Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
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CICLO XXIX

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NK cells expressing KIR2DL2 inhibitory receptor: effect on HHVs infection control in MS disease

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1. Introduction

1.1 Multiple sclerosis disease

Multiple sclerosis (MS) is a disease of the Central Nervous System (CNS) characterized by acute focal inflammatory demyelination and axonal damage, that leads to the formation of chronic multifocal sclerotic plaques[1]. MS is characterized by a relapsing-remitting (RR) or a progressive course with multifocal CNS dysfunctions. MS patients show a huge variety of recurring symptoms and an unpredictable course of the disease that often flows into a chronic progressive disability[1].

The disease onset usually happens between 20 and 40 years and affects most frequently women than men. The precise etiology of multiple sclerosis remains unknown, but in the past few years even more genetic variants involved in MS disease have been discovered and recent studies suggest that the interaction between genetic susceptibility and environmental factors could modulate the disease pattern[2].

1.1.1 Diagnosis

MS clinical manifestations involve motor, sensory, visual and autonomic systems [3].

Weakness, sensory disturbance, optic neuritis, diplopia, ataxia and gait instability could represent the early symptoms of the disease. Furthermore, fatigue, heat sensitivity and bladder dysfunction might occur. Typical clinical features of MS are represented by the Lhermitte's symptom (an electrical sensation running down the spine or into the limbs on neck flexion) and the Uhthoff phenomenon (transient worsening of the symptoms when the body temperature increases). Again, an involvement of the anterior visual pathway is a common sign due to lesions of the brainstem and cerebellar pathways that result in the bulbar musculature and axial muscles disruption[2,3]. The symptoms and signs often reflect the result of an impaired saltatory nervous conduction at affected sites.

Since the aspecific set of symptoms that characterized MS, clinicians refer to standardized criteria to identify a diagnosis. McDonald criteria consist in parameters series that allow to diagnose MS when a clinical evidence is lacking, basing on MRI to define dissemination

of lesions in space (DIS) and time (DIT) and to exclude other diseases[4-10]. A summary of the 2010 McDonald Criteria for diagnosis of MS has been reported in Table 1[4].

Table 1 The 2010 McDonald Criteria for Diagnosis of MS[4]

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥ 2 attacks[a]; objective clinical evidence of ≥ 2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack	None[c]
≥ 2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥ 1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d] or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥ 2 lesions	Dissemination in time, demonstrated by: simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions at any time; or a new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: for DIS: ≥ 1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or await a second clinical attack[a] implicating a different CNS site; and for DIT: simultaneous presence of asymptomatic gadolinium-enhancing and non-enhancing lesions at any time; or a new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥ 1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥ 2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is ''MS''; if suspicious, but the Criteria are not completely met, the diagnosis is ''possible MS''; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is ''not MS.''

a) An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

b) Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

c) No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

d) Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS: multiple sclerosis; CNS: central nervous system; MRI: magnetic resonance imaging; DIS: dissemination in space; DIT: dissemination in time; PPMS: primary progressive multiple sclerosis; CSF: cerebrospinal fluid; IgG: immunoglobulin G.

1.1.2 Disease mechanism and pathogenesis

The oligodendrocyte in the CNS and the Schwann cells in Peripheral Nervous System (PNS) produce and maintain the myelin membranes that surround the axons with the purpose to facilitate the transmission of electrical impulses. This process is regulated by defined growth factors that manage proliferation, migration and differentiation of oligodendrocyte precursors into myelinating cells. In particular, the myelin sheath consists of a condensed membrane spiraled around axons needed to axonal conduction. The portions of the axon uncovered by myelin are called nodes of Ranvier, whereas the myelinated axons segments are internodes. Myelin is an electrical insulator that allows conduction in axons known as saltatory conduction, that results in an increased speed of local circuit spreading (Fig.1)[11].

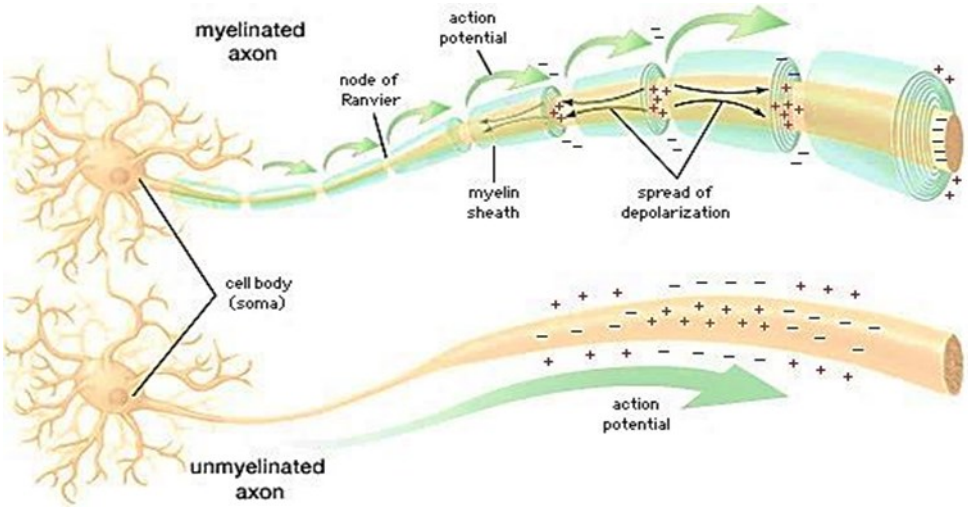


Figure 1 Conduction of the action potential in myelinated and demyelinated axon. Encyclopædia Britannica, Inc. 2002

Demyelination in saltatory conduction clarifies many clinical and laboratory features of MS. Partially demyelinated axons show a reduced speed in the impulses conduction. Demyelinated axons can discharge spontaneously, causing an increased mechanical sensitivity, flashes of light on the eye movement. In fact, MS patients are typically tire during physical and cognitive tasks and take longer to recover. The sclerotic plaques in

demyelinating disease result from a process that involves inflammation, demyelination, remyelination, astrocytosis and neuronal degeneration[3].

Autoreactive T cell responses against antigens derived from CNS are thought to trigger several diseases, such as MS, neuromyelitis optica and encephalomyelitis. It is believed that myelin specific T cells are activated in periphery, cross the blood-brain barrier and consequently are reactivated by CNS resident APCs presenting myelin antigens (Fig.2).

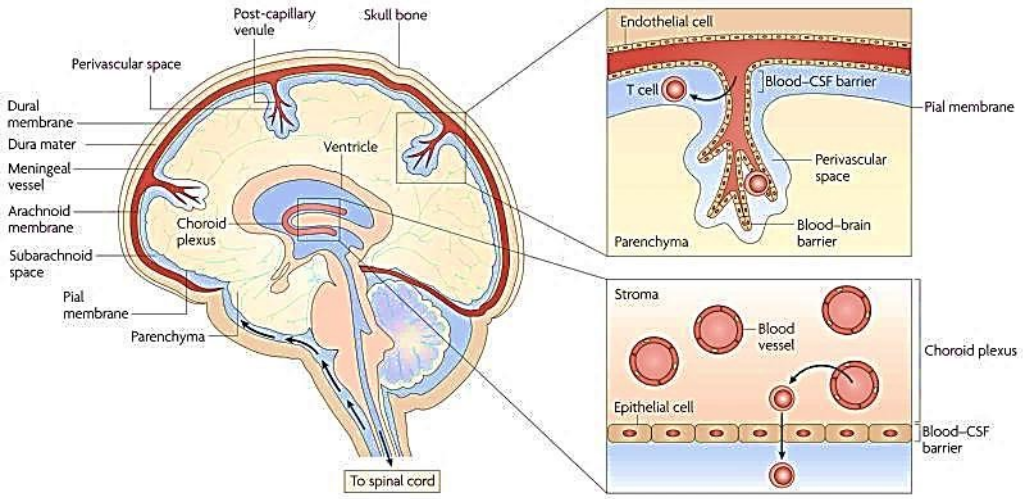


Figure 2 Anatomical organization of the brain and the possible routes of activated T cell entry[12]

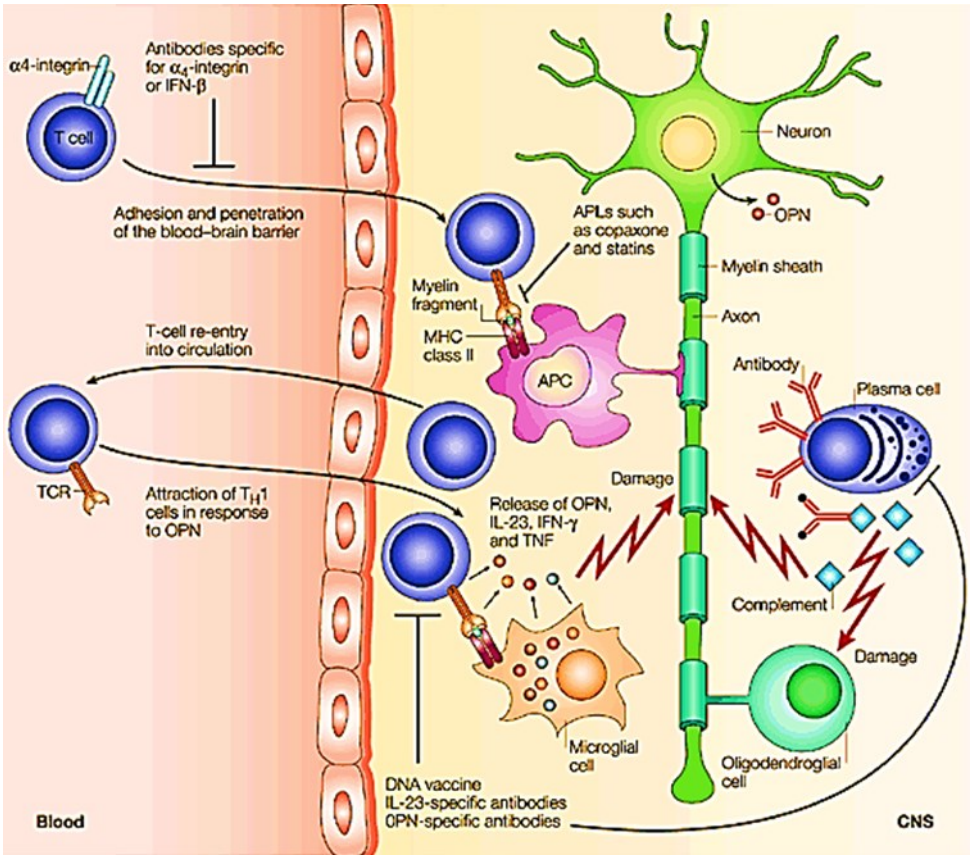


Figure 3 T cells, B cells and antigen-presenting cells (APCs), including macrophages, secrete in the CNS certain cytokines that damage the oligodendroglial cells. These cells damage the myelin that insulates the neuronal axon. The injured myelin cannot conduct electrical impulses normally. Once the blood–brain barrier is breached, other inflammatory cells accumulate in the white matter where release osteopontin (OPN), interleukin-23 (IL-23), IFN-gamma and tumor-necrosis factor (TNF), all of which damage the myelin sheath. The OPN might lead to the attraction of T helper 1 (TH1) cells. Moreover, B cells produce myelin-specific antibodies, which interact with the terminal complex in the complement cascade to produce membrane-attack complexes, damaging oligodendroglial cells. DNA vaccination can be used to tolerize T- and B-cell responses to myelin[13].

This reactivation induces the recruitment of innate immune cells, that produce several soluble mediators. In normal conditions, CNS is protected from cellular infiltration by blood-brain barrier and by the blood-cerebrospinal fluid (CSF) barrier and the access to the CNS by circulating cells is controlled through tight junctions. However, T cells can escape immune surveillance of the CNS and cross the barriers activating an immune response within the brain (Fig.3)[12].

This immune-cell trafficking into the brain is essential to understand not only the onset but also the progression of the disease. Once crossed the blood-brain barrier, myelin-reactive T cells can induce neuronal and tissue damage and degeneration[14]. The lymphocytes migration into CNS parenchyma is a process that requires a close contact between lymphocytes and brain endothelial cells. The firm adhesion of T cells and their trans-migration process across the blood-brain barrier is carried out by upregulating the expression of several adhesion molecules on brain endothelial cells surface, such as

ICAM-1 (Intercellular Adhesion Molecule), VCAM-1 (Vessel Cell Adhesion Molecule) and ALCAM (Activated Leukocyte Cell Adhesion Molecule). However, the complex mechanism behind the induction and interaction of these molecules remains poorly understood[15]. This is the cellular process that allows to set up an immune response within the brain. Focal inflammatory demyelinating lesions are characterized by the presence of leukocytes, lymphocytes and macrophages that form cuff-like aggregations, preferentially around small blood vessel, and swarm out into parenchyma[16,17]. After monocytes, that are the most represented infiltrating cell type found in MS lesions, the second major infiltrating cellular group is composed by T cells lymphocytes. The perivascular infiltrates predominately contain $CD8^+$ T cells, followed by $CD4^+$, B cells, monocytes and plasma cells[18].

Among $CD4^+$ T lymphocytes, known as T helper (Th), Th1 pro-inflammatory pathway was considered the most important mediator of the MS pathogenesis, while Th2 anti-inflammatory pathway plays a positive role in the disease process.

The composition of the infiltrating cells in MS plaques represents an active immune process. According by previous studies that have analyzed T cell receptors (TCRs) using infiltrating $CD8^+$ T cells, it has been shown that MS lesions contain clusters of T cells with identical TCR sequences, indicating a clonal offspring of the same ancestor T cell. Obviously, the cells of the same clone share identical TCR and then bind the same antigen (or autoantigen), but the nature of the putative target antigen remains poorly understood[19]. No specific structure or $CD8^+$ T cells autoantigen has been identified. In contrast, it was described an antigen binding sequence motif in TCR beta chain identical to a myelin basic protein (MBP)-specific motif found in a T cell line isolated from a MS patient[20].

In addition, B cells may give an important contribute to the immune pathogenesis of MS. Indeed, these cells could influence the immune process producing myelin specific auto-antibodies. Furthermore, B cells present auto-antigen to T cells, increasing T cells differentiation and activation and release of soluble factors that can modulate immune response[21]. In particular B cells release immunoglobulins that form typical oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF). Since the OCBs occur frequently in MS pathology, they have become an important diagnostic biomarker[16].

Numerous evidences support an autoimmune base early MS pathogenesis, but the nature of a putative target autoantigen remains still unknown. Different study models have reported that the presence of brain specific autoimmune T cells could be normally part of the immune system, but this kind of T cells are maintained in an inactive state and show their pathological potential only when activated[16]. Moreover, it was demonstrated that only

activated myelin-specific $CD4^+$ T cells accumulated in CNS parenchyma and MHC-II expression in the brain seems to be required for CNS infiltration[22]. In normal conditions, BECs (brain endothelial cells) express MHC-I, whereas MHC-II seems to be absent. It was observed that inflammation can increase expression of MHC-II as well as of the co-stimulatory CD40 molecule, stimulating allogenic T-cells proliferation in vitro[23].

In addition, pro-inflammatory cytokines amplify the immune response by recruiting naïve microglia that, together with infiltrated T cells, conduct to acute demyelinating lesions formation.

Remyelination, the process that creates new myelin sheaths, is responsible of shadow plaques in MS disease. This process is particularly active during the acute inflammation, due to the phagocytic removal of myelin debris, and during the progressive phase. A pool of undifferentiated oligodendrocyte precursors shall be maintained in the mature nervous system in order to migrate and surround the lesions that occur during MS[24,25]. During this process, plaques are eventually remyelinated, but these cycles of demyelination and remyelination affect the tissue repair ability[3].

Nowadays, MS can be treated in three possible ways: 1. by treating the exacerbations, 2. slowing disease progression with disease-modifying therapies (DMTs) and 3. using symptomatic therapy. Anti-inflammatory agents, such as high dose of intravenous or oral cortical steroids, are the first line treatment for MS exacerbations. Cortical steroids can improve motor functions and reduce symptoms, moreover can reduce recovery time after acute attacks[26,27].

DMTs are a component of the long-term management of MS patients that aims to reduce the early clinical and subclinical disease activity associated to long-term disability. All the DMTs work only with relapsing MS, but new drugs are being tested to find a cure also for progressive MS (non-relapsing). All DMTs work to reduce the immune response rather than to turn off inflammation. Moreover, efficacy, tolerability, and safety can change among agents.

An additional crucial element in the treatment of MS patients is the management of MS-related symptoms. A wide range of symptoms can affect the daily routine and are classified as primary, secondary and tertiary. The first group is referred to symptoms that are a direct consequence of the nerve damage, such as spasticity, paresthesia and optic neuritis. The second one arises from complications of primary symptoms. Tertiary symptoms consist of social and psychological complications[28]. The aim of symptoms management is to ameliorate or eliminate symptoms that affect and reduce life quality[29].

1.2 Multiple sclerosis etiology and environmental risk factors

The etiology of multiple sclerosis is not yet completely understood, but it is known that there is a combination of both genetic and environmental factors. The following is a summary of the main and important factors involved.

1.2.1 Multiple sclerosis and HLA genetics

The first evidence of a correlation between pathogenesis of MS and a genetic component was found through studies on families of affected individuals, in particular in monozygotic twins. Despite neither the recurrence rate nor the twin concordance supported the probability of a Mendelian inheritance, it has become evident that MS is a multifactorial disease and MS phenotype results from several polymorphic genes, with different alleles that can contribute to MS risk with a small, moderate or strong effect[30].

The factors involved in MS pathogenesis are still poorly understood, but since they are heterogeneous, an interplay between genetics and environmental causal factors was suggested.

According to MS severity, different disease phenotypes have been described. Primary progressive MS (PPMS) is the phenotype associated with a worse prognosis than relapse-onset MS (RMS) phenotype. However, the rate of progression is still variable among individuals with the same phenotype, depending also on other factors such as sex ratio and the age at disease onset. Despite some evidences suggest that shared polymorphisms underlie the genetic risk for both phenotypes, there is the possibility that the genetic structure associated with disease severity in PPMS and RMS could be different[31].

There were many explanations for the heritability in MS disease, including population and disease gene heterogeneity, environment interactions and cis/trans regulators of allelic expression. Anyway, genes within MHC region represent the largest genetic component for the MS risk. The influence of HLA genes in MS susceptibility was the first important evidence of a genetic association with MS and represents 10% of the genetic risk associated with MS disease. In particular, it was observed that the allele HLA-DRB1*15:01 in the class II region of MHC was associated to MS higher risk. It has also been seen that DRB1*15:01 showed an average odds ratios of 3.08, while all additional DRB1 associations represent less than 2% of the residual variance. It was also found, in addition to an increased risk in presence of DRB1*15:01 homozygous genotypes, an epistatic effect in DRB1*15:01/*08:01 heterozygous genotypes. The allele DRB1*08:01 does not seem to show any effect on its own, but it has been seen to enhance the effect of

DRB1*15:01 when the two alleles were in the same genotype. DRB1*15:01 was often found in association with markers of disease severity, such as gender (especially female) and age of onset (an earlier one). In addition, also these alleles are associated with an increased risk: HLA-DQA1*01:02, HLA-DQB1*06:02. All these three alleles establish the haplotype that mostly influences disease severity, suggesting that it plays a specific role in disease pathogenesis, differing from other associated HLA alleles and haplotypes[30].

Some HLA class II alleles are also associated to protection from MS in European population, such as DRB1*14:01, that seems to have a dominant protective role, abrogating DRB1*15:01 susceptibility effects. There are additional class II protective alleles that were found, such as DRB1*11, DRB1*13 and DQB1*06:03[32-34].

Regarding HLA class I, several alleles have been shown to be implicated in MS risk. In particular, HLA-A3 allele has been found associated to MS susceptibility since it has been found in LD (linkage disequilibrium) with DRB1*15:01~DQB1*06:02. Furthermore, HLA-A3 is in strong LD with HLA-B7, another HLA class I allele that has prevalence in MS patients[35].

On the contrary, some HLA class I alleles showed a protective effect in multiple such as HLA-A*02:01 and HLA-B*44:02[36]. Moreover, other interesting observation were reported by studying class I alleles in relation with their role as ligand for killer immunoglobulin-like receptor (KIR).

1.2.2 Natural Killer cells, Killer immunoglobulin-like (KIR) receptors and multiple sclerosis

Natural Killer (NK) cells are cytotoxic lymphocytes and represent an important component of the innate immune response also involved in the regulation of adaptive response. NK cells accomplish their functions by cytolytic activity and production of cytokines[37]. It has been described a typical NK-immunophenotype identified as $CD3^-$, to distinguish them from T cells, and their expression level of CD56 and presence/absence of CD16. The $CD16^+$ $CD56^{dim}$ (low expression) NK cell subset has a potent cytotoxic activity and cytokines production in response to appropriate stimuli while $CD16^-$ $CD56^{bright}$ (high expression) NK cells release high amounts of cytokines, but show a low cytotoxicity[38].

Several data suggest an involvement of NK cells in MS pathogenesis and protection, supporting an important role of KIR polymorphisms in MS susceptibility. NK cells can contribute in this process through their immune-regulatory activity or cytotoxicity towards self-tissues. Certainly, the literature data on NK cell role in MS onset are still

controversial. In fact, on one hand NK activation seems to down-regulate MS progression, while on the other hand the production of several cytokines by NK cells seems to worsen disease condition[39-43].

NK cells have been originally described for their ability to kill tumors or virally infected cells without previous stimulation[44, 45]. They are able to recognize aberrant/transformed cells through different types of receptors that, normally, lead to NK activation. In particular, there are two distinct families of receptors that can regulate NK cells activity: the immunoglobulin-like NK receptors (KIR, LILR, NCRs, p75/AIRM1, IRp60, 2B4/CD244, NTB-A, DNAM1/CD226 and LAIR) and the C-type lectin-like NK receptors (CD94/NKG2, NKG2D, NKp80, NKRP1 and the rodents Ly49 receptors)[37, 46].

Killer Ig-like receptors (KIR) belong to the immunoglobulin superfamily and are able to interact with human leukocyte antigen (HLA) class-I molecules. These receptors prevent the attack of NK cells against normal autologous cells, while promote the killing of aberrant cells, recognizing the presence or absence/modification of HLA class-I molecules[38].

KIR receptor can be inhibitory or activating receptors (Fig.4).

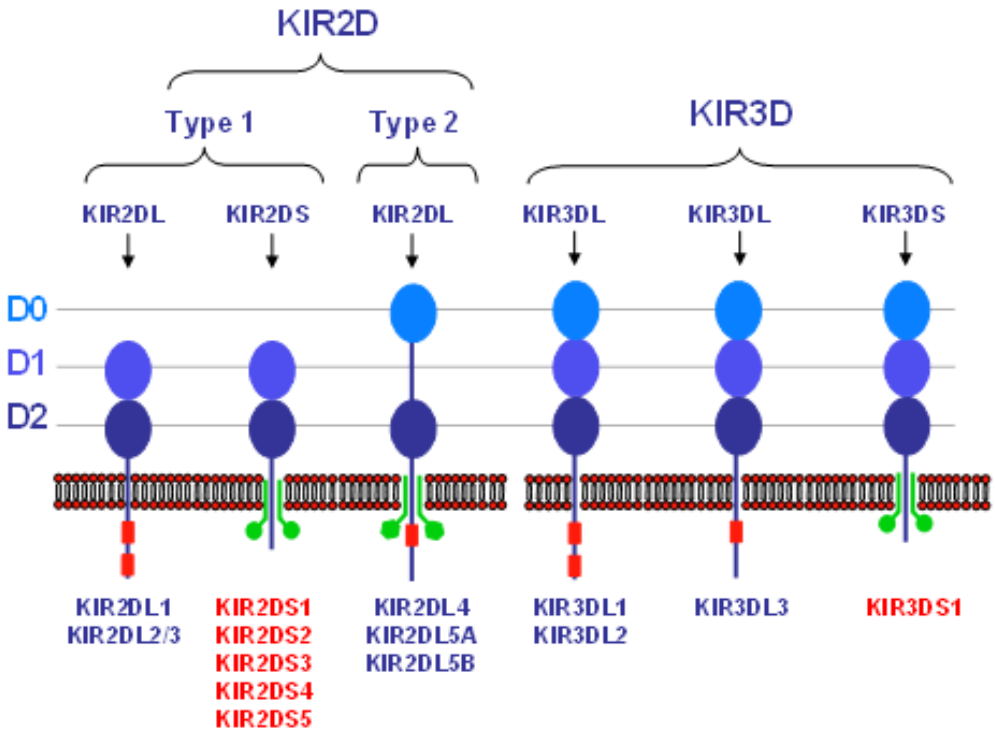


Figure 4 Killer Ig-like receptors (KIR) are characterized by two (KIR2D) or three (KIR3D) extracellular immunoglobulin domains and a short (S) or long (L) intracytoplasmic tails which transduce activating or inhibitory signals[47].

Inhibitory KIRs are characterized by two or three (KIR2D/KIR3D) extracellular immunoglobulin domains and a long (L) intracytoplasmic tail in which is present a immunoreceptor tyrosine-based inhibitory motif (ITIM) that can be phosphorylated and associated with Src-homology domain-bearing tyrosine phosphatases (SHP-1, -2)[37,38]. These receptors bind the polymorphic HLA-A, -B, -C molecules. In particular, KIR2DL1 recognizes HLA-C allotypes carrying a lysine at position 80 (C2 epitope), whereas KIR2DL2/L3 bound HLA-C allotypes carrying an asparagine at the same position (C1 epitope). These two receptors are also able to recognize C2 epitope, but with a low affinity. Regarding inhibitory KIRs with three extracellular domains, KIR3DL1 interacts with HLA- A and HLA-B (Bw4) alleles, while KIR3DL2 bounds HLA-A3 and HLA-11. KIR2DL4 recognizes HLA-G molecules but, despite the presence of a long cytoplasmic tail, typical of inhibitor receptors, displays a very weak inhibitory potential[48-52]. Activating KIRs are characterized by the same two or three extracellular immunoglobulin domains, but have a short (S) intracytoplasmic tail with an Immunoreceptor Tyrosine Activating Motif (ITAM). Upon phosphorylation, this tail can transduce activation signals, through p72syk and ZAP70 cytoplasmic PTK[37, 53]. KIR2DS1, -2DS2, -2DS3, -2DS4, -2DS5 and -3DS1 belong to this group of KIRs, but only some of these receptors have been demonstrated to recognize HLA class I molecules. In particular, C2 is the ligand of 2DS1, 2DS4 can bind HLA-A*11 and also HLA-C alleles while 3DS1 can bind HLA-B^{bw4}. No ligands have been yet identified for the others activating KIRs[37, 54]. Inhibitory and activating KIRs are clonally distributed on NK cells, except for KIR2DL4 that is constitutively present in all NK cells and in all KIR haplotypes. The KIR genes are located on chromosome 19 and are inherited as a haplotype. In particular, there are two distinct KIR haplotypes in humans: A and B. The first one shows the presence of a fixed number of genes encoding inhibitory receptors and the activating receptor KIR2DS4, while the haplotype B has a variable number of genes and in addition one or more B-specific genes such as KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR2DL2, and KIR2DL5[55-57].

In vivo experiments confirmed that NK cytotoxicity was correlated with the absence of MHC class I molecules on the target cells (Fig. 5)[58].

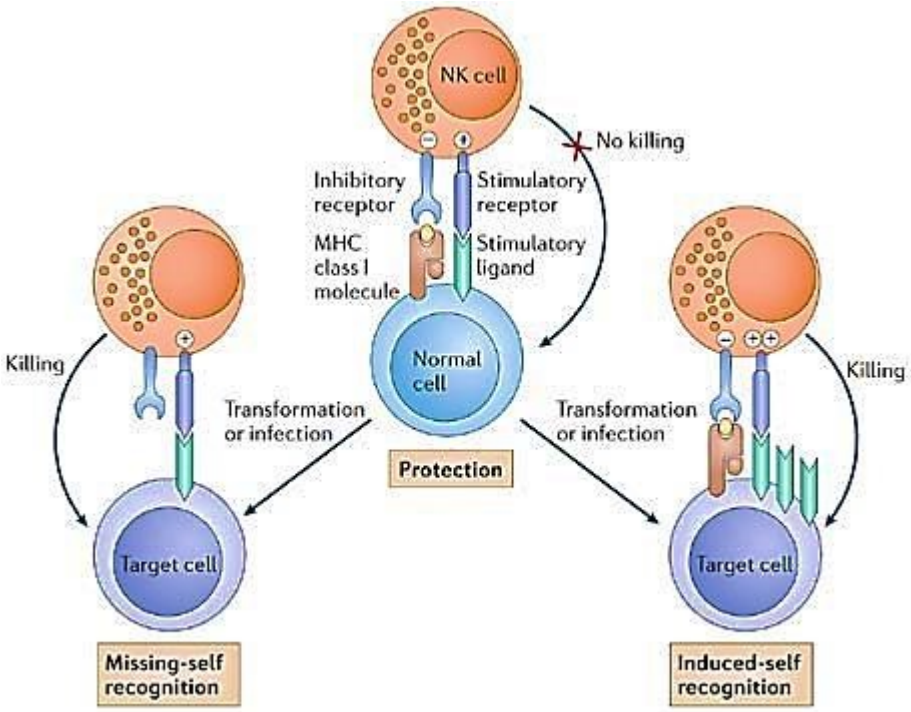


Figure 5 Normal target cells are protected from killing by natural killer (NK) cells when signals delivered by stimulatory ligands are balanced by inhibitory signals delivered by self MHC class I molecules. However, the loss of self MHC class I molecules expression resulting in NK-cell activation and target-cell lysis (known as missing-self recognition). Transformation or infection might also induce expression of stimulatory ligands such that constitutive inhibition delivered by inhibitory receptors is overcome (known as induced-self recognition)[59].

However, the integration of molecular signals is necessary to determine NK cell cytotoxic[60-62]. NK cytotoxicity is based on granule exocytosis of performed cytoplasmic granules which contain perforin and granzymes that directly kill the target cell. Furthermore, NK cells can induce other immune cells to kill the target cells releasing pro-inflammatory cytokines, in particular, IFN-γ and TNF-α. IFN-γ plays a main role towards microbial infections or tumor development. IFN- γ stimulates $CD4^+$ T cells that polarize to Th1 response. TNF-α stimulates adaptive immune response, promoting B cell proliferation and monocyte and macrophages differentiation[60].

KIRs have been associated with immune-mediated diseases, such as MS[60,63].

In particular, the absence of the KIR2DL3, and consequently the presence of the KIR2DL2/KIR2DS2 alleles, was associated with predisposition to MS[64]. Moreover, it was also observed a correlation between the disease and the presence of KIR2DL5, KIR3DS1 and KIR2DS4*001/002, while KIR2DS1 seems to have a protective function[65, 66]. The simultaneous analysis of KIRs together with HLA class I molecules have shown interesting associations with MS. In a Norwegian cohort the presence of HLA-B^{Bw4} allele, associated

with its KIR receptors, assumed a protective role[67]. Similarly, HLA-C*05 and its receptor KIR2DL1 presented a protective function[36].

