

KIR2DS2/KIR2DL2/HLA-C1 haplotype is associated with Alzheimer's disease: implication for the role of herpesvirus infections.

Authors: Roberta Rizzo¹, Daria Bortolotti¹, Valentina Gentili¹, Antonella Rotola¹, Silvia Bolzani¹, Elisabetta Caselli¹, Maria Rosaria Tola², Dario Di Luca¹.

¹University of Ferrara, Department of Medical Sciences, Ferrara, Italy; ²University Hospital, Arcispedale S. Anna, Department of Neurosciences and Rehabilitation, Ferrara, Italy.

Running title: KIR2DL2 and herpesvirus in Alzheimer's disease

Corresponding authors

Prof. Dario Di Luca

University of Ferrara- Department of Medical Sciences -Section of Microbiology and Medical Genetics; Via Luigi Borsari, 46 - 44121 Ferrara – Italy

Tel: +39 0532455318; Fax: +390532974470; e-mail: dario.diluca@unife.it

Prof. Roberta Rizzo

University of Ferrara- Department of Medical Sciences -Section of Microbiology and Medical Genetics; Via Luigi Borsari, 46 - 44121 Ferrara – Italy

Tel: +39 0532455382; Fax: +390532974470; e-mail: rbr@unife.it

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, where neuroinflammation and immune cells are key pathological factors. Recently it was suggested a possible association between AD and human herpesvirus 6 (HHV-6) infection. Since we recently observed that Multiple sclerosis patients with KIR2DL2 expression on Natural Killer (NK) cells are more susceptible to herpesvirus infection, we tested the possible implication of KIR/HLA genetic for HHV-6A infection. We identified, for the first time, a possible implication of a specific KIR/HLA subset in AD. The combination KIR2DS2/KIR2DL2/C1 correlated with a lower MMSE_D score, representative of a severe AD status and an increased susceptibility to HHV-6A infection. Therefore, the results seem to converge on the hypothesis that herpesvirus infection might play a role in AD. If this hypothesis finds experimental confirmation, a new therapeutic strategy, modulating KIR2DL2 expression on NK cells, for AD might be envisaged.

Key words: Alzheimer, Herpesvirus; HHV-6A; KIR; NK cell

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease worldwide, representing 60–70 % of all dementia cases [1]. Neuroinflammation is a key factor in AD pathology, and immune cells play a functional role in modulating soluble molecules including neuroendocrines, neurotransmitters and cytokines [2, 3]. Several studies have reported that the presence of proinflammatory cytokines and lymphocyte subset distribution can be disturbed in AD [2, 3]. Natural killer (NK) cells are key effectors of the immune system, due to their human leukocyte antigen (HLA)-restricted cytotoxic activity. NK inhibitory receptors prevent NK cell-mediated attack against normal cells whereas cells with compromised HLA class I expression (e.g. by tumor transformation or viral infection) are susceptible to NK cell-mediated killing. There are two main types of NK inhibitory receptors : (i) killer Ig-like receptors (KIRs), specific for determinants shared by groups of HLA-A, HLA-B or HLA-C allotypes [4] and (ii) CD94/NKG2A, recognizing HLA-E [5]. Inhibitory KIRs have long cytoplasmic tails (KIR-L) containing two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that, upon phosphorylation, shut down NK cell activation. Each KIR displays two (KIR2DL) or three (KIR3DL) extracellular Ig-domains conferring specificity for HLA allotypes. The inhibitory KIR2DL1, KIR2DL2 and KIR2DL3 receptors recognize distinct HLA-C allotypes, KIR3DL1 ligands are HLA-B molecules with the Bw4 epitope and KIR3DL2 binds HLA-A3 and HLA-A11.

Although the immunobiology of NK cells in neurodegenerative diseases such as Multiple sclerosis (MS) is well studied, little is known regarding their role in AD [3]. NK cells have two main functions: killing of target cells and cytokine production. NK cells killing takes place by secretion of perforin and granzymes and mediators of apoptosis (TNF-related apoptosis-inducing ligand and FasL). NK cells may secrete both immunostimulatory and immunomodulatory cytokines such as interferon (IFN)- γ , TNF- α , granulocyte monocyte colony-stimulating factor, IL-5, IL-13, IL-10 and transforming growth factor (TGF)- β . The immune system in AD patients is severely affected and several studies reported that proinflammatory circulating cytokines and lymphocyte subset

distribution can be disturbed in AD [3]. The implication of NK cells in AD immunopathogenesis was observed by Krishnaraj [6] who showed that choline-esterase inhibitors could suppress expansion and cytotoxic function of NK cells in AD patients, with an improvement of clinical status. Araga et al. [7] showed that the functional potential of NK cells in AD patients was significantly lower than in normal controls. Also other researchers reported decreased NK cytotoxic function [3]. Thus, evaluating the role of NK cells in the immunopathogenesis of AD may open new horizons for designing novel therapeutic methods.

In our recent studies on MS, we observed that patients with KIR2DL2 expression are more susceptible to herpesvirus infection [8, 9]. In particular, MS patients positive for KIR2DL2 expression on NK cells failed to control herpes simplex virus 1 (HSV-1) infection and secreted high levels of Th17 cytokines, while MS patients negative for KIR2DL2 on NK cells had an efficient killing of virus-infected cells and released Th1 cytokines. Furthermore, we have evidence that the lack of activation of KIR2DL2 positive NK cell takes place in the presence of other human herpesviruses such as EBV and HHV-6 (Rizzo R et al., manuscript in preparation). A lower NK cells intracerebral surveillance triggered by herpesvirus infection could have an important role in MS pathogenesis.

AD has been associated with a possible infective aethiology [10], and almost three decades ago was suggested for the first time a possible involvement of herpes simplex virus type 1 (HSV-1) [11]. The ever-increasing number of these studies warrants re-evaluation of a possible association between HSV infection and AD. In humans, herpesvirus brain infection has been associated to AD-like pathology [12-14]. In mice and in cell culture, A β deposition and tau abnormalities typical of AD are observed after infection with HSV-1 [15-18], and a direct interaction between A β PP and HSV-1 has been reported [19]. Antivirals, including acyclovir, block *in vitro* HSV-1-induced A β and tau pathology [20]. HSV-1 reactivation and seropositivity correlate with incident AD [12, 13] and the use of antiherpetic drugs reduces the relative risk of senile dementia by a factor of 10 [14]. Apolipoprotein E (APOE), a risk factor for AD, increases HSV-1-induced brain damage [21]. Inside the brain, ApoE,

characterized by three allelic forms: APOE2, APOE3, APOE4, helps to clear beta-amyloid, a plaque component. ApoE ϵ 2 appears to perform this function more effectively than ApoE ϵ 4, with ApoE ϵ 3 in the middle. Interestingly, ApoE ϵ 4 is a risk factor for vascular causes of cognitive impairment and dementia [22, 23], can increase the risk of atherosclerosis and stroke [24], may induce the response to stress or injury [24], increases the risk for AD and lowers the age of onset [25].

