0.36; 95% CI, 0.18–0.73; P = 0.005; Fig. 1J) as well as in the context of HLA-C1/C2, where median and restricted mean survival extended to 14.2 and 17.2 months, respectively (HR 0.34; 95% CI, 0.16–0.70; P = 0.004; Fig. 1J). No patients were KIR2DS4*00101$^+$ in the context of HLA-C2/C2.

As KIR2DS4*00101 binds strongly only to a select group of HLA-C1 (C*1601, C*0102, C*1402) and HLA-C2 epitopes (C*0501, C*0201, C*0401; refs. 15, 16), we investigated association of these specific HLA-C1/C2 epitopes with KIR2DS4*00101 in glioblastoma patients and controls from the Norwegian cohort. We also investigated the association of KIR2DS4*00101 with the weakly binding HLA epitopes (C*0304 and C*0702). 47.22% (17/36) of patients carried KIR2DS4*00101 in context of both strongly binding HLA-C1/C2 epitopes compared to 67.86% (38/56) of patients lacking the KIR2DS4*00101 allele (χ^2 = 3.84; OR 0.423; 95% CI, 0.17–1.027; P = 0.051; Fig. 1K). Strikingly, none of the healthy controls carried the KIR2DS4*00101 allele in context of the strongly or weakly binding HLA-C1/C2 epitopes (data not shown). Eight KIR2DS4*00101 bearing patients, and seven patients without KIR2DS4*00101 allele lacked the binding epitopes. These findings demonstrated a cancer-specific positive association of the KIR2DS4*00101 allele with selective HLA-C1/C2 binding alleles.

CMV infection imprints NK cells from glioblastoma patients to coexpress CD94/NKG2C and KIR2DS4

We next investigated the hypothesis that CMV infection in glioblastoma patients might lead to redistribution of the NK cells' KIR–HLA ligand repertoire. Intriguingly, KIR2DS4*00101 was in 100% concordance with CMV seropositivity in glioblastoma patients but not in healthy controls (Fishers exact P = 0.031; Fig. 2A). Sixty-seven percent (18/27) of glioblastoma patients were IgG seropositive compared to 50.5% (46/91) of healthy Norwegian controls (OR 11.25, exact logistic regression; P = 0.044). Two patients and controls were additionally IgM seropositive. Of the glioblastoma patients, 30.5% (33/108) were positive for *pp65* DNA in tumor tissue and/or blood, whereas 12% (12/100) were positive for *pp65* DNA in blood, which could be an indication of the reactivation of CMV (data not shown). Seropositivity correlated with increased presence of *pp65* CMV DNA in the tumor microenvironment in only 11.1% (3/27) cases (Fig. 2B). NK cells from all glioblastoma patients were more differentiated than those from controls (Fig. 2C), as more NK cells expressing differentiation markers, CD57, CD16, NKG2D, and CD94/NKG2C, were present in glioblastoma patients who were CMV seropositive (Fig. 2C and D). Thus, there was a dual effect of cancer and CMV seropositivity on expression of these NK-cell differentiation markers and expansion of the CD94/NKG2C subset.

We hypothesized that association of seropositivity with KIR2DS4*00101 might thus be due to CMV imprinting of CD94/NKG2C$^+$ NK-cell compartment. Therefore, we investigated whether KIR2DS4$^+$ NK-cell subsets were associated with the CD94/NKG2C subsets acquired in response to CMV infection. In the KIR2DS4*00101$^+$, CMV$^+$ glioblastoma patients, KIR2DS4 was coexpressed with 35.8 ± 10.1% of NKG2C$^+$ NK cells (Fig. 2E). In KIR2DS4*00101$^+$, CMV$^+$ controls, the percentage of coexpressing NK cells was similar (32.44 ± 9.1%; P > 0.05; Fig. 2E). CD94/NKG2C subset analysis further confirmed that elevated expression of CD16 (P < 0.01) and NKG2D (P < 0.05, two-way ANOVA, df = 1; Fig. 2F and G) was a characteristic of more NK cells in CMV$^+$ glioblastoma patients compared to controls. However, no difference in the levels of CD16 was apparent in CMV$^+$KIR2DS4*00101 bearing patients and controls (Fig. 2H), indicating that CD16 expression was increased because of CMV infection and not KIR2DS4*00101 status (Fig. 2H and J). In contrast, expansion of KIR3DL2/1$^+$ subsets in healthy controls was irrespective of their CMV status (two-way ANOVA, df = 1, P < 0.0001; Fig. 2I and J). Taken together, these data indicate that selective expansion of CD94/NKG2C$^+$/CD16$^+$ NK-cell subsets was a result of CMV infection, and expansion of this subset in the context of KIR2DS4*00101 with HLA-C1/C2 was associated with improved survival in glioblastoma patients.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