1.2.3 KIR's promoter regulation

The development and the activation of NK cells is tightly regulated by KIR expression. In particular, specific molecular mechanisms, based on transcription factors and post-transcriptional modifications, can regulate KIRs expression in NK cells.

1.2.3.1 Transcriptional factors.

A proximal bidirectional promoter is present upstream each KIR gene. The affinity of binding sites for transcription factors lead to sense or antisense promoter activity, generating sense transcript for gene activation or antisense transcript for gene silencing, respectively (Fig 6,7)[68]. In particular, it has been suggested that antisense transcription could be responsible for KIR gene silencing by producing ds-RNA[69]. The bidirectional KIR promoter is adjacent to the first coding exon and it is responsible for the control of KIR expression.

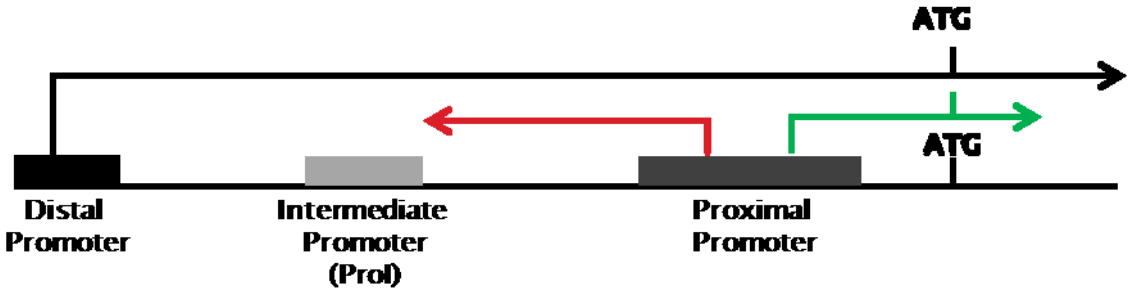


Figure 6 Distal and proximal promoter region.

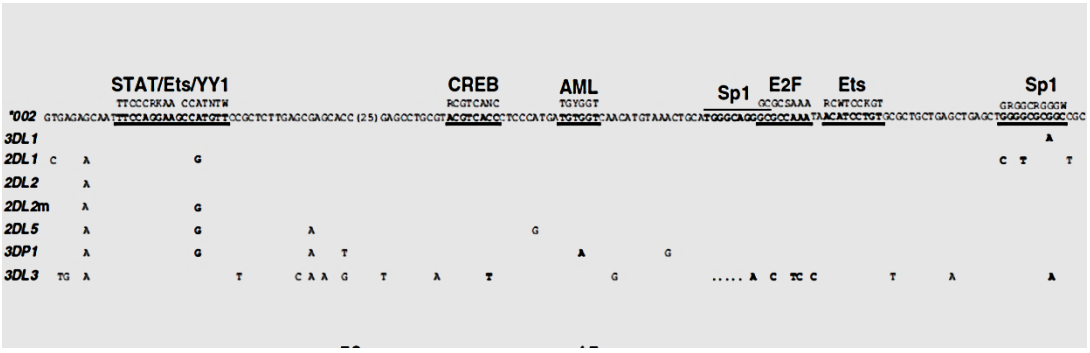


Figure 7 KIR proximal promoters with distinct forward- and reverse-promoter activities[69].

In addition to the bidirectional proximal promoter, an upstream distal promoter is required for protein expression, as reported for KIR2DL4, where was reported the presence of an intermediate and a distal promoter. The transcripts from these promoters are spliced at 190bp upstream of the initiation codon, bypassing bidirectional action of proximal promoter. Recently, the presence of this intermediate KIR promoter (ProI) was described in KIR2DL1 gene and then ProI elements were found in all KIR genes. In particular, there are four different classes of ProI elements that correlate with four different phenotypes of KIR subgroups: KIR2DL2/S2/L3; KIR2DL4; KIR3DL3; the remaining KIRs (Fig.8,9).

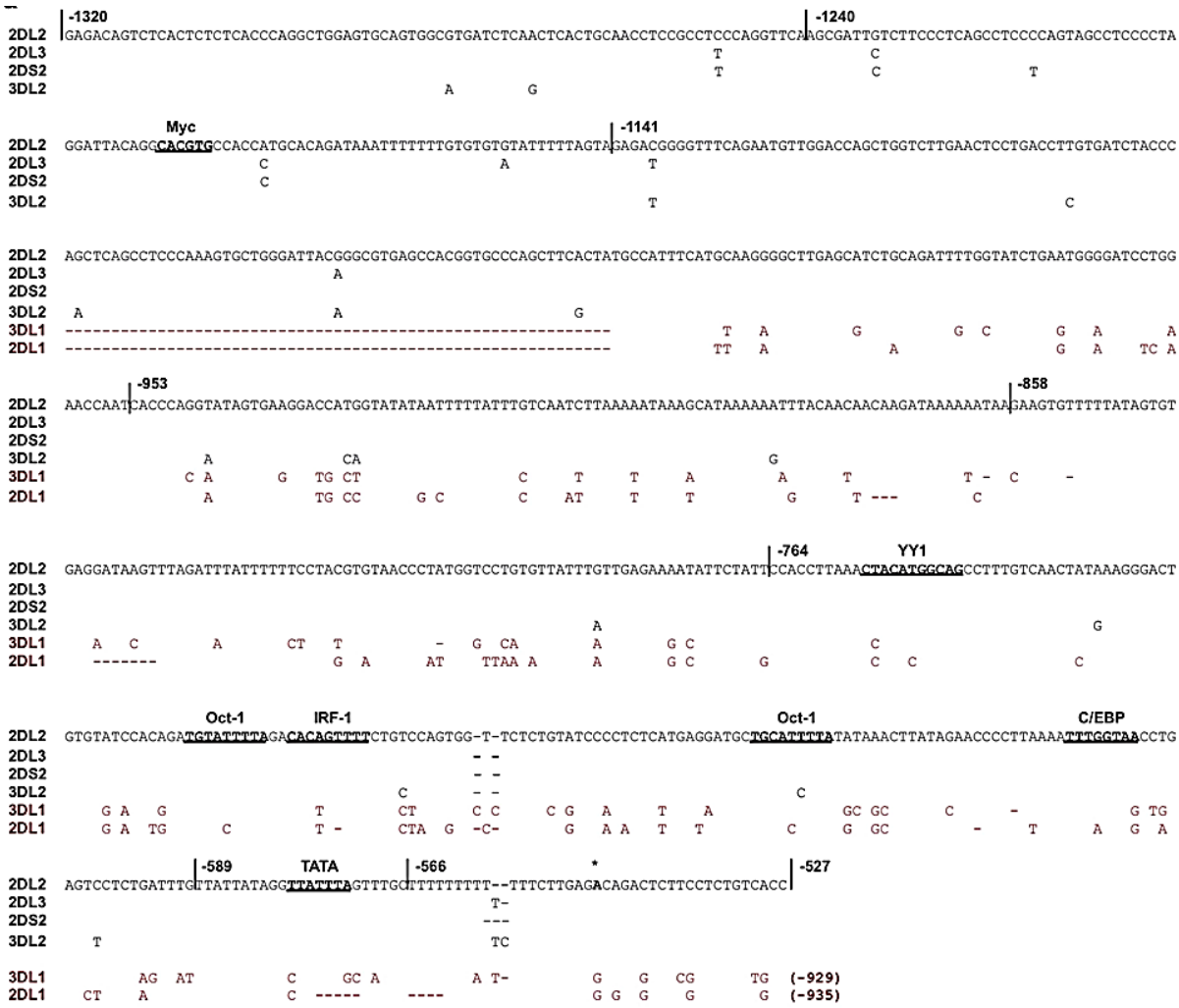


Figure 8 Identification of the KIR2DL2/L3/S2 and KIR3DL2 ProI region. The sequence of the 793 bp KIR2DL2 region for ProI activity is shown with only nucleotide differences for the other KIR genes[68].

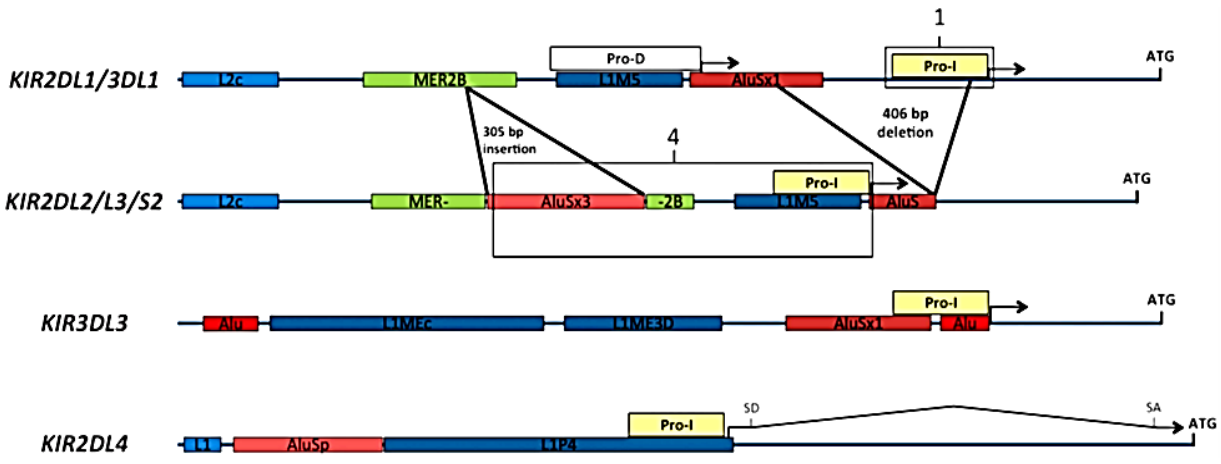


Figure 9 The four classes of KIR genes based on ProI analysis is shown. The start codon of each gene is indicated by (ATG) over a vertical line[68].

The comparison of the intergenic regions of KIR family members (Fig.9) shows that the different ProI elements have been generated as a result of insertion/deletion of repetitive elements. The KIR2DL1-related ProI elements are in a region of non-repetitive DNA, whereas three other classes are all associated with repetitive elements. In particular, the KIR2DL2 intermediate promoters has been located within LINE elements[68]. These four classes of ProI elements show different properties and contribute to the tissue/cell-type specificity of KIR transcription[68]. Recent studies reported a correlation between intermediate promoter activity and protein expression, suggesting the possibility that ProI elements act as regulating elements on KIR espression[70]. However, whether methylation of the proximal promoter has been shown to regulate KIR expression by silencing, methylation of ProI elements does not appear to silence gene expression[71,72]. On the other hand, it is supposed a collaboration between ProI and the proximal promoter in gene demethylation and activation[68, 73].

KIR expression is finely regulated by different specific transcriptional factors. In particular, Sp1 and RUNX are involved in KIR expression regulation[74].

Transcription factor Sp1 (specificity protein 1) is a zinc-finger protein that belongs to the Sp/KLF family, involved in many cellular processes, including differentiation, immune response, apoptosis and chromatin remodeling. Its activity is regulated by different post-translational modifications such as phosphorylation, acetylation, glycosylation, SUMOylation, and ubiquitylation[75]. Sp1 binding sites are located in a short core promoter common to all KIR genes[76]. Mutations and methylation in Sp1 binding site have a great effect on

KIR promoter functions[77]. Furthermore, Wang *et al*[78] have shown that there is a correlation between Sp1 and MS risk by showing specific binding of this transcription factor to CD24 promoter. CD24 gene encodes for a small glycosyl-phosphatidyl inositol (GPI)-anchored glycoprotein that is involved in adaptive and innate immunity. However, our knowledge on Sp1 involvement in KIR expression is still controversial and requires further investigations to explore its precise role in KIR promoter activation.

The RUNX (runt-related transcription factor) protein family is constituted of three members: RUNX-1, -2 and -3. RUNX proteins act with CBFβ protein. Core binding factors (CBFs) are dimeric transcription complexes composed of an α unit (Cbfa) that binds to DNA and a β subunit (Cbfb)[79-81]. There is only one Cbfβ subunit while the α subunit is encoded by RUNX1, RUNX2, andRUNX3 and they all specifically require Cbfβ for their functions[82, 83]. Core-binding factor subunit forms a heterodimer with RUNX family proteins. This subunit is a non-DNA binding regulatory subunit and increases the affinity of RUNX proteins for DNA through a conserved Runt domain. RUNX1 plays an important role in hematopoiesis, whereas RUNX3 has important functions in thymogenesis and neurogenesis[84]. RUNX1 and RUNX3 work together in the establishment of lineage determination of T lymphocytes[84]. RUNX3 is expressed in NK cell lineage from NKPs (natural killer progenitor) to mature NK cells where it seems to be involved in the modulation of NKP marker (CD122) and NK cell maturation marker (CD43) expression.[85-88]. RUNX proteins seem necessary for NK cell cytokines production and tumor immunity[85]. The role of RUNX proteins in KIR gene expression regulation has aroused even more interest. It has been reported that a putative binding site for RUNX family proteins is located near to the position -98 bp in almost all KIR genes, and previous studies have suggested the involvement of RUNX proteins in autoimmune diseases[89-93].

1.2.4 Methylation

The regulatory function exerted by transcriptional factors is strictly dependent on promoter epigenetic modifications, such as methylation.

DNA methylation is an epigenetic mechanism induced by DNA methyltransferase (DNMT) enzymes, with S-adenosylmethionine (SAM) as the methyl donor (Fig.10).

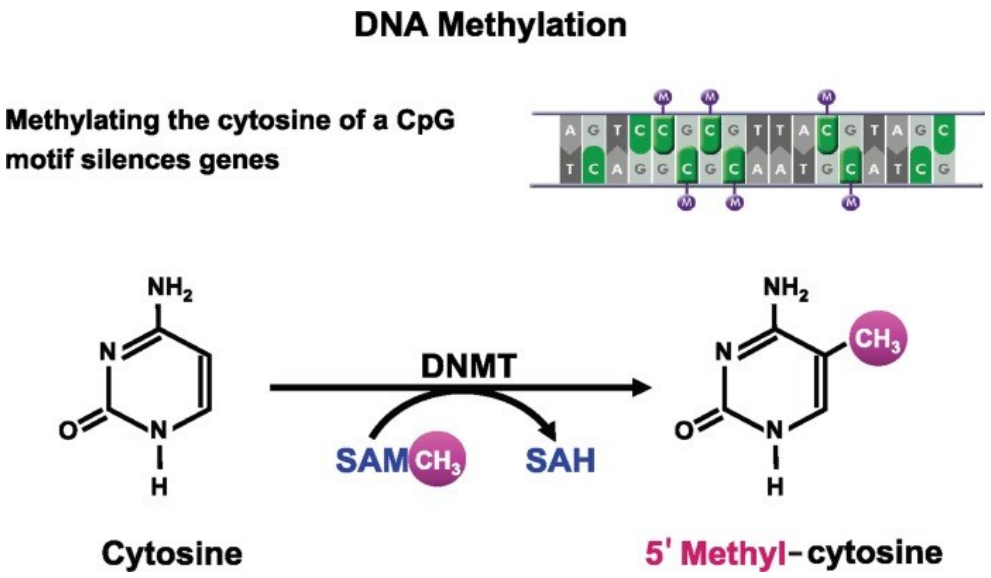


Figure 10 DNA methylation. The methyl group (CH3) is transferred onto the C5 position of cytosines to form 5-methylcytosine (5mC)[94]

In particular, there are five types of DNMT enzymes, which include de novo methylating DNMTs (DNMT3a, DNMT3b, and DNMT3L) and maintenance DNMTs (DNMT1 and DNMT2).

During methylation, the methyl group (CH3) is transferred onto the C5 position of cytosines to form the 5-methylcytosine (5mC). There are three different types of methylation: the methylation of CpG islands within promoter region of genes, methylation of CpG island shores (located up to 2 Kb from CpG islands) of gene bodies throughout the gene, and methylation of repetitive sequences (Quintero-Ronderos and Montoya-Ortiz, 2012)[95, 96].

As previously described, NK cells are involved in early control of infections as well as elimination of aberrant cells. These functions are mediated by a critical balance of signals controlled by inhibitory and activating receptors, such as KIR receptors. In particular, each NK cell clone expresses a different set of inhibitory and activating KIR receptors and their distribution appears to be stochastic. This peculiar distribution correlates with a wide range

of functional NK cell specificities, which are necessary for NK cell activation. Since KIR promoter regions are highly conserved, it has been hypothesized that not only genetically defined factors can be involved in this fine regulation, but also epigenetic modifications could promote and maintain different expression patterns[71]. In particular, methylation of cytosines within CpG islands is one of the most common modifications that correlates with repression of transcription[97]. Particularly, it has been demonstrated that DNA methylation is critical in regulating clonal KIR expression patterns in NK cells[71], since this modification inhibits the binding of transcription factors to the promoter binding-site, blocking the transcription machinery[98-102].

Furthermore, DNA methylation is a relevant element in MS pathogenesis and several studies have been made to investigate this mechanism in MS patients[103-105]. It has been found that RRMS patients showed particular disease-specific alterations in the methylation pattern of cell-free plasma DNA. Graves *et al.*[104], analyzed the methylation status in peripheral blood $CD4^+$ T cells from MS patients and healthy individuals and found 74 significantly different methylated CpG sites between patients and controls. Among these 74 sites, 19 were located in the MHC region and 10 were found into the human leukocyte antigen (HLA)-DRB1 region. The remaining CpG sites belonged to non-MHC region[104].

Moreover, a study performed on the DNA methylation status in different cell types from whole blood of MS patients and healthy controls, showed a different pattern of methylation in $CD4^+$ and $CD8^+$ T cells[106]. Furthermore, analysis of DNA samples from white matter of affected central nervous tissue, revealed that CpG islands cytosines have a 30 % reduction in the methylation rate compared to controls[107]. These data point out that the epigenetic regulation in immune system cells, as well as neurodegenerative process, leads to a dysregulation of gene expressions that modifies protein function, playing a crucial role in MS pathogenesis.

1.3 Environmental risk factors: infection

Epidemiological studies have shown that, in addition to the genetic predisposition, the exposure to an infectious agent may be involved in MS pathogenesis[108-110].

In particular, it has been observed that in MS patients, non-specific systemic infections represent a risk factor for relapse[111, 112] and oligoclonal bands have been found in MS cerebrospinal fluid (CSF) in presence of viral, fungal and bacterial CNS chronic infections[113, 114]. Furthermore, MS patients treated with antiviral agents, such as IFN-β,

have noticed improvements, suggesting a possible involvement of an infective agent in MS pathogenesis[115, 116]. Nowadays, there are two main hypotheses on the role of microbial infections in MS development: infections could be implicated in MS autoimmunity or alternatively infections might accelerate subclinical autoimmune processes[112, 117].

1.3.1 Herpes viruses and multiple sclerosis

The development of MS autoimmunity could involve the presence of a latent viral infection, characterized by periodical viral reactivation, typical of herpes viruses[118]. Several authors suggested that viruses could lead to exacerbation in the relapsing-remitting form of MS, hypothesizing that asymptomatic viral infection or reactivation, typical of herpes simplex virus, could be implicated in MS relapses[119, 120]. Several evidence of a possible association between increased frequency of herpes viruses' infections and MS disease are reported, particularly involving Epstein-Barr virus (EBV)[121], herpes simplex virus (HSV)[120], human herpes virus-6 (HHV-6)[122].

Herpesviridae encompasses a family of DNA viruses causing a latent or lytic infection and is subdivided into three subfamilies, the Alpha-, Beta-, and Gamma-herpesvirinae[123, 124]. In particular, herpesvirus family includes nine members (Tab.2) that present a similar molecular structure formed by a double-stranded DNA core and an envelope comprising glycoproteins conferring distinct differences to each member[125, 126]. The herpesviruses persist in latent status in different tissues, including the CNS, and are able to reactivate during periods of relative immunosuppression[127]. In particular, these viruses remain latent in neural tissue and, when reactivated, is able to spread to the CNS in a retrograde manner along nerves or through endothelial cells of cerebral vessels[128].

Table 2 Human herpesviruses classification and basic characterization[129].

Scientific name	Genome size (Kbp) and type	Target cells	Tissue tropism	Pathophysiology	Common modes of transmission	Global prevalence (%)
Alpha herpesviruses: short replicative cycle.						
HHV-1 (HSV-1)	152 dsDNA	Mucoepithelial cells (predominantly oro-facial tract)	Nerve ganglia	Oral-genital herpes, latency	Close contact, sexual contact	50-90
HHV-2 (HSV-2)	155 dsDNA	Mucoepithelial cells (predominantly genital tract)	Nerve ganglia	Oral and/or genital herpes, latency	Sexual/direct contact	20-60
HHV-3 (VZV)	125 dsDNA	Mucoepithelial cells and T cells	Nerve ganglia	Chickenpox and Shingles, latency	Close contact, respiratory route	50-95
Beta herpesviruses: Long replicative cycle						
HHV-5 (CMV)	235 dsDNA	Epithelial cells, lymphocytes, fibroblast	Leukocytes, epithelial cells	Multiple organs are involved, mild to life threatening manifestations	Respiratory, body fluids, sexual contact, blood transfusion, organ transplants	40-100
HHV-6 (variant A and B)	168-170 dsDNA	Epithelial cells, T-lymphocytes, fibroblast	T-lymphocytes	Sixth disease (Roseola infantum or exanthema subitum)	Salivary	60-100
HHV-7	145 dsDNA	Epithelial cells, T-lymphocytes, fibroblast	T-lymphocytes	Sixth disease (Roseola infantum or exanthema subitum)	Salivary	40-100
Gamma herpesviruses: variable replicative cycle						
HHV-4 (EBV)	172 dsDNA	Mucoepithelial cells, B-lymphocytes	B-lymphocytes	Infectious mononucleosis, several types of lymphomas and carcinomas	Salivary	80-100
HHV-8 (KSHV)	145 dsDNA	Lymphocytes	Lymphocytes	Kaposi-sarcoma (KS), primary effusion lymphoma (PEL)	Salivary, sexual contact	2-50
HHV: Human herpes viruses, VZV: Varicella zoster virus, CMV: cytomegalovirus, EBV: Epstein-Barr virus, KSHV: Kaposi sarcoma associated herpes virus.						

Previous studies have shown the strong association between Epstein-Barr virus infection and autoimmune diseases, including MS[130]. EBV belongs to human γ-herpes viruses with primary tropism for B cells, in particular, it infects B lymphocytes, persists latently for the lifetime of the infected individual. A huge amount of worldwide population is positive for the presence of EBV: EBV is the cause of infectious mononucleosis, often asymptomatic, is transmitted via saliva and infects naïve B cells in the tonsils. The attachment occurs by

the interaction between EBV glycoprotein gp350 and the complement receptor type 2 (CR2/CD21) on B cells. The binding takes place in a position very close to the binding site of the natural ligand of CR2, C3dg, a fragment of complement. Moreover, the virus is endocytosed into a low pH compartment, where fusion occurs by the core fusion machinery, formed by several proteins[131]. Subsequently, the virus infects naïve B cells, activating them into proliferating latently infected B blast, which take advantage for the normal pathway of B cell differentiation to progress through a germinal central (GC) to participate to GC reaction, where they become memory B cells that exit from the tonsil and migrate in the blood[132, 133]. In these conditions, they express a more restricted pattern of latent protein. These cells can leave the latency by expressing only the viral genome protein EBNA1, that is an EBV nuclear antigen, which engages the host cell DNA polymerase allowing the transmission of viral genome to the daughter cells. On the other hand, they can express no viral proteins choosing latency program. In that condition the virus is quiescent and invisible to the immune response. Every time a subset of latently infected memory B cells, through terminal differentiation signals, initiate lytic reactivation[134, 135]. Immediately, the transcription factors for viral replication are expressed, followed by the production of proteins involved in the next stage of replication, at least the viral DNA and structural protein are assembled into virions. The infectious virus is released and it can spread into saliva or infect other naïve B cells[136].

EBV is implicated in the pathogenesis of several diseases, mainly cancer and autoimmune diseases[130].

Considerable evidence has been found on the association between EBV infections with MS risk, in particular, a similar epidemiology has been shown for infective mononucleosis and MS.

Again, several studies based on immunological and pathological observations, draw attention to a possible link between EBV infections and MS risk[137]. Higher titers of anti-EBV antibodies were found in MS patients compared to those found in healthy individuals infected with EBV. Moreover, it has recently shown a clear association between exposure to EBV infection and MS, documented by anti-VCA IgG, anticomplex EBNA IgG, and anti-EBNA1 IgG antibodies[138]. Furthermore, it has been seen a significant increase in anti-EBV antibodies in healthy individuals infected with EBV few years prior to the onset of the MS, suggesting a role of infection as an early event in the development of the disease[139]. An association between EBV-MS age-related has also been observed. In pre-symptomatic MS patients younger than 20 years, anti-EBNA titers were similar to those of controls and increased about two-threefold in patients older than 25 years[140].

Significant alterations have been described also in cellular immunity. Certain studies have reported a higher EBV-specific $CD8^+$ T cell responses in MS patients than in controls or in patients with other neurological inflammatory disease[141]. Moreover, previous studies have found EBV-encoded small nuclear mRNA (EBER) transcripts in B and plasma cells infiltrating the brain of MS patients, in particular in acute and chronic active white matter lesions[137].

Despite the evidences reported about the role of EBV infections in the pathology of MS, the mechanisms involved remain poorly understood and many hypotheses have been made. For example, a molecular mimicry between EBV and myelin antigens could exist. In fact, an immune response against EVB antigens cross-react with myelin antigens, engaging B and T cells[142, 143].

EBV could lead to B cells immortalization, auto-antibody production and antigen presentation to pathogenic T cells. Indeed, a report shows how EBNA1 (the main EBV latency protein)-specific T cells recognize myelin antigens more frequently than other auto-antigens, leading to production of IFN-γ[144]. EBV-infected B cells in the white or gray matter of MS brains could lead to a direct effect of infection on CNS, inducing T cells to a cytotoxic response that damage the surrounding tissues[145]. These observations emphasize the important role of EBV in the pathogenesis of MS, suggesting that prevent EVB infection could help in reducing the risk of disease[145].

Human herpes simplex viruses, are divided in two types, HSV-1 and HSV- 2, and cause a lasting infection with recurrent lesions. HSV-1 has been seen associated with oro-labial disease, whereas HSV-2 with genital disease[146]. In particular, HSV-1 is a ubiquitous human pathogen able to cause an infection that lasts for the lifetime of the host as primary, latent, recurrent or persistent infection. The HSV-1 genome is constituted by a double-stranded linear DNA that encodes for 84 genes[147]. This genome is contained in a nucleocapsid that is surrounded by the tegument, a heterogeneous group of proteins. It is externally covered by a lipid envelope that allows to bind to and enter cells. The HSV-1 infection begins when HSV-1 glycoprotein B and C (gB and gC) interact with the heparin sulfate proteoglycans (HSPGs), allowing the virion attachment to host cells[148]. Virions can also infect cells lacking HSPGs because gB is able to interact even with paired immunoglobulin like type 2receptor alpha (PILRα)[149]. After the attachment to the host cell, the virus enters through fusion at the plasma membrane or endocytic vesicle. This step is led by gD glycoprotein that interacts with cellular receptors and this interaction allows a conformational shift in gD, resulting in the formation of the multiprotein fusion complex consisting of gD, gB, gH and gL[150]. As mentioned earlier, the virus can also enter through

fusion following endocytosis, but this mechanism has not been fully understood. Following the fusion, the tegument proteins are able to enter into the cytosol of the target cell. These proteins play several roles such as regulating cell processes[151], evading the immune system[152] and promoting transcription of viral genes. Tegument protein VP16, associating with host cell factor 1 (HCF-1) and octamer-binding transcription factor 1 (OCT-1), forms a transcription factor complex to allow the transcription of immediate-early (IE) viral genes[153]. The transcription of HSV-1 genes is finely regulated following a temporal pattern, for this reason IE genes have been expressed at first, and their products serve as activators for early-genes expression, which are necessary for replication of viral DNA. When the late-genes, which encode for virion structural components, are expressed, packaged virions are released and spread to neighbor uninfected cells. Besides HSV virus infects neighboring peripheral sensory neurons during primary infection, establishing the condition for a latent infection.

HSV-1 virions bind and enter the axons and proceed in retrograde direction toward the nucleus of the neuron, where the viral genome, is translocated and remains in latency[154, 155]. It has been observed that some neurons are more susceptible to productive infection, whereas in some neurons productive infection may be prevented or aborted leading the virus into a latent state. Viral gene expression could be enhanced by selected stimuli and consequently induce viral production first in the ganglia and later at the peripheral sites. Reactivation can be both asymptomatic or symptomatic. In particular, the rupture of vesicular lesions leads to the spread of the virus in different anatomical regions or allows the transmission to a new host. In immuno-competent individual the disease is auto-limiting, while in immuno-deficient subjects may result in serious disease[156, 157].

Herpes simplex virus type 1 (HSV-1) is known to be implicated in many neuropsychiatric diseases, like bipolar disorder and schizophrenia, as well as in beta-amyloid deposition, tau protein phosphorylation, demyelination and cognitive deficits related to Parkinson's disease, Alzheimer or multiple sclerosis[120,158-162]. In particular, in multiple sclerosis it has been shown that HSV-1 seropositivity is associated with an increased risk in HLA-DRB1-negative individuals[163]. HSV-1 also plays a role in promoting demyelination, as demonstrated in studies on animal models and by experiments on human oligodendrocyte cell lines[118, 164]. Ferrante *et al.*[120] have investigated the possible correlation between MS acute phase and herpes viruses, by studying the prevalence of DNA and mRNA of several herpes viruses in relapsing-remitting MS patients and healthy controls. The most interesting data they had was the presence of HSV-1 and HSV-2 in MS patients, but not in healthy controls.

The same authors reported that the presence of viral DNA in the PBMCs (peripheral blood mononuclear cells), collected on the first day of the acute attack, and the finding of HSV-1 mRNA in a certain percentage of patients, but not in controls, indicate a possible role of viral reactivation as a risk factor in sustaining the acute clinical attack. This result suggests also the use of antiviral drug in the treatment of MS patients that present HSV-1 or HSV-2 reactivation, in order to decrease the frequency and the gravity of relapses[120].

It has been found that the pathogenesis of MS can also be correlated with human herpes virus 6 (HHV-6)[165]. HHV-6 was isolated for the first time in 1986 from interleukin2-stimulated PBMCs of patients with AIDS or lymphoproliferative disorders[166]. HHV-6 belongs to *Roseolovirus* genus, from the β-herpesvirus subfamily. This strain of virus is divided in two variants: HHV-6A and HHV-6B. The virion particle has the morphological features typical of herpes viruses: the viral linear double stranded DNA is contained in a central core, whereas a tegument layer is surrounded by a membrane structure[167]. Seven genes blocks are common for all Herpesviridae, these are the herpes virus core genes, whereas another block, that includes open reading frames (ORFs) from U2 to U14, is specific to β-herpesviruses, and from U15 to U25 it has been found a region specific to *Roseolovirus* genus. There is a high homology between HHV-6 and HHV-7, but it has been shown that three genes, U22, U83, U94, are specific to HHV-6[165]. There are several differences between the two variants of HHV-6. In particular, differences in biological properties have been found concerning in vitro cell tropism, regulation of transcription and reactivity to some moAb directed to specific variants of epitopes[168, 169].