Furthermore, it has been suggested that there is a cumulative correlation between multiple herpesvirus infections and cognitive decline in patients with vascular disease [26]. Also, a connection between cytomegalovirus (CMV) and AD was made, showing that CMV IgG and IFN- γ levels in cerebrospinal fluid correlate with neuropathologic characteristics of AD [27] and with an increased rate of cognitive decline [28].

Recently it was suggested a possible association between AD and human herpesvirus 6 (HHV-6) infection [29]. The Authors, constructing multiscale networks of late-onset AD, observed increased presence of HHV-6A in AD patients than in controls, suggesting viral activity as a general feature of AD. HHV-6 is classified in two distinct species, HHV-6A and HHV-6B [30], but the term HHV-6 remains in usage for both species. The two viral species are quite similar, sharing many similarities, but differ for cell receptors, specific tropism and disease association. HHV-6 preferentially replicates in activated CD4⁺ T lymphocytes [31, 32], but can infect also NK cells, endothelial cells, astrocytes, oligodendrocytes, and microglial cells [31]. The *in vivo* host tissue range of HHV-6 includes brain, tonsils, salivary glands, kidneys, liver, lymph nodes, endothelial cells, and monocytes/macrophages [31, 33-35].

Interestingly, similarly to MS, also AD patients show decreased NK cell activity [2]. These two observations (i.e. HHV-6A presence and NK cell decreased activation) might support the hypothesis that NK cells and herpesvirus infection might contribute to AD immune-pathogenesis.

Our aim was to test the possible implication of KIR/HLA genetic background in AD, and its association with HHV-6A infection.

Materials and Methods

1. Study subjects

We recruited 98 patients (55-87 years old) with an AD clinical diagnosis in accordance with the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [36]. These criteria include a mild-to-moderate severity, neuroimaging analysis, and a Mini-Mental State Examination (MMSE_i) [37] score of 11.0 to 29.0 at diagnosis (Table 1). MMSE values were corrected by age and educational level as proposed by Magni et al. [38]. After correction, the MMSE_i scores ranged from 12.3 to 24.7. We recruited 1 severe, 42 moderate and 55 mild patients. All patients were recruited at the Alzheimer Center of the University Hospital, Arcispedale S. Anna, during the 2004-2006 period. Disease severity was re-evaluated after one year of follow up. The study was approved by the Ethics Committee of the University of Ferrara, and the subjects and the caregivers consented to the study. Healthy members of societies for retired people were recruited for the cognitively-normal control group. Only ethnic Italians without known dementia in first degree relatives were included.

2. Genotyping KIR and HLA

Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany). Genotyping of KIR and HLA alleles was performed by Olerup typing kit (West Chester, PA, USA) following manufacturer's protocols.

3. Haplotype reconstruction

Maximum-likelihood KIR haplotype frequencies were estimated using an expectation-maximization (EM) algorithm implemented in Arlequin 3.1 [39]. KIR-inferred haplotypes were compared with

those available on the web site of the official allele nomenclature (<https://www.ebi.ac.uk/ipd/kir/nomenclature.html>).

4. Genotyping APOE

APOE alleles were determined by genotyping at two single nucleotide polymorphisms (SNPs), rs7412 and rs429358. Genotyping was performed using predesigned Taqman Assays (C_904973_10 and C_3084793_20, Applied Biosystems) on the Applied Biosystems 7900 HT Fast Real Time PCR. Deviation from Hardy Weinberg Equilibrium (HWE) was calculated for the APOE locus, and both cases ($p = 0.86$) and controls ($p = 0.36$) were found to be in HWE.

5. NK92 cell nucleofection

NK92 (ATCC CRL-2407) cell line was grown in MEM-Alpha medium (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 20% of FCS (fetal calf serum), 0.1 mM 2-Mercaptoethanol, 100 U/mL of penicillin and streptomycin and 150 U/mL of IL-2. Cell cultures were maintained at 37° C in humidified atmosphere of 5% CO₂.

KIR2DL2-GFP-tagged was inserted in the plasmid pCMV6-AC-GFP (OriGene Technologies, Rockville, MD USA). The recombinant plasmid was amplified in One Shot® TOP 10F' E. Coli (Invitrogen). Plasmid DNA was extracted with plasmid MAXI kit (QIAGEN, Hilden, Germany) and nucleofected in NK92 cells (Ingenio® Electroporation Kit, Mirus Bio LLC, WI USA). Stable cell colonies were selected in the presence of 1000 µg/mL of Geneticin® (G418 Sulfate, Gibco®). KIR2DL2 NK92 cells and NK92 control cells were co-cultured with infected cells in a 1:5 ratio and collected for subsequent analysis [8, 9]. Each experiment was performed in triplicate.

Images were acquired with Nikon Eclipse TE2000S fluorescence microscope equipped with a digital camera.

6. KIR2 e beta-actin mRNA analysis

Total RNA was extracted from PBMC by TRIzol (Invitrogen). RNA samples were digested with DNase (1U/ug RNA) and stored at -85° C in ethanol until cDNA synthesis. Two micrograms of total RNA were reverse transcribed using SuperScript™ First-Strand Synthesis System (Invitrogen). cDNA was amplified by specific oligonucleotide primers for KIR2DL2 [40] and house-keeping control gene beta-actin [41, 42]. Amplification products were analysed by ethidium bromide–stained agarose gel electrophoresis.

7. HHV-6 infection

The human T cell line J-Jhan was cultured in RPMI-1640 (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) with 10% FCS, supplemented with 100 U/ml each of penicillin and streptomycin and maintained at 37° C in humidified atmosphere of 5% CO₂.

J-Jhan cells were infected with a HHV-6A (U1102) cell free inoculum as previously described [42]. The day after infection, J-Jhan cells were harvested for DNA and RNA quantification and for APOE mRNA analysis or NK92 cells were added at a 1:5 ratio. Each experiment was performed in triplicate.

8. HHV-6 analysis

HHV-6 DNA viral load was analyzed by real time quantitative (qPCR) in duplicate, as described [43]. RNA cell extraction was performed using the RNeasy kit (Qiagen, Hilden, Germany). No DNA contaminated the RNA samples, as shown by control β -actin PCR without retrotranscription [41, 42]. Reverse transcription was performed by the RT2 First strand kit (Qiagen, Hilden, Germany). cDNA aliquots corresponding to 200 ng RNA were used for virus transcription analysis, performed by qPCR detecting the expression of U42 gene, as previously reported [44] .

9. Sp1 phosphorylation status analysis

Sp1 phosphorylation was evaluated by Western-Blot. Proteins were extracted by RIPA buffer and ready-to-use cocktails of protease and phosphatase inhibitors (Abcam, UK). Protein concentration was quantified by Bradford assay (Bio-Rad Laboratories). Similar amounts of protein were loaded in 12% TGX-Pre-cast gel (Biorad, Segrate, MI, Italy), with subsequent electroblotting.. The membrane was incubated with 1:200 Sp1 (ProSci Incorporated; Poway, CA, USA) or anti-phospho Sp1 (pThr) (Sigma-Aldrich; Saint Louis, MO, USA) monoclonal antibodies. A horseradish peroxidase (HRP)-conjugated antimouse antibody (1:5000; Amersham Biosciences, NJ, USA) was added and developed with the ECL kit (Amersham Biosciences, NJ, USA). The images were acquired by GelDoc EZ System (BioRad; Milano; Italy).