Table 2. Median survival, crude HR, 95% confidence intervals, and probability

Variable	n	Median survival (days)	Crude HR	95% CI (HR)	*P*-value log rank	*P*-value Cox regression by KIR2DS4
Gender			0.84	(0.534–1.322)	0.45	
Males	62	326				
Females	46	291				
Age			1.37	(1.121–1.681)	**0.002**	**0.001**
<55 years	31	426				
55–65 years	31	271				
>65 years	43	291				
MGMT			2.0	(1.198.3–3.387)	**0.008**	**0.005**
Unmethylated	31	339				
Methylated	46	359				
Pre-op steroids						
Unknown	9	385	0.990	(0.442–2.219)	0.982	
1 day	26	345	1			
2–7 days	23	291	1.06	(0.579–1.965)	0.836	
8–14 days	14	310	0.761	(0.369–1.569)	0.460	
Over 14 days	27	306	0.779	(0.419–1.449)	0.431	
KIR2DS4						
Genotype positive	42	354	0.6	(0.385–0.963)	**0.034**	
Genotype negative	57	291	1.0			
Post-op treatment						**0.049**
Surgery only	6	218	3.835	(1.451–10.1339)	**0.007**	**0.015**
39 Gy IR only	6	162	6.314	(2.332–17.094)	<**0.001**	<**0.001**
Temodal + 60 Gy IR	52	358	1			
(Temodal ± additional chemo)	29	339	1.706	(1.025–2.839)	**0.04**	0.089
Unknown	6	175	5.750	(1.928–17.143)	**0.002**	**0.004**

NOTE: Median survival and 95% confidence interval (CI) of HR. Numbers in bold are significant *P* values.

KIR2DS4*00101 is associated with diminished risk for the development of glioblastoma

Given the favorable prognosis of *KIR2DS4*00101* in the context of its *HLA-C1/C2* ligands and CMV infection in the Norwegian glioblastoma patients, we hypothesized that the *KIR2DS4*00101* allele might increase inflammation and be protective against glioblastoma development. We thus investigated the risk of ending up in the glioblastoma group as opposed to the noncancer group if the individual carries two alleles of *KIR2DS4*00101 (KIR2DS4FUNC/FUNC)* relative to the deletion alleles (*KIR2DS4-DEL/DEL)*. The number of mixed American glioblastoma patients possessing *KIR2DS4FUNC/FUNC* was significantly diminished compared to healthy multinational controls (13.33%, 40/300 vs. 18.77%, 470/2504, respectively, $P = 0.029$; Fig. 3A). Likewise, white Caucasian glioblastoma patients possessing *KIR2DS4-FUNC/FUNC* was significantly diminished compared to healthy multinational controls (10.19%, 27/265 vs. 18.77%, 470/2504, respectively, $P = 0.001$; Fig. 3A), Multinational controls possessing *KIR2DS4FUNC/FUNC* allele relative to *KIR2DS4DEL/DEL* had 37% reduced risk of ending up in mixed glioblastoma patient group (OR 0.63; 95% CI, 0.41–0.98%; $n = 2804$; $P = 0.0038$), in contrast to 50% reduced risk of ending up in the white glioblastoma patient group (OR 0.496; 96% CI, 0.327–0.752%; $n = 2769$; $P = 0.001$; Fig. 3A).

Likewise, American Caucasian controls carrying *KIR2DS4-FUNC/FUNC* (20.46%, 71/347) had 33% reduced risk of ending up in mixed glioblastoma patient group (OR 0.67; 96% CI, 0.47–0.96%; $n = 647$; $P = 0.029$; Fig. 3A). However, risk of American Caucasian controls ending up in the white glioblastoma patient group was substantially reduced by 53% (OR 0.466; 95% CI, 0.287–0.755%; $n = 612$; $P = 0.002$). This finding underscores the importance of controlling for ethnicity as potential confounder.

Associations with age, Karnofsky performance and treatment were also examined in order to validate our overall statistical approach and our cohorts. First, age was prognostic (HR 1.042; 95% CI, 1.03–1.06%; $P < 0.001$). The median survival was 10.5 months in glioblastoma patients <55 years, 9.6 months for patients between 55 and 65 years, and 5.4 months in patients >65 years (Fig. 3B). Second, Karnofsky performance status of at least 80 was associated with improved survival by 29 days (HR 1.85; 95% CI, 1.28–1.66%; $P = 0.001$; Fig. 3C). Third, the standard treatment of Temozolomide combined with 60 Gy ionizing radiation (IR) significantly improved median survival compared to chemotherapy only (10.9 months vs. 5.5 months, respectively; HR 2.86; 95% CI, 1.78–4.60%; $P < 0.0001$; Fig. 3D). The standard treatment was also superior to Temozolomide + low-dose IR (median survival, 6.8 months; HR 2.43; 95% CI, 1.09–4.11%; $P = 0.018$), IR alone (median survival, 6.9 months; HR 2.11; 95% CI, 1.09–4.11%; $P = 0.028$), unknown treatment (median survival, 3.7 months; HR 4.35; 95% CI, 3.06–6.19%; $P < 0.0001$), or no treatment (median survival, 2.3 months; HR 11.84; 95% CI, 5.53–25.35%; $P < 0.0001$; Fig. 3D). Finally, the standard treatment was not better than IR combined with other alkylating chemotherapy drugs (median survival, 10.9 months; HR 1.61; 95% CI, 0.90–2.89%; $P = 0.108$; Fig. 3D). These results corroborate established findings in the field, and therefore support our analysis regarding the *KIR2DS4*00101* allele.