Infection with HHV-6A and -B is very common. High antibody titers are found in newborn children, decrease at 3 to 9 months after birth and rise shortly after, and remain elevated until 60 years. This pattern suggests the presence in newborns of maternal anti-HHV6 antibody and a primary infection within the first three years of life[170, 171]. Three different stages of HHV-6 infection have been identified: the first one, is the acute primary infection in infants; the second is represented by the infection in children and adult, in which the virus replicates in the salivary glands and remains in latency in lymphocytes and monocytes; the third stage concerns the infection in immunocompromised individuals and is related to reactivation of virus from latency[165]. HHV-6A and HHV-6B can infect many cells in vivo: T cells, mainly $CD4^+$ T cells, monocytes-macrophages, epithelial cells of the kidney and salivary glands, microglial cells, endothelial cells, astrocytes and oligodendrocytes[172]. HHV-6A and HHV-6B glycoproteins gH, gL, gQ and gB bind the host cell surface receptor CD46. The nucleocapsid of the virus is conducted into the cell's nucleus, where the viral DNA is released[173]. Thereafter, the virus uses the cell's

transcriptional and translational machinery to express the viral genome. In particular, initial early (IE) proteins are encoded by U86, U89, U95, U16-U-19, and U3 genes, that are expressed directly after infection and regulate the expression of the following viral genes. Early proteins (E), encoded by U27, U41, U43/U74/U77, U73, and U94 genes, are instead necessary for the viral genome's replication and the virion assembly. After that, late stage proteins (L) are included into mature virions, such as the protein encoded by U83, that is involved in the organization of latency stage. Virions packaged and assembled in the host cell nucleus is released in the cytoplasm, where gain tegument protein encoded by U31 and U54. Finally, the virions reach the Golgi, where is covered with glycoproteins gH, gL, gQ and gB. HHV-6 particles can be released from the cell[173-176]. During latency viral replication persists in salivary glands, explaining HHV-6 isolates in saliva samples and the possible transmission to seronegative individuals[177]. HHV-6 is the only herpes virus capable to persist in latency by integration into specific regions of the host chromosomes[178]. HHV-6 possesses also an important neuro-tropism. In fact, neuro-invasion has been identified in infants with primary infection as well as in children and adults with AIDS or in immunologically competent individuals[179, 180].

These data explain the highest levels of HHV-6 expression found in MS brain in comparison with controls brain[181, 182]. Moreover, it has been found also an increased level of viral DNA and mRNA in demyelinated plaques, in particular associated to acute lesions, suggesting a possible role of HHV-6 in MS pathogenesis[183, 184]. An evidence that HHV-6 could be an important component in MS pathology comes from the HHV-6 specificity found in oligoclonal bands (OCB)[185, 186]. The finding of increased levels of anti-HHV-6 antibody and the presence of HHV-6 DNA in the serum of the MS patients suggested a connection between an immune response to the virus and the pathogenesis of MS[187, 188]. A study of a Latvian MS cohort reported the presence of HHV-6 DNA in the plasma of MS patients during relapse in correlation with higher levels in the serum of several inflammatory cytokines such as IL-12 and TNF-alpha, compared with the remission period[189, 190]. Previous studies in a Tunisian and Italian cohort, analyzed the titers of antibody against the latency-promoting protein U94/REP, finding an increased response in MS patients during the relapsing phases, compared to controls[191, 192].

Mimicry is a typical tactic through which virus can elude the immune system taking advantage by homology between a viral sequence and a self-molecule. It is known that HHV-6 U24 protein[193] presents homology with human myelin basic protein (MBP)[181], and a huge amount of T cells responding to both HHV-6 U24 (residues 1 to 13) and MBP (residues 93 to 105) have been reported in MS patients respect to healthy controls[194]. This

observation could explain the demyelination mechanism or oligodendrocytes injury observed in MS, suggesting a possible role of mimicry in the connection among MS pathology and the virus.

2. Aim

In previous studies we evaluated the role of NK cells towards herpes viruses (HHVs) infection in Multiple Sclerosis (MS) patients. We observed that a 40% MS patients were unable to counteract Human Herpes simplex 1 (HSV-1) infection and were characterized by NK cells that failed to activate towards HSV-1 and up-modulated KIR2DL2 expression.
The goal of this project was to investigate the molecular mechanisms that differentiate NK cell behavior to HHVs infection in association with KIR2DL2 expression. For this reason, two different cell models have been created to establish a standardized in vitro system, in which it is possible to analyze the expression and effect of KIR2DL2 receptor during HHVs infections. We have analyzed NK cells activation (degranulation), cytokine production, transcription factors expression. We have established an *in vitro* model to evaluate KIR2DL2 promoter interaction with transcription factors. Moreover, we have investigated the role of epigenetic modifications, as methylation, in modulating the expression of KIR2DL2 in MS patients.
This study will increase our knowledge on KIR2DL2 receptor involvement in the activation of NK cells in MS patients during viral infections, allowing new potential strategies to counteract viral infections and disease follow up.

3. Material and methods

3.1 Cell lines

NK92 (ATCC CRL-2407) is a human NK cell line derived from a malignant non-Hodgkin's lymphoma. It was grown in MEM-Alpha medium (Minimal Essential Medium, Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 20% of FCS (fetal calf serum, Euroclone, Pero, MI, Italy), 0.1 mM 2-Mercaptoethanol (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin and 150 U/mL of IL-2. Cell cultures have been maintained at 37° C in humidified atmosphere of 5% CO_2 in air.

NK92-MI cell line (ATCC CRL-2408), characterized by a stable IL-2 expressing plasmid, was cultured in MEM-Alpha medium (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 20% of FCS (fetal calf serum), 0.1 mM 2-Mercaptoethanol (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, and was maintained at 37° C in humidified atmosphere of 5% CO2 in air.

HeLa cell line (human epithelial carcinoma) (ATCC CCL-2) was cultured as monolayers in DMEM-F12 medium (Dulbecco's Minimal Essential Medium, Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with L-glutamine in presence of 10% FCS (fetal calf serum), 100 U/mL penicillin and 100 µg/mL streptomycin, and was maintained at 37ºC and 5% CO2.

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

SUP-T1 human cell line (ATCC CRL-1942) was derived from T-Cell Lymphoblastic Lymphoma. The cell line was cultured in RPMI-1640 medium (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% FCS (fetal calf serum) and 100 U/mL penicillin, 100 µg/mL streptomycin, and was maintained at 37° C in humidified atmosphere of 5% CO2 in air.

Lymphoblastoid cell lines (LCL) were obtained by infecting peripheral blood mononuclear cells (PBMC) from normal donors with EBV strain B95.8[195]. Donors were negative for antibodies against EBV viral capsid antigen and early antigen (EA), and PBMC were negative for EBV type 1 and type 2 by PCR analyses of EBNA2and EBNA3 genes[196]. The

source of EBV was the culture medium of marmoset cell line B95.8. Cells were grown in RPMI-1640 medium (Celbio, Rock-ford, IL), supplemented with 2% glutamine and 10% Fetal Calf Serum (FCS; Gibco, Milano, Italy) (standard medium)[197].

3.2 NK92 cell line demethylation and HHVs Infection

3.2.1 Demethylation: 5-Aza treatment

NK92 cells were treated with the DNA-demethylating compound 5-Aza (Sigma-Aldrich, St Louis, MO, USA). NK92 cells were placed in 75 cm^2 flask at a density of $1x10^6$cells/mL with supplemented 20% FCS medium and were treated with 2.5 μM 5-azacytidine[100]. MEM-alpha-20% FCS medium supplemented with 5-Aza treatment were changed every 24 h for 48 h. NK92 treated cells and NK92 control cells were harvested and replaced at a density of $1x10^6$ cells/mL in 75 cm^2 cultures flasks, with fresh MEM-alpha-20% FCS medium with or without 25 μg/mL CpG (ODN 2006 type B, TIB MOLBIOL, Genova, Italy)[198]. The selection of KIR2DL2 expressing cells were performed with Dynabead-CD158b conjugated as manufacturer's recommendations (ThermoFisher Scientific). The dynabeads were incubated for 24h at 37°C with anti-CD158b moAb (107 dynabeads/3 μg anti-CD158b moAb). After several washes in specific buffers, the dynabead-CD158b conjugated were stored in PBS, pH 7.4, 0.1% BSA, 0.02% sodium azide at 4°C. Later demethylated NK92 cells were incubated with 10^6 dynabead-CD158b conjugated for 1 h at 37°C, washed twice with PBS and placed in culture after bead release[199].

3.2.2 Infection with HHVs

The day before the infection, HeLa, J-Jhan and SupT1 cells were seeded in 6-well plates at density of $3.5x10^5$ cells/mL. HeLa cells were infected with HSV-1 strain F, J-Jhan cells were infected with HHV-6A, SupT1 were infected with HHV-6B, at a multiplicity of infection of 0.1 plaque forming unit (PFU)/cell for 24 h. To induce EBV production, LCL B95-8 cells were stimulate with 20ng/ml tetradecanoyl phorbol acetate (TPA) for 1 hour at 37°C. Cells were washed three times with RPMI 1640 to remove TPA and resuspended in RPMI1640.

The day after infection, NK92 cells were co-cultured with infected cells at a 1:5 ratio[116]. NK92 cells supernatants and pellets were collected for subsequent analysis.

3.2.3 HHVs analyses

HeLa cells were infected with a green fluorescent protein (GFP)- tagged HSV-1[199] (provided by Prof. Manservigi Roberto, Dep. of Life Sciences and Biotechnology, section of Microbiology, University of Ferrara, Italy).
Fluorescent virus was visualized by Nikon fluorescence microscope Nikon Eclipse TE2000Sequipped with a digital camera.
HSV- titration was performed on VERO cells in 96-wells culture plates (Nunc Multidish, USA). Cells supernatants were ten-fold diluted and inoculated in each plate well. The micro plate was incubated for seven days at 37 °C in CO2 (5%), and examined daily for evidence of CPE. Titers of all cases were determined by Reed Muench's method and reported as PFU/mL in infected cells[116].

DNA from JJahn, SupT1 and LCL B95-8 cell cultures, infected with HHV-6A, HHV-6B and EBV respectively, was extracted using spin column technique of QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. In each PCR reaction, were used at least two negative controls by using sterile double-distilled water instead of the sample.
HHV-6 DNA viral load were analyzed by real time quantitative (qPCR) specific for the U94 gene. Real-time PCR for HHV-6 DNA was performed as previous described from Comar et *al*[200] with the following set of primers/probe: HHV6 U94(+) (5′-GAG CGC CCG ATA TTA AAT GGA T-3′); HHV6 U94(−) (5′-GCT TGA GCG TAC CAC TTT GCA-3′); HHV6 U94 PROBE (5′-FAM-CTG GAA TAA TAA AAC TGC CGT CCC CAC C-TAMRA-3′)[201] where FAM serves as reporter fluorochrome and 6-carboxytetramethylrhodamine, TAMRA, as the quencher. Each sample was run in duplicate.
HHV-6 variant A or B identification was obtained by restriction enzyme digestion with HindIII enzyme of the U31 nested PCR amplification product, as reported previously by Di Luca *et al.*[202]
EBV DNA content was measured using a real-time quantitative PCR as reported from Righetti *et al.*[203]. For the EBNA2 gene of EBV were used the forward and the reverse primers combinations: 5'-CTG CCC ACC CTG AGG ATT TCC-3' and 5'-CTG CCA CCT GGC GGC AAC-3'. Moreover, it was used a fluorogenic probe, 5'-FAM-AAT CCT CCT ACC CT C TCT TTA TGC CAT GTG TGT-TAMRA-3', where FAM serves as reporter fluorochrome and 6-carboxytetramethylrhodamine, TAMRA, as the quencher.

Each PCR was performed in a 50 µL reaction mixture containing 25 µL 2 x Taq Man Universal PCR master Mix (PE Applied Biosystems), 300 nM each primer, and 100 nM fluorogenic probe. Amplifications were carried out in a 96-well reaction plate (PE Applied Biosystems) in a 7300 Real-time PCR machine (Applied Biosystems). The conditions were: 2 min at 50°C, a denaturation step of 10 min at 95°C; after that 45 cycles were run, each consisting of 15 s at 95°C and 60 s at 60°C. Each sample was run in duplicate.

3.2.4 Fluorescence microscopy

Image acquisition was performed on a Nikon fluorescence microscope Nikon Eclipse TE2000S equipped with a digital camera.

3.2.5 CD107a degranulation assay and CFSE assay

NK92 cells KIR2DL2^{+} were characterized with a specific anti-CD panel (CD3-PerCp-Cy5.5, CD56-FITC, CD107a-PE) (e-Bioscience, Frankfurt, DE), anti-KIR2DL2-2DS2-2DL3/CD158b-PE (ThermoScientific, Erembodegem, BE) monoclonal antibodies (moAb). After an incubation with the moAb for 30 min in ice, cells were washed and analyzed with FACSVantage flow cytometer and CellQuest software (Becton Dickinson, Milano, Italy), acquiring 10,000 events. NK cells (CD3^{-}/CD56^{+}) were defined and gated within the lymphocyte gate. CD158b levels were measured in the CD3^{-}/CD56^{+} gated cells. Cell viability was assessed by propidium iodide staining. For the CD107a degranulation assay, the cells were incubated 1 h at 37 °C with anti-CD107a moAb and treated with GolgiStop solution (Becton Dickinson, San Jose, CA, USA) for 3 h. Ten thousand events were acquired.

The infected cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) to assess cell-mediated cytoxicity, using 7AAD/CFSE Cell-mediated cytoxicity assay kit (1180 E. Ellsworth Rd Ann Arbor, MI, USA). Cells pellet was resuspended at a concentration of 10^{7} cells/ml in CFSE staining solution and incubated for 15 minutes at 37°C. Control target cells were resuspended in 0.1% BSA. After that cells were washed two times with culture medium and finally were replaced at a density of 10^{5} cells/ml. Labelled cells were incubated for 30 minutes at 37°C. At this point NK KIR2DL2^{+} and KIR2DL2^{-} cells were put in co-culture with CFSE-labelled infected cells at a 1:5 ratio. The cell mixture was incubated for 4h. After that cells were centrifuged and resuspended in 7-AAD staining solution. Control target cells were resuspended in Assay Buffer. Cells were incubated for 15 minutes in the dark at 4°C. Then cells were centrifuged and

resuspended in Assay buffer. Subsequently labelled cells were analyzed with FACSVantage flow cytometer and CellQuest software (Becton Dickinson, Milano, Italy).

3.3 NK92 cell line nucleofection: cytokines and transcription factors analysis

3.3.1 Nucleofection

The plasmid pCMV6-AC-GFP (RG224797, OriGene Technologies, Inc. 9620 Medical Center Drive Suite 200 Rockville, MD 20850 USA), has been used to insert KIR2DL2 (GFP- tagged- Human killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2) into NK92 cells. The plasmid was amplified by transforming an aliquot of One Shot® TOP 10F' E. Coli competent cells (Invitrogren). The plasmid DNA was extracted from competent cells using QIAGEN plasmid MAXI kit (QIAGEN, Hilden, Germany). The amplified products were analyzed by ethidium bromide–stained agarose gel electrophoresis. In order to establish the optimal antibiotic concentration for selecting stable cell colonies, we have performed a dose-response experiment. NK92 cells were seeded at density of $5x10^5$ cells/mL in 24-well cultures plate and treated with 0 µg, 100 µg, 250 µg, 500 µg, 750 µg, 1000 µg and 1250 µg/mL of Geneticin® Selective Antibiotic (G418 Sulfate, Gibco®), in MEM-alpha-20% FCS medium. Media were changed every two days. Viability of cells was monitored and scored by cell counting with trypan blue staining. $5x10^6$ NK92 cells were re-suspended in a volume of 98 µL of Ingenio solution (Ingenio®Electroporation Kits and Solution, Mirus Bio LLC, WI 53711 USA). Immediately IngenioSolution/cell mixture was added to 20 µg (4µL) of plasmid pCMV-6-AC-GFP. After that, 102 µL of Ingenio solution/plasmid DNA/cell mixture was transferred in 0.2 cm cuvettes and was electroporated at room temperature, using program A-24 of Amaxa® Nucleofector™ I device (Amaxa GmbH, 50829 Koeln, Germany). Immediately, 500 µL of pre-warmed MEM-alpha-20% FCS medium were added to electroporated cells, which were placed in 12-well plates, containing 1.5 mL/well of pre-warmed medium. After 24h transfected cells were harvested, centrifuged and placed in a 75 cm^2 flask with 35 mL of MEM-alpha-20% FCS and Geneticin at the concentration of 1000 µg/mL. The medium was replaced every two days to allow the selection of transfected cells during 12 days. NK92 transfected cells and NK92 control cells were collected and seeded at a density of $1x10^6$ cells/mL in 75 cm2 cultures flasks, with or without 25 µg/mL CpG (ODN 2006 type B, TIB MOLBIOL, Genova, Italy)[198]. NK92 transfected and control cells were co-cultured

with infected cells (see 3.2.2 Infection with HHVs paragraph) in a 1:5 ratio. 24 h later NK92 cells supernatants and pellets were collected for subsequent analysis.

3.3.2 Cytokine levels analysis

Culture supernatants were analyzed for the expression of 3 cytokines, representative of Th1, Th2 and Th17 response: tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), and intrleukin-22 (IL-22). The detection was performed using the MyBioSource ELISA kit (Inc. San Diego, CA 92195-3308 USA) according to the manufacture's protocol. The data were collected and analyzed by Víctor Multilabel Plate Reader (PerkinElmer, USA).

3.3.3 Transcription factors evaluation

Total RNA of NK92 cells (transfected/controls) was extracted using RNase MiniKit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. RNA samples were digested with DNase (Fermentas, ON, Canada) (1 U/μg RNA) and stored at −85 °C until cDNA synthesis. After that RNA samples were retro-transcribed to cDNA using SuperScript™ First-Strand Synthesis System (Invitrogen, San Giuliano Milanese, MI, Italy) as manufacturer's instructions. The relative expression of the transcription factor Sp1 (specificity protein 1) was evaluated by polymerase chain reaction (PCR) with specific primers. 100 ng of cDNA in a final volume of 25 μL with final concentration of each reagent as follows: PCR buffer 1x; 1,5 mM MgCl2; 0.2 mM of each dNTP; 1.25 unit of Taq-polymerase and 0.5 μM of each primer (Sp1 For 5' – CTA AGT CCT AGC TAA GTA TCA GG -3'; Sp1 Rev 5' – AGA GGA TGA CAG ACT TAG GAA GG – 3'). Thermocycling conditions were assessed as follows: initial denaturation for 3 min at 95° C, followed by 30 cycles at 95° C for 30 s, annealing at 58° C for 30 s, extension at 72°C for 60 s and a final extension at 72° C for 4 min. Each polymerase chain reaction was performed using the ProFlex PCR System (Applied Biosystems). Amplification products were analyzed by ethidium bromide–stained agarose gel electrophoresis.

Moreover, Sp1 level was also detected in culture supernatants. The detection was performed using the MyBioSource ELISA kit (Inc. San Diego, CA 92195-3308 USA) according to the manufacture's protocol. The data were collected and analyzed by and Victor Multilabel Plate Reader (PerkinElmer, USA).

3.4 KIR2DL2 promoter activity analysis

3.4.1 Plasmid construction

A sequence of human KIR2DL2 promoter was amplified using genomic DNA isolated from Jurkat cell line (KIR2DL2$^+$ cells). A 208-bp fragment was sub-cloned from the full-length KIR2DL2 promoter upstream from the first translation codon using forward (5'- TAA GCA GGT ACC ATT CTA GTG AGA ACA ATT- 3') and reverse (5'- TGC TTA AAG CTT ACC AAC ACA CGC CAT GCT- 3') primers (described previously by Xu J et al., 2005)[76]. 100 ng of the Jurkat's genomic DNA (provided by I. Taniuchi, Laboratory for Transcriptional Regulation, Riken IMS Institute, Japan) was used in a polymerase chain reaction (PCR) in a final volume of 50 µL with PCR buffer 1x, 2.5 mM MgCl2, 0.4 mM of each dNTP, 2.5 unit of Taq-polymerase and 0.4 µM of each primer. Thermocycling conditions were as follows: initial denaturation for 1 min at 95° C, followed by 35 cycles at 95° C for 30 s, annealing at 52° C for 30 s, extension at 72°C for 30 s and a final extension at 72° C for 10 min. Amplification products were analyzed by 1.5 % ethidium bromide–stained agarose gel electrophoresis. After that it has been conducted the gel's purification by using QIAquick Gel Extraction Kit (Qiagen, USA) following the manufacture's manual. PCR purified product and pGL3-basic Luciferase reporter vector (Promega) were digested with HindIII and KpnI enzymes to produce a blunt end fragment and vector, performing a reaction of 30 µL final volume with 1 unit of each enzyme, 1 µg of PCR product and pGL3-basic Luciferase reporter vector and 1x buffer, at a temperature of 37 °C for 1 hour. Then the blunt end PCR product and pGL3-basic Luciferase reporter vector products were analyzed by 1.5 % ethidium bromide–stained agarose gel electrophoresis and were purified using the QIAquick Gel Extraction Kit (Qiagen,) following the manufacture's manual. Subsequently they were ligated in the ligation reaction performing with 1 µg of vector and PCR product insert and 4 µL of I ligation mix (Takara Bio, Japan) put over night at 16 °C. The ligation product was amplified, according to the following procedure: 100 ng of pGL3-Luc-KIRpro (pGL3-basic Luciferase vector containing KIR2DL2 promoter fragment) were mixed into 100 µL of XL1 blue competent bacteria and incubated on ice for 30 minutes. The ligation-bacteria mix was heat-shocked by placing at 42 °C for 40 sec and subsequently put on ice for 2 min. 500 µL LB broth was added to the mixture in a micro-centrifuge tube, that was incubated at 37 °C for 1 hour with shacking at 400 rpm (revolutions per minute). After that different concentration of transformed competent cells were plated on LB agar plates (containing 100 µg/mL ampicillin) over night. The following day individual colonies were picked and cultures were grown overnight in LB-containing 100 µg/mL ampicillin broth at 37 °C. Purified

pGL3-Luc-KIRpro from 7 colonies was obtained using Miniprep kit (Qiagen) and digested as previously described. Integrity of the sequences and correct orientation of the plasmids was confirmed by direct sequencing. The bacterial colony containing the correct copy of the pGL3-Luc-KIRpro plasmid has been grown overnight to proceed with plasmidic DNA extraction with Midiprep kit (Qiagen).

3.4.2 Luciferase reporter assay

NK92MI cells were seeded in 24-well plates at a density of 2.5 x 10^5 cells/well and were transiently transfected with TransIT-X2® Dynamic Delivery System (Mirus Bio LLC, WI 53711 USA). NK92MI cells were co-transfected respectively with pGL3 Luciferase reporter vector empty or ligated with KIR2DL2 promoter fragment and pRL (Renilla Luciferase control vector, provided by I. Taniuchi, Laboratory for Transcriptional Regulation, Riken IMS Institute, Japan). In addition, a pCDNA3 expression vector containing RUNX1 sequence (provided by I. Taniuchi, Laboratory for Transcriptional Regulation, Riken IMS Institute, Japan) was added to pGL3-Luc-KIRpro co-transfected cells. A pCMV-6 empty plasmid (Origene, USA) was used in order to ensure the same amount of DNA in each well. Untransfected NK92MI-cells were used as experimental control. The cells were cultured in complete MEM-alpha medium with 10% FCS, to avoid interference with luciferase assay. 24 h later all samples were transferred in a 96-well plate, using 75 µL of each cell cultures, and assayed for luciferase activity using Dual-Glo® Luciferase Assay System (Promega, USA) according to the manufacturer's protocol. The amount of firefly luciferase activity was normalized against renilla luciferase activity. The results from each experimental setting was normalized with control samples. The data were acquired using Victor Multilabel Plate Reader (PerkinElmer, USA).

3.5 Methylation studies

3.5.1 Patients and controls

We enrolled 18 control health donors, from the Regional Blood Center, Region Emilia Romagna, AUSL Bologna – Maggiore Hospital, and 18 unrelated patients affected by definite MS according to the classification of McDonald[4], followed at the Neurology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliero-Universitaria, Arcispedale S. Anna, Ferrara (Italy).

The genomic DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The DNA was genotyped for the inhibitory KIR2DL2 receptor using specific primers (KIR2DL2 For 5'-CCA TGA TGG GGT CTC CAA A-3', KIR2DL2 Rev 5'-GCC CTG CAG AGA ACC TAC A-3')[204].

3.5.2 NK cell isolation

NK cells were isolated by depletion of non-NK cells (negative selection) using NK Cell Isolation Kit (Milteny Biotec GmbH, Germany) according to the manufacturer's instructions. 3 x 10^7 total cells (PBMC) for each patient/healthy donor were centrifuged and resuspended in required buffer. After that was added NK Cell Biotin-Antibody Cocktail (10 µL/ 10^7 cells) and the mixture was refrigerated for 10 minutes at 4 °C. Then cells were mixed well with buffer and successively with NK Cell MicroBead Cocktail (20 µL/ 10^7 cells). The mixture was refrigerated for additional 15 minutes at 4 °C. Cells were washed by adding 1-2 mL of buffer/ 10^7 cells and centrifuged. The pellet was resuspend in 500 µL of buffer/ 10^7 cells and this mixture was applied onto a column in the magnetic field, previously rinsed with appropriate amount of buffer. Unlabeled cells were collected and the column was washed three time with buffer. This fraction represented the magnetically non-labeled NK cells which were collected and used for following analyses.

3.5.3 The methylation status of the KIR2DL2 promoter

The DNA from KIR2DL2 positive subjects (N= 6 controls and 6 MS patients) was analyzed for the methylation status of the KIR2DL2 promoter using Epitect® Bisulfite Kit (QIAGEN, Hilden, Germany). In particular, the process consisted of bisulfite-mediated conversion of unmethylated cytosines (providing by a bisulfite thermal cycling program with an optimized series of incubation steps necessary for DNA denaturation and subsequent sulfonation and cytosine deamination), binding of the converted single-stranded DNA to the membrane of an specific spin column, washing and desulfonation of membrane-bound DNA, washing to remove desulfonation agent and finally the elution of the converted and purified DNA. The thermal cycling program has been realized by using ProFlex PCR System (Applied Biosystems). The quantitative Methylation-Specific PCR (qMSP) assays were designed to amplify bisulphite-converted methylated/unmethylated DNA of KIR2DL2 promoter sequences. The qMSP assay, a real-time PCR assay, was performed as briefly described: 1 µg of DNA was put in a final reaction volume of 20 µL,

containing 2x SYBR Select Master Mix (Thermo Fisher Scientific Inc., Asheville, NC) and 0.4 μM of KIR2DL2 Met Forward 5'-TAG GGC GTT AAA TAA TAT TTT GTG C- 3' and KIR2DL2 Met Reverse 5' - GCC ATA CTA ACG ACC ATA AAC G- 3' primers, or 2x SYBR Select Master Mix and 0.4 μM of KIR2DL2 Unmet Forward 5' - GGG TGT TAA ATA ATA TTT TGT GTG T – 3' and KIR2DL2 Unmet Reverse 5' - CAC ACC ATA CTA ACA ACC ATA AAC AAC – 3' primers, respectively to amplify methylated or unmethylated sequences. The data were acquired and analyzed by 7300 Real-time PCR machine (Applied Biosystems).

3.6 Statistical analysis

Statistical analysis was performed with Stat View (SAS Institute Inc.). Biological data were analyzed by Student t test, as characterized by a normal distribution (Kolmogorov-Smirnov test). Statistical significance was assumed for $p<0.05$ (two tailed).

4. Results

In our previous studies we evidenced the role of NK cells towards herpesvirus infection in MS patients, observing a subgroup of MS patients unable to counteract HSV-1 infection[116]. In particular, NK cells failed to activate towards HSV-1 because of the up-modulation of KIR2DL2 expression. Hence, we focused our interest on the molecular mechanisms that differentiate NK cell behavior towards herpes virus infection in association with KIR2DL2 expression:

- we evaluated the effect of KIR2DL2 expression on NK cell activation towards HSV-1, HHV-6A and B, and EBV infections;
- we analyzed cytokine secretions and transcription factors expression by NK cells in the presence or absence of KIR2DL2 receptor;
- we evaluated KIR2DL2 promoter interaction with transcription factors;
- we analyzed KIR2DL2 promoter methylation status in MS patients.

4.1 Effect of KIR2DL2 expression during HSV-1, HHV-6A, -6B, and EBV infections

KIR2DL2 positive NK92 cells were obtained by DNA-demethylation with 5-Aza (2.5uM) and purification with Dynabead-CD158b conjugated. The immunofluorescence analysis showed the expression of KIR2DL2 in the 90% of the purified NK92 cells (Fig.12).

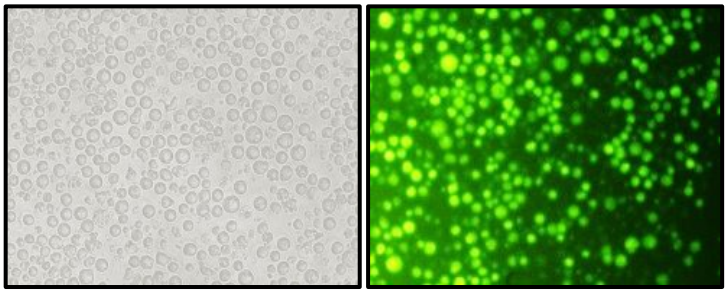


Figure 12 Immunofluorescence for KIR2DL2 expression by NK92 cell line treated with DNA-demethylating compound 5-Aza (2.5uM) and purified with Dynabead-CD158b conjugated.

KIR2DL2 positive and negative NK92 cells were co-cultured with HSV-1, HHV-6A, HHV-6B and EBV infected cells. NK92 cell activation, evaluated as percentage of NK92 cells expressing CD107a marker, showed an increase in activated KIR2DL2$^-$ NK cells in the presence of HSV-1, HHV-6A, HHV-6B and EBV infected cells (Fig. 13b) ($p<0.001$;

Student T test). On the contrary, KIR2DL2^{+} NK92 cells did not activate in the presence of HSV-1, HHV-6A, HHV-6B and EBV infected cells (Fig. 13a).