10. PKA analysis

PKA was extracted from 10^7 cells with PepTag® Non-Radioactive Protein Kinase Assays (Promega, Milano, Italy). The inhibition of PKA activity was obtained with increasing concentrations (0.25, 0.5, 1.0, 2.0, 2.5 μ M) of KT 5720 (Sigma-Aldrich, S. Louis, MO, USA), a specific inhibitor of protein kinase A.

11. Carboxyfluorescein diacetate succinimidyl ester (CFSE) analysis

Infected cells were assessed for cell-mediated cytotoxicity, using 7AAD/CFSE Cell-mediated cytotoxicity assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's protocol. Labelled cells were analyzed with FACSCantoII flow cytometer (Becton Dickinson, Milano, Italy) and FlowJo software (FlowJo LLC, OR, USA).

12. APOE expression

J-Jhan cells, infected with HHV-6A (U1102) were harvested after 1, 3, 7, 14, 21 day post infection. Total RNA was extracted (RNeasy kit, Qiagen, Hilden, Germany) and checked for the absence of contaminating DNA[41]. RNA reverse transcription was performed as described above. ApoE

expression analysis was performed on DNA aliquots corresponding to 200 ng RNA using Applied Biosystems Gene expression analysis (Hs00171168_m1).

13. Statistical analyses

To detect significant departure from Hardy–Weinberg equilibrium, we used the extended version of Fisher’s exact test implemented in Arlequin 3.01 [39]. Tests of association between genotypes and cognitive changes were performed with a logistic regression adjusted for age, sex, baseline MMSE_i, to test the possible association between disease status and KIR. Biological variables were evaluated by Student t test by Graph pad software. p -value < 0.05 was considered to be statistically significant. p values were corrected (p_c) for multiple comparisons, using Bonferroni correction.

Results

1. KIR/HLA combinations

Patient characteristics are shown in Table 1. Frequencies and distributions of KIR alleles were in Hardy Weinberg equilibrium and are summarized in Table 2. Patients with AD showed a significantly increased frequency of the activating receptor KIR2DS2 (62% vs. 37.5%; $p=2.2 \times 10^{-3}$) and a reduced frequency of the activating receptor KIR3DS1 (16.6% vs. 32.7%; $p=2.9 \times 10^{-3}$). A higher number of AD patients had increased inhibitory receptor KIR2DL2 (62.2% vs 36.4%; $p=2.2 \times 10^{-3}$). No significant differences were found in the distribution of KIR genotypes (AA or Bx) but there was an increase in HLA-C1/C2 genotype in AD patients (45.9% vs 22.7%; $p=2 \times 10^{-3}$). When analyzing KIRs and their respective ligands, the combination KIR2DS2/KIR2DL2 present/C1 present, was significantly increased in AD patients (48% vs 28.2%; $p=0.037$) while the combination KIR2DS2/KIR2DL2 absent/C1 present was significantly decreased in AD patients (6.1% vs 30.9%; $p=5.1 \times 10^{-5}$). After including all variables in logistic regression, the increased frequency of KIR2DL2 and increased frequency of the combination KIR2DS2/KIR2DL2 present/C1 present maintained a strict correlation with AD ($p=0.02$; $p=0.01$ respectively).

There was a significant lower MMSE_I score at diagnosis in patients with the KIR2DS2/KIR2DL2/C1 combination ($p=0.0002$) (Figure 1a). Disease severity at diagnosis, ADL_I and IADL_I did not present any correlation with KIR/HLA combinations (Figure 1b, c). Interestingly, the worsening of the disease status after one year follow-up was more evident in patients with the KIR2DS2/KIR2DL2/C1 combination. In fact, 14 of 20 patients with a worsened disease status had KIR2DS2/KIR2DL2/C1 ($p=0.0045$).

2. KIR/HLA combinations and APOE alleles and expression

Frequencies and distributions of APOE alleles were in Hardy Weinberg equilibrium. The frequency of APOE $\epsilon 4$ allele was highest in AD patients ($p=0.00018$) (Table 3). APOE $\epsilon 4$ allele was

significantly more frequent in AD patients with the KIR2DS2/KIR2DL2/C1 combination ($p=3.9 \times 10^{-5}$) (Table 4).

We evaluated the possible effect of HHV-6A infection on apoE $\epsilon 4$ expression. The results, shown in Figure 1, evidence that apoE $\epsilon 4$ mRNA synthesis is enhanced by HHV-6A infection, in a time dependent correlation. In particular, there is a clear correlation between the enhancement in HHV-6A RNA production and the increase in apoE $\epsilon 4$ mRNA at day 3 p.i. with a peak at day 14 p.i. (Figure 1).

2. KIR2DL2 receptor and HHV-6 infection

Since KIR2DS2/KIR2DL2/C1 combination has been shown to increase the susceptibility to HSV-1 infections [9], we evaluated if this might be the case also for HHV-6A. NK92 cell line, negative for KIR expression, was nucleofected with KIR2DL2 or control plasmid (GFP) (Figure 2d). Efficiency of transfection was 89% (Figure 2e). NK92-GFP and NK92-KIR2DL2 cells were co-cultured with HHV-6A infected T cells, expressing HLA-C1 allele. NK92-GFP cells were able to control HHV-6A infection, reducing viral titre, while NK92-KIR2DL2 cells did not (Figure 2f) ($p<0.001$). The blocking of KIR2DL2 receptor with CD158b moAb, specific for KIR2DL2 receptor, reverted the effect of KIR2DL2 expression on NK92 cells, reducing HHV-6A titre to values similar to those obtained in NK92-GFP cells (Figure 2f). To ensure that NK92-GFP cells killed HHV-6A infected cells and not only to prevent viral replication, cells were analyzed by flow cytometry. We observed no modification in HHV-6A infected cell percentage when co-cultured with NK92-GFP cells (Figure 2g). On the contrary, NK92-KIR2DL2 cells reduced CFSE⁺ cell percentage, indicating a higher proliferation of the cells and suggesting the inability of NK92-KIR2DL2 cells to kill infected cells.

3. Involvement of PKA/Sp1 pathway

Since KIR2DL2 expression is controlled by specific transcription factors [45], we evaluated the modulation of Sp1 phosphorylation (pThr), highly represented in NK92 cell line [46, 47]. We

observed an increase in Sp1 phosphorylation in NK92 cells co-cultured with HHV-6A infected cells (Figure 3a). Since Sp1 DNA binding properties are stimulated by cAMP-dependent protein kinase (PKA) [48], we looked at possible differences in PKA activity. PKA activity increased in NK92 cells co-cultured with HHV-6A infected cells (Figure 3b). Interestingly, Sp1 phosphorylation was decreased in the presence of KT 5720, a PKA inhibitor, even in the presence of HHV-6A infected cells (Figure 3c). These data suggest that the PKA/Sp1 pathway might be involved in KIR2DL2 expression on NK cells, inhibiting their activation during HHV-6A infection.