Discussion

Immunotherapy is beginning to take hold as a viable option for the treatment of some solid tumor types. Unfortunately, the modality has been so far ineffective in the treatment of human glioblastoma, a clinically aggressive and

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

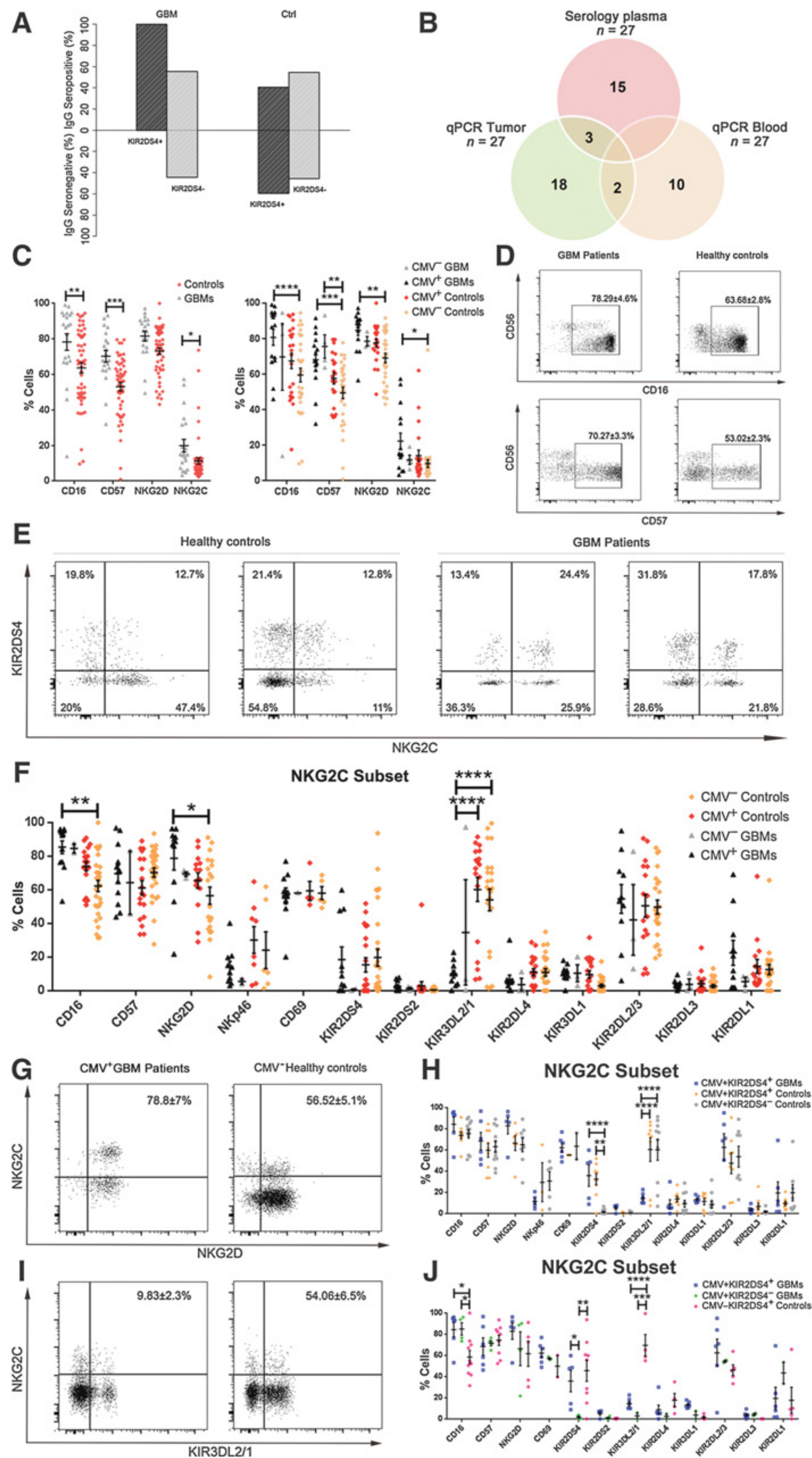

Figure 2.
CMV serostatus and imprinting on NK cell subsets from glioblastoma patients and controls. **A,** percent frequency of CMV seropositive vs. seronegative glioblastoma patients and controls based on *KIR2DS4*00101*. **B,** Venn diagram showing seropositive glioblastoma patients that are qPCR positive for pp65 in patient tumor vs. blood. (**C**, left) NK-cell phenotype of patients vs. controls and (**C**, right). NK-cell phenotypes of CMV$^+$ and CMV$^-$ patients vs. controls. **D,** representative dot plots of data in **C** showing CD56$^+$ CD57$^+$ (below) vs. CD56$^+$CD16$^+$ (above). **E,** representative dot plots showing KIR2DS4$^+$ NKG2C$^+$ double positive NK cells from CMV$^+$ controls (left) and glioblastoma patients (right). **F,** percentage of cells within CD94/NKG2C$^+$ subsets from CMV$^+$ or CMV$^-$ patients and controls expressing phenotypic markers. **G,** representative dot plots of NKG2D expression within CD94/NKG2C$^+$ subsets from CMV$^+$ glioblastoma and CMV$^-$ controls. **H,** percentage of CD94/NKG2C$^+$ NK cells subsets