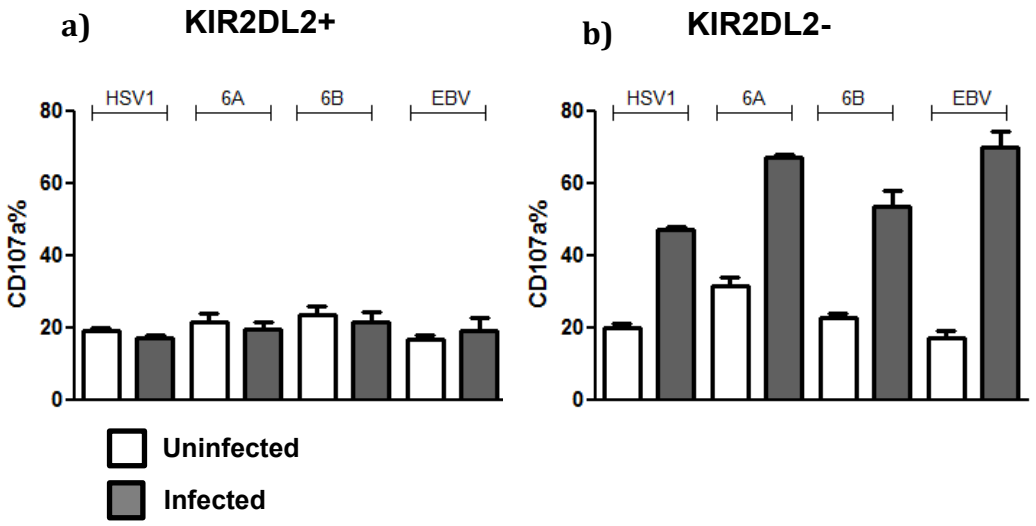
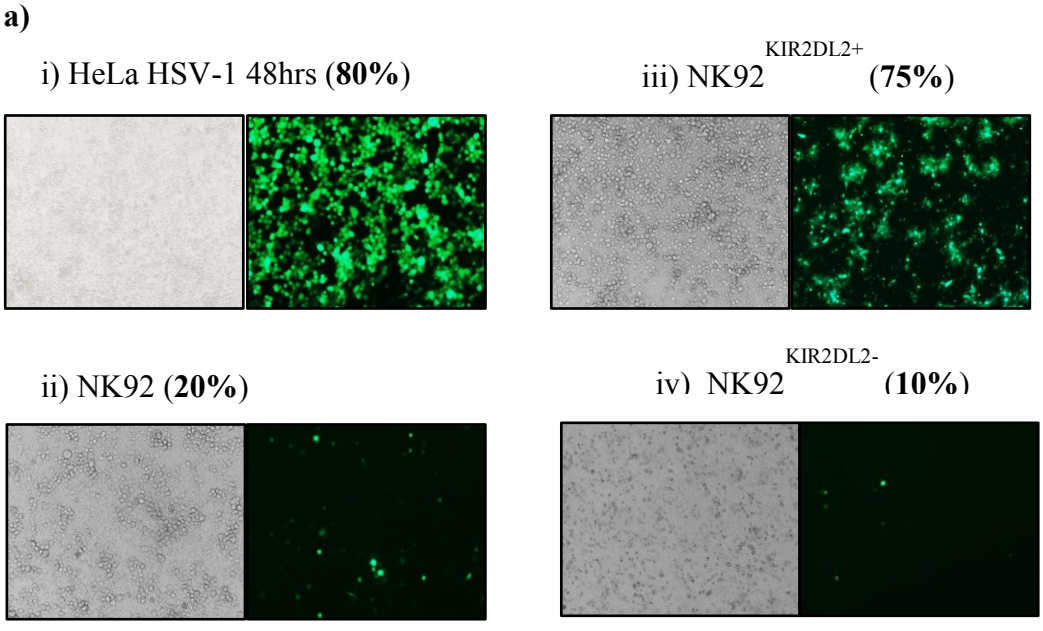


Figure 13 NK cell activation as expression of CD107a reported as percentage of positive cells KIR2DL2^{+} (a) or KIR2DL2^{-} (b) co-cultured with HeLa HSV-1 infected, J-Jhan (T cell line) HHV-6A infected, SupT1 (T cell line) HHV-6B infected, LCL (B cell) EBV infected cells. Means±SD are reported.

When we looked at viral titres, we observed that KIR2DL2^{+} NK92 cells were not able to control HSV-1 infection in HeLa cells, maintaining a 75% infected cells in comparison with KIR2DL2^{-} NK92 cells, that reduced the infected cells to a 10% (Fig.14a). Similarly, HSV-1 titration documented a decrease in PFU/ml in the co-culture with KIR2DL2^{-} NK92 cells (Fig.14b).



b)

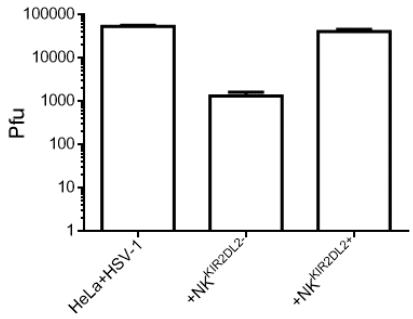


Figure 14 (a) Percentage of HSV-1 HeLa infected cells alone (i) or in co-culture with wild type (ii), KIR2DL2$^+$ (iii) and KIR2DL2$^-$ (iv) NK92 cells. (b) The histogram shows means ± SD of virus titration (PFU/mL). The results were obtained from four independent experiments on HeLa infected cells, 2 days post infection. ** significant p values (Student *t* Test).

Similarly, KIR2DL2$^+$ NK92 cells were unable to counteract HHV-6A and HHV-6B infections (Fig.15a), that are controlled by KIR2DL2$^-$ NK92 cells (Fig.15b).

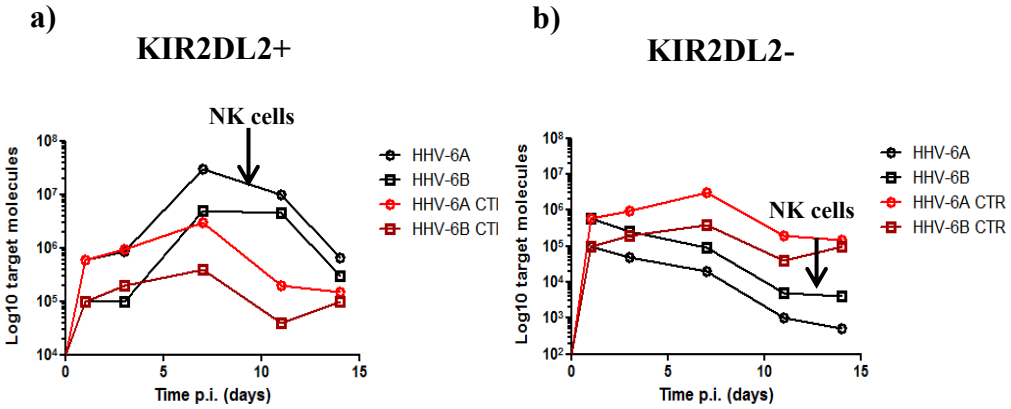


Figure 15 HHV-6 infection of J-Jhan and SupT1 after co-culture with KIR2DL2+ (a) and KIR2DL2$^-$ (b) NK92 cells. Virus DNA presence was evaluated using an amount of 100 ng of DNA. Results are expressed as mean Log10 target molecules.

When we looked at EBV infection, we observed the inability of KIR2DL2$^+$ NK92 cells to counteract EBV infection, that maintained a high titre even in NK92 cell co-culture (Fig.16a). On the contrary, KIR2DL2$^-$ NK92 cells maintained a low EBV titre (Fig.16b).

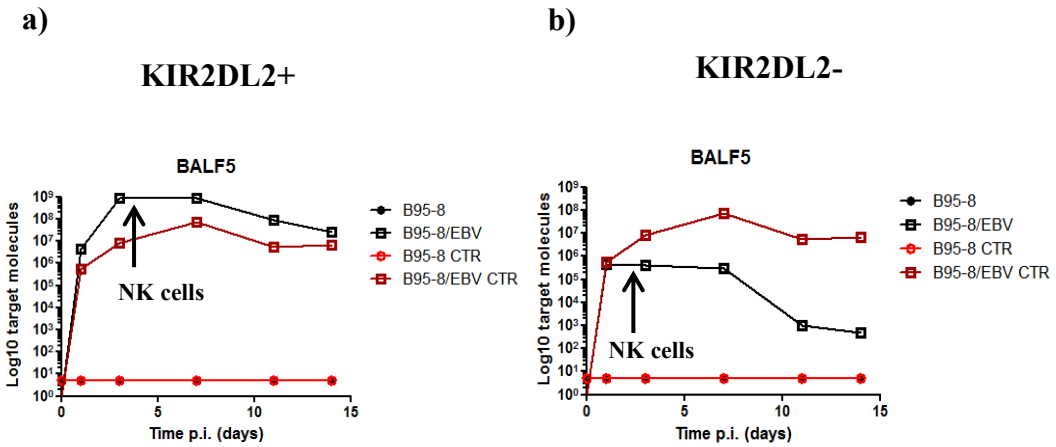


Figure 16 EBV activation of B95-8 in co-culture with KIR2DL2+ (a) and KIR2DL2- (b) NK92 cells. Virus DNA was evaluated using an amount of 100 ng of DNA. Results are expressed as mean Log10 target molecules.

To be sure that KIR2DL2$^-$ NK92 cells were able to kill HHV infected cells and not only to prevent the viral replication, CFSE (carboxyfluorescein diacetate succinimidyl ester) staining of target cells was detected by flow cytometry . We observed, for HSV-1 infected cells, no modification in CFSE$^+$ cell percentage in the co-culture with KIR2DL2$^-$ NK92 cells (Fig.17). HHV-6A, HHV-6B and EBV infected cells increased CFSE positivity after 5 days post infection (data non shown). On the contrary, KIR2DL2$^+$ NK92 cells reduced CFSE$^+$ cell percentage, indicating a higher proliferation of infected cells and suggesting the inability of KIR2DL2$^+$ cells to kill infected cells.

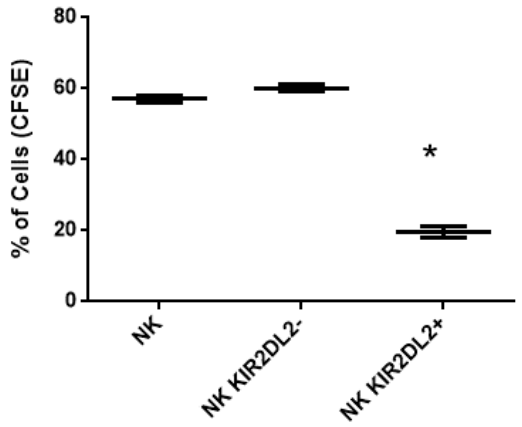


Figure 17 CFSE Fluorescent cell staining dye. Mean results obtained from killing of HSV-1, HHV-6A, HHV-6B and EBV infected cells respectively by control, KIR2DL2$^-$ and KIR2DL2$^+$ NK92 cells. KIR2DL2 negative NK92 cells were able to kill HHV infected cell, whereas KIR2DL2 positive NK92 cells were inactive toward infection. *significant p values, $p \leq 0.05$

4.2 Analysis of cytokine secretions and transcription factors expression

4.2.1 NK92 cell line nucleofection and infection

We established an in vitro system by the transfection of the NK92 cell line with the expression vector pCMV6-AC-GFP, which contains the KIR2DL2 DNA sequence (Fig.18).

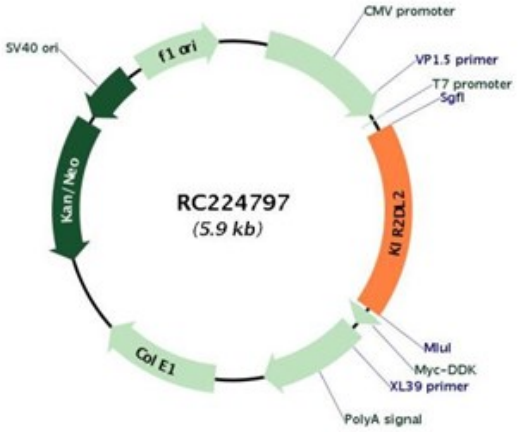


Figure 18 pCMV6-AC-GFP vector, KIR2DL2 Human cDNA ORF Clone (GFP-tagged) - Human killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 (KIR2DL2)

NK92 cell line exhibits a KIR2DL1, 2DL2, 2DL3, 3DL1, 3DL2, 2DS2, 2DS4, 2DS5 genotype[71] and maintains functional characteristics of $CD56^{bright}$ NK cells[205]. Nevertheless, the KIR's expression is restricted to a low percentage of cells[71], by reason of the methylation patterns of CpG islands, related to transcription activity of the respective KIR genes. For this reason, we chosen to transfect an expression plasmid, in order to obtain predominantly the expression of KIR2DL2 in NK92 cell cultures. We checked the efficiency of transfection at several times (Fig.19), through the green fluorescent protein (GFP) used as a reporter gene into the plasmid. After 24 h transfected cells were put in selection with Geneticin (at 1000µg/mL, minimum concentration able to kill untransfected cells) and were maintained in selection for 12 days to obtain a stable transfection. 24 h before the infection, NK92 cells were treated with CpG, necessary to obtain pre-activated status[116,206].

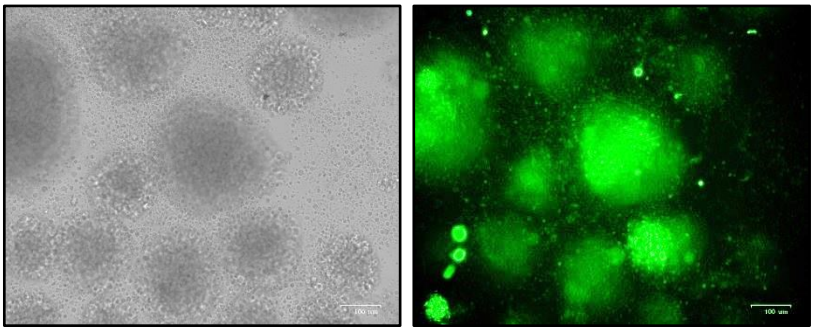


Figure 19 NK92 transfected cells with pCMV6-AC-KIR2DL2 plasmid. GFP expression after 24 h.

To be sure that the in vitro model is representative of the effect of KIR2DL2 expression in the presence of HHVs infection, we put NK92 cells ($KIR2DL2^+$ and $KIR2DL2^-$) in co-culture with HeLa cells, which were previously infected with HSV-1. We noticed a wide spread of infection 24 hours post-infection in HeLa-infected cells (Fig. 20a), HeLa-infected cells in co-culture with $KIRDL2^-$ NK92 cells presented a decrease in infected cells (Fig. 20b). On the contrary, HeLa-infected cells in co-culture with $KIR2DL2^+$ clones showed a strong spread of infection, supporting the inability to activate of NK92 $KIR2DL2^+$ cells (Fig.20c).

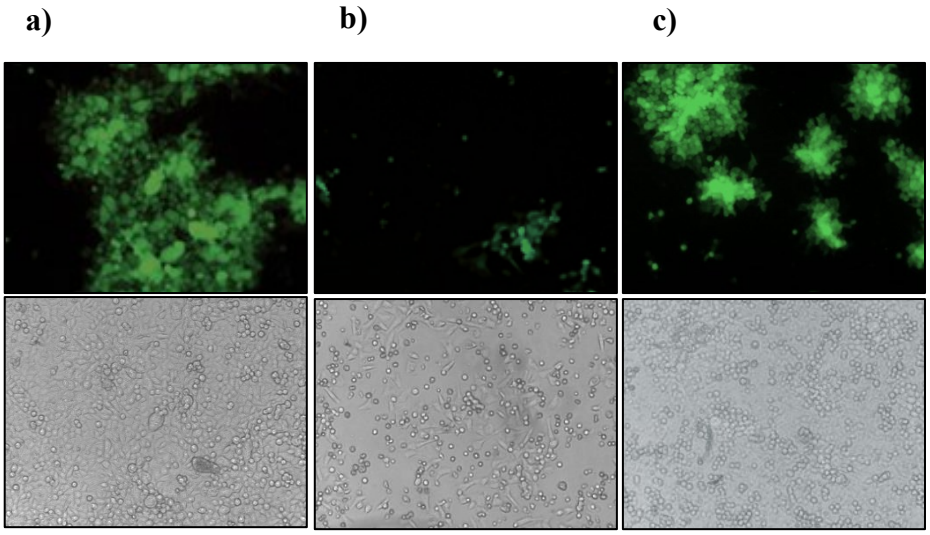


Fig.20 HSV-1-GFP HeLa infected cells alone (a), presenting a spread of infection, or in co-culture with $KIR2DL2^-$ (b) and $KIR2DL2^+$ NK92 cells (c). $KIR2DL2^-$ NK92 cells were able to counteract the infection, whereas KIR2DL2 positive NK92 cells seem to be inactive towards HSV-1 infection.

4.2.2 Analysis of cytokine secretions and transcription factors expression

Since NK cell activation sustain cytokine secretion, we looked at the production of cytokines involved in the immune response during infection, selecting TNF-α, IL-10 and IL-22 as representative for Th1, Th2 and Th17 response, respectively.

We observed that in the medium of KIR2DL2^{-} NK92 cells co-cultured with HeLa HSV-1 infected cells, there was an increase in TNF alpha, IL-10 and IL-22 levels, in comparison with co-cultures of KIR2DL2^{+} NK92 cells and HeLa HSV-1 infected (Fig.21). Interesting, KIR2DL2^{+} NK92 cells in co-culture with HeLa HSV-1 infected cells decreased the secretion of IL-10, supporting the inability of KIR2DL2^{+} NK cells to engage an immune control towards HHVs infections.

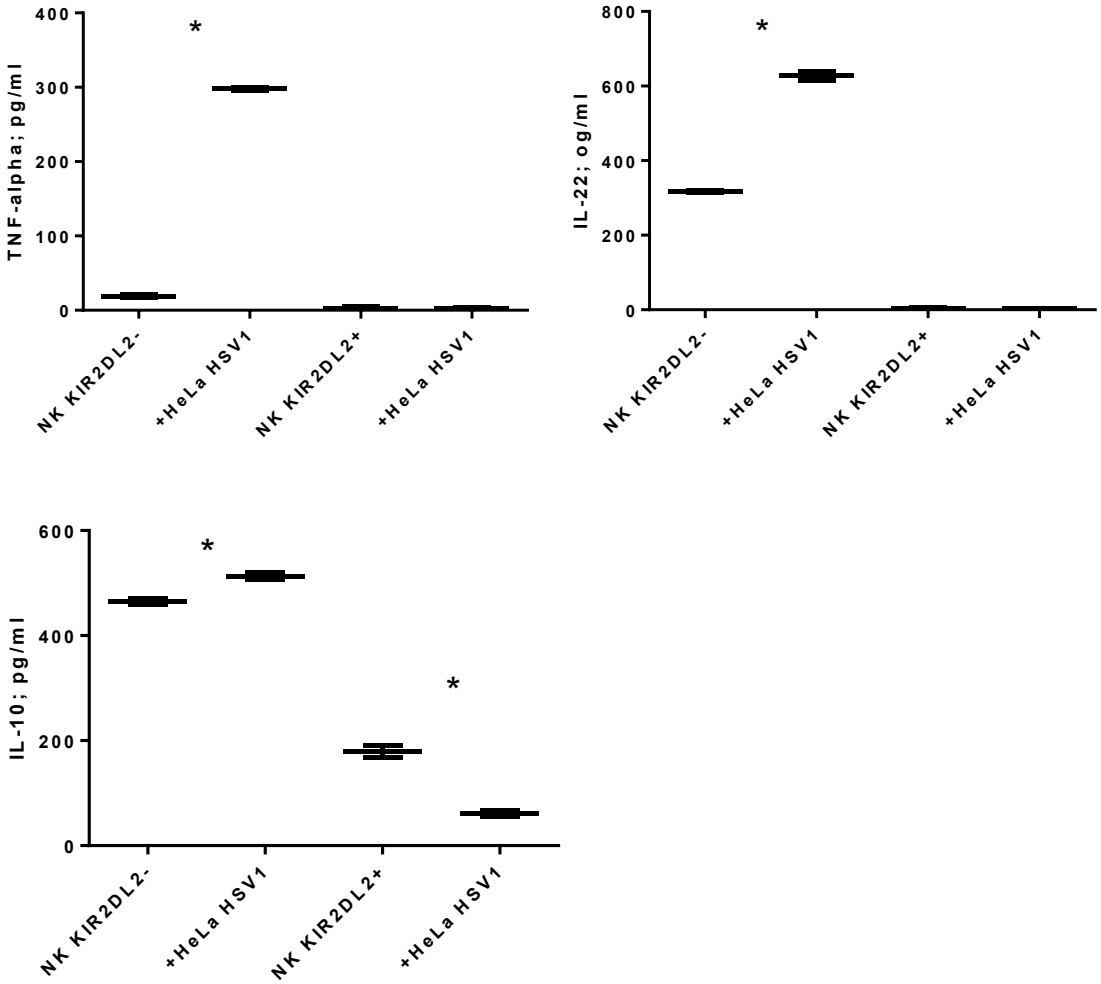


Fig. 21 Mean cytokines levels in KIR2DL2^{-} and KIR2DL2^{+} NK92 cells with or without HeLa-infected cells. *significant p values, $p \leq 0.05$ (Student T test).

4.2.3 Transcription factors

The literature reports several evidences of transcription factors that modulates KIR promoters' activity. In particular, two transcription factors have shown a peculiar role in the regulation of KIR promoters: Sp1 and RUNX1[207].
For this reason, we analyzed their role in controlling the expression of KIR2DL2 receptor in NK cells.

4.2.3.1 Sp1 transcription factor and KIR2DL2

We analyzed the expression of the mRNA encoding Sp1 transcription factor in NK92 cells with CpG treatment and in the presence of HeLa cells and HSV-1-infected HeLa cells. We found that it is expressed in KIR2DL2$^-$ NK92 cells, whereas KIR2DL2$^+$ NK92 cells up-modulated Sp1 expression in the presence of HeLa-infected cells (Fig.22). These data suggest to deepen the role of Sp1 transcription factors in the presence of KIR2DL2 expression.

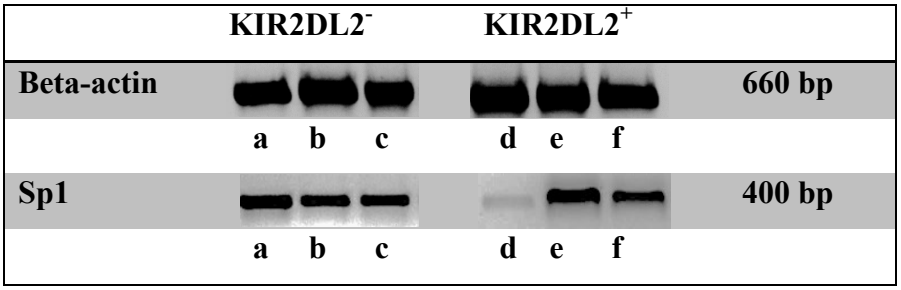


Fig.22 Sp1 mRNA expression in KIR2DL2$^+$NK92 and KIR2DL2$^-$NK92 cells. Beta-actin is used as loading control. Sp1 in KIR2DL2$^-$ NK92 cells is equally expressed in NK92 cells with CpG treatment (a), in the presence of HeLa cells (b) and HSV-1-infected HeLa cells (c). Whereas Sp1 results up- modulated in KIR2DL2$^+$ NK92 cells with CpG treatment in the presence of HeLa cells (e) and HSV-1-infected HeLa cells (f). Sp1 resulted with a lower expression in KIR2DL2$^+$ NK92 cells without HeLa infected cells (d).

We evaluated the expression of Sp1 protein in the three different culture conditions. We observed an induced expression of Sp1 in KIR2DL2$^+$ NK92 cells with CpG-ODN treatment and in the presence of HeLa-infected cells (Fig. 23). KIR2DL2$^-$ NK92 cells up-modulated Sp1 expression in the presence of HeLa-infected cells, but reaching lower levels. These data suggest that the presence of Sp1 mRNA does not always correlate with the protein expression. In fact, KIR2DL2$^-$ cells constitutively expressed Sp1 mRNA in all conditions, whereas protein was detected only in the presence of infected cells.

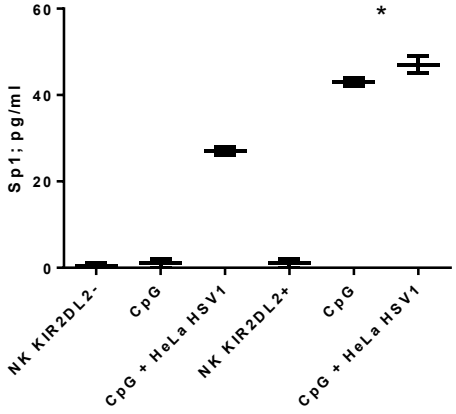
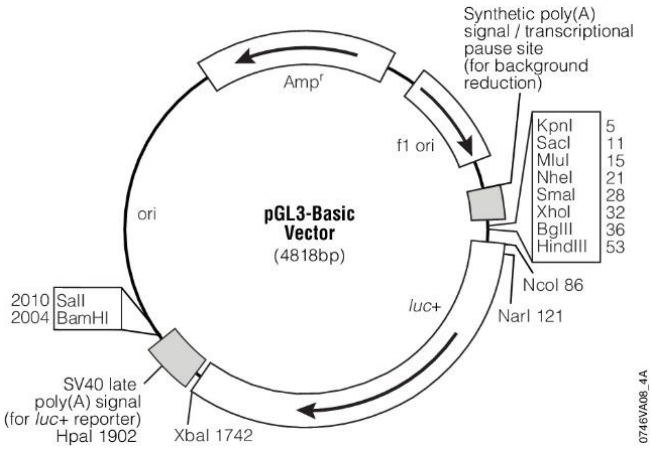


Figure 23 Sp1 expression levels in KIR2DL2$^-$ and KIR2DL2$^+$ NK cells with or without HeLa infected cells. KIR2DL2$^+$ NK cells treated with CpG-ODN and co-cultured with HeLa-infected cells present an increased level of Sp1 production. *significant p values, $p \leq 0.05$ (Student t test).

4.3 RUNX1 transcription factor and KIR2DL2

On the basis of literature data, we decided to investigate the effect of RUNX1 in activating or repressing KIR2DL2 promoter and performed a reporter transfection assay. Because a 208-bp KIR2DL2 promoter gene construct was shown to be sufficient to drive promoter activation in the Jurkat (T cells) and NK92 cell lines (Xu J et al., 2005)[76], we decided to perform a reporter luciferase vector that would contain this 208-bp fragment, that seems to be a region with a higher concentration of binding sites for activating/inhibitory elements. In particular, RUNX1-binding site has been identified in this region (at position 98bp). Therefore, we amplified this sequence from Jurkat DNA (previously genotyped for KIR2DL2) and put it in pGL3 luciferase reporter vector (Fig.24a). Thence this pGL3-Luc-KIRpro construct was co-transfected with or without the RUNX1-pCDNA3 expression vector in NK92MI cells (Fig.24b).

a)



b)

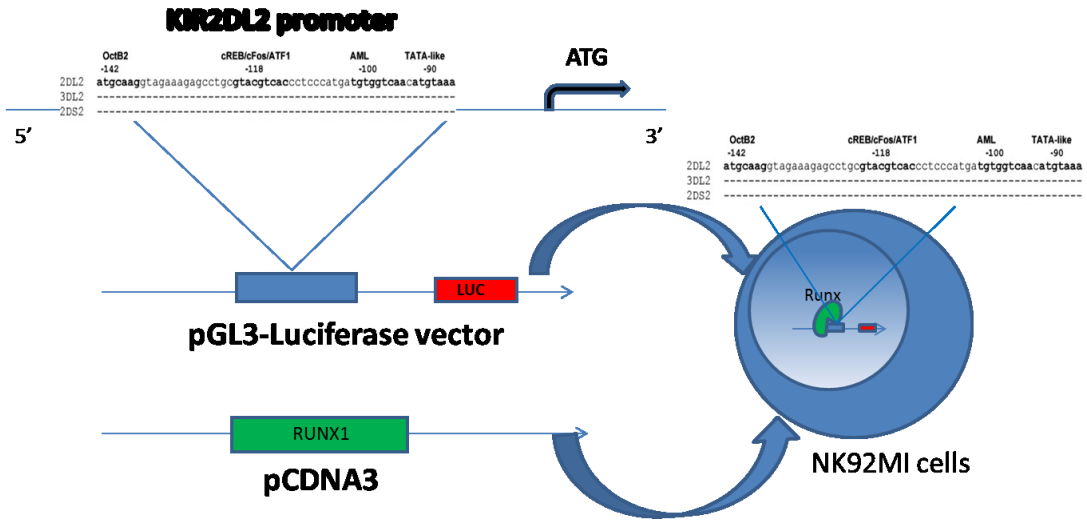


Figure 24 pGL3 Luciferase Basic vector, Promega (a). Cloning of KIR2DL2 promoter and construction of Luciferase reporter vector (b).

The analysis of results clearly showed a higher activation of the pGL3-Luc-KIRpro co-transfected with RUNX1-pCDNA3 compared to promoter-less pGL3 control construct and pGL3-Luc-KIRpro without RUNX1 expression vector in NK92MI cells (Fig. 25). In particular, we found a ~50-fold higher activation in pGL3-Luc-KIRpro/RUNX1 co-transfected cultures. These results showed that KIR2DL2 promoter is positively influenced

by the presence of RUNX1, as shown by luciferase assay, so it is likely that the binding of this factor to the position 98 of the promoter leads to its activation.

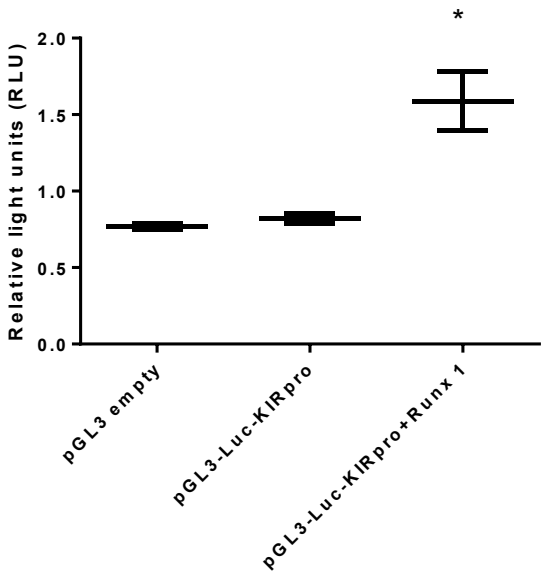


Figure 25 Relative luciferase activity from each construct, in particular pGL3 promoter-less luciferase reporter vector, pGL3-Luc-KIRpro alone and with RUNX1 pCDNA3 vector. Every construct is compared to pGL3 promoter-less luciferase reporter vector. Values are shown in relative light units (RLU). *significant p values, $p \leq 0.05$ (Student t test).