Conclusions

In this study, we describe a possible role for a specific KIR/HLA subset in AD. We observed an increased frequency of KIR2DS2/KIR2DL2/C1 in AD patients that correlates with a lower MMSE_i score at diagnosis and a worsening of the disease status in the one year follow-up. Since we have previously shown that the combination KIR2DS2/KIR2DL2/C1 results in a higher susceptibility of MS patients to herpesvirus infections, this genetic background might have a role also in herpesvirus infection in AD. Interestingly, both activating KIR2DS2 and inhibiting KIR2DL2 receptors resulted increased in AD patients. KIR2DL2 and KIR2DS2 are in linkage disequilibrium, implying they are “preferentially associated” with one another. However, when KIR2DS2 and KIR2DL2 are co-expressed, NK cell inhibition via KIR2DL2 over-rides NK cell activation via KIR2DS2 [49]. We hypothesized that AD patients with KIR2DS2/KIR2DL2/C1 might be less prone to control HHV-6A infection and, in more general terms, herpesvirus infection. The *in vitro* experiments on NK92-KIR2DL2 cells showed an impaired control of HHV-6A infection in comparison with wild type NK92-GFP. Interestingly, NK92 cells challenged with HHV-6A infected cells increased the phosphorylation status of Sp1 due to both PKA activity and the direct transactivating effect of HHV-6 on Sp1 binding sites [50]. Since KIR2DL2 promoter has a Sp1 binding site [45], the activation of Sp1 could affect the expression of KIR2DL2 on the surface of NK cells in the presence of herpesvirus infected cells [9]. Meanwhile, the activation of PKA could have a role in AD neurofibrillary pathology, directly participating in the tau pathological hyperphosphorylation [51]. We hypothesize that HHV-6A infection might induce PKA activation, tau hyperphosphorylation, and Sp1 phosphorylation, thereby increasing its binding with the KIR2DL2 promoter. Overexpression of KIR2DL2 will consequently inhibit NK cells [9], possibly with a direct role in AD. Previous studies have already described the possible involvement of NK cells in AD immune-pathogenesis [7], showing a lower NK cell functional potential in AD patients [2]. Solerte *et al.* suggested that the overactivity of NK cells AD patients could be related to the dysregulation of PKC (protein kinase C) expression [52], similarly to what we observed for PKA.

Taking into consideration the genetic background of AD patients, it was necessary to look at APOE allelic distribution. Inside the brain, ApoE, characterized by three allelic forms: APOE2, APOE3, APOE4, helps to clear beta-amyloid, a plaque component. We confirmed that APOE ϵ 4 allele has an increased frequency in AD patients (Table 3 and 4), and, interestingly, this was more frequent in KIR2DS2/KIR2DL2/C1 AD patients. These results suggest that APOE ϵ 4 allele and KIR2DS2/KIR2DL2/C1 could have a combined effect in a multi-gene form of AD. In particular, APOE is able to inhibit the activation of immune cells, and therefore it could enhance the NK inhibition associated to KIR2DS2/KIR2DL2/C1. We also observed that HHV-6A infection up-modulates apoE ϵ 4 expression. Moreover, apoE increases PKA activity [53] and, conversely, the secretion of apoE occurs via a PKA-dependent pathway [54]. Therefore a loop could be established, with HHV-6 increasing apoE ϵ 4 expression and this would further decrease an already reduced KIR2DS2/KIR2DL2/C1 NK patrolling activity.

The involvement of NK cell in AD could include their defensive role towards HHV infections, leading to the deregulation of immune system and resulting in the AD progression [55]. Our results with NK92-KIR2DL2 cells, expressing only KIR2DL2 and not other KIR receptors that could confound the analysis, supports this possibility. Higher herpesvirus infection might lead to hyperphosphorylation and polymerization of tau proteins, induce amyloid- β peptide (A β) fibrilization and deposition [56], suggesting that herpesviruses might have an important role also in several features of AD pathology.

In conclusion, the possible role of herpesvirus infection in AD development/control is supported by: i) the increase of KIR2DL2/C1 genotype in AD patients (Table 2), ii) a lower anti-herpetic activity of KIR2DL2 positive NK cells (Fig 1f) [9], iii) a lower NK activity in AD [2], iv) an increased abundance of neurotropic herpesviruses in AD [29] that promote A β deposition [56], and the creation of a molecular environment favourable for tau fibrilization (Fig 2b).

To confirm this hypothesis, further studies are needed on *ex vivo* samples from AD patients. If this hypothesis finds experimental confirmation, a new therapeutic strategy for AD might be envisaged.

In fact, modulation of KIR2DL2 expression on NK cells, as already proposed for other pathological conditions [57] [58], could restore their anti-herpetic surveillance.

Acknowledgements

We thank Kristin Loomis and Linda Sartor for the support in paper writing and English revision. This research was supported by University of Ferrara FAR (RR, DDL) and FISM grant 2015/R/20 (RR).

Conflict of Interest/Disclosure Statement

The authors have no conflict of interest to report.

References

- [1] Hort J, O'Brien JT, Gainotti G, Pirttila T, Popescu BO, Rektorova I, Sorbi S, Scheltens P, Dementia ESPo (2010) EFNS guidelines for the diagnosis and management of Alzheimer's disease. *Eur J Neurol* **17**, 1236-1248.
- [2] Richartz-Salzbunger E, Batra A, Stransky E, Laske C, Kohler N, Bartels M, Buchkremer G, Schott K (2007) Altered lymphocyte distribution in Alzheimer's disease. *J Psychiatr Res* **41**, 174-178.
- [3] Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P (2007) Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. *Neurobiol Aging* **28**, 1163-1169.
- [4] Thielens A, Vivier E, Romagne F (2012) NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Curr Opin Immunol* **24**, 239-245.
- [5] Cheent KS, Jamil KM, Cassidy S, Liu M, Mbiribindi B, Mulder A, Claas FH, Purbhoo MA, Khakoo SI (2013) Synergistic inhibition of natural killer cells by the nonsignaling molecule CD94. *Proc Natl Acad Sci U S A* **110**, 16981-16986.
- [6] Krishnaraj R (1991) Immunomodulation by 9-amino-1,2,3,4-tetrahydroacridine (THA): 1. Down-regulation of natural cell-mediated cytotoxicity in vitro. *Immunopharmacology* **22**, 69-76.
- [7] Araga S, Kagimoto H, Funamoto K, Takahashi K (1991) Reduced natural killer cell activity in patients with dementia of the Alzheimer type. *Acta Neurol Scand* **84**, 259-263.
- [8] Rizzo R, Bortolotti D, Fainardi E, Gentili V, Bolzani S, Baldi E, Casetta I, Granieri E, Rotola A, Furlan R, Di Luca D (2016) KIR2DL2 inhibitory pathway enhances Th17 cytokine secretion by NK cells in response to herpesvirus infection in multiple sclerosis patients. *J Neuroimmunol* **294**, 1-5.