expressing phenotypic markers from CMV$^+$*KIR2DS4*00101* glioblastoma patients compared with CMV$^+$*KIR2DS4*00101* or *KIR2DS4*$^-$ controls. **I,** representative dot plots of *KIR3DL2/1* expression within CD94/NKG2C$^+$ subsets from CMV$^+$ glioblastoma and CMV$^-$ controls. **J,** percentage of CD94/NKG2C$^+$ NK cells subsets expressing phenotypic markers from CMV$^+$*KIR2DS4*00101*$^+$ or KIR2DS4$^-$ glioblastoma patients compared to CMV$^-$*KIR2DS4*00101* controls. Two-way ANOVA with Bonferoni correction for multiple testing, $df = 1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, data represents mean ± SEM.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

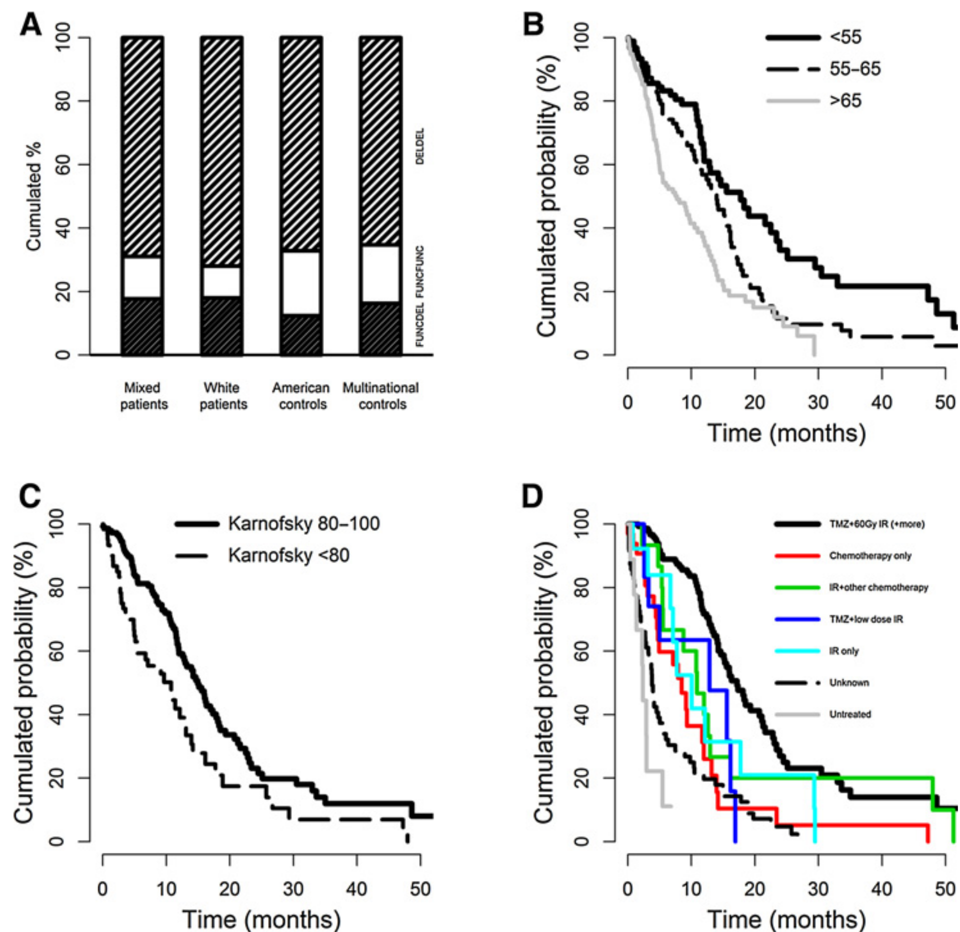

Figure 3.
Homozygous functional *KIR2DS4*00101* is protective against glioblastoma diagnosis. **A,** frequency of *KIR2DS4FUNCFUNC*; *FUNCDEL* and *DELDEL* among mixed American glioblastoma patients, White caucasoid American glioblastoma patients, American Caucasians controls, and multinational controls. Kaplan–Meier cumulative survival curves for glioblastoma patients by age (**B**), where 31.44% (94/299) were under 55 years old, 29.77% (89/299) were between 55 and 65 years old, and 38.80% (116/299) were older than 65 years; Karnofsky performance status, where the majority had score of 80 and 100 (70.62%, 149/211; **C**); and postoperative, anticancer treatment (**D**).