4.4 Epigenetic modification: methylation status of KIR2DL2 promoter

DNA methylation has been associated with MS as a critical factor involved in the regulation of gene expression. For this reason, we decide to study the methylation/demethylation status of KIR2DL2 promoter in MS patients, in order to understand the role in the regulation of this gene and consequently in the behavior of NK cells and possibly in disease status.

In particular, the methylation status of a KIR2DL2 promoter's sequence was investigated in 6 health control subjects and 6 MS patients characterized by a KIR2DL2 homozygous genotype.

Three MS patients displayed a higher level of methylation than the other 3 MS patients and healthy controls (Fig.26). The correlation with disease status revealed that these three MS patients are characterized by a stable disease (Mean EDSS: 1.5 vs EDSS: 2.5), suggesting a possible association between a lower expression of KIR2DL2 and a better disease prognosis.

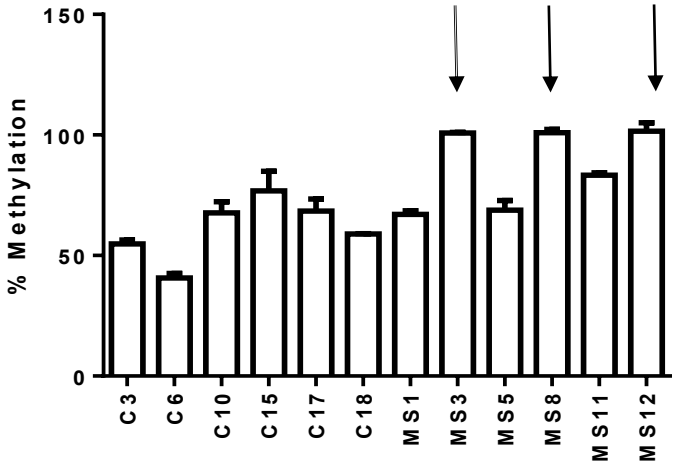


Figure 26 Methylation status percentage in healthy donors (C3, C6, C10, C15, C17, C18) and in MS patients (MS1, MS3, MS5, MS8, MS11, MS12). MS3, MS8 and MS12 patients presented a higher status of methylation that was correlated with a better prognosis.

Table 3 Clinical data of MS patients

Patient	Gender	MS onset	MS type	EDSS	Clinical activity 0=no; 1=relapse in the last 6-12 months	RM activity 0=NO,1= Gd+;
MS1	M	2007	RR	2,5	0	1
MS3	F	2006	RR	1	0	0
MS5	F	2000	RR	2,5	1	1
MS8	F	2005	RR	1	0	0
MS11	M	2002	RR	2,5	0	0
MS12	F	2009	RR	2	0	0

5. Discussion

NK cells control viral infections killing infected cells that down-modulates human leukocyte antigen (HLA) class I surface expression, while sparing cells with HLA-I via recognition by killer-cell inhibitory receptors (KIR). In previous studies, we evaluated the role of NK cells towards herpesvirus (HHVs) infection in Multiple Sclerosis (MS) patients. We observed that a 40% MS patients were unable to counteract Human Herpes simplex 1 (HSV-1) infection and were characterized by NK cells that failed to activate towards HSV-1 and up-modulated KIR2DL2 expression[116].

Based on this background, we explored the molecular mechanisms that differentiate NK cells behavior to HHVs infection in association with KIR2DL2 expression. Above all we focused the experiments and studies to understand which molecular mechanisms regulate KIR2DL2 expression in NK cells.

First of all, this thesis clearly showed that the expression of KIR2DL2 in NK cells was associated with the inability of NK cell to activate and kill HHVs infected cells. In fact, we evaluated the effect of KIR2DL2 expression during HSV-1, HHV-6A, -6B and EBV infection and we observed that NK cell activation, evaluated as percentage of NK cells expressing CD107a marker, increased in $KIR2DL2^-$ NK cells in the presence of HSV-1, HHV-6A, HHV-6B and EBV infected cells. On the contrary, $KIR2DL2^+$ NK cells did not activate in the presence of HSV-1, HHV-6A, HHV-6B and EBV infected cells. Moreover, CFSE assay showed a decrease in $CFSE^+$ percentage in HHVs infected cells co-cultured with KIR2DL2 positive NK cells, supporting the inability of NK cell to kill infected cells.

On the basis of these results, we wanted to identify the possible alteration in KIR2DL2 positive and negative NK cell cytokine expression profile. From literature, we know that these molecules are involved in cell signaling and are able to modulate the balance between various immune responses. In fact, the $CD4^+$ lymphocyte, known as helper T (Th) cells, can differentiate into different subset according to the cytokine production, which lead to a specific response. In particular, we analyzed cytokines involved in Th1, Th2 and Th17 responses[208, 209]. Amongst these, Th1 (pro-inflammatory) response appears as the most involved pathway in MS pathogenesis and is mainly promoted by IL-12 secretion and involves the production of several cytokines as TNF-α, IFN-γ, IL-2, whereas Th2 (anti-inflammatory) response results as an antagonist on Th1 response and is represented by the production of IL-4, IL-5[208]. Recently a new phenotype of Th cells made its first appearance as an additional factor involved in the development of CNS autoimmunity[208]. Th17 pathway is induced by IL-23, that stimulates IL-17a production[210]. Several studies have

recently supported the role of Th17 response in MS pathogenesis, as well as in the development of EAE[211]. Apart from IL-23 and IL-17a, several cytokines seem to be involved in this pathway: IL-22, IL-21, IL-8, IL-6 and others[210]. We found a peculiar cytokine profile in KIR2DL2$^+$ NK92 cells. In fact, the co-cultures of KIR2DL2$^-$ NK92 cells with HeLa HSV-1 infected cells presented an increased level of TNF-α and IL-22, suggesting a Th1 and Th17 secretion. On the contrary, there was a decrease of TNF-α, IL-22 and IL-10 in KIR2DL2$^+$ NK92 cells co-cultured with HeLa HSV-1 infected cells, supporting the inability of KIR2DL2$^+$ NK cells to counteract HHVs infection.

Moreover, this thesis addressed the role of transcription factors and methylation status in the control of KIR2DL2 expression. Hence, we proceeded focusing our studies on the analysis of two transcription factors involved in the regulation of the KIR genes expression (Sp1 and RUNX1).

Sp1 is an ubiquitously DNA-binding protein which modulate transcription, activating or inhibiting, in response to physiological or pathological conditions and can contributes to increase KIR receptor expression, modulating promoter function. RUNX transcription factor was reported to control NK cell development and functions and it becomes evident that the RUNX-binding site in KIR promoter region plays a significant role[212]. On the basis of this knowledge, we investigated the involvement of Sp1 in KIR2DL2 expression by analyzing mRNA and protein release in KIR2DL2 positive and negative NK92 cells with or without CpG treatment and HeLa HSV-1 infected cells. Sp1 mRNA expression was up-modulated in KIR2DL2$^+$ NK92 cells treated with CpG-ODN and co-cultured with HeLa infected cells, whereas its expression resulted stable in KIR2DL2$^-$ NK92 cells. These data were confirmed by protein analyses that showed again a significant increase of Sp1 expression in KIR2DL2$^+$ - NK92 cells treated with CpG-ODN and co-cultured with HeLa HSV-1 infected cells. Moreover, these data suggest that the presence of Sp1 mRNA does not always correlate with the protein expression. In fact, KIR2DL2$^-$ cells constitutively expressed Sp1 mRNA in all conditions, whereas protein was detected only in the presence of infected cells.

Afterwards, by analyzing the effect of RUNX1 on KIR2DL2 promoter, we observed a higher activation of the KIR2DL2 promoter in association with RUNX1 expression. We can speculate that RUNX1 can act as an activating factor on KIR2DL2 promoter, by supporting KIR2DL2 receptor expression.

It is almost clear that the interplay between genetic susceptibility and environmental factors influences the MS disease pathogenesis. In particular it is evident from literature that epigenetic mechanisms , in particular DNA methylation, result involved in MS[213]. For

this reason, we focused our studies on the role of methylation in the modulation of KIR2DL2 proximal promoter activity in NK cells from health donors and MS patients. In particular, our goal was to find whether there was a difference in methylation status between healthy subjects and MS patients. Our results showed that all the healthy subjects presented almost the same degree of methylation in CpG islands of the KIR2DL2 gene promoter region in NK cells. On the other hand, MS patients are characterized by different levels of methylation related to a better or a worsen MS disease status. The correlation between clinical conditions and degree of methylation showed that three MS patients, characterized by a stable disease, presented a higher level of methylation compared to other patients. Considering the involvement of the epigenetic mechanisms in MS disease and their contribution to MS pathogenesis, these data suggested a possible correlation between a lower expression of KIR2DL2 and a better MS disease prognosis, endorsing our initial assumption.

Altogether these data provide a novel point of view in the landscape of the immune response toward HHVs infection in MS disease, in particular in relation to NK cell activation. Based on our results, we have confirmed the possible effect of KIR2DL2 on viral infection in MS patients. At the same time our data showed that there is an accurate transcriptional control of KIR2DL2 expression, which involves not only several transcription factors, but also epigenetic modifications.

These data will lead to a better knowledge of NK cells in particular of their KIR2DL2 inhibitor receptor. Important achievements will be made in the management of viral infections in MS patients seizing the opportunity to exploit KIR2DL2 receptor expression as a target for modulating NK cell activation.

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HLA-G molecules in autoimmune diseases and infections

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Human leukocyte antigen (HLA)-G molecule, a non-classical HLA-Ib molecule, is less polymorphic when compared to classical HLA class I molecules. Human leukocyte antigen-G (HLA-G) was first detected on cytotrophoblast cells at the feto-maternal interface but its expression is prevalent during viral infections and several autoimmune diseases. *HLA-G* gene is characterized by polymorphisms at the 3′ un-translated region and 5′ upstream regulatory region that regulate its expression and are associated with autoimmune diseases and viral infection susceptibility, creating an unbalanced and pathologic environment. This review focuses on the role of HLA-G genetic polymorphisms, mRNA, and protein expression in autoimmune conditions and viral infections.

Keywords: HLA-G, inflammation, autoimmunity, infection, regulation

INTRODUCTION

Human Leukocyte Antigen-G (HLA-G) is a functional molecule belonging to class Ib human leukocyte antigens (HLA) characterized by a non-covalent link between β_2-microglobulin (β2m) and glycoprotein heavy chain. The gene is located within Major Histocompatibility Complex (MHC) locus on chromosome 6 (1, 2). HLA-G products show some peculiar features for which they are considered as non-classical HLA-I antigens: (1) the limitation of their allelic polymorphism (3); (2) the expression of seven isoforms represented by four membrane-bound (G1, G2, G3, and G4) and three soluble (G5, G6, and G7) proteins (4); and (3) the restriction of their tissue distribution (5). Polymorphisms at the 5′ upstream regulatory region and at the 3′ UTR of the *HLA-G* gene play an important role in the regulation of HLA-G production (6). Mainly, two polymorphisms at the 3′ UTR: a deletion/insertion (*DEL/INS*) of 14 base pairs (*14bp*) polymorphism (rs371194629) and a $C > G$ single-nucleotide polymorphism (SNP) at the *+3142bp* position (rs1063320) (7) (**Figure 1**) are able to affect mRNA stability *in vivo* and protein production and implicated in pathological conditions: *14bpINS* allele is associated with mRNA instability (8, 9); *+3142G* allele creates a binding site for three microRNAs (miRNAs) (*miR-148a*, *miR-148b*, and *miR-152*) reducing soluble protein production (10). These observations suggest that *14bpINS/INS* and *+3142G/G* genotypes are associated with a lower HLA-G production than *14bpDEL/INS* and *DEL/DEL*, *+3142C/G*, and *C/C* genotypes (8, 10).

Membrane-bound HLA-G1 and soluble HLA-G5 (HLA-G5) represent the mainly expressed and investigated HLA-G isoforms (1) and are currently supposed to be the most important and functional isoforms (11). However, while HLA-G5 molecules are actively secreted as soluble isoforms, HLA-G1 proteins could be released by proteolytic shedding from cell surface (sHLA-G1) via matrix metalloproteinase-2 (MMP-2) (12–16). HLA-G can exist as β2m-associated and -free monomers (17, 18) and as disulfide-linked dimers or multimers (17, 19, 20). HLA-G disulfide-linked dimers are linked by disulfide bonds between two cysteine residues at position 42 of the HLA-G alpha-1 domain (19–21) and present higher affinity for ILT-2 and ILT-4 receptors compared to monomers (22, 23). Placental trophoblast cells (24), thymus (25), cornea (26), nail matrix (27), pancreas (28), erythroid, and endothelial precursors (29) present a physiological expression of HLA-G molecules. However, HLA-G can be ectopically expressed also on monocytes (30), in transplantation, tumors, viral infections, and autoimmune diseases (1, 2). HLA-G antigens are currently considered as immune-modulatory molecules due to their role in preserving immune tolerance at the feto-maternal interface (31), promoting graft tolerance (32), reducing inflammatory and immune responses (33), favoring tumors (34), and virus infection via immune escape (35). Both membrane-bound and soluble HLA-G antigens exert their immune-suppressive properties: (a) inhibiting the activity and inducing apoptosis of cytotoxic $CD8^+$ T cells and NK cells (36–38); (b) inhibiting the proliferation of $CD4^+$ T cells that are shifted to an immune-suppressive profile (39, 40); (c) inhibiting antigen-presenting cells and B cell differentiation (41, 42); (d) inducing a Th2 polarization (43); and (e) inducing regulatory T cells (44) and Interleukin (IL)-10 secreting dendritic cells (DC10) (45) (**Figure 2**). The interactions between HLA-G proteins and their specific inhibitory receptors ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), and KIR2DL4 (CD158d) expressed by immune cells (46) account for the effects of these molecules on immune cells.

Moreover, HLA-G expression is up-regulated by the secretion of anti-inflammatory cytokines such as IL-10 which, in its turn, is enhanced by HLA-G (30). For these reasons, the implication of HLA-G molecules in inflammatory, immune-mediated, and infective conditions has been investigated (47, 48). The knowledge of

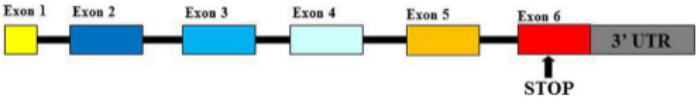

FIGURE 1 | Human leukocyte antigen-G gene. UTR, un-translated region.

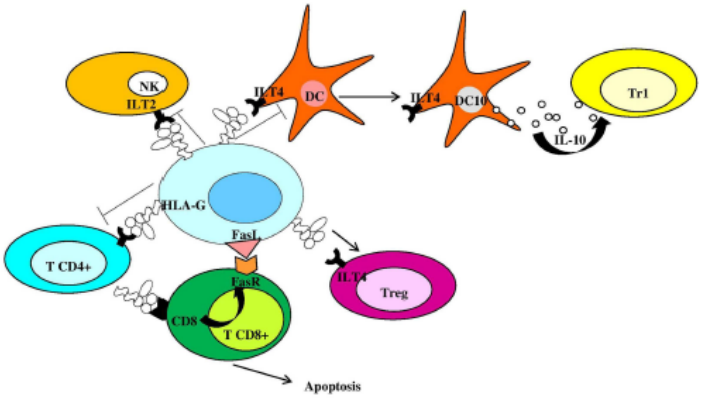

FIGURE 2 | Human leukocyte antigen-G is an anti-inflammatory molecule inhibiting and controlling immune cell activation. NK, natural killer cells; Tr1, type 1 regulatory T cells; DC, dendritic cell; Treg, regulatory T cell; FasR, Fas receptor; DC10, IL-10-differentiated dendritic cells.

the interactions between HLA-G molecules and immune mechanisms and their implication in pathological conditions may assist in improving our knowledge on the mechanisms at the basis of several autoimmune diseases and viral infections.

HLA-G AND GASTROINTESTINAL DISEASES

Celiac disease is a gluten sensitivity, which induces an inflammation that damages the villi in the small intestine of genetically predisposed subjects. Both genetic and environmental factors contribute to the development of celiac disease (CD). Torres and coauthors (49) have shown the presence of HLA-G in biopsies from celiac patients and have observed higher sHLA-G amounts in comparison with control subjects. The evaluation of the *14bp INS/DEL* polymorphism in a group of 522 celiac patients (50), subdivided accordingly with the presence of HLA-DQ2 molecule, encoded by *DQA1*05/DQB1*02* genes, has demonstrated an increased frequency of the *14bp INS/INS* genotype in comparison with controls. These data suggest that the *14bp INS* allele may increase the risk of gut inflammation, most likely leading to chronicity. Ulcerative colitis (UC) and Crohn's disease are characterized by a different sHLA-G expression pattern (51) by peripheral blood mononuclear cells. Non-activated peripheral blood mononuclear cells from Crohn's disease patients secrete spontaneously sHLA-G while those from UC patients and healthy donors do not. Furthermore, after stimulation with LPS, both cells from Crohn's disease and healthy subjects show sHLA-G production, while this does not happen in UC patients. The different HLA-G expression profiles in UC and Crohn's disease patients sustain the different aethiopathogenesis at the origin of these two diseases. In particular, the responses to therapies in UC and Crohn's disease correspond to different sHLA-G secretion levels (52). The immunosuppressant therapy normalizes the production of HLA-G molecules in Crohn's disease while it starts the release of HLA-G in UC patients. These data confirm the diversity in the behavior of these two pathologies and propose the analysis of sHLA-G levels with the final goal of distinguishing between UC and Crohn's disease patients and to monitor therapy.

HLA-G AND RHEUMATOLOGIC DISEASES

Rheumatic diseases are inflammatory and autoimmune diseases, which are the second most common cause of disability after musculoskeletal injuries. Rheumatoid arthritis (RA) is an autoimmune disease caused by the immune system attacking synovial cells. A combination of genetic and environmental factors may increase the risk of RA. Gene expression profiles (GEPs) in bone marrow-derived RA mononuclear cells (53) have shown 1,910 down-regulated and 764 up-regulated gene, which include the *HLA-G* gene. Several studies have evaluated the role of *HLA-G* polymorphisms in RA susceptibility without reaching a final common result. The evaluation on 256 RA patients and 356 healthy controls genotyped for the *HLA-G 14bp INS/DEL* polymorphism has reported no differences in allelic and genotypic

frequencies and no correlation with disease characteristics (54). The analysis of two SNPs (rs1736936, −1305G/A and rs2735022, −689A/G) in *HLA-G* promoter in the Korean population has not presented any connection to the development of RA (55). The evaluation in a Brazilian cohort documented the implication of 3′ UTR polymorphisms in RA follow-up (56). The authors have observed a significant association of the *−762C > T*, *−716T > G*, *−689A > G*, *−666G > T*, *−633G > A*, *−486A > C*, and *−201G > A* (*rs1632946*; *rs2249863*; *rs2735022*; *rs35674592*; *rs1632944*; *rs1736933*; and *rs1233333*) SNPs with the disease. The analysis of 106 patients with juvenile idiopathic arthritis (JIA) has shown an association between JIA female susceptibility and the 14 bp DEL allele. These different associations support the presence of different pathogenic elements between RA and JIA (54). RA (57) and JIA patients present lower serum sHLA-G concentration than in controls (58), with a possible contribution to the chronicity of the inflammation. On the contrary, JIA synovial fluids showed higher sHLA-G levels than controls (SF) (56). Since we have observed that HLA-G molecules are enhanced in synovial fibroblasts from inflamed joints (59) and that high sHLA-G levels correlate with disease activity (57), we may suggest an impaired control of immune reaction at joint, which characterizes JIA disease. The *HLA-G 14bp INS/DEL* polymorphism has also been evaluated as a marker for RA therapy. Methotrexate (MTX), a disease-modifying anti-rheumatic drug (DMARD), induces an increased production of IL-10 in RA patients with a better therapeutic response (60) and is able to enhance HLA-G secretion by peripheral blood mononuclear cells (61). Interestingly, the *14bp DEL/DEL* genotype is increased in RA patients with a good response to MTX therapy (62), with a possible implication in the control of immune activation. It must be underlined, however, that contrasting results have been obtained (63, 64), possibly due to a different dosage of MTX, a different cut-off value for RA therapy response assessment. Scleroderma (SSc) is an autoimmune rheumatic disease of the connective tissue (65). Only SSc patients with a longer survival, lower frequency of vascular cutaneous ulcers, telangiectasias, and inflammatory polyarthralgia present HLA-G molecule expression in skin biopsies (66) suggesting an implication of this molecule on the control of immune response at the skin level.

Systemic lupus erythematosus is a systemic autoimmune disease of the connective tissue that can affect any part of the body. The immune response is mainly characterized by Th2-cell predominance. Rosado and coauthors (67) and Chen and coauthors (68) have shown higher sHLA-G and IL-10 levels in systemic lupus erythematosus (SLE) patients in comparison with healthy controls, while Rizzo and coauthors (69) have observed lower sHLA-G concentrations in SLE patients (70). Interesting, the analysis of monocytes and mature CD83 positive dendritic cells from SLE patients has evidenced a diminished expression of HLA-G in comparison with healthy controls (71), a lower HLA-G expression in response to IL-10 and a lower HLA-G trogocytosis from autologous monocytes compared with controls. Using the SNPs mapping approach, *HLA-G* gene is recognized as a novel independent locus for SLE (72). In particular, *HLA-G 14bp INS/DEL* polymorphism and *HLA-G +3142C > G* SNP have been analyzed in a SLE population. SLE patients showed a higher frequency of *14bp INS* allele and *14bp INS/INS* genotype (69) and the heterozygote group showed lower systemic lupus erythematosus disease activity index (SLEDAI) indexes than homozygous groups (73). On the contrary, the evaluation of *HLA-G 14bp INS/DEL* polymorphism in a SLE Brazilian population did not present an association (74), while the *+3142G* allele and the *+3142 GG* genotype frequencies were increased among SLE patients as compared with controls (75, 76). These data sustain a possible role of HLA-G expression in modifying SLE condition. Behçet (BD) and Kawasaki diseases are autoimmune vasculitis. The *HLA-G*01:01:01* allele is associated with a reduced risk of BD while *HLA-G*01:01:02* and *G*01:05N* alleles are associated with an increased risk of BD (77, 78). Non-synonymous SNP *(+755A/C)* of the *HLA-G* gene (rs12722477, *G*01:04*) is significantly associated with Kawasaki disease (79). These data suggest an influence of *HLA-G* polymorphisms in determining disease risk, possibly affecting HLA-G production and consequently inflammation status.

HLA-G AND CUTANEOUS DISEASES

The skin is characterized by a "skin immune system (SIS)," where immune cells and humoral components support cutaneous inflammation. The deregulation of skin defense mechanisms is evident in a large variety of inflammatory disorders of the skin, such as psoriasis, atopic dermatitis, pemfigo, vitiligo, and systemic sclerosis (80). HLA-G protein is not expressed in the skin from healthy controls (81, 82). Ectopic HLA-G expression has been described in skin pathologies (83–86).

Psoriasis is a chronic inflammatory skin disease with an autoimmune component. Both membrane-bound and soluble HLA-G proteins have been detected in psoriatic skin lesions with the main compound characterized by macrophage lining at the dermo-epidermal junctions (82). The up-regulation of HLA-G molecules by macrophages could represent an attempt to control auto-reactive T cells, induced by activated keratinocytes-derived cytokines/chemokines. HLA-G may prevent keratinocyte destruction by modulating the activity of cytotoxic lymphocytes and promoting the development of Treg cells (87). Interestingly, significantly lower plasma sHLA-G levels have been found in psoriatic patients compared with controls (88), suggesting a difference in systemic HLA-G expression that could be associated with the IL-10 deficiency typical of psoriasis. Psoriasis management can be divided into three main types: topical drugs, light therapy, and systemic medications. Evaluation of therapeutic effects on sHLA-G expression has shown an increase in plasmatic levels of systemic treated patients (efalizumab, cyclosporin A, and acitretin) (88) and a significant association between *HLA-G 14bp DEL* allele and *14bp DEL/DEL* genotype with acitretin clinical outcome (89). We can suppose a possible direct effect of HLA-G in antagonizing systemic T helper 1 activation and with a potential role as a marker of response to acitretin in psoriatic patients.

Pemphigus vulgaris is a blistering disease caused by autoantibodies to desmoglein skin adhesion proteins. Skin tissue sections from pemphigus vulgaris (PV) patients express detectable HLA-G molecules at both transcriptional and translational levels, while control sections present only HLA-G transcription (90). Moreover, the HLA-G *14bp DEL* allele has been observed with higher frequency in PV patients in comparison with controls in a Jewish

population (91). These data suggest that HLA-G expression could be a detrimental factor for the development of PV.

HLA-G AND DIABETES

Type 1 and type 2 diabetes present immunologic defects that enhance insulin resistance as a result of genetics sedentary lifestyle, obesity, and other conditions, such as chronic inflammation or infection. It has been shown that higher levels of sHLA-G are frequent in subjects with an impaired glucose metabolism (92). These data suggest a possible implication of HLA-G antigens in the diabetic condition. In fact, SNPs *rs4122198*, *rs2394186*, *rs1619379*, and *rs1611133* near the *HLA-G* gene have been associated with type 1 diabetes (93); dendritic cells from type 1 diabetic patients produce lower HLA-G molecules in response to IFN-beta (94) in comparison with control subjects and the *HLA-G 14bp INS-INS* genotype might contribute to the development of high blood pressure in type 2 diabetes (95).

Interestingly, HLA-G has been found in some secretory granules and on the cell surface of primary islet cells induced to secrete insulin (28). On the basis of these data, it could be hypothesized that an impaired HLA-G expression at pancreatic islets could sustain T cell activation and onset of diabetes.

HLA-G IN MULTIPLE SCLEROSIS

Multiple sclerosis is the prototypic autoimmune disease of the central nervous system (CNS) characterized by chronic inflammatory demyelination and neurodegeneration of unidentified origin (96). Multiple sclerosis (MS) typically occurs in young adults and manifests in women twice as frequently as in men with neurological symptoms and signs, called relapses, which are usually disseminated in space and time (97). About the 80% of MS patients present a disease onset with a relapsing–remitting (RR) form followed by a secondary progressive (SP) course that arises after years, whereas MS starts with a primary progressive (PP) form in approximately the 20% of subjects (98). However, the recent proposed criteria (99) suggest that the coexistence of multi-focal lesions in the periventricular white matter on T2-weighted Magnetic Resonance Imaging (MRI) scans with or without Gadolinium (Gd) enhancement on T1-weighted MRI scans are needed for the diagnosis of MS. Based on epidemiological studies, exposure to an environmental factor, e.g., an infectious agent, in genetically predisposed individuals is currently thought to be crucial for MS pathogenesis (100) in which the traffic into the CNS of activated auto-reactive $CD4^+$ T helper 1 (Th1) cells plays a central role (96, 101, 102). The initiation of brain inflammation is due to the activation of microglia by infiltrating $CD4^+$ T cells leading to the generation of Th1-mediated immune responses (IL-12/IFN-γ and IL-23/IL-17), while the resolution of neuroinflammation is triggered by astrocytes, which promote anti-inflammatory Th2-polarized responses (IL-10 and TGF-β) and the elimination of infiltrating immune cells through Fas/FasL-dependent apoptosis (96, 101) (**Figure 3**).

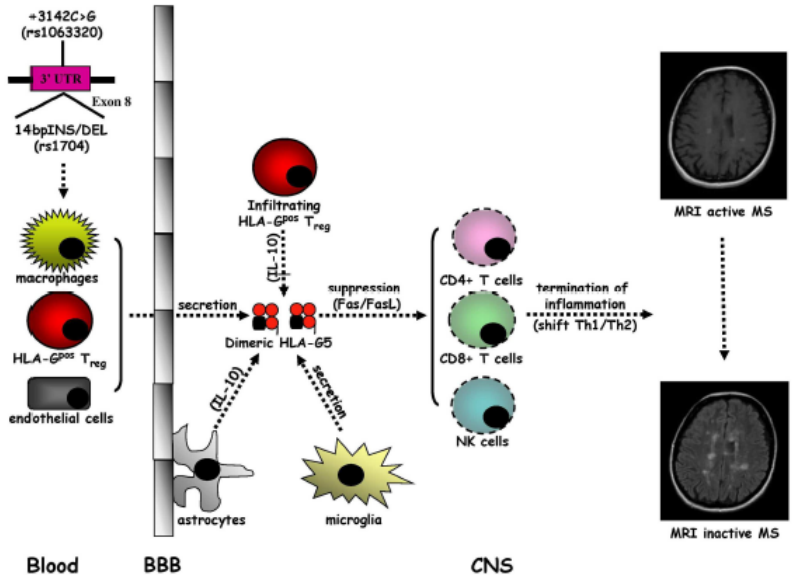

FIGURE 3 | Intrathecal immune milieu in MS. The secretion of HLA-G5 in dimeric form by macrophages and HLA-G^{pos} T_{reg} infiltrating the central nervous system (CNS) across the blood–brain barrier (BBB), endothelial cells, and microglia, sustained by a IL-10 release by astrocytes, may promote the suppression of $CD4^+$ Th1 cell activity and the apoptotic removal of $CD8^+$ T cells and NK cells that favor the formation of an anti-inflammatory intrathecal microenvironment leading to the termination of MS inflammation.