- [9] Rizzo R, Gentili V, Casetta I, Caselli E, De Gennaro R, Granieri E, Cassai E, Di Luca D, Rotola A (2012) Altered natural killer cells' response to herpes virus infection in multiple sclerosis involves KIR2DL2 expression. *J Neuroimmunol* **251**, 55-64.
- [10] Itzhaki RF, Lathe R, Balin BJ, Ball MJ, Bearer EL, Braak H, Bullido MJ, Carter C, Clerici M, Cosby SL, Del Tredici K, Field H, Fulop T, Grassi C, Griffin WS, Haas J, Hudson AP, Kamer AR, Kell DB, Licastro F, Letenneur L, Lovheim H, Mancuso R, Miklossy J, Otth C, Palamara AT, Perry G, Preston C, Pretorius E, Strandberg T, Tabet N, Taylor-Robinson SD, Whittum-Hudson JA (2016) Microbes and Alzheimer's Disease. *J Alzheimers Dis* **51**, 979-984.
- [11] Jamieson GA, Maitland NJ, Wilcock GK, Craske J, Itzhaki RF (1991) Latent herpes simplex virus type 1 in normal and Alzheimer's disease brains. *J Med Virol* **33**, 224-227.
- [12] Letenneur L, Peres K, Fleury H, Garrigue I, Barberger-Gateau P, Helmer C, Orgogozo JM, Gauthier S, Dartigues JF (2008) Seropositivity to herpes simplex virus antibodies and risk of Alzheimer's disease: a population-based cohort study. *PLoS One* **3**, e3637.
- [13] Lovheim H, Gilthorpe J, Adolfsson R, Nilsson LG, Elgh F (2015) Reactivated herpes simplex infection increases the risk of Alzheimer's disease. *Alzheimers Dement* **11**, 593-599.
- [14] Tzeng NS, Chung CH, Lin FH, Chiang CP, Yeh CB, Huang SY, Lu RB, Chang HA, Kao YC, Yeh HW, Chiang WS, Chou YC, Tsao CH, Wu YF, Chien WC (2018) Anti-herpetic Medications and Reduced Risk of Dementia in Patients with Herpes Simplex Virus Infections- a Nationwide, Population-Based Cohort Study in Taiwan. *Neurotherapeutics* **15**, 417-429.
- [15] Wozniak MA, Itzhaki RF, Shipley SJ, Dobson CB (2007) Herpes simplex virus infection causes cellular beta-amyloid accumulation and secretase upregulation. *Neurosci Lett* **429**, 95-100.
- [16] De Chiara G, Marcocci ME, Civitelli L, Argnani R, Piacentini R, Ripoli C, Manservigi R, Grassi C, Garaci E, Palamara AT (2010) APP processing induced by herpes simplex virus

type 1 (HSV-1) yields several APP fragments in human and rat neuronal cells. *PLoS One* **5**, e13989.

- [17] Martin C, Aguila B, Araya P, Vio K, Valdivia S, Zambrano A, Concha MI, Otth C (2014) Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation. *J Alzheimers Dis* **39**, 849-859.
- [18] Piacentini R, Li Puma DD, Ripoli C, Marcocci ME, De Chiara G, Garaci E, Palamara AT, Grassi C (2015) Herpes Simplex Virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-beta protein accumulation. *Sci Rep* **5**, 15444.
- [19] Cheng SB, Ferland P, Webster P, Bearer EL (2011) Herpes simplex virus dances with amyloid precursor protein while exiting the cell. *PLoS One* **6**, e17966.
- [20] Wozniak MA, Frost AL, Preston CM, Itzhaki RF (2011) Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with herpes simplex virus type 1. *PLoS One* **6**, e25152.
- [21] Harris SA, Harris EA (2018) Molecular Mechanisms for Herpes Simplex Virus Type 1 Pathogenesis in Alzheimer's Disease. *Front Aging Neurosci* **10**, 48.
- [22] Dwyer R, Skrobot OA, Dwyer J, Munafo M, Kehoe PG (2013) Using Alzgene-like approaches to investigate susceptibility genes for vascular cognitive impairment. *J Alzheimers Dis* **34**, 145-154.
- [23] Sun JH, Tan L, Wang HF, Tan MS, Tan L, Li JQ, Xu W, Zhu XC, Jiang T, Yu JT (2015) Genetics of Vascular Dementia: Systematic Review and Meta-Analysis. *J Alzheimers Dis* **46**, 611-629.
- [24] Liu CC, Liu CC, Kanekiyo T, Xu H, Bu G (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nat Rev Neurol* **9**, 106-118.
- [25] Michaelson DM (2014) APOE epsilon4: the most prevalent yet understudied risk factor for Alzheimer's disease. *Alzheimers Dement* **10**, 861-868.

- [26] Strandberg TE, Pitkala KH, Linnavuori KH, Tilvis RS (2003) Impact of viral and bacterial burden on cognitive impairment in elderly persons with cardiovascular diseases. *Stroke* **34**, 2126-2131.
- [27] Lurain NS, Hanson BA, Martinson J, Leurgans SE, Landay AL, Bennett DA, Schneider JA (2013) Virological and immunological characteristics of human cytomegalovirus infection associated with Alzheimer disease. *J Infect Dis* **208**, 564-572.
- [28] Aiello AE, Haan M, Blythe L, Moore K, Gonzalez JM, Jagust W (2006) The influence of latent viral infection on rate of cognitive decline over 4 years. *J Am Geriatr Soc* **54**, 1046-1054.
- [29] Readhead B, Haure-Mirande JV, Funk CC, Richards MA, Shannon P, Haroutunian V, Sano M, Liang WS, Beckmann ND, Price ND, Reiman EM, Schadt EE, Ehrlich ME, Gandy S, Dudley JT (2018) Multiscale Analysis of Independent Alzheimer's Cohorts Finds Disruption of Molecular, Genetic, and Clinical Networks by Human Herpesvirus. *Neuron* **99**, 64-82 e67.
- [30] Ablashi D, Agut H, Alvarez-Lafuente R, Clark DA, Dewhurst S, DiLuca D, Flamand L, Frenkel N, Gallo R, Gompels UA, Hollsberg P, Jacobson S, Luppi M, Lusso P, Malnati M, Medveczky P, Mori Y, Pellett PE, Pritchett JC, Yamanishi K, Yoshikawa T (2014) Classification of HHV-6A and HHV-6B as distinct viruses. *Arch Virol* **159**, 863-870.
- [31] De Bolle L, Naesens L, De Clercq E (2005) Update on human herpesvirus 6 biology, clinical features, and therapy. *Clin Microbiol Rev* **18**, 217-245.
- [32] Braun DK, Dominguez G, Pellett PE (1997) Human herpesvirus 6. *Clin Microbiol Rev* **10**, 521-567.
- [33] Donati D, Akhyani N, Fogdell-Hahn A, Cermelli C, Cassiani-Ingoni R, Vortmeyer A, Heiss JD, Cogen P, Gaillard WD, Sato S, Theodore WH, Jacobson S (2003) Detection of human herpesvirus-6 in mesial temporal lobe epilepsy surgical brain resections. *Neurology* **61**, 1405-1411.