psychologically devastating disease with poor treatment options. Understanding the molecular factors that drive immunogenicity in glioblastoma patients is further required for designing effective immunotherapy strategies that might be based on the specific immunobiological characteristics of each patient's tumor. Here, we have used the unique approach of characterizing large cohorts of glioblastoma patients and healthy controls based on NK-cell receptor repertoires and associated these profiles with risk of acquiring the disease and progression with endpoint patient survival. We are the first to report *KIR2DS4*00101* allele as an independent prognostic marker for improved survival in glioblastoma patients, where the effect was further modified by the presence of its cognate HLA-ligands in the context of CMV. Our findings should inspire greater focus on molecular characterization of immune of cell types in solid tumors to not only devise appropriate immunotherapy strategies but importantly, to identify those patients most likely to respond. Because *KIR2DS4*00101* associated with protection from developing highly aggressive glioblastoma, we identified also a prognostic factor that may inform loss of health life years. Our findings highlight a new paradigm that may advance research within solid tumors, where focus is still on subtyping tumors based on their intrinsically aberrant molecular profiles.

Indeed, few studies have investigated the potential of NK-cell therapy for glioblastoma patients (47). We have previously demonstrated that NK cells infiltrating patient glioblastoma tumors highly express activating receptor NKG2D (43) and that NK cells derived from aKIRs KIR2DS4$^+$ and KIR2DS2$^+$ donors were more potent against glioblastoma stem-like cells (44). Thus, the specific association of *KIR2DS4*00101* with *HLA-C1* in the context of CMV seropositivity in glioblastoma patients but not healthy controls has intriguing implications for immune response, although the mechanisms underlying their association remain unknown. We speculate that licensing of *KIR2DS4* in the context of CMV might modulate receptor specificity and that these NK-cell subsets might bind preferentially and more strongly to *HLA-C1* and *HLA-C2* ligands. Indeed, nearly half of the *KIR2DS4*00101* bearing CMV$^+$ patients had the *KIR2DS4*00101* allele in context of strongly binding *HLA-C1/C2* epitopes in contrast to healthy controls, where none had *KIR2DS4*00101* in context of these strong epitopes. This may indicate a cancer and/or CMV-specific association, as well as an important role HLA-C in cancer as opposed to HLA-A because no significant difference in association of *KIR2DS4*00101* with *HLA-A11* ligands was uncovered. Unfortunately, because diminished sample size in these subanalyses, we could not determine whether *KIR2DS4*00101* association with specific *HLA-C1/C2* binding epitopes further enhanced patient survival.

A possible mechanism for the appearance in patients, but not controls, of *KIR2DS4*00101* NK cells in context of strongly

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

binding *HLA-C1/C2* epitopes may be through CMV peptides generated in post-childhood infections, which enhance the specificity and strength of *KIR2DS4*00101* binding to *HLA-C1/C2* epitopes and ultimately impact NK cell response. Indeed peptides have been shown to influence the specificity of particular KIR–HLA ligand interactions (48). Unfortunately, the associative nature of our study falls short in providing precise mechanistic insights of how the *KIR2DS4*00101–HLA-C1/C2* interactions may be contributing to the immune regulation in glioblastoma patients. Indeed, a caveat that the observed effect of *KIR2DS4*00101* may potentially be mediated by linked genes cannot be entirely ruled out. However, because glioblastoma patients lacking *KIR2DS4*00101* exhibited poor prognosis despite frequency of strongly binding *HLA-C1/C2* epitopes, the importance of *KIR2DS4*00101* may be accentuated. *KIR2DL2* that shares these epitopes had no significant impact on patient survival.

HLA-C2/C2 was associated with a shorter median survival of 10.5 months, whereas patients with *HLA-C1/C2* had modest survival 10.9 months. In other cancer types, *HLA-C2* has been associated with higher relapse rates (46, 49), and diminished progression free and overall survival. However, in the context of CMV seropositivity, *HLA-C1/C2* plus *KIR2DS4*00101* was associated with an extended median survival of 14.2 months. An interpretation of these findings might be that *HLA-C1/C2* educated KIR2DS4 subsets in context of CMV antibodies override inhibitory effects of *KIR2DL1* ligation to *HLA-C2* and subsequent tolerance to self. However, our findings do not rule out the possibility of *KIR2DS4*00101* recognizing similar non-class I HLA ligands in the glioblastoma tumors that were previously reported in melanoma (19). The possible significance of our findings is that the immune system of *KIR2DS4*00101* glioblastoma patients might be capable of generating potent proinflammatory NK-cell subsets in response to CMV infection that effectively impede malignant progression. Indeed, the uncovered protection against glioblastoma development in the independent populations of the OKG and TCGA networks implies a causal benevolent effect of *KIR2DS4*00101* allele against malignancy. Such NK-cell subsets carrying the *KIR2DS4*00101* allele may promote inflammation through potent IFNγ release and cytotoxic responses. Further studies are underway to delineate the precise role of NK cells in glioblastoma patients in the context of CMV infection with a particular focus on elucidating whether NK cells from individuals bearing *KIR2DS4*00101* allele display distinct functional activity towards glioblastoma cells with or without CMV infection. Furthermore, it would be intriguing to investigate whether the strong protection against glioblastoma might be apparent in other solid tumors with a strong anti-inflammatory signature and in other carefully controlled ethnic groups.