A growing body of evidence indicates that sHLA-G antigens may have a tolerogenic role in MS (102, 103). Cerebrospinal fluid (CSF) detectable sHLA-G has been detected in RRMS patients with higher levels in comparison with other inflammatory neurological disorders (OIND), non-inflammatory neurological disorders (NIND), and controls (104). Furthermore, higher CSF sHLA-G levels have been detected in RRMS without MRI evidence compared to those with MRI active disease. Notably, a positive correlation between CSF concentrations of sHLA-G and IL-10 has been found in MS patients without MRI evidence of active disease. Therefore, CSF levels of sHLA-G may act, together with IL-10, as anti-inflammatory molecules to regulate MS disease activity. The association between elevated CSF sHLA-G levels and clinical and MRI appearance of MS stable disease is supported by the intrathecal synthesis of sHLA-G in MS clinically and MRI inactive patients (105). We have found higher CSF levels of HLA-G5 and not of sHLA-G1 isoforms compared with controls and in presence rather than in absence of MRI Gd enhancing lesions (106) and an as well as inverse correlation between CSF levels of sHLA-G and anti-apoptotic sFas molecules in MS patients without MRI disease activity (107). Collectively, these results suggest a strong correlation between high CSF levels of sHLA-G antigens and the resolution of MS autoimmunity probably related to the anti-inflammatory properties of these molecules. The impact of HLA-G in MS pathogenesis was recently confirmed by other studies, which demonstrated that: (a) Th1 and Th2 cytokine production and $CD4^+$ T cell proliferation are suppressed by HLA-G from MS patient peripheral blood monocytes during the first month of treatment with IFN-β (108); (b) MS disease activity during pregnancy may be modulated by tolerogenic properties of sHLA-G since post-partum serum sHLA-G levels are higher in MS patients without clinical attacks (109); and (c) microglia, macrophages, and endothelial cells located within and around MS lesions present a strong immunohistochemical expression of HLA-G and its inhibitory receptors (ILT-2 and ILT-4), with an elevated protein HLA-G expression on cultured human microglial cells after activation with Th1 pro-inflammatory cytokines (110). Meanwhile, a novel subpopulation of naturally occurring $CD4^+$ and $CD8^+$ regulatory T cells of thymic origin expressing HLA-G (HLA-G^{pos} T_{reg}), has been characterized in MS patients with a suppressive activity through the secretion of HLA-G5 and the shedding of sHLA-G1 (111–113). Overall, these data sustain anti-inflammatory properties of sHLA-G molecules, and in particular HLA-G-5 isoform, which could lead to the remission of MS autoimmunity. Although it has been demonstrated that SNP rs4959039, a SNP in the downstream un-translated region of HLA-G gene is independently associated with MS susceptibility (114), the possible link between *HLA-G* genetic polymorphisms and MS has not been intensively explored (102, 103). Conflicting results have been obtained. Although no association between *HLA-G* gene polymorphism and MS or severity of the disease has been initially found (115), *14bpINS* and *−725G* (rs1233334) alleles have been shown to be related to MS (116). However, a recent study, evaluating the influence of *14bpDEL/INS* and *+3142C > G* HLA-G polymorphisms on CSF and serum sHLA-G production, has documented a correlation between HLA-G genetic polymorphisms and sHLA-G concentrations in both CSF and serum (117). These findings indicate that CSF and serum sHLA-G levels in MS could be affected by two main HLA-G polymorphisms. Moreover, preliminary results from our laboratory have demonstrated that, MS patients present dimeric sHLA-G form more frequently than control, in particular in MRI inactive MS patients (unpublished data), suggesting that large amounts of biologically active dimeric sHLA-G form could be released in CSF of MS patients, possibly induced by pharmacological treatment (118). Nevertheless, in a recent study no association was found between serum sHLA-G levels, disability progression, disease MRI activity, and time to conversion from clinically isolated syndrome (CIS) to clinically definite MS (119). These findings suggest that the use of sHLA-G levels in CSF should be taken into consideration as a prognostic marker for monitoring disease conversion, activity, progression, and response to therapy.

HLA-G IMPACT IN VIRAL INFECTIONS

Even if host immune system present several mechanisms to control viral infections, the viruses have developed several strategies to counteract host immune defenses (120). HLA-G seems to be implicated in viral immune-escape from Natural Killer cells (121).

Human immunodeficiency virus type 1 (HIV-1) up-regulates HLA-G molecules and down-regulates classical HLA-A and -B. Studies have focused on the expression of HLA-G in monocytes, which are relevant as reservoirs of HIV-1, and in lymphocytes, which are more susceptible to infection by HIV-1. Monocytes from HIV-1 seropositive patients express HLA-G (122) with a possible association with antiretroviral therapy (HAART), since patients undergoing HAART present higher levels of HLA-G expression on monocytes in comparison with untreated and healthy subjects (122, 123). T cells obtained from HIV-1 seropositive individuals have been found to express HLA-G at a higher proportion (124) and behave like HLA-G+ Treg. Furthermore, on the basis of *HLA-G* genetics, it would seem that the *HLA-G 14bpINS* and *+3142G* polymorphisms affect the susceptibility to HIV (125) but not mother–child transmission (126) in African population.

Human cytomegalovirus is a herpes virus that persists in the host (127) by means of several strategies to evade the immune system. HLA-G expression is evidenced during viral reactivation in macrophages and astrocytoma cells (35) and the levels of expression on monocytes an in serum is higher during active human cytomegalovirus (HCMV) infection (128). This up-regulation is proposed to be associated with virus-encoded homologs of humanIL-10 (cmvIL-10) (129), which prevents NK cell recognition of infected cells.

There is also evidence to support also a role of HLA-G molecules in susceptibility and outcome of human papilloma virus (HPV) infections. The alleles HLA-G *14bp INS*, *+1537C* (rs12722477), *G*01:01*, *G*01:04*, and *G*01:06* have been associated with both high-grade squamous intraepithelial lesions and cervical cancer, while *HLA-G 14bp DEL* and *+3142C* alleles have been identified as protective (130–135). These results are in agreement with the low levels of HLA-G5 expression in cervical cancer (136). On the other hand, two researches recognized *HLA-G 14bp DEL* allele and *+3142C* as associated with increased risk of cervical cancer (137, 138), in agreement with increased expression

of HLA-G in cervical cancer tissues (139) and with the spontaneous de-methylation of *HLA-G* promoter that allows immune-evasion and the development of precancerous cervical lesions (140). HLA-G has been also implicated in nasal polyposis development in the presence of HPV infection (141). Nasal polyps with HPV11 infection have shown HLA-G expression on epithelial cells, while no HLA-G expression has been observed in HPV negative polyps.

Neurotropic viruses such as herpes simplex virus-1 (HSV-1) and Rabdovirus (RABV) (142) induce the expression and up-regulation of membrane and soluble HLA-G molecules in actively infected neurons with a consequent protection toward host NK cells.

Hepatitis C virus (HCV) and Hepatitis B virus (HBV) seems to induce HLA-G expression to control host immune response (125, 143–148).

On the basis of these results, HLA-G proteins are expressed by virally infected cells as a mechanism to evade host immune control, preventing T cell and NK cell activation. The main challenge would be to block HLA-G up-regulation by viral infection, in order to allow the recognition by immune cells.

INTERACTION OF HLA-G MOLECULES WITH OTHER HLA-Ib MOLECULES

Other HLA-Ib molecules have been identified: HLA-E and HLA-F (149, 150) characterized by a low genetic diversity as well as by a particular expression pattern, structural organization and functional profile.

Similar to HLA-G, HLA-E forms a complex with β2-microglobulin. HLA-E is known to play an important role as immune-modulator during pregnancy and transplantation (151), inhibiting immune responses by its interaction with $CD8^+$ T cell receptors (TCRs) (152) and with the CD94/NKG2A inhibitory receptors of NK cells (153). Meanwhile, this molecule may present non-self antigens activating immune response (154).

Similar to other HLA molecules, HLA-F can form a complex with beta2 microglobuli and three splicing variants have been described. While the presence of HLA-G and HLA-E has been recently correlated with physiological and pathological conditions, the clinic-pathological significance of HLA-F is limited. HLA-F is expressed by peripheral blood B cells upon activation (155) and is detected in embryonic tissues, including the extravillous trophoblasts invading maternal deciduas, and in spermatozoids (156, 157) and in the serum of patients affected by tumors (158).

Only few data are available on the interaction of HLA-G molecules with the other HLA-Ib antigens. In physiological conditions, HLA-G molecules interact with HLA-E and co-operate to inhibit NK cells, mainly at feto-maternal interface, via interaction with ILT-2 and CD94/NKG2A, respectively (159). In pathological condition, the interaction between these two molecules facilitates the escape of tumor cells from NK cell recognition (160). In MS, HLA-G and HLA-E molecules are expressed by resident CNS cells and interact with NK cell and cytotoxic lymphocytes (161). HLA-G, -E, and -F expression by trophoblasts correlates with the protection of the fetus from destruction by the maternal immune system, suggesting a co-operation for fetal tissue preservation.

CONCLUSION

This review aims to focus on the key role of HLA-G molecules in autoimmune diseases and viral infections. The data herein summarized suggest that HLA-G may have a crucial role in the creation of an impaired immune response that characterizes these pathological conditions.

In fact, it appears even more evident that HLA-G proteins are involved in the regulation of the immune system during autoimmunity, such as gastrointestinal, skin, rheumatic and neurological diseases and in the immune-escape mechanisms during viral infections.

Here, we have reviewed a series of experimental and epidemiological studies that support the direct influence of HLA-G proteins on the balance of immune settings. On this basis, understanding the function of HLA-G in these disorders could help in the identification of new approaches to control HLA-G production.

For example, it is interesting to note that inflammatory cutaneous diseases present a disproportional expression of HLA-G molecules with respect to controls and that this could generate autoimmunity. Thus it appears that down/over-expression of HLA-G may not only act as an immunosuppressive and beneficial molecule but may also sustain an unbalanced immune stimulation and autoimmunity. With reference to bowel diseases especially, it appears clear that the different HLA-G expression levels could help in the differential diagnosis and consequently in the choice of appropriate treatment.

Furthermore, several studies have evidenced the possible role of sHLA-G antigens as a tolerogenic molecules in MS since their intrathecal production is associated with disease remission. It is of extreme importance to evaluate the role of HLA-G antigens in MS pathogenesis, in particular if they are implicated in disease progression or if they represent an indirect manifestation of MS inflammation of CNS. Still to be clarified are the functional differences between HLA-G5 and sHLA-G1, and whether dimers and monomers exert a different function in MS inflammatory disease activity. As far as viral infections are concerned, HLA-G could be considered a target for anti-viral treatment, so increased knowledge in this field could contribute to identifying different therapeutic strategies.

Collectively, the results emerging from the literature confirm the importance of the HLA-G molecule in the pathogenesis and progression of immune-based diseases and infections, underlining the relevance of its investigation with the aim to developing new therapeutic strategies and clinical markers. Meanwhile, the analysis of the interactions between HLA-G and other HLA-Ib molecules may be useful to understand the mechanisms for the creation of immune-suppressive microenvironments.

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Fetal cell microchimerism: a protective role in autoimmune thyroid diseases

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Abstract

Objective: The physiological persistence of fetal cells in the circulation and tissue of a previously pregnant woman is called fetal cell microchimerism (FCM). It has been hypothesized to play a role in systemic autoimmune disease; however, only limited data are available regarding its role in autoimmune thyroid disease (AITD).
Design: Circulating FCM was analyzed in a large series of previously pregnant women with Graves' disease (GD), Hashimoto's thyroiditis (HT), or no disease (healthy controls (HCs)). To exclude the possible bias related to placental factors, the polymorphic pattern of human leukocyte antigen-G (*HLA-G*) gene, which is known to be involved in the tolerance of fetal cells by the maternal immune system, was investigated.
Methods: FCM was evaluated by PCR in the peripheral blood, and the Y chromosome was identified by fluorescence *in situ* hybridization in some GD tissues. *HLA-G* polymorphism typing was assessed by real-time PCR.
Results: FCM was significantly more frequent in HC (63.6%) than in GD (33.3%) or HT (27.8%) women ($P=0.0004$ and $P=0.001$ respectively). A quantitative analysis confirmed that circulating male DNA was more abundant in HC than it was in GD or HT. Microchimeric cells were documented in vessels and in thyroid follicles. In neither GD/HT patients nor HC women was the *HLA-G* typing different between FCM-positive and FCM-negative cases.
Conclusion: The higher prevalence of FCM in HC as compared to GD and HT patients suggests that it plays a possible protective role in autoimmune thyroid disorders. Placental factors have been excluded as determinants of the differences found. The vascular and tissue localization of microchimeric cells further highlights the ability of those cells to migrate to damaged tissues.

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Introduction

During pregnancy, a bidirectional exchange of cells has been observed between the fetus and the mother, which starts between the 4th and 6th weeks of gestation (1). The passage of cells from the fetus to the mother is called fetal cell microchimerism (FCM), whereas that occurring from the mother to the fetus is named maternal cell microchimerism (MCM). Both fetal and maternal microchimeric cells have been shown to persist in the maternal circulation or tissue for decades and to have progenitor-like properties, because they are able to differentiate into different cell types (2, 3, 4). The most widely used procedure for evaluating FCM is assessing by PCR the

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presence of male cells in a woman with a previous male pregnancy. By this and other methods, FCM has been extensively studied in autoimmune diseases, particularly systemic ones, because they are more often observed in females and appear to be modulated by pregnancy (5, 6, 7, 8). As far as autoimmune thyroid disease (AITD) is concerned, FCM has been documented in Hashimoto's thyroiditis (HT) and Graves' disease (GD) (9, 10, 11, 12, 13). These diseases occur more frequently in women, with a peak incidence during the fertile age. They appear to be related to pregnancy and often have a spontaneous resolution after delivery and an onset or exacerbation in the *postpartum* period, although their relation with parity is still controversial (14, 15, 16). In the few available series, which include a limited number of cases, FCM was found to be represented more at the tissue level in HT and GD as compared to non-AITDs (4, 9, 10, 11, 12, 13, 17). Even at the peripheral level, scanty and discrepant results have been reported. In particular, one study that used a PCR-based technique showed that the number of circulating fetal microchimeric cells did not differ between GD and controls (12), whereas another study that used a fluorescence *in situ* hybridization (FISH) analysis demonstrated that a higher number of circulating fetal cells were found in GD and HT patients as compared to healthy volunteers (18).

Thus, in the present study, we compared the prevalence of FCM in two large populations of parous women with a previous male pregnancy, one including women affected with GD or HT and the other including healthy controls (HCs). Moreover, in order to investigate if possible differences in the prevalence could be the result of placental factors, we assessed the polymorphic pattern of the human leukocyte antigen-G (*HLA-G*) gene. Indeed, HLA-G molecules are non-classical HLA class I antigens that are expressed by villous trophoblasts and are involved in the reprogramming of the local maternal immune response because they inhibit the activation of decidual T and NK cells; they have been proposed to positively influence the outcome of pregnancy (19, 20). The HLA-G expression profile can be modulated by genetic polymorphisms, mainly two variants at the 3′ UTR, the 14 bp insertion/deletion (rs1704), and the +3142C>G (rs1063320), which influence mRNA stability and protein production and thereby lead to increased/reduced HLA-G expression and soluble HLA-G protein amounts in body fluids (21, 22, 23). In particular, the 14 bp deletion in hetero- or homozygosis is associated with a higher HLA-G expression (high secretor profile), whereas both the 14 bp insertion and the +3142C>G polymorphism in exon 8 decrease HLA-G expression (low secretor profile). We speculated that a high secretor HLA-G genetic profile, which allows for a stronger control of maternal immune response, might favor the passage of fetal cells to the mother during pregnancy and result in a higher prevalence of FCM. Thus, the *HLA-G* profile has been correlated with the microchimeric status in GD, HT, and HC.

Subjects and methods

Patients

Peripheral blood was collected from 105 female patients with AITDs treated at our institution. To be enrolled, patients must have had at least one male pregnancy preceding the diagnosis of AITD. In particular, 69 women had GD, with a mean age at enrollment of 53.3 years (range 27–85), and 36 had HT, with a mean age at enrollment of 53.1 years (range 33–88). Graves' orbitopathy (GO) was diagnosed in 58/69 GD and in 4/36 HT patients. Peripheral blood was also collected from 66 HCs with one or more male children without neoplastic or autoimmune diseases (mean age 48 years, range 29–80). Moreover, none of the control women had a thyroid disease, as assessed by FT_4, TSH, anti-thyroglobulin and anti-thyroperoxidase autoantibodies, and neck ultrasound. None of the included subjects had a history of other potential sources of chimerism, such as blood transfusion, organ transplant, twin siblings, or abortion. All of the patients gave informed consent to enter the trial, which was approved by the local ethical committee.

DNA extraction from whole-blood and formalin-fixed paraffin-embedded tissues

Genomic DNA was extracted from blood samples using an Illustra DNA Extraction Kit BACC2 (GE Healthcare, Buckinghamshire, UK). DNA was also extracted from the formalin-fixed paraffin-embedded thyroid tissues of two women with GD submitted to thyroidectomy by means of a commercial kit (Puregene Core Kit A, Qiagen Sciences).

PCR for SRY sequence

For the detection of the human Y chromosome, DNA was submitted to two rounds of PCR (35 cycles each) using primers specific for the SRY locus and corresponding to the region upstream of the SRY-coding region. We previously demonstrated that this method has a sensitivity of 1 male cell/1 million female cells (24). Each PCR analysis was

repeated twice, two negative controls (DNA from two prepubertal girls) and two positive controls (DNA from two men) were included, and special care was taken to avoid external contamination. In particular, all of the samples were handled by a female technician, positive displacement micropipettes were used, and DNA extraction, PCR preparation, and analyses were conducted in separate rooms.

Male DNA quantification

DNA pools were obtained by mixing the same amount of DNA extracted from ten women with HT, ten women with GD, and ten HC who were previously found to be positive for the presence of FCM by PCR amplification. Each DNA pool was precipitated and concentrated to 1 μg/μl, and sequential dilutions were conducted from 6600 ng (1 000 000 gEq) to 0.0066 ng (1 gEq). Male DNA quantities were expressed in DNA genome equivalents (gEq) by using a conversion factor of 6.6 pg DNA per cell (1 gEq = DNA content of a single cell). Dilutions obtained for each pool were subjected to two rounds of PCR. Triplicate analyses were performed for dilutions ranging from 66 to 0.0066 ng in order to improve accuracy and test reliability. Samples were electrophoresed in 3% agarose gels and visualized and photographed on an u.v. trans-illuminator (VisiDoc-It Imaging System, UVP, Cambridge, UK).

Fluorescence *in situ* hybridization

The presence of microchimeric cells was also tested at the tissue level in two GD women who were analyzed for FCM at the peripheral blood level and for whom thyroid tissue was available. In those cases, we performed both the PCR amplification of the SRY gene and the FISH analyses on 4 μm-thick paraffin-embedded tissue sections, as previously described (25). In particular, the DNA probes used were specific for the α-satellite regions of the X (PAC probe pDMX1, locus DXZ1) and Y (band region Yp11.1-q11.1, locus DYZ3) chromosomes. The X probe was labeled with digoxigenin and was detected using FITC-conjugated antibody anti-digoxigenin (green signal; Roche Diagnostics GmbH), whereas the Y probe was labeled with a SpectrumOrange fluorochrome (Vysis, Downers Grove, IL, USA) and was detected as red signal.

Image acquisition was performed on a Leica DMRA2 fluorescence microscope (Leica, Wetzlar, Germany) equipped with Leica filters specific for DAPI, FITC, and Cy3. Images were acquired using a charge-coupled device (CCD) camera (Leica) with a magnification factor of 100×. Image analysis was performed using Leica CW4000-FISH software version Y1.3.1.

HLA-G polymorphism typing

In 96/105 AITD cases and in 58/66 controls, the *HLA-G* 14 bp (14 bp Ins/Del) and the +3142C>G polymorphisms were genotyped by real-time PCR, as previously described (26, 27). Briefly, 100 ng of genomic DNA were amplified in a 25 μl reaction, and the analysis performed using a 7300 Real-Time PCR System (Applied Biosystems). To analyze the 14 bp polymorphism, the forward primer 5′-GTGATGGGCTGTTTAAAGTGTCACC-3′ and the reverse primer 5′-GGAAGGAATGCAGTTCAGCATGA-3′ were used. The probe used for detection of the 14-bp deletion allele was 5′-VIC-GAGTGGCAAGTCCCTTTGTG-BHQ-3-3′, and the probe for the 14-bp insertion allele was 5′-FAM-CAAGATTTGTTCATGCCTTCCC-BHQ-1-3′. To genotype samples for the +3142C>G polymorphism, the forward primer 5′-CCTTTAATTAACCCATCAATC-TCTCTTG-3′ and the reverse primer 5′-TGTCTCCGTC-TCTGTCTCAAATTT-3′ were used. The MGB probe used for the detection of the 3142C allele was 5′-VIC-TAAGTTA-TAGCTCAGTGGAC-3′, and the MGB probe for the 3142G allele was 5′-FAM-TAAGTTATAGCTCAGTGCAC-3′. Each probe had a non-fluorescent quencher at the 3′ end. Amplification was performed with 0.625 μl Assay Mix 40× (Applied Biosystems) and 12.5 μl PCR master mix 2×. Before the amplification, a pre-read run was performed for 1 min at 60 °C. The amplification protocol was: an initial step for 10 min at 95 °C followed by amplification for 15 s at 92 °C and for 60 s at 60 °C for 40 cycles. A post-run reading was performed for 1 min at 60 °C.

Statistical analysis

Possible differences in the clinical features (age at diagnosis/enrollment, age at the first male child birth, number of sons, and period between the birth of the first male child and diagnosis/enrollment) between GD or HT patients and HC were assessed by *t*-test and χ^2 test, as appropriate. The differences in the prevalence of FCM between women with GD, HT, or HC and the association of FCM with either clinical features or the *HLA-G* profile were tested by Fisher or χ^2 tests. Odds ratios (ORs) and 95% CI were used to assess the strength of the association between the presence/absence of FCM and the presence/absence of the disease. Differences between values were considered significant when $P<0.05$. All of the tests were performed using MedCalc Software version 13.2.2 (B-8400, Ostend, Belgium).

Results

Clinical features and blood FCM in patients with GD, patients with HT, and controls

Neither the GD nor the HT group significantly differed from the HC group with respect to age at the first male birth, the period between the birth of the first male child and the diagnosis of AITD, the age at diagnosis or enrollment, and the number of male children (data not shown). The presence of male DNA of presumed fetal origin had a significantly higher prevalence in HC cases than it was in either GD cases (63.6 vs 33.3%, $P=0.0004$, OR 3.5, 95% CI 1.723–7.11), HT cases (63.6 vs 27.8%, $P=0.0005$, OR 4.55, 95% CI 1.877–11.03), or AITD women as a whole (63.6 vs 31.4%, $P<0.0001$, OR 3.818, 95% CI 1.995–7.308) (Fig. 1A and B). Among either AITD patients or HC, the cases that were positive for the presence of FCM (FMC +ve) were not significantly different from those without FCM (FMC −ve) in terms of age at the birth of first male child, number of male children, interval between the birth of the first male child and diagnosis or enrollment, age at diagnosis or enrollment, and, for patients, the interval between the diagnosis and the enrollment and the association with orbitopathy (Supplementary Table 1, see section on supplementary data given at the end of this article). In GD cases, the type of treatment (anti-thyroid drugs, thyroidectomy, or radiometabolic treatment) and the interval between the thyroidectomy or radioactive iodine ablation and enrollment were also considered, because the ablation of the thyroid tissue could have determined the disappearance of circulating fetal cells, but no differences were noted between FMC +ve and FMC −ve cases (data not shown).

Interestingly, the SRY amplification was successful during the first round PCR in the majority of HCs, but only after a two-round PCR in GD and HT cases, which indicates that there is a higher amount of circulating male DNA in controls than there is in AITD patients. Thus, with the aim of performing a quantitative evaluation, FCM +ve DNA of ten HT patients, ten GD patients, and ten HCs were pooled, and sequential dilutions were done. Interestingly, this quantitative analysis confirmed that male DNA was more abundant in HCs than it was in either GD or HT patients. In particular, in HCs, male DNA could be clearly detected up to a dilution that corresponded to 66 ng total DNA (10 000 genome equivalents), whereas it was hardly or not detected at that dilution in GD and HT cases (Fig. 2).

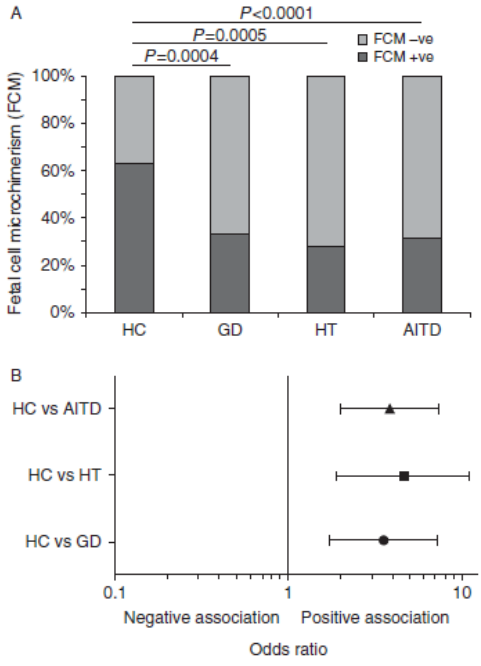

Figure 1
(A) The prevalence of fetal cell microchimerism (FCM) in healthy controls (HCs) with respect to Graves' disease (GD), Hashimoto's thyroiditis (HT), or autoimmune thyroid disease (AITD) patients. FCM was significantly more prevalent in HCs than in either GD or HT cases or in AITD women considered as a whole. (B) Forest plot for FCM and AITD risk. The geometric figures and horizontal lines correspond to the odds ratios (ORs) and 95% CIs respectively. In all of the cases, OR values indicated that there was a positive association between the presence of FCM and the absence of AITDs, which indicates its protective role in the development of these diseases.

Identification of FCM in the thyroid tissue of women affected with GD

The thyroid tissue of two GD women, one of whom was positive and one of whom was negative for FCM at the peripheral level, was studied. In both cases, male cells of presumed fetal origin were documented in the tissue by both PCR and FISH analysis. Interestingly, microchimeric cells were detected as forming part of thyroid follicles, interposed between female maternal cells (Fig. 3A, B and C).

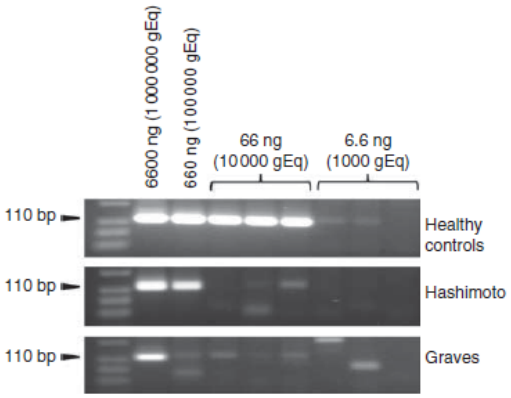

Figure 2

Quantitative evaluation of circulating male DNA in patients with autoimmune thyroid diseases (AITDs) and in healthy controls (HCs). The DNA of ten Hashimoto's thyroiditis (HT) patients, ten Graves' disease (GD) patients, and ten HCs were pooled, and sequential dilutions were obtained. In controls, male DNA could be clearly detected up to a dilution that corresponded to 66 ng total DNA (10 000 genome equivalents), whereas it was hardly or not at all detected at that dilution in GD or HT cases.

Moreover, a microchimeric male cell was identified in a blood vessel (Fig. 3D).

HLA-G genetic polymorphism typing

The *HLA-G* genetic polymorphism typing was not different between FCM +ve and −ve patients of the GD, HT, or HC groups or in the AITD cases considered as a whole. In particular, no differences were found in the prevalence of the homozygous genotype (Del/Del) and the Del allele, which correspond to a high secretor profile (Table 1). As far as the +3142C>G variant was concerned, no differences were observed between the HT or GD patients and the HCs for both the genotype distribution and the allele frequencies (data not shown).

Discussion

In the present large series of AITD cases, the prevalence of FCM was found to be significantly higher in HCs than it was in AITD patients, which suggests a protective role for FCM toward the onset of these diseases. Most of the previous studies in systemic and non-systemic autoimmune diseases were done at the peripheral blood level, and contrasting data have been obtained about the possible role of FCM. Some authors considered it to be pathogenic because they found a higher prevalence of FCM in affected women as compared to controls (5, 6, 7, 8) and because of the hypothesis that microchimeric cells might elicit an intra-organ graft vs host rejection (GvHR) (5, 9, 28, 29). Other authors did not confirm these data (30, 31, 32, 33) and did not support the causative role for this phenomenon, which is also observed in non-autoimmune diseases (17, 24, 25, 34, 35, 36, 37, 38, 39).

The protective role of FCM in AITD that was suggested by the present data is consistent with what has been reported for neoplastic diseases, and in particular for thyroid and breast cancers (24, 25, 35, 37). Based on the immunophenotypic characterization of FMC in neoplastic diseases, we and others hypothesized that fetal cells could migrate from bone marrow niches through blood vessels to reach the injured areas and to differentiate into cells that are able to repair the diseased tissues because at least some of the engrafted fetal cells have stem cell potential (4, 25). Moreover, further evidence to support this 'protective' hypothesis has come from several studies in animal models. In particular, the selective homing of fetal cells to the site of organ injury and not to healthy tissues suggests that fetal cells sense specific signals, which enables them

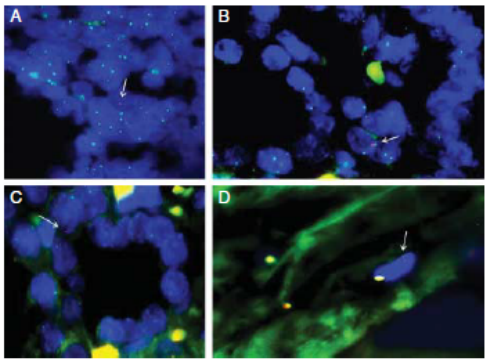

Figure 3

FISH analysis on two Graves' disease tissues. (A, B and C) Male microchimeric cells (red and green signals, indicated by arrows) were detected interposed between female maternal cells (green signals) to form parts of thyroid follicles. (D) A microchimeric male cell (red and green signal, indicated by an arrow) was identified in a blood vessel. Magnification 100×.

Table 1 *HLA-G* genetic polymorphism typing in healthy controls (HC), Hashimoto's thyroiditis (HT) patients, and Graves' disease (GD) patients as either positive or negative for fetal cell microchimerism (FCM).

14 bp insertion/deletion polymorphism	GD *n* (%)			HT *n* (%)			HC *n* (%)		
	FCM +ve	FCM −ve	*P*	FCM +ve	FCM −ve	*P*	FCM +ve	FCM −ve	*P*
Genotype			0.41			0.12			0.97
Ins/Ins	4 (18.1)	9 (23.1)		2 (20)	1 (4)		7 (19.4)	4 (18.2)	
Del/Del	6 (27.3)	10 (25.6)		2 (20)	13 (52)		7 (19.4)	5 (22.7)	
Ins/Del	12 (54.5)	20 (51.3)		6 (60)	11 (44)		22 (61.1)	13 (59.1)	
Total	22	39		10	25		36	22	
Allele			0.57			0.10			0.9
Ins	20 (45.5)	40 (51.3)		10 (50)	13 (26)		36 (50)	21 (47.7)	
Del	24 (54.5)	38 (48.7)		10 (50)	37 (74)		36 (50)	23 (52.3)	
Total	44	78		20	50		72	44	

+ve, positive; −ve, negative.

to target diseased tissue (40, 41, 42). Consistently in the GD cases in the present study, male cells were documented to 'travel' into a blood vessel and were found interposed between female cells and forming follicles in the thyroid. Although we cannot exclude a role for fetal microchimeric cells as being innocent bystanders that do not have any significant biological effect at the thyroid tissue level in GD, two tentative hypotheses can be drawn. First, male-activated fetal T cells, monocytes, macrophages, and NK cells could be the effectors of autoimmunity and could be involved in the initiation of autoimmune diseases (allo-autoimmunity), but this hypothesis seems to be unlikely because we have not observed male cells within lymphocytic infiltration in the affected thyroid gland. Alternatively, these cells may be recognized as being partially alloimmune, that is, as giving rise to an immune reaction (auto-alloimmunity) in which the effectors are maternal immune cells. Nevertheless, the number of male cells observed in the thyroid glands of these women was too low to support this hypothesis. Second, male microchimeric cells could be involved in the repair/repopulation process because of their follicular localization and their morphology, which resembles that of thyrocytes. Indeed, the presence of male cells interposed between female cells in thyroid follicles, which has already been observed in thyroid cancer (25), in nodular goiter specimens (11), and in the normal thyroids of healthy women (17), seems to reinforce the findings at the blood level and to suggest a protective/repairing role for FCM. Nevertheless, it should be noted that the microchimeric status at the peripheral level may or may not reflect that of the thyroid tissue, as was already observed in thyroid cancer (24). Consistently, Y chromosome cells were found in both of the GD tissues that were analyzed, even though only one of those patients was positive for FCM at the peripheral level. The possibility of extensively studying microchimeric status at the tissue level in AITD is strongly limited by the fact that thyroidectomy is not indicated for the treatment of HT, and it is seldom used for GD patients, who are instead mainly treated with radioiodine.