- [34] Roush KS, Domiati-Saad RK, Margraf LR, Krisher K, Scheuermann RH, Rogers BB, Dawson DB (2001) Prevalence and cellular reservoir of latent human herpesvirus 6 in tonsillar lymphoid tissue. *Am J Clin Pathol* **116**, 648-654.
- [35] Fox JD, Briggs M, Ward PA, Tedder RS (1990) Human herpesvirus 6 in salivary glands. *Lancet* **336**, 590-593.
- [36] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-944.
- [37] Folstein MF, Folstein SE, McHugh PR (1975) "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* **12**, 189-198.
- [38] Magni E, Binetti G, Bianchetti A, Rozzini R, Trabucchi M (1996) Mini-Mental State Examination: a normative study in Italian elderly population. *Eur J Neurol* **3**, 198-202.
- [39] Excoffier L, Laval G, Schneider S (2007) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online* **1**, 47-50.
- [40] Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, Tyan D, Lanier LL, Parham P (1997) Human diversity in killer cell inhibitory receptor genes. *Immunity* **7**, 753-763.
- [41] Caselli E, Bortolotti D, Marci R, Rotola A, Gentili V, Soffritti I, D'Accolti M, Lo Monte G, Siculo M, Barao I, Di Luca D, Rizzo R (2017) HHV-6A Infection of Endometrial Epithelial Cells Induces Increased Endometrial NK Cell-Mediated Cytotoxicity. *Front Microbiol* **8**, 2525.
- [42] Rizzo R, Soffritti I, D'Accolti M, Bortolotti D, Di Luca D, Caselli E (2017) HHV-6A/6B Infection of NK Cells Modulates the Expression of miRNAs and Transcription Factors Potentially Associated to Impaired NK Activity. *Front Microbiol* **8**, 2143.

- [43] Comar M, D'Agaro P, Horejsh D, Galvan M, Fiorentini S, Andolina M, Caruso A, Di Luca D, Campello C (2005) Long-lasting CD3⁺ T-cell deficiency after cord blood stem cell transplantation in a human herpesvirus 6-infected child. *J Clin Microbiol* **43**, 2002-2003.
- [44] Caselli E, Zatelli MC, Rizzo R, Benedetti S, Martorelli D, Trasforini G, Cassai E, degli Uberti EC, Di Luca D, Dolcetti R (2012) Virologic and immunologic evidence supporting an association between HHV-6 and Hashimoto's thyroiditis. *PLoS Pathog* **8**, e1002951.
- [45] Liu Y, Kuick R, Hanash S, Richardson B (2009) DNA methylation inhibition increases T cell KIR expression through effects on both promoter methylation and transcription factors. *Clin Immunol* **130**, 213-224.
- [46] Xu J, Vallejo AN, Jiang Y, Weyand CM, Goronzy JJ (2005) Distinct transcriptional control mechanisms of killer immunoglobulin-like receptors in natural killer (NK) and in T cells. *J Biol Chem* **280**, 24277-24285.
- [47] Guo L, Eviatar-Ribak T, Miskimins R (2010) Sp1 phosphorylation is involved in myelin basic protein gene transcription. *J Neurosci Res* **88**, 3233-3242.
- [48] Rohlf C, Ahmad S, Borellini F, Lei J, Glazer RI (1997) Modulation of transcription factor Sp1 by cAMP-dependent protein kinase. *J Biol Chem* **272**, 21137-21141.
- [49] David G, Djaoud Z, Willem C, Legrand N, Rettman P, Gagne K, Cesbron A, Retiere C (2013) Large spectrum of HLA-C recognition by killer Ig-like receptor (KIR)2DL2 and KIR2DL3 and restricted C1 SPECIFICITY of KIR2DS2: dominant impact of KIR2DL2/KIR2DS2 on KIR2D NK cell repertoire formation. *J Immunol* **191**, 4778-4788.
- [50] Wang J, Jones C, Norcross M, Bohnlein E, Razzaque A (1994) Identification and characterization of a human herpesvirus 6 gene segment capable of transactivating the human immunodeficiency virus type 1 long terminal repeat in an Sp1 binding site-dependent manner. *J Virol* **68**, 1706-1713.

- [51] Jicha GA, Weaver C, Lane E, Vianna C, Kress Y, Rockwood J, Davies P (1999) cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease. *J Neurosci* **19**, 7486-7494.
- [52] Solerte SB, Fioravanti M, Pascale A, Ferrari E, Govoni S, Battaini F (1998) Increased natural killer cell cytotoxicity in Alzheimer's disease may involve protein kinase C dysregulation. *Neurobiol Aging* **19**, 191-199.
- [53] Zhu Y, Hui DY (2003) Apolipoprotein E binding to low density lipoprotein receptor-related protein-1 inhibits cell migration via activation of cAMP-dependent protein kinase A. *J Biol Chem* **278**, 36257-36263.
- [54] Kockx M, Guo DL, Huby T, Lesnik P, Kay J, Sabaretnam T, Jary E, Hill M, Gaus K, Chapman J, Stow JL, Jessup W, Kritharides L (2007) Secretion of apolipoprotein E from macrophages occurs via a protein kinase A and calcium-dependent pathway along the microtubule network. *Circ Res* **101**, 607-616.
- [55] Holtzman DM, Morris JC, Goate AM (2011) Alzheimer's disease: the challenge of the second century. *Sci Transl Med* **3**, 77sr71.
- [56] Eimer WA, Vijaya Kumar DK, Navalpur Shanmugam NK, Rodriguez AS, Mitchell T, Washicosky KJ, Gyorgy B, Breakefield XO, Tanzi RE, Moir RD (2018) Alzheimer's Disease-Associated beta-Amyloid Is Rapidly Seeded by Herpesviridae to Protect against Brain Infection. *Neuron* **99**, 56-63 e53.
- [57] Vey N, Karlin L, Sadot-Lebouvier S, Broussais F, Berton-Rigaud D, Rey J, Charbonnier A, Marie D, Andre P, Paturel C, Zerbib R, Bennouna J, Salles G, Goncalves A (2018) A phase 1 study of lirilumab (antibody against killer immunoglobulin-like receptor antibody KIR2D; IPH2102) in patients with solid tumors and hematologic malignancies. *Oncotarget* **9**, 17675-17688.
- [58] Yalniz FF, Daver N, Rezvani K, Kornblau S, Ohanian M, Borthakur G, DiNardo CD, Konopleva M, Burger J, Gasior Y, Pierce S, Kantarjian H, Garcia-Manero G (2018) A Pilot

Trial of Lirilumab With or Without Azacitidine for Patients With Myelodysplastic Syndrome.

Clin Lymphoma Myeloma Leuk.

Figure legends

Figure 1. J-Jhan T cell lines were infected with HHV-6A at a multiplicity of infection of 100 genome equivalent/cell. Virus presence (DNA) and transcription (RNA) were evaluated respectively by qPCR and RT-qPCR performed on U94 and U42 virus genes respectively, at 1, 3 and 7, 14, 21 d.p.i., as already detailed. mRNA apoE ϵ 4 expression was evaluated in J-Jhan cells at 1, 3 and 7, 14, 21 d.p.i. RQ: relative quantity of apoE ϵ 4 mRNA.