Activating KIR receptors have been associated with delayed progression of various diseases, including respiratory papillomatosis, human immune deficiency virus to AIDS, and Hepatitis C virus (20, 23, 50). A critical role for CD94/NKG2C in antiviral response was revealed in several studies performed on AIDS patients, where a natural chromosomal variation, which has a deletion of *KLRC2*, the gene that codes for CD94/NKG2C, was associated with increased risk of contracting HIV and more aggressive disease progression to AIDS (51). It is therefore possible that a heightened state of anti-CMV immune surveillance in carriers of *KIR2DS4*00101* forestalls development and progression of tumors.

CD94/NKG2C$^+$ NK-cell subsets have previously been correlated with CMV seropositivity in healthy individuals (29, 52), and these subsets have been found to be highly cytotoxic against viral targets and antibody-coated cells. Ordinarily, 10% of NK cells in peripheral blood express CD94/NKG2C. Thus, the observed three-fold increase of these NK-cell subsets in *KIR2DS4*00101*, CMV seropositive glioblastoma patients, and controls was a consequence of CMV imprinting. The role of CMV imprinting in stimulating development of these subsets is further supported by elevated expression of differentiation markers CD16, CD57, and NKG2D in both CMV$^+$ patients and controls. Loss of CD16 and NKG2D is characteristic of NK cells from cancer patients and is a hallmark of immune escape. Changes in these receptors confer poor cytotoxicity, reduced cytokine production, and the inability to execute ADCC onto NK cells. Our finding of coexpression of CD94/NKG2C with KIR2DS4$^+$ on NK-cell subsets from CMV-seropositive glioblastoma patients also highlights the role of aKIRs in response to CMV infection. The association of *KIR2DS4*00101* with enhanced survival in glioblastoma patients underscores this argument.

CMV seropositivity in glioblastoma patients was high compared to healthy Norwegian controls (67% vs. 50.5%, respectively), but may be explained by a higher median age (61 years vs. 49 years; glioblastoma patients and controls, respectively) as CMV seropositivity has been correlated with age, race/ethnicity, gender, and socioeconomic status (53). However, the serostatus of our Norwegian cohort corroborates results reported from a German cohort of similar social demographics (54). Of our entire glioblastoma patients, 30.5% were pp65 positive in tumor, whereas 12% had CMV DNA in blood. The latter may be indicative of reactivation as a consequence of changes in the immune function of patients as CMV generally establishes latency after primary infection during childhood or adolescence (55). One difficulty is that there is currently no consensus regarding the best method for demonstrating CMV positivity in glioblastoma patients. Others have reported positivity for IE1 protein in 11% of tumors using immunohistochemistry and fluorescence *in situ* hybridization (56).

In summary, KIR2DS4$^+$NKG2C$^+$–*HLA-C1/C2* interactions emerged as a novel, positive prognostic indicator for the glioblastoma patients undergoing standard treatment. Further work will characterize whether the CD94/NKG2C$^+$ NK cells correspond to adaptive NK subsets exhibiting long-term persistence in blood and whether these subsets can effectively assist in the execution of immunotherapy targeting glioblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Chekenya

Development of methodology: M. Dominguez–Valentin, A. Gras Navarro, A.M. Rahman, S. Kumar

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gras Navarro, E. Ulvestad, M. Lund-Johansen, B.A. Lie, P. Øyvind Enger, G. Njølstad, E. Kristoffersen, M. Chekenya

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Dominguez–Valentin, A. Gras Navarro, A.M. Rahman, S. Kumar, E. Ulvestad, V. Kristensen, P. Øyvind Enger, S.A. Lie, M. Chekenya

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

Writing, review, and/or revision of the manuscript: M. Dominguez–Valentin, A. Gras Navarro, A.M. Rahman, S. Kumar, E. Ulvestad, V. Kristensen, M. Lund-Johansen, B.A. Lie, G. Njølstad, E. Kristoffersen, S.A. Lie, M. Chekenya
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Retière, B.A. Lie
Study supervision: S.A. Lie, M. Chekenya
Other (Statistical analyses): S.A. Lie
Other (Provided funding for study): M. Chekenya

Acknowledgments

We are grateful to Bendik Nordanger, Ingrid Sandvik Gavlen, Varathalingam Sharanga, and Guro Gundersen for technical assistance, and neuropathologist Dr. Hrvoje Miletic for scoring viable tumor/necrosis fraction. We thank the patients, controls, and the staff at the neurosurgical unit at Haukeland University Hospital for providing tumor tissue, blood samples, and for the Brain Tumor Biobank. We thank the Norwegian Bone Marrow Donor Registry and The Blood Transfusion Bank, Bergen, for providing healthy controls. Flow cytometry was performed at the molecular imaging centre at UiB. The results presented here are in part based upon data generated by TCGA and The 1000 Genome research networks. We thank Mr. Tore Linde at the Dept. Information and Technology at UiB for secure analysis and supercomputing platform. We are grateful to Dr. Janice Nigro for manuscript editing.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 25, 2016; revised June 2, 2016; accepted June 22, 2016; published OnlineFirst July 12, 2016.