It should be noted that contradictory data to those presented here were previously obtained by Lepez *et al.* (18) in a more limited AITD series. Four hypotheses can be drawn to tentatively explain these contrasting results. The first involves the different intervals between diagnosis and enrollment, which was a maximum of 5 years in the Lepez *et al.* study and ranged from a few months to 30 years in the present study. This longer latency could theoretically explain the differences in the amount of circulating male cells, which could be progressively eliminated over time by the autoimmune process. However, we did not find any correlation between the presence/absence of FCM and the length of the interval between diagnosis and enrollment. The second hypothesis involves the interval between the birth of the youngest son and the age at diagnosis, which was 3–12 months in the Lepez *et al.* study and 1–60 years in the present study. This also could have contributed to differences in the amount of circulating male cells, which may progressively lessen after delivery. Nevertheless, we did not find any correlation between the presence/absence of FCM and the length of this interval either in previous studies (24) or in the present study. Third, Lepez *et al.* used FISH analysis, which allowed them to identify intact male cells, whereas our PCR-based method may have also led to the detection of free-DNA released from damaged cells. Still, our qualitative results were also nicely confirmed in the quantitative analysis, which demonstrated a higher amount of male DNA in HCs, and we estimate that our

analysis was derived mostly from intact cells. Finally, the discordancy could be the result of the low number of patients enrolled by Lepez *et al.* (11 women) or to the non-selection of control cases. On the contrary, control cases were well selected in the present study by biochemical and ultrasound examinations in order to exclude non-clinically evident thyroid diseases.

In the present study, the 3′ UTR polymorphisms 14 bp insertion/deletion (rs1704) and +3142C>*G* SNP (rs1063320) that control HLA-G expression (21, 22) were studied for the first time in AITD. Indeed, because high levels of HLA-G expression seem to be crucial for successful implantation, we hypothesized that a high secretor genetic profile in the mother could be associated with a higher tolerance of fetal cells and is thus associated with higher levels of FMC. No differences in the *HLA-G* typing or, in particular, in the secretor genetic profile, were found between FCM +ve and −ve cases in the GD, HT or HC groups, which is consistent with data previously obtained in HTs and controls (43). Thus, the present findings allowed us to exclude the suggestion that the higher prevalence of FCM in HCs could be the result of a high secretor genetic profile that leads to a facilitated passage of fetal cells. Along these lines, a recent study tested the same hypothesis by analyzing *HLA-G* polymorphisms in children from women with scleroderma (44) and showed that a high secretor genetic profile does not allow more fetal-to-mother chimerism traffic.

In conclusion, circulating fetal cells are significantly more abundant in HCs than they are in patients with thyroid autoimmunity, which suggests a protective role for FCM in AITDs. The possible contribution to these findings of a high secretor *HLA-G* genetic profile has been excluded. The localization studies confirmed that microchimeric cells have the ability to migrate to diseased tissues and may have a possible protective/repairing function.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-15-0028.

Dedaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Short Report

Cerebrospinal fluid amounts of HLA-G in dimeric form are strongly associated to patients with MRI inactive multiple sclerosis

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Abstract

Background: The relevance of human leukocyte antigen (HLA)-G in dimeric form in multiple sclerosis (MS) is still unknown.

Objective: To investigate the contribution of cerebrospinal fluid (CSF) HLA-G dimers in MS pathogenesis.

Methods: CSF amounts of 78-kDa HLA-G dimers were measured by western blot analysis in 80 MS relapsing–remitting MS (RRMS) patients and in 81 inflammatory and 70 non-inflammatory controls.

Results: CSF amounts of 78kDa HLA-G dimers were more frequent in RRMS than in inflammatory ($p<0.01$) and non-inflammatory controls ($p<0.001$) and in magnetic resonance imaging (MRI) inactive than in MRI active RRMS ($p<0.00001$).

Conclusion: Our findings suggest that HLA-G dimers may be implicated in termination of inflammatory response occurring in MS.

Keywords: Multiple Sclerosis, HLA-G, dimeric form, CSF levels, MRI activity

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Introduction

Human leukocyte antigens (HLA)-G are immunonologically functional non-classical HLA-I proteins which have recently been indicated as potential immunomodulatory molecules in multiple sclerosis (MS). In fact, an overexpression of HLA-G and its inhibitory receptors (Ig-like transcript-2 (ILT-2) and Ig-like transcript-4 (ILT-4)) was found in MS plaques and on cerebrospinal fluid (CSF) monocytes from MS patients.[1] In addition, high levels of intrathecally produced soluble HLA-G (sHLA-G) were observed in CSF of MS patients with magnetic resonance imaging (MRI) inactive disease,[2,3] in whom they were correlated positively with CSF concentrations of anti-inflammatory IL-10[2] and inversely with CSF concentrations of anti-apoptotic sFas molecules.[4] These findings argue for a tolerogenic role of sHLA-G in MS, favoring the remission of the disease. In this setting, the immunosuppressive function could be exerted by actively secreted HLA-G5, which was the preponderant sHLA-G isoform detectable in CSF of MS patients without evidence of MRI activity unlike sHLA-G1, the other most important functionally active sHLA-G isoform released after shedding of membrane-bound HLA-G1 from the cell surface.[5] However, this hypothesis remains to be demonstrated since no data are currently available on the presence of HLA-G disulfide-linked dimers in the CSF of MS patients. Indeed, HLA-G in dimeric form exhibits higher affinity for ILT2 and ILT4 receptors and promotes a more efficient inhibitory receptor signaling than monomers[6,7] and, therefore, it is considered as the biologically active HLA-G form with the strongest immunosuppressive effects.[8] Based on these considerations, in this study we sought to investigate the distribution of CSF HLA-G dimers in MS and controls.

*Affiliations are indicated between brackets in Appendix 1

Materials and methods

CSF samples were prospectively collected in 80 consecutive newly diagnosed[9] definite relapsing–remitting (RRMS) patients (55 women and 25 men; mean age=38.7±10.9 years) and 151 age- and sex-matched neurological controls represented by 81 patients (55 women and 26 men; mean age=38.5±9.9 years) with other inflammatory neurological disorders (OINDs) and 70 subjects (49 women and 21 men; mean age=39.9±10.3 years) with other non-inflammatory neurological disorders (NINDs) (Supplementary Table), who were followed by the MS Centre of Ferrara (Italy) during the period from July 2010 to October 2014. All patients were imaged with a 1.5-tesla MRI unit within 48 hours after sampling. MS patients were considered as clinically and MRI disease active if they had evidence of a relapse at admission and lesions showing Gd-enhancement on T1-weighted scans, respectively. The Median Expanded Disability Status Scale was 2.0 (interquartile range=1.0–3.5; mean=2.1±1.5). Median duration of the disease was 7.0 years (interquartile range=1.0–39; mean=28±38.3). None of the patients had fever or other symptoms or signs of acute infections and none had received any potential disease-modifying therapies during the 6 months before the study. None of the female MS patients was pregnant. All OIND and NIND patients were free of immunosuppressant drugs, including steroids. Informed consent was given by all patients before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research. As previously reported,[2–5] CSF levels of sHLA-G were measured by enzyme-linked immunosorbent assay using the monoclonal antibody MEM-G/9 as the capture antibody (Supplementary Material). After biotinylation and immunoprecipitation of CSF samples, CSF HLA-G monomers and dimers were identified by western blot analysis under reducing and non-reducing conditions in all sHLA-G positive RRMS, OIND, and NIND patients as described elsewhere[10] (Supplementary Material). Western Blot analysis was standardized on cell culture supernatants and cell lysates from 721.221 HLA-G1 transfected cells (Supplementary Figure). After checking data for normality by using the Kolmogorov–Smirnov test, continuous variables were compared using the Kruskal–Wallis and the Mann–Whitney U tests; categorical variables were compared by means of the chi-square test. Bonferroni correction was utilized for multiple comparisons. A value of $p<0.05$ was assumed to be statistically significant.

Results

Detectable CSF levels of sHLA-G were more frequent ($p<0.00001$; chi-square) in RRMS (58/80; 72.5%) than in OIND (23/81; 28.4%) and in NIND (12/70; 17.1%) patients. As shown in Table 1, CSF concentrations of sHLA-G were different among RRMS and controls ($p<0.00001$; Kruskal–Wallis) since they were higher in RRMS than in OIND and NIND ($p<0.00001$) and equivalent between OIND and NIND. In sHLA-G-positive patients, while a 39-kDa monomeric band was present in 100% of RRMS and controls, a 78-kDa dimeric HLA-G band was more represented in RRMS than in OINDs ($p<0.001$) and NINDs ($p<0.01$). When RRMS patients were stratified according to clinical and MRI activity, CSF concentrations of sHLA-G and CSF monomers and dimers distribution did not differ between clinically active and clinically stable RRMS. Conversely, CSF concentrations of sHLA-G were more elevated ($p<0.001$) in RRMS patients without than in those with Gd-enhancing lesions, whereas CSF HLA-G dimers were more frequent in MRI-inactive than in MRI-active sHLA-G-positive RRMS ($p<0.00001$). An additional HLA-G like band with a molecular weight of 53 kDa was detected, under reducing conditions, in six clinically and MRI active RMMS, in four OIND and three NIND sHLA-G-positive patients (Figure 1).

Discussion

In this study, we confirmed previous data[2–5] showing that CSF levels of sHLA-G were higher in MS than in controls and predominated in MS patients without MRI evidence of active disease. These results further strengthen the possibility that sHLA-G can be implicated in immunomodulation of the CNS inflammatory response operating in MS. However, whether sHLA-G molecules detected at intrathecal level in MS are functionally active is still to be proven. Therefore, the main finding of our investigation was the demonstration, for the first time, that HLA-G in dimeric form was present in the CSF of MS patients, and its distribution reflected the fluctuations of sHLA-G antigens because HLA-G dimers were more frequent in MS than in controls and in MRI-inactive than in MRI-active MS. In fact, the unique characteristic of both membrane-bound and soluble isoforms of HLA-G is the ability to form disulfide-linked dimers that are created through the generation of disulfide bonds between two cysteine residues at position 42 of the HLA-G alpha-1 domain. As these structures interact with high affinity with HLA-G-specific receptors and, thus, are believed to represent the biologically active form of HLA-G generating immunosuppressive effects.[6–8] These data strongly support the involvement of HLA-G in MS autoimmunity as anti-inflammatory molecules mediating the termination of inflammation. Intriguingly, elevated amounts of a 53-kDa HLA-G-like protein were

Table 1. CSF levels of sHLA-G in all patients with other non-inflammatory neurological disorders (NINDs), other inflammatory neurological disorders (OINDs), and relapsing–remitting (RRMS) categorized according to clinical and magnetic resonance imaging (MRI) activity, and distribution of CSF monomers and dimers in HLA-G positive NIND, OIND, and RRMS.

	CSF sHLA-G (ng/mL) median, IQR, mean ± SD (total)	39-kDa HLA-G monomer % (sHLA-G+)	78kDa HLA-G dimer % (sHLA-G+)	HLA-G like 53kDa % (sHLA-G+)
NIND (total *n*=70; sHLA-G+ *n*=12)	0, 0–0, 1.2±7.2	12/12 (100%)	3/12 (25%)	0/12 (0%)
OIND (total *n*=81; sHLA-G+ *n*=23)	0, 0–1.2, 5.1±12.1	23/23 (100%)	6/23 (26.1%)	2/23 (8.7%)
RRMS (total *n*=80; sHLA-G+ *n*=58)	16, 0–32.3, 19.4±19.5[c]	58/58 (100%)	40/58 (69%)[a,b,c]	6/58 (10.3%)
CA RRMS (total *n*=49; sHLA-G+ *n*=36)	15, 0–26, 16.4±15.2	40/40 (100%)	25/40 (62.5%)	6/40 (15%)
CS RRMS (total *n*=31; sHLA-G+ *n*=13)	18.6, 0–39.7, 24.1±24.3	22/22 (100%)	15/22 (68.2%)	0/22 (0%)
Gd+ RRMS (total *n*=34; sHLA-G+ *n*=19)	2.7, 0–18.4, 10.8±14.1	19/19 (100%)	6/19 (31.6%)	6/19 (31.6%)
Gd- RRMS (total *n*=46; sHLA-G+ *n*=39)	21, 11.0–41.7, 25.7±20.6[f]	39/39 (100%)	34/39 (87.2%)[d]	0/39 (0%)

Total: total patients; sHLA-G+: HLA-G positive patients; IQR: interquartile range; SD: standard deviation; Gd+: MRI appearance of gadolinium-enhancing lesions; Gd−: no MRI evidence of gadolinium-enhancing lesions; CA: RRMS clinical active; CS: RRMS clinical stable.
78-kDa HLA-G dimer (chi square): [a]RRMS vs. OIND and NIND ($p<0.00001$), [b]RRMS vs. OIND ($p<0.001$), [c]RRMS vs. NIND ($p<0.01$), [d]Gd− vs. Gd+ RRMS ($p<0.00001$).
CSF sHLA-G levels (Mann–Whitney): [e]RRMS vs. OIND and NIND ($p<0.00001$).
[f]Gd− vs. Gd+ RRMS ($p<0.001$).

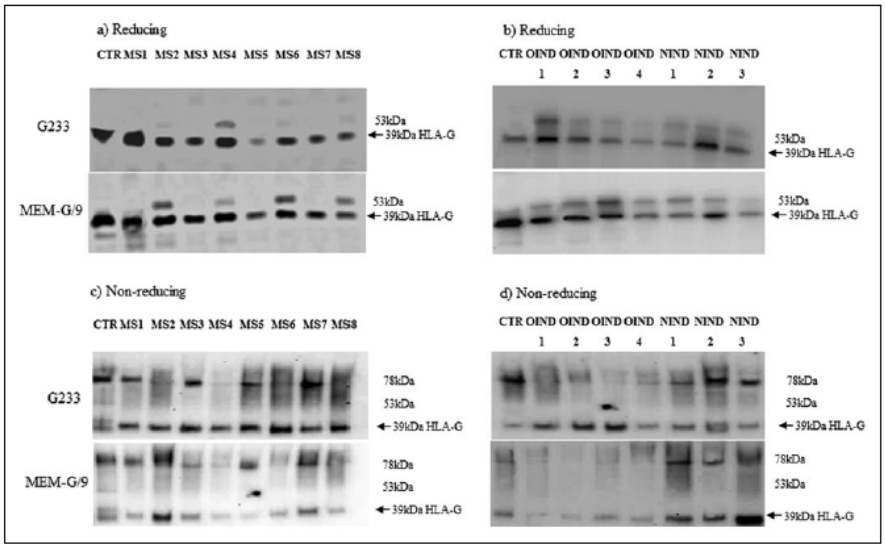

Figure 1. Representative western blot profiles of enzyme-linked immunosorbent assay sHLA-G positive CSF samples from eight patients with relapsing–remitting MS (MS), four patients with other inflammatory neurological disorders (OINDs) and three patients with non-inflammatory neurological disorders (NINDs). The samples were immunoprecipitated with both G233 and MEM-G/9 antibodies and analyzed under reducing (denaturating) and non-reducing (non-denaturating) conditions. The molecular weights were determined with the BenchMark (Invitrogen, CA, USA) (Mk) pre-stained protein ladder (range 10–200 kD). 721.221 HLA-G transfected cell culture supernatants are the positive control (CTR). sHLA-G monomers and dimers migrate at 39 kDa and 78 kDa, respectively, whereas additional HLA-G like molecules are recognized at 53 kDa. Upper blots show results obtained under reducing conditions in MS (a) and OIND and NIND (b) patients after G233 and MEM-G/9 immunoprecipitation. Lower blots indicate the staining under non-reducing conditions in MS (c) and OIND and NIND (d) patients immunoprecipitated with G233 and MEM-G/9.

found in the CSF of a small proportion of clinically and MRI-active RRMS patients and, to a lesser extent, OIND and NIND patients. However, the actual biological significance of this molecule is currently elusive.[10] Future studies are warranted to elucidate the actual significance of CSF HLA-G dimers in MS, with particular attention to the choice of control subjects, which, in our study, were slightly more heterogeneous than recently recommended.[11] In fact, although the search for a potential biomarker for MS was beyond the scope of the current investigation, this limitation could affect the consistency of our data. On the other hand, further efforts are required to obtain quantitative measurements of CSF HLA-G dimers in MS. In this way, a recently proposed densitometry analysis of chemoluminescent signals[8] could represent a reliable option.

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Conflict of interest

None declared.

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Appendix 1

The Emilia-Romagna network for MS (ERMES) study group

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Short communication

KIR2DL2 inhibitory pathway enhances Th17 cytokine secretion by NK cells in response to herpesvirus infection in multiple sclerosis patients

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ABSTRACT

We have previously demonstrated that multiple sclerosis (MS) patients with KIR2DL2 expression on Natural killer (NK) cells are more susceptible to herpes simplex virus 1 (HSV-1) infection. We explored cytokine expression by NK cells during HSV-1 infection in association with KIR2DL2 expression. MS KIR2DL2^{+} NK cells failed to control HSV-1 infection and secreted high levels of Th17 cytokines, while MS KIR2DL2^{-} NK cells released Th1 cytokines, mainly IFN-gamma. Our data showed, for the first time, a peculiar Th17 cytokine secretion by MS KIR2DL2^{+} NK cells in the presence of HSV-1 infection, that could be implicated in MS pathogenesis.
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1. Introduction

Natural killer (NK) cells exert a fundamental role in the control of viral infections. In particular, NK cells recognize viral infected cells where the virus has induced the lack of human leukocyte antigen (HLA) class I surface molecules, used as one microbial immune escape mechanisms. This recognition mechanism is mainly due to killer-cell inhibitory receptors (KIR), specific for HLA-C (CD158a and CD158b) (Moretta et al., 1990; 1993), HLA-B (p70) (Litwin et al., 1994), and HLA-A (p140) (Pende et al., 1996; Döhring et al., 1996) antigens. These receptors are characterized by an intracytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) that maintains inactive NK cells. The loss of HLA-I expression is a rare event that occurs in certain tumor and virus-infected cells to avoid recognition by cytolytic T cells, which use T-cell receptor recognition of antigenic peptides bound to MHC-I molecules to identify abnormal target cells. In this way, NK cells eliminate HLA-I-deficient target cells and KIR-mediated regulation of NK cells can significantly impact their responsiveness during viral infections and cancer. We have recently demonstrated that multiple sclerosis (MS) patients characterized by the presence of KIR2DL2 expression on NK cells are more susceptible to herpes simplex virus 1 (HSV-1) infection (Rizzo et al., 2012; Ben Fredj et al., 2014). In particular, we showed a reduced NK cell activation and, consequently, a low HSV-1 clearance in MS patients with KIR2DL2 allele. These data are in agreement with previous results on the correlation between KIR2DL2 and the susceptibility to HSV-1 infection (Estefania et al., 2007; Moraru et al., 2012), identifying receptor/ligand pair KIR2DL2/HLA-C1 as predisposing factor to symptomatic HSV-1 infection and to frequently recurring forms of the disease.

We planned to explore the possible modification in NK cell cytokine secretion in response to HSV-1 infection in association with KIR2DL2 expression. We focalized our interest on the MS population, where we observed the most significant effect of KIR2DL2 on HSV-1 susceptibility (Rizzo et al., 2012).

2. Results

2.1. KIR2DL2 expression increase HSV-1 susceptibility in MS patients

Twenty definite relapsing–remitting MS (RRMS) consecutive patients (10 males and 10 females; mean age 37.1 ± 11.9 years) newly diagnosed according to the currently accepted criteria (Polman and Rudick, 2010; Lublin, 2014) and followed at the MS Centre of the Department of Neurology, University of Ferrara were enrolled in the

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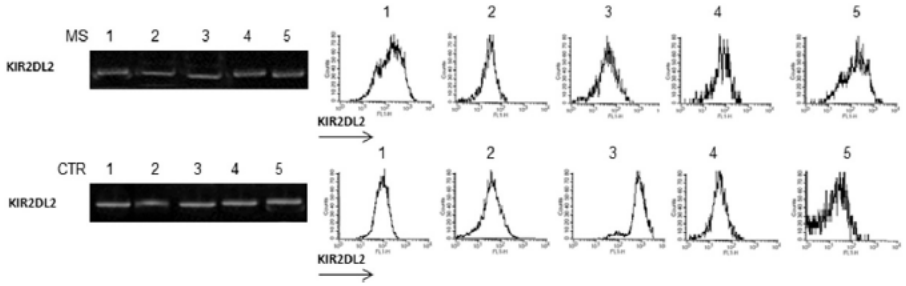

Fig. 1. KIR2DL2 mRNA (left panel) and protein (right panel) expression in NK cells from 5 representative MS patients (MS) and 5 representative control subjects (CTR).

study. Six patients were clinically and MRI active, 4 patients were clinically inactive and MRI active and 10 patients were clinically and MRI inactive. Disease severity, measured by the Expanded Disability Status Scale (EDSS) was 1.9 ± 1.0 (range 0.5–3.5) (Kurtzke, 2015). Disease duration was 15.7 ± 8.7 months (range 4–28). At the time of sample collection, none of the patients had fever or other signs of acute infections, nor had they been receiving any disease-modifying therapies during the 6 months before the study. Ten MS patients were characterized by the positivity for KIR2DL2 gene, where we confirmed the presence of KIR2DL2 expression on CD3-CD56+ NK cells (Fig. 1a), identified as lymphocytes according to forward/side scatter profile and gated as CD3−/CD56+ within the lymphocyte gate. Ten MS patients were negative. Purified $CD3^+$ lymphocytes were infected with HSV-1 and co-cultivated with autologous purified NK cells pre-activated with CpG-ODN. We confirmed a lower $KIR2DL2^+$ NK cell activation in the presence of HSV-1 infected autologous lymphocytes in comparison with $KIR2DL2^-$ MS patients (Fig. 2b) ($p = 0.002$). The treatment with both anti-KIR2DL2 moAb and NSC87877, a specific inhibitor of SHP1/2 tyrosine phosphatases mediating the intracellular signaling of KIRs (Abeyweera et al., 2011), reconstituted NK cell activation (Fig. 2b), that restored the ability of NK cells to reduce virus titer (Fig. 2c). As previously reported, we found no differences in NK cells from twenty control subjects. $KIR2DL2^+$ ($n = 10$) and $KIR2DL2^-$ ($n = 10$) NK cells from control subjects are both able to activate towards HSV-1 infection (Fig. 2d) and reduce virus titer (Fig. 2e). These data confirm the implication of KIR2DL2 receptor in the failure to counteract

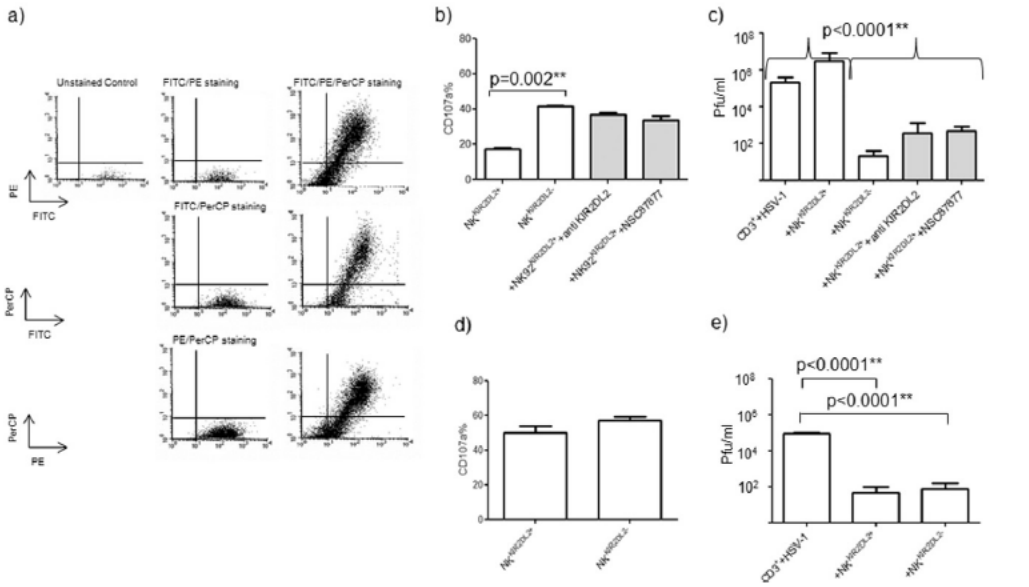

Fig. 2. a) A fluorescence-minus-one (FMO) control was performed to optimize the identification of CD3-CD56 + CD107a+ NK cells. The unstained control, the FMO control and the fully stained cell sample (test stain) — stained with anti-CD3–FITC (fluorescein isothiocyanate), anti-CD56–PE (phycoerythrin), anti-CD107a–PerCP (Peridinin chlorophyll) (Affymetrix eBioscience, CA, USA) — are shown after compensation. b) CD107a expression, reported as percentage of positive cells, by CD3-CD56+ NK cells from ten $KIR2DL2^+$ and ten $KIR2DL2^-$ MS patients co-cultured with HSV-1 infected autologous $CD3^+$ cells. The addition of NSC87877 (inhibitor of SHP1/2) was performed to evaluate the role of KIR2DL2 transduction pathway on NK control of HSV-1 infection. Means ± SD are obtained from 4 independent experiments. **significant p values, obtained with Student *t* test. c) The histogram shows means ± SD of virus titration (Pfu/ml) obtained from 4 independent experiments. **significant p values, obtained with Student *t* test. d) CD107a expression, reported as percentage of positive cells, by CD3-CD56+ NK cells from $KIR2DL2^+$ and $KIR2DL2^-$ healthy control subjects co-cultured with HSV-1 infected autologous $CD3^+$ cells. Means ± SD are obtained from 4 independent experiments. **significant p values, obtained with Student *t* test. e) The histogram shows means ± SD of virus titration (Pfu/ml) obtained from 4 independent experiments. **significant p values, obtained with Student *t* test.

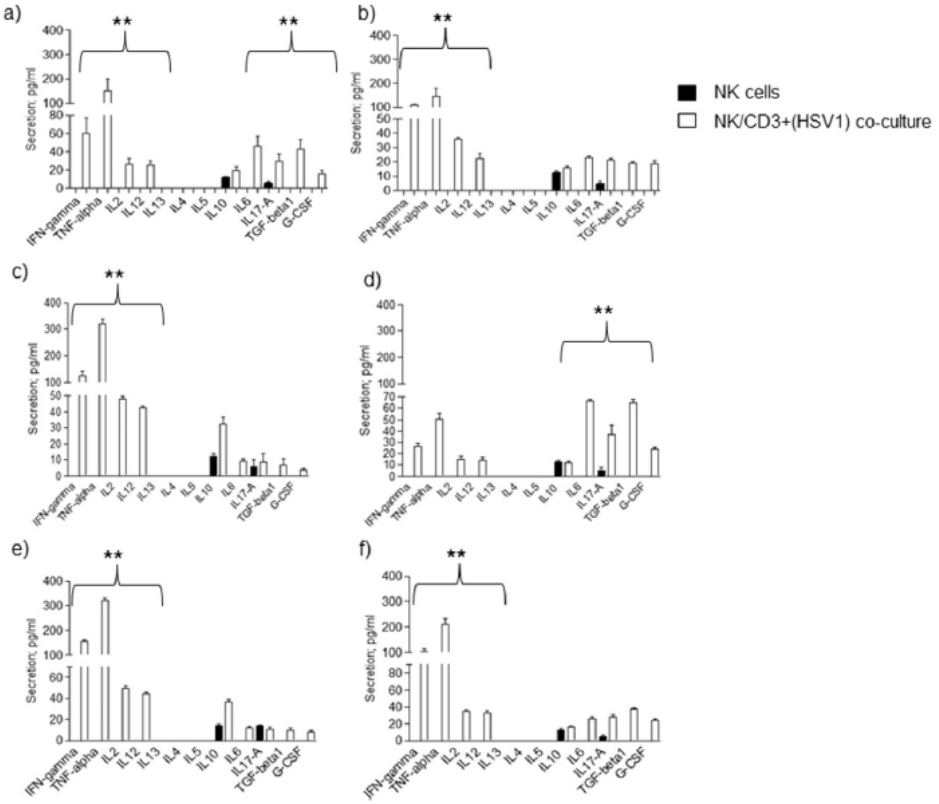

Fig. 3. Levels of secretion of 12 cytokines (Th1/Th2/Th17) by NK cells alone (black histogram) or b) in co-culture with HSV-1 infected $CD3^+$ cells (white histogram) in a) MS patients and b) control subjects; in c) KIR2DL2 negative and d) KIR2DL2 positive MS subjects and e) KIR2DL2 negative and f) KIR2DL2 positive control subjects. Means ± SD are obtained from 4 independent experiments. **significant p values, obtained with Student *t* test.

HSV-1 infection in MS patients, identifying the SHP1/2 intracellular inhibitory signaling.

2.2. KIR2DL2 expression on NK cells from MS patients influences cytokine expression

Since KIR2DL2 expression affects NK cell activation, we asked if cytokine production profile was also modified. We analyzed the levels of 12 different cytokines (Th1 (IFN-gamma, TNF-alpha, IL-2, IL-12, IL-13), Th17 (IL-17A, IL-6, G-CSF, TGF-beta) and Th2 (IL-4, IL-5, IL-10)) in the culture supernatants of NK cells co-cultured with autologous purified $CD3^+$ lymphocytes infected with HSV-1. We observed increase of Th1 cytokines (IFN-gamma, TNF-alpha, IL-2, IL-12, IL-13) in the culture supernatants of both MS patients and control subjects (Fig. 3a, b). Interestingly, MS patients presented also an increase in Th17 cytokines (IL-17A, IL-6, G-CSF, TGF-beta) ($p < 0.001$) (Fig. 3a). When we subdivided MS patients and control subjects in accordance with the KIR2DL2 expression, we observed that $KIR2DL2^-$ MS patients increased Th1 cytokines ($p < 0.001$) (Fig. 3c) while $KIR2DL2^+$ MS patients showed a prevalent expression of Th17 cytokines ($p < 0.0001$) (Fig. 3d). On the contrary, the co-culture of $KIR2DL2^+$ NK cells of control subjects with autologous $CD3^+$ cells infected with HSV-1 induced a slight increase in Th17 cytokines, that is not significantly different from the cytokine pattern observed in $KIR2DL2^-$ control subjects (Fig. 3e, f). To confirm the polarization of NK cells into secreting Th1 or Th17 cytokines, NK cells were analyzed by intra-cellular staining for IL-17 and IFN-gamma. IFN-gamma levels were higher in $KIR2DL2^-$ MS patients ($p = 0.028$), while $KIR2DL2^+$ MS patients showed a higher IL-17 expression ($p = 0.012$) (Fig. 4b). Interesting, the differences in cytokine secretion is not associated with a different distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cell subtypes. In particular, $KIR2DL2^-$ and $KIR2DL2^+$ MS patients did not present differences in the percentage of $CD56^{dim}$ ($18.23 \pm 7.15\%$ and $17.16 \pm 5.89\%$, respectively) and $CD56^{bright}$ NK cells (0.56% and 0.60%, respectively) ($p = 0.45$, $p = 0.36$, respectively; Student *t* test). When we co-cultured $KIR2DL2^+$ NK cells with autologous HSV-1-infected $CD3^+$ cells pre-treated with NSC87877, the SHP1/2 inhibitor, we observed a significant decrease in $IL\text{-}17^+$ NK cells ($p < 0.0001$; Fig. 4c).