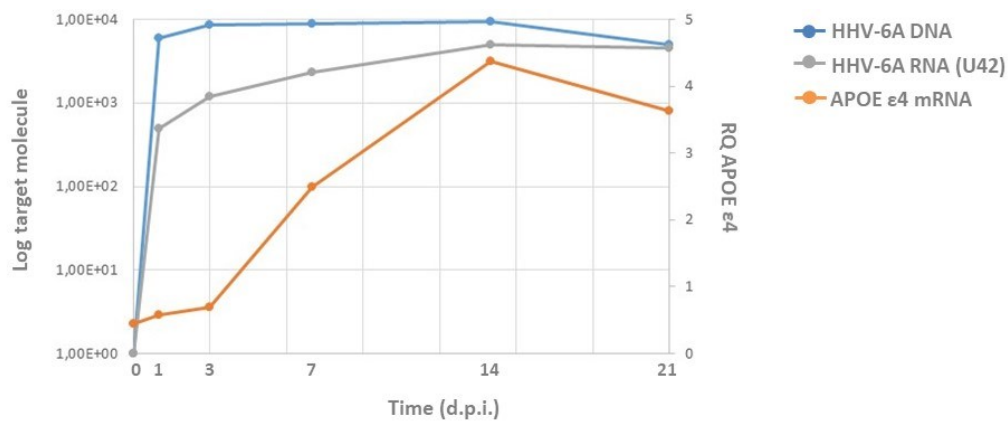


Figure 2. a) MMSE_I (mini-mental state examination at diagnosis), b) ADL_I (activities of daily living at diagnosis) and c) IADL_I (instrumental activities of daily living at diagnosis) distribution according with the KIR2DS2/KIR2DL2 present/C1 present combination. **p value <0.05, obtained by logistic regression. d) NK92 nucleofected cells with pCMV6-AC-GFP (RG224797, OriGene Technologies, Inc. 9620 Medical Center Drive Suite 200 Rockville, MD 20850 USA) (NK92-GFP) or pCMV6-AC-KIR2DL2 (GFP- tagged- Human killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2) (NK92-KIR2DL2) plasmid. GFP expression was evaluated after 12 days of selection. e) Beta actin and KIR2DL2 mRNA expression in NK92 (WT), NK92 nucleofected cells with pCMV6-AC-GFP (GFP) or pCMV6-AC-KIR2DL2 (KIR2DL2) plasmid. f) HHV-6A infection of J-Jhan cells and co-culture with NK92 nucleofected cells with pCMV6-AC-GFP (NK92-GFP) or pCMV6-AC-KIR2DL2 plasmid with (NK92-KIR2DL2 + CD158b) or without (NK92-KIR2DL2) the addition of anti-KIR2DL2 moAb (CD158b). Virus DNA presence was evaluated using an amount of 100 ng of DNA. Results are expressed as mean Log10 target molecules. g) Mean results obtained for CFSE Fluorescent cell staining of HHV-6A infection of J-Jhan cells co-cultured with NK92 nucleofected cells with pCMV6-AC-GFP (NK92-GFP) or pCMV6-AC-KIR2DL2 plasmid with (NK92-KIR2DL2 + CD158b) with (NK92-KIR2DL2 + CD158b) or without (NK92-KIR2DL2) the addition of anti-KIR2DL2 moAb (CD158b). * p value <0.05, obtained by Student t test. Each experiment was performed in triplicate.

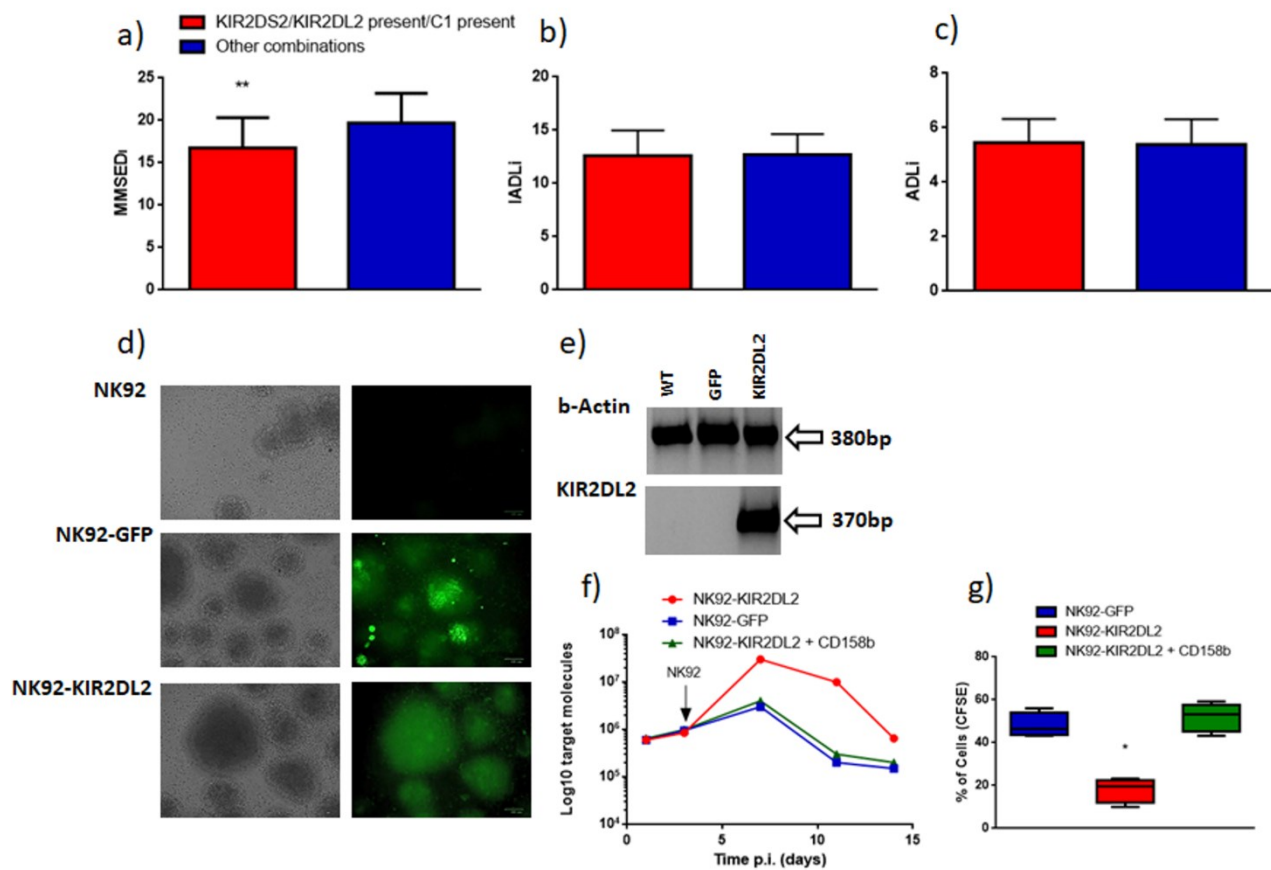


Figure 3. a) Western Blot analysis for the evaluation of Sp1 and pThrSp1 expression in NK92 cells in co-culture with HHV-6A infection of J-Jhan cells (HHV-6A). b) PKA activity in NK92 cells in co-culture with HHV-6A infection of J-Jhan cells (HHV-6A). c) Fold increase of pThrSp1 in NK92 cells in co-culture with HHV-6A infection of J-Jhan cells (HHV-6A) in the presence of increasing concentrations (0.25, 0.5, 1.0, 2.0, 2.5 μ M) of KT 5720, a specific, cell-permeable inhibitor of protein kinase A. * p value <0.05, obtained by Student t test. Each experiment was performed in triplicate.