References

1. Engels EA, Pfeiffer RM, Fraumeni JFJr, Kasiske BL, Israni AK, Snyder JJ, et al. Spectrum of cancer risk among US solid organ transplant recipients. JAMA 2011;306:1891–901.
2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711–23.
3. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369:122–33.
4. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
5. Hegi ME, Diserens AC, Godard S, Dietrich PY, Regli L, Ostermann S, et al. Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 2004;10:1871–4.
6. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer 1975; 16:230–9.
7. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol 1975;5: 112–7.
8. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 1986;319:675–8.
9. Ljunggren HG, Karre K. In search of the `missing self': MHC molecules and NK cell recognition. Immunol Today 1990;11:237–44.
10. Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. Science 2004;306:1517–9.
11. Khakoo SI, Rajalingam R, Shum BP, Weidenbach K, Flodin L, Muir DG, et al. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. Immunity 2000;12:687–98.
12. Stewart CA, Laugier-Anfossi F, Vely F, Saulquin X, Riedmuller J, Tisserant A, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. Proc Natl Acad Sci U S A 2005;102: 13224–9.
13. Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, Michaelsson J. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. Blood 2010;115:1166–74.
14. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimer M, Parham P. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J Immunol 2008;180:3969–79.
15. Graef T, Moesta AK, Norman PJ, Abi-Rached L, Vago L, Older Aguilar AM, et al. KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. J Exp Med 2009;206:2557–72.
16. Katz G, Markel G, Mizrahi S, Arnon TI, Mandelboim O. Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4. J Immunol 2001;166:7260–7.
17. Benton MJ, Donoghue PC. Paleontological evidence to date the tree of life. Mol Biol Evol 2007;24:26–53.
18. Middleton D, Gonzalez A, Gilmore PM. Studies on the expression of the deleted KIR2DS4*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. Hum Immunol 2007;68:128–34.
19. Katz G, Gazit R, Arnon TI, Gonen-Gross T, Tarcic G, Markel G, et al. MHC class I-independent recognition of NK-activating receptor KIR2DS4. J Immunol 2004;173:1819–25.
20. Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, et al. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS 2008;22:595–9.
21. Gaudieri S, DeSantis D, McKinnon E, Moore C, Nolan D, Witt CS, et al. Killer immunoglobulin-like receptors and HLA act both independently and synergistically to modify HIV disease progression. Genes Immun 2005;6:683–90.
22. Bao XJ, Hou LH, Sun AN, Qiu QC, Yuan XN, Chen MH, et al. The impact of KIR2DS4 alleles and the expression of KIR in the development of acute GVHD after unrelated allogeneic hematopoietic SCT. Bone Marrow Transplant 2010;45:1435–41.
23. Bonagura VR, Du Z, Ashouri E, Luo L, Hatam LJ, DeVoti JA, et al. Activating killer cell immunoglobulin-like receptors 3DS1 and 2DS1 protect against developing the severe form of recurrent respiratory papillomatosis. Hum Immunol 2010;71:212–9.
24. Chen C, Busson M, Rocha V, Appert ML, Lepage V, Dulphy N, et al. Activating KIR genes are associated with CMV reactivation and survival after non-T-cell depleted HLA-identical sibling bone marrow transplantation for malignant disorders. Bone Marrow Transplant 2006;38:437–44.
25. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 2002;31:429–34.
26. Amirian ES, Zhou R, Wrensch MR, Olson SH, Scheurer ME, Il'yasova D, et al. Approaching a scientific consensus on the association between allergies and glioma risk: a report from the glioma international case-control study. Cancer Epidemiol Biomarkers Prev 2016;25:282–90.
27. Wilkinson GW, Tomasec P, Stanton RJ, Armstrong M, Prod'homme V, Aicheler R, et al. Modulation of natural killer cells by human cytomegalovirus. J Clin Virol 2008;41:206–12.
28. Muntasell A, Vilches C, Angulo A, Lopez-Botet M. Adaptive reconfiguration of the human NK-cell compartment in response to cytomegalovirus: a different perspective of the host-pathogen interaction. Eur J Immunol 2013;43:1133–41.
29. Guma M, Cabrera C, Erkizia I, Bofill M, Clotet B, Ruiz L, et al. Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients. J Infect Dis 2006;194:38–41.
30. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. Blood 2012;119:2665–74.
31. Lopez-Verges S, Milush JM, Schwartz BS, Pando MJ, Jarjoura J, York VA, et al. Expansion of a unique CD57(+)NKG2Chi natural killer cell subset