Since $CD3^+$ lymphocyte might be a source of the cytokines as well, we evaluated intra-cellular staining for IL-17 and IFN-gamma. We observed no differences in IFN-gamma and IL-17 intracellular positivity in $CD3^+$ cells of $KIR2DL2^-$ and $KIR2DL2^+$ MS patients ($p = 0.54$, $p = 0.69$, respectively) (Fig. 4d).

3. Discussion

The highly variable clinical course of HSV-1 infections suggests that susceptibility host genes are involved in its control. We previously

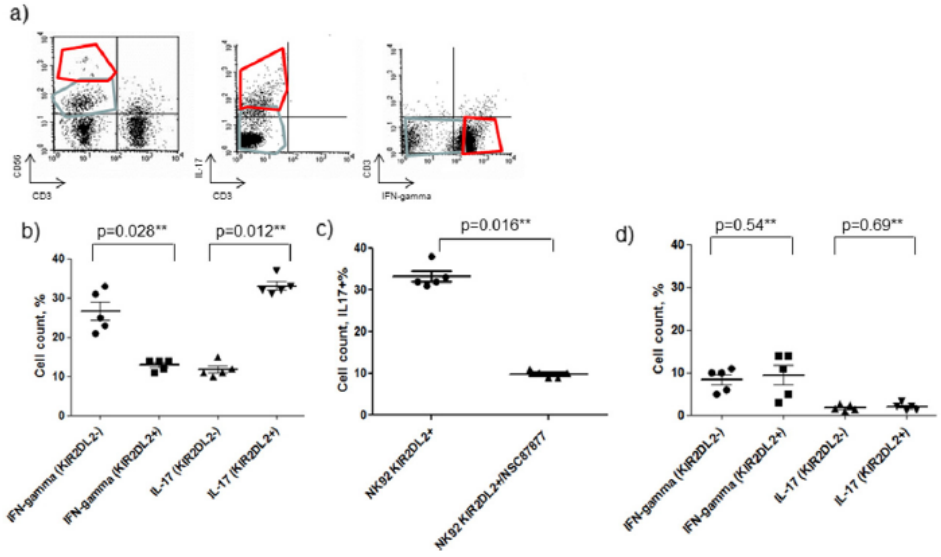

Fig. 4. a) Gating strategy for intra-cellular staining for IL-17 and IFN-gamma of $CD56^{dim}$ (blue gate) and $CD56^{bright}$ (red gate) NK cells. b) Percentage of IFN-gamma and IL-17A intracellular positive $KIR2DL2^+$ and $KIR2DL2^-$ NK cells in co-culture with HSV-1 infected lymphocytes. c) Percentage of IL-17A intracellular positive $KIR2DL2^+$ NK cells in co-culture with HSV-1 infected lymphocytes. The addition of NSC87877 (inhibitor of SHP1/2) was performed to evaluate the role of KIR2DL2 transduction pathway on IL-17A secretion. d) Percentage of IFN-gamma and IL-17A intracellular positive HSV-1 infected $CD3^+$ cells of $KIR2DL2^+$ and $KIR2DL2^-$ MS patients. Means ± SD are obtained from independent experiments. **significant p values, obtained with Student *t* test.

observed a reduced NK cell activation and HSV-1 clearance in MS patients with *KIR2DL2* gene (Rizzo et al., 2012), confirming previous data on the implication of *KIR2DL2* as a susceptible gene for HSV-1 infection (Estefania et al., 2007; Moraru et al., 2012).

In this study we wanted to identify the possible modification in cytokine expression profile that could be responsible for the implication of KIR2DL2 in HSV-1 infection control. In particular, we focused on MS population, where we observed the most significant effect of KIR2DL2 on HSV-1 susceptibility (Rizzo et al., 2012). We showed that KIR2DL2 expression in MS patients is enough to modify the ability of NK cells to counteract HSV-1 infection. In fact, the blockade of the SHP1–SHP2 signaling pathway of KIR2DL2 receptor is able to re-establish the NK cell activation towards HSV-1 infected cells, showing this inhibitory intracellular pathway at the basis of KIR2DL2 effect.

Our data suggest that KIR2DL2 and consequently SHP1–SHP2 signaling pathway make NK cells less efficient in HSV-1 infection control. On the contrary, the absence of KIR2DL2 or the blockage of SHP1–SHP2 signaling pathway seems to be protective against viral infections allowing to set up a defense against viral infection (Cassidy et al., 2015).

The analysis of the cytokine profile modifications due to KIR2DL2 expression on NK cells showed, for the first time, a peculiar cytokine secretion in $KIR2DL2^+$ MS patients. $KIR2DL2^+$ NK cells from MS patients secreted high levels of Th17 cytokines, mainly IL-17A during HSV-1 infection. On the contrary, $KIR2DL2^-$ NK cells from MS patients and NK cells from both $KIR2DL2^+$ and $KIR2DL2^-$ control subjects released Th1 cytokines, mainly IFN-gamma. Interesting, the differences in cytokine secretion in MS patients is not associated with a different distribution of $CD56^{bright}$ and $CD56^{dim}$ NK cell subtypes in $KIR2DL2^+$ and $KIR2DL2^-$ MS subjects. Moreover, the secretion of IFN-gamma and IL-17 by CD3+ cells from MS patients did not present any difference when we subdivided the patients in $KIR2DL2^+$ and $KIR2DL2^-$ subjects. These points sustain a peculiar implication of NK cells suggesting a possible role in MS pathogenesis, if we consider the detection of a NK17 subset in cerebrospinal fluid from MS patients (Pandya et al., 2011). It has been proposed that central nervous system (CNS)-resident NK cells are responsible for the control of CNS inflammation suppressing myelin-reactive Th17 cells (Hao et al., 2010). Moreover, IL-17 up-regulates anti-apoptotic molecules and, consequently, increases persistent infection by enhancing the survival of virus-infected cells and by blocking target cell destruction by cytotoxic T cells (Hou et al., 2009). The presence of a NK17 subtype could increase the effect of Th17-polarizing cytokines on disease status (Han et al., 2015) and viral infection persistence. We can hypothesize a similar effect in $KIR2DL2^+$ NK cells, where the secretion of Th17 cytokines and the maintenance of an inactive status in NK cells could facilitate the persistence of HSV-1 infected cells. We can speculate that the maintenance of an inactive status in $KIR2DL2^+$ NK cells is evident in MS patients and not in $KIR2DL2^+$ control subjects, who behave as $KIR2DL2^-$ subjects, because of the peculiar NK cell characteristics in MS disease (Kaur et al., 2013). Anecdotic observation supporting this concept is that in our very small cohort, $KIR2DL2^+$ MS patients were more active at MRI (9/10), as compared to and $KIR2DL2^-$ MS patients (2/10) over a 5 years follow-up. MS patients are characterized by increased levels of regulatory NK cells (Rodríguez-Martín et al., 2015) that control activated T cells (Jiang et al., 2011) via cytokine secretion with a beneficial effect for autoimmune responses and viral infections (Strowig et al., 2008; Williams et al., 2005). The increase in KIR2DL2 expression and the induction of SHP1–SHP2 signaling pathway could shift NK cell cytokine secretion towards a Th17 phenotype, with a possible implication on disease and HSV-1 infection. Indeed, we observed an increased in vivo susceptibility to HSV-1 infection in $KIR2DL2^+$ MS patients (Ben Fredj et al., 2014).

Altogether, these data provide novel insights into the biology of NK cells in relation with HSV-1 infection in MS disease, identifying peculiar modifications in cytokine secretion profile, and might lead to the design of NK cell-based approaches for intervention in viral infection onset and relapse in autoimmune disorders of the CNS.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2016.03.007.

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RESEARCH ARTICLE

Presence of HHV-6A in Endometrial Epithelial Cells from Women with Primary Unexplained Infertility

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Abstract

To elucidate the roles of human herpesvirus (HHV)-6 primary unexplained infertile women, a prospective randomized study was conducted on a cohort of primary unexplained infertile women and a cohort of control women, with at least one successful pregnancy. HHV-6 DNA was analyzed and the percentage and immune-phenotype of resident endometrial Natural Killer (NK) cells, as the first line of defense towards viral infections, was evaluated in endometrial biopsies. Cytokine levels in uterine flushing samples were analyzed. HHV-6A DNA was found in 43% of endometrial biopsies from primary unexplained infertile women, but not in control women. On the contrary, HHV-6B DNA was absent in endometrial biopsies, but present in PBMCs of both cohorts. Endometrial NK cells presented a different distribution in infertile women with HHV6-A infection compared with infertile women without HHV6-A infection. Notably, we observed a lower percentage of endometrial specific CD56brightCD16- NK cells. We observed an enhanced HHV-6A-specific endometrial NK cell response in HHV-6A positive infertile women, with a marked increase in the number of endometrial NK cells activating towards HHV-6A infected cells. The analysis of uterine flushing samples showed an increase in IL-10 levels and a decrease of IFN-gamma concentrations in infertile women with HHV6-A infection. Our study indicates, for the first time, that HHV-6A infection might be an important factor in female unexplained infertility development, with a possible role in modifying endometrial NK cells immune profile and ability to sustain a successful pregnancy.

Introduction

HHV-6 is an ubiquitous virus that was first discovered in 1986 [1]. It has been identified as the etiological agent of roseola infantum, and has been implicated (with various degrees) in a number of conditions such as liver disease [2], pneumonitis [3], myocarditis [4], multiple sclerosis [5], drug induced hypersensitivity syndrome [6, 7], the nodular sclerosis subset of Hodgkin's

lymphoma [8], and autoimmune diseases [9]. Since early times after HHV-6 discovery, the existence of the viral variants (HHV-6A and HHV-6B) was recognized [10]. Recently, HHV-6 variants have been recognized as different viral species, on the basis of specific biological, immunological, pathological and molecular characteristics [11].

Although both HHV-6 variants infect mainly T-cells it has wide tropism are important differences in cell tropism between HHV-6A and HHV-6B, HHV-6A but not HHV-6B reproduces in human neural stem cells [12], oligodendrocyte progenitor cells [13] and hepatocytes [14] while HHV-6B infection in astrocytes and hepatocytes result in abortive infection. HHV-6A but not HHV-6B can productively infect CD8+ T cells, natural killer cells and gamma/delta T cells. Some evidence suggests that HHV-6 can also infect and replicate in the human genital tract [15, 16]. In fact: HHV-6 DNA has been detected in genital tract secretions from pregnant and non-pregnant women [17–19]; several studies have reported low-level HHV-6 shedding from the genital tract in up to 25% of women [18–21], with pregnant women characterized by the highest prevalence of shedding [19]; HHV-6 DNA sequences and antigens have been detected in biopsies in archived cervical samples [22–26]. More specifically, the *in-vitro* HHV-6A infection of cervical carcinoma cell lines [27, 28] raises the possibility that the detection HHV-6 footprints reflect the ability of the virus to infect cervical cells, instead of being simply attributed to infected lymphocytes present in the tissue.

These data suggest that the female genital tract may be a secondary site for HHV-6 infection or persistence, although this needs to be confirmed. The possible pathogenic relevance for the genital presence of HHV-6 deserves careful evaluation.

In an attempt to elucidate this understudied aspect of HHV-6 biology, we analyzed the presence of HHV-6 infection in two cohorts of women with differing levels of fertility. Specifically, we studied the prevalence of HHV-6A and HHV-6B infection in the uterine flushing and endometrium biopsies of a randomized group of women with primary infertility group attending an infertility clinic in Italy and a cohort of fertile women. In addition, we assessed the possibility that HHV-6 infection might affect NK cells and cytokine secretion in the uterine environment.

Materials and Methods

Clinical Samples

Endometrial tissues were collected from patients that were recruited at admission for tubal patency assessment by Hystero-sono contrast sonography. Inclusion criteria for the study group were: 21–38 years old, regular menstrual cycle (24–35 days), body mass index (BMI) ranging between 18 and 26 Kg/m2, FSH (day 2–3 of the menstrual cycle) <10 mUI/mL, 17-β-Estradiol < 50 pg/ml (day 2–3 of the menstrual cycle), normal karyotype. Women with endometritis, endometriosis, tubal factor, ovulatory dysfunction, anatomical uterine pathologies and recurrent miscarriage were excluded. The stage of the menstrual cycle was categorized into secretory (days 14–28). Five women were enrolled for the collection of endometrial samples in three time points of the mestrual cycle: proliferative (days 5–14), ovulatory (days 11–21) and secretory (days 14–28) phases. Tissue samples collected in HEPES-buffered Dulbecco modified Eagle medium/ Hams F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA) with 1% antibiotic- antimycotic solution (final concentrations: 100 μg/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B; Invitrogen), and 5% newborn calf serum (NCS; CSL Ltd., Parkville, VIC, Australia), stored at 4°C, and processed within 2 hrs.

Ethics Statement

Informed written consent was obtained from each patient and ethics approval was obtained from the Ferrara Ethics Committee.

Preparation of Endometrial Epithelial and Stromal Cells

The endometrium was dissociated in Ca2+ and Mg2+ free phosphate buffered saline (PBS, pH 7.4) containing 300 μg/ml collagenase type III (Worthington Biochemical Corporation, Freehold, NJ) and 40 μg/ml deoxyribonuclease type I (Roche Diagnostics, Mannheim, Germany) in a shaking incubator (Bioline 4700; Edwards Instrument Company, Narellan, NSW, Australia) rotating at 150 rpm at 37°C [29]. At 15-min intervals, the digests were pipetted vigorously and dissociation was monitored microscopically. After 45 min, the cell suspensions were filtered using a 40-μm sieve (Becton Dickinson Labware, Franklin Lakes, NJ) to separate single cells from debris. Further dissociation of the filtrate was prevented by the addition of HEPES-buffered DMEM/F-12/5%FCS. To isolate mononuclear, stromal and epithelial cells we used Ficoll-Paque (Pharmacia Biotechnology, Uppsala, Sweden) and centrifuged for 8–10 min at 390 × *g*. Endometrial epithelial cells were removed from the Ficoll-Paque-medium interface by positive selection using BerEP4-coated magnetic Dynabeads (Dynal Biotech, Oslo, Norway).The beaded epithelial cells were recovered, washed several times in HEPES-buffered DMEM/F-12/1%FCS using a magnetic particle collector (Dynal Biotech) and seeded on basement membrane extract (BME) (Matrigel®, Collaborative Biomedical Products, Bedford, MA, USA) culture plates. The supernatant containing mononuclear and stromal cells were collected and seeded on 100 mm plastic tissue culture dishes. After 12hrs culture the supernatant cells were depleted from the culture, to eliminate mononuclear non adherent cells. Purity of epithelial and stromal components was assessed by morphological determination by light microscopy and reassessed by cytokeratin-18 (CK18) and vimentin staining for epithelial and stromal cells respectively. Each cell population was routinely over 98% purity.

DNA Analysis

DNA samples were extracted as described [30]. HHV-6 DNA presence and load were analyzed by PCR and real time quantitative (qPCR) specific for the U94 and U42 genes [30], and samples were considered positive when 1 μg of cell DNA harbored more than 100 copies of viral DNA. Amplification of the house-keeping human RNase P or beta-actin genes was used as a control. All clinical samples were analyzed in a randomized and blinded fashion. In addition, when there was enough material to repeat the analysis, the samples were tested again in a randomized and blinded fashion at a distant time from the first analyses. HHV-6 variant A or B identification was obtained by restriction enzyme digestion with HindIII enzyme of the U31 nested PCR amplification product, as reported previously [30]. Digestion products were then visualized on ethidium bromide stained agarose gel after electrophoresis migration.

Immunofluorescence Assay

Immunofluorescence for HHV-6 antigen expression was performed with a mouse monoclonal antibodies (mAb) directed against p41 and IE2 (early antigen) and glycoprotein gp116 (late antigen) of HHV-6 A and B (ABI, Columbia, MD, USA), as previously described [30]. Epithelial and stromal cells were respectively stained with mouse anti-Cytokeratin- 18, a heterotetramer cytoskeleton protein (CK18) and rabbit anti-vimentin, an intermediate filament of the cytoskeleton proteins moAbs (Abcam, Cambridge, UK), respectively. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole-dihydrochroride) (Sigma-Aldrich, S.Louis, MO, USA).

Peripheral Blood Mononuclear Cell Purification

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll gradient (Cederlane, Hornby, Ontario, Canada) and resuspended in RPMI medium (EuroClone,

Milano, Italy) with 10% FCS, 100U/ml penicillin and 100 U/ml streptomycin (Sigma-Aldrich, S.Louis, MO, USA).

NK Cell Purification

Peripheral and endometrial NK cells were purified from PBMC and endometrial samples, respectively, through negative magnetic cell separation (MACS) system (Miltenyi Biotech, Gladbach, Germany) [31]. As determined by flow cytometry with CD3-PerCp-Cy5.5, CD56-FITC moAbs (e-Bioscience, Frankfurt, DE), the procedure resulted in >90% pure NK cells (data non shown). Freshly isolated NK cells were cultured for 24 h in medium supplemented with suboptimal doses of IL-12 (1 ng/ml) [31].

HHV Cell Infection

HeLa cell line (ATCC CCL-2) was grown in DMEM F12 medium (Euroclone) supplemented with L-glutamine in presence of 1% penicillin-streptamycine and 10% of FCS at 37°C with the 5% of CO2. HeLa cell lines were infected with HHV-6A and HSV-1 to evaluate NK cell activation. We used HeLa cells as a good in vitro model of epithelial cells to perform and obtain HSV-1 and HHV-6A in vitro infection.

We used HHV-6A (strain U1102) cell free virus inocula [30] and infected with 10 genome equivalents per 1 cell. We used HSV-1 strain F at a multiplicity of infection of 0.1 PFU (plaque forming unit)/cell for 48 hrs [31]. We used HSV-1 and HHV-6A UV-inactivated viral preparations as controls.

Infected cells were then collected to perform co-culture experiment with NK cells.

Flow Cytometry

NK cells were defined as CD3-/CD56+ cells. CD158b levels were measured in the CD3-/CD56 + gated cells. Cell viability was assessed by propidium iodide staining. Anti-isotype controls (Exbio, Praha, CZ) were performed. For the CD107a degranulation assay, the cells were incubated 1 h at 37°C with anti-CD107a moAb and treated with Golgi Stop solution (Becton Dickinson, San Jose, CA, USA) for 3 hrs [31]. The CD107a mobilization assay was performed using HHV-6A infected or non-infected HeLa cells as target cells and NK cells from controls or patients as effector cells, with an effector:target ratio of 2:1. 10 ng/ml LPS (Calbiochem, La Jolla, Calif., USA), 25 μg/ml CpG-ODN (ODN-C, TIB MOLBIOL, Genova, Italy) and HSV-1 infected HeLa cells were used as positive control of NK activation. Degranulation was assessed in triplicate after 4 hours of co-culture by staining with PE-Cy5-conjugated anti-CD107a mAb (e-Bioscience, Frankfurt, DE) [31]. Ten thousand events were acquired.

Statistical Analysis

Statistical analysis was performed with Stat View (SAS Institute Inc). Biological data were analysed by Student T test because they presented a normal distribution (Kruskal Wallis test). Statistical significance was assumed for $p<0.05$ (two tailed).

Results

HHV-6 in Clinical Specimens

We enrolled 30 women with unexplained primary infertility characterized by the absence of previous pregnancies or pathological determinant, and 36 fertile women with at least one previous successful pregnancy. As reported in Table 1, no significant differences were present between the two cohorts, except a slight increase in estradiol levels in infertile patients

Table 1. Women cohorts: demographical and clinical parameters.

Parameters (medain; mean±SD)	Infertile (30)	Control (36)	p value*
Age (yrs)	34.3(31.2±3.2)	34.4(32.5±3.1)	0.98
Duration of infertility (yrs)	3.0(2.7±2.1)	2.1(2.2±1.6)	0.66
Length of menstrual cycle (days)	4.1(3.6±2.4)	4.2(3.9±2.1)	0.45
FSH (mUI/mL) (day 3)	8.7(8.3±2.1)	7.2(7.1±3.1)	0.43
LH (mUI/mL) (day 3)	7.1(6.9±2.1)	6.9(6.5±2.)	0.43
Estradiol (pg/mL) (day 3)	79.9(75.4±65.2)	66.3(56.2±45.2)	**0.05****
TSH (uUI/mL)	3.0(2.9±1.2)	2.5(2.4±2.1)	0.46
FT4 (pg/mL)	2.3(2.1±1.4)	2.5(2.2±1.5)	0.89
Progesterone (pg/mL) (day 21)	16.3(14.6±5.7)	14.0(13.6±6.7)	0.56
Smoke habits (%)	20	19.3	0.88
Day (mestrual cycle) of sample collection	14.1(13.9±1.9)	13.8(13.2±1.8)	0.84

*Student T test

** Significant p value

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(p = 0.05; Student T test). High estradiol levels could indicate that this hormone is artificially suppressing FSH levels and there could be fertility problems. No differences in progesterone levels were found.

All clinical samples were analyzed for the presence of HHV-6 infection and the results are reported in Table 2. We observed the presence of HHV-6B DNA in the 25% and 28% of the PBMC of women with primary infertility and control women, respectively (p = 0.75; Fisher exact test). These results are in agreement with literature data [32], that reported the presence of the HHV-6B variant in a 25–30% of peripheral blood samples. HHV-6A DNA was not detected in PBMC of all the subjects. Surprisingly, the 43% (13/30) of the endometrial epithelial cells from women with primary infertility were positive for HHV-6A DNA, while the cohort of control women did not present HHV-6A viral DNA in their endometrial epithelial cells ($p = 5.8x10^{-6}$; Fisher exact test). HHV-6B DNA was not present in endometrial epithelial cells from either group.

Endometrial epithelial cells from HHV6-A positive primary infertile women showed an average viral load of 450.000 copies/ug of cellular DNA (range 670.000–250.000 copies/ug DNA), corresponding to about 4 copies of viral DNA per diploid cell (Fig 1A). Also stromal cells obtained from the purification of epithelial cells were analyzed for HHV-6 presence and,

Table 2. HHV-6 DNA results in peripheral blood mononuclear cells (PBMC) and endometrial biopsies.

Samples (N)	Infertile (30)	Control (36)	p value*
HHV6-A			
Endometrial epithelium	13	0	**$5.8x10^{-6}$****
Endometrial stroma	0	0	NA
PBMC	0	0	NA
HHV-6B			
Endometrial epithelium	0	0	NA
Endometrial stroma	0	0	NA
PBMC	8	10	0,75

*Student T test

** Significant p value

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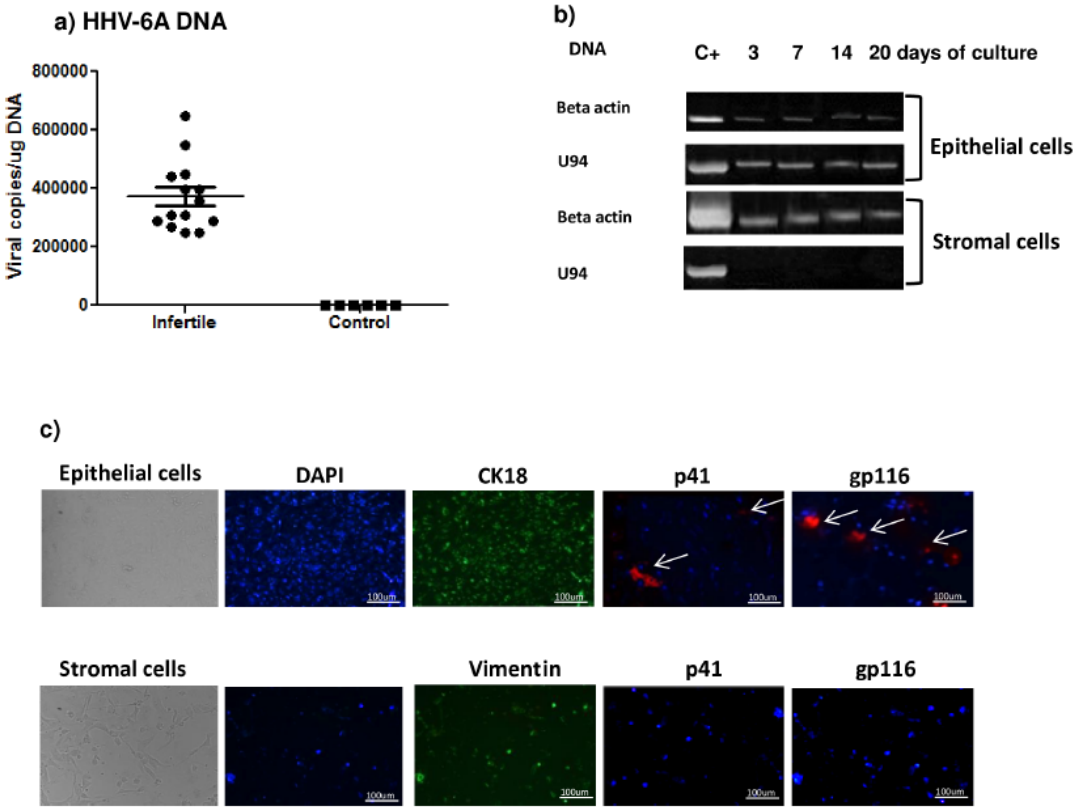

Fig 1. HHV-6 analysis in endometrial biopsies. a) HHV-6 DNA was searched by real time qPCR specific for U42 gene in endometrial biopsies. Results are expressed in viral copies/ug DNA and represent the mean copy number ± SD referred to duplicates of 2 independent assays. Infertile: primary unexplained infertile women; Control: women with at least one successful pregnancy. b) Cell fractions derived by immunomagnetic separation were characterized by PCR specific for HHV-6A U94 DNA after 3, 7, 14 and 20 days of culture. C+: HHV-6A infected J-Jahn cells. c) HHV-6A p41 (early protein) and gp116 (late protein) immunofluorescence (PE) (white arrow) in epithelial and stroma cells from endometrial biopsies of primary infertile women. CK18 and vimentin staining was used to confirm epithelial and stromal cell types, respectively. DAPI staining indicated DNA. Images were taken in bright field (*left panels*) or fluorescence (*right panels*) (Nikon Eclipse TE2000S) equipped with a digital camera. Original magnification 100×.

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interestingly, no HHV-6 DNA was detected (Table 2; Fig 1B), supporting a localized site of infection for HHV-6A into endometrial epithelium of primary unexplained infertile women.

To be sure of the specific HHV-6A infection in endometrial epithelial cells, we cultured both epithelial and stromal cells and evaluated the presence of HHV-6A proteins. The use of CK18 and vimentin staining in immune-fluorescence confirmed the purification of epithelial cells from stromal cells respectively. We observed the expression of HHV-6A p41 (early) and gp116 (late) proteins after 14 days of culture in approximately 15% of the explanted epithelial cells (Fig 1C).

Since we performed all the experiments on endometrial cells derived from the secretory phase of the menstrual cycle, we analyzed HHV-6A protein expression by endometrial cells

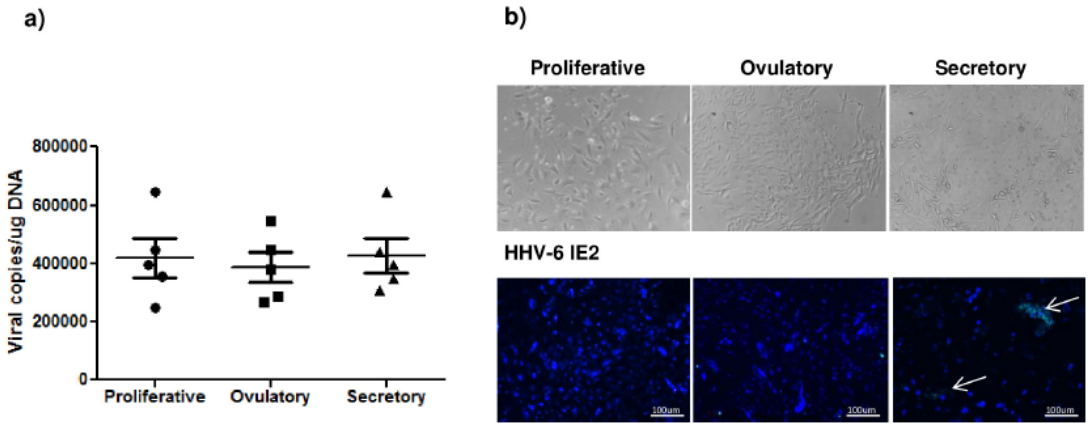

Fig 2. HHV-6A infection and menstrual cycle phases. a) HHV-6 DNA was searched by real time qPCR specific for U42 gene in endometrial biopsies of infertile women during proliferative, ovulatory and secretory phases. Results are expressed in viral copies/ug DNA and represent the mean copy number ± SD referred to duplicates of 2 independent assays. b) HHV-6A IE2 (early protein) immunofluorescence (FITC) (white arrow) in epithelial cells from endometrial biopsies of infertile women during proliferative, ovulatory and secretory phases. DAPI staining indicated DNA. Images were taken in bright field (*left panels*) or fluorescence (*right panels*) (Nikon Eclipse TE2000S) equipped with a digital camera. Original magnification 100×.

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also from proliferative and ovulatory phases. As reported in Fig 2A, we observed HHV-6A DNA in all three menstrual cycle phases while the expression of HHV-6A IE2 early protein is evident only in the secretory phase (Fig 2B), suggesting a sub-clinical reactivation of HHV-6A latent infection during this specific menstrual cycle phase.

HHV-6A Modifies Endometrial eNK Cell Immune-Phenotype and Uterine Cytokine Environment

Due to the high prevalence of HHV-6A active infection in a percentage of infertile women, we searched for the presence of differences in clinical and immunological parameters, subdividing infertile women on the basis of the presence/absence of HHV-6A infection (**Tables 3 and 4**). Estradiol levels are higher in infertile women with HHV-6A infection in comparison with those HHV-6A negative ($p = 0.045$) (Table 3). The previous identification of a correlation between estrogen