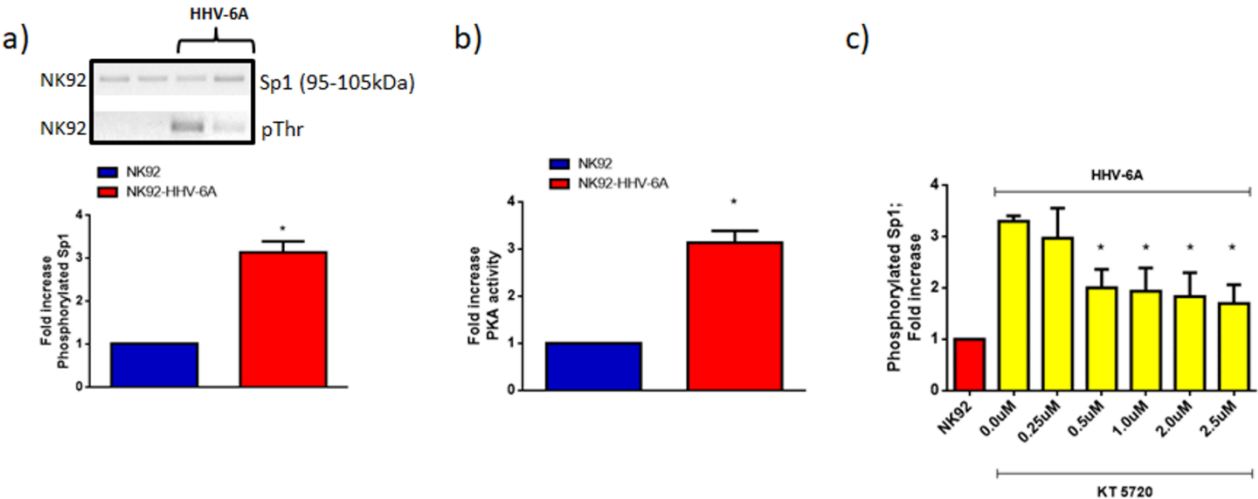


Table 1. Baseline characteristics

Baseline characteristics	AD (mean±SD)	Control (mean±SD)
Female : Male	65:33	66:34
Age (years)	75.9±5.5	74.3±4.2
Age at diagnosis (years)	73.3±5.5	-
MMSE _D score	19.4±3.6	-
ADL _D score/6 ^a	5.4±0.88	-
IADL _D score/19 ^a	13.1±2.3	-
MMSE _D score	19.3±3.2	-

MMSE_D: mini-mental state examination at diagnosis, corrected by age and education; ADL_D: activities of daily living at diagnosis; IADL_D: instrumental activities of daily living at diagnosis. Higher score indicates better performances in normal daily activities (ADL, IADL) or better cognitive functions.

Table 2. KIR/HLA frequency

KIR and HLA			AD (N=98)	%	Contro l (N=110)	%	p	p _c
<i>Activating KIR</i>								
KIR2DS1			49	50	45	40.9	0.21	
KIR2DS2			61	62	41	37.2	2.8x10 ⁻⁴	2.2 x10 ⁻³
KIR2DS3			35	35.7	31	28.2	0.29	
KIR2DS4			85	87	91	82.7	0.45	3.6x10 ⁻⁴
KIR2DS5			46	46.9	33	30	0.015	
KIR3DS1			12	16.6	36	32.7	4.9x10 ⁻⁴	2.9x10 ⁻³
<i>Inhibitory KIR</i>								
KIR2DL1			88	89.8	95	86.4	0.52	
KIR2DL2			61	62.2	40	36.4	2.8x10 ⁻⁴	2.2 x10 ⁻³
KIR2DL3			71	72.4	85	77.3	0.52	
KIR2DL4			98	100	110	100	0	
KIR2DL5			59	60.2	50	45.5	0.038	
KIR3DL1			92	93.9	91	82.7	0.018	
KIR3DL2			98	100	110	100	0	
KIR3DL3			98	100	110	100	0	
<i>KIR genotype</i>								
AA			26	26.5	32	29	0.35	
Bx			72	73.5	65	59.1		
<i>HLA genotype</i>								
C1/C1			25	25.5	22	20	0.41	
C1/C2			45	45.9	25	22.7	4x10 ⁻⁴	2 x10 ⁻³
C2/C2			28	28.5	47	42.7	0.042	
HLA-Bw4			77	78.6	78	70.9	1	
HLA-Bw6			91	92.9	92	83.6	0.054	
<i>KIR and their ligands</i>								
KIR2DS1/KIR2DL1	pres/HLA-C2	39	39.8	31	28.2	0.08		
pres								
KIR2DS1/KIR2DL1	pres/HLA-C2	10	10.2	9	8.2	0.64		
abs								
KIR2DS1/KIR2DL1	abs/HLA-C2	2	2	1	0.9	0.60		
pres								
KIR2DS2/KIR2DL2	pres/HLA-C1	47	48	31	28.2	0.0041	0.037	
pres								
KIR2DS2/KIR2DL2	pres/HLA-C1	14	14.3	15	13.6	1		
abs								
KIR2DS2/KIR2DL2	abs/HLA-C1	6	6.1	34	30.9	5.7x10 ⁻⁶	5.1x10 ⁻⁵	
pres								
KIR3DS1/KI3DL1	pres/ HLA-Bw4	29	29.6	27	24.5	0.88		
pres								
KIR3DS1/KI3DL1	pres/ HLA-Bw4	9	9.2	8	7.3	0.80		
abs								
KIR3DS1/KI3DL1	abs/ HLA-Bw4	1	1	3	2.7	0.62		
pres								

p: p value obtained by Fisher's exact test. p_c : corrected p value for multiple comparisons (Bonferroni correction).

Table 3. APOE allele frequency

		Allele frequencies		
		$\epsilon 2$	$\epsilon 3$	$\epsilon 4$
Alzheimer	patients	7.2	52.4	40.4
(98)				
Control subjects (110)		11.4	74.6	14.0

Table 4. KIR/HLA frequency in combination with APOE allele frequency

			AD (N=98)			Control (N=110)		
			$\epsilon 2$	$\epsilon 3$	$\epsilon 4$	$\epsilon 2$	$\epsilon 3$	$\epsilon 4$
KIR2DS2/KIR2DL2	pres/HLA-C1		6.4	42.6	46.8	9.7	64.5	16.9
pres								
Other combinations			7.8	62.7	35.3	10.1	68.4	11.4