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025

during acute human cytomegalovirus infection. Proc Natl Acad Sci U S A 2011;108:14725–32.
32. Costa-Garcia M, Vera A, Moraru M, Vilches C, Lopez-Botet M, Muntasell A. Antibody-mediated response of NKG2Cbright NK cells against human cytomegalovirus. J Immunol 2015;194:2715–24.
33. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002;62:3347–50.
34. Lucas KG, Bao L, Bruggeman R, Dunham K, Specht C. The detection of CMV pp65 and IE1 in glioblastoma multiforme. J Neurooncol 2011;103:231–8.
35. Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro Oncol 2008;10:10–8.
36. Rahbar A, Stragliotto G, Orrego A, Peredo I, Taher C, Willems J, et al. Low levels of human cytomegalovirus infection in glioblastoma multiforme associates with patient survival—a case-control study. Herpesviridae 2012;3:3.
37. Soderberg-Naucler C, Peredo I, Stragliotto G. Valganciclovir in patients with glioblastoma. N Engl J Med 2013;369:2066–7.
38. Laurent S, Queirolo P, Boero S, Salvi S, Piccioli P, Boccardo S, et al. The engagement of CTLA-4 on primary melanoma cell lines induces antibody-dependent cellular cytotoxicity and TNF-alpha production. J Transl Med 2013;11:108.
39. Karlsen TH, Boberg KM, Olsson M, Sun JY, Senitzer D, Bergquist A, et al. Particular genetic variants of ligands for natural killer cell receptors may contribute to the HLA associated risk of primary sclerosing cholangitis. J Hepatol 2007;46:899–906.
40. Griscelli F, Barrois M, Chauvin S, Lastere S, Bellet D, Bourhis JH. Quantification of human cytomegalovirus DNA in bone marrow transplant recipients by real-time PCR. J Clin Microbiol 2001;39:4362–9.
41. Habbal W, Monem F, Gartner BC. Comparative evaluation of published cytomegalovirus primers for rapid real-time PCR: which are the most sensitive? J Med Microbiol 2009;58:878–83.
42. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966;50:163–70.
43. Kmiecik J, Poli A, Brons NH, Waha A, Eide GE, Enger PØ, et al. Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level. J Neuroimmunol 2013;264:71–83.
44. Gras Navarro A, Kmiecik J, Leiss L, Zelkowski M, Engelsen A, Bruserud Ø, et al. NK cells with KIR2DS2 immunogenotype have a functional activation advantage to efficiently kill glioblastoma and prolong animal survival. J Immunol 2014;193:6192–206.
45. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity 2006;25:331–42.
46. Babor F, Manser AR, Fischer JC, Scherenschlich N, Enczmann J, Chazara O, et al. KIR ligand C2 is associated with increased susceptibility to childhood ALL and confers an elevated risk for late relapse. Blood 2014;124:2248–51.
47. Ishikawa E, Tsuboi K, Saijo K, Harada H, Takano S, Nose T, et al. Autologous natural killer cell therapy for human recurrent malignant glioma. Anticancer Res 2004;24:1861–71.
48. Cassidy S, Mukherjee S, Myint TM, Mbiribindi B, North H, Traherne J, et al. Peptide selectivity discriminates NK cells from KIR2DL2- and KIR2DL3-positive individuals. Eur J Immunol 2015;45:492–500.
49. Cariani E, Pilli M, Zerbini A, Rota C, Olivani A, Zanelli P, et al. HLA and killer immunoglobulin-like receptor genes as outcome predictors of hepatitis C virus-related hepatocellular carcinoma. Clin Cancer Res 2013;19: 5465–73.
50. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872–4.
51. Thomas R, Low HZ, Kniesch K, Jacobs R, Schmidt RE, Witte T. NKG2C deletion is a risk factor of HIV infection. AIDS Res Hum Retroviruses 2012;28:844–51.
52. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood 2004;104:3664–71.
53. Staras SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988–1994. Clin Infect Dis 2006;43:1143–51.
54. Hecker M, Qiu D, Marquardt K, Bein G, Hackstein H. Continuous cytomegalovirus seroconversion in a large group of healthy blood donors. Vox Sang 2004;86:41–4.
55. Yamashita Y, Ito Y, Isomura H, Takemura N, Okamoto A, Motomura K, et al. Lack of presence of the human cytomegalovirus in human glioblastoma. Mod Pathol 2014;27:922–9.
56. Sabatier J, Uro-Coste E, Pommepuy I, Labrousse F, Allart S, Trémoulet M, et al. Detection of human cytomegalovirus genome and gene products in central nervous system tumours. Br J Cancer 2005;92:747–50.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/76/18/5326/2739265/5326.pdf by guest on 27 March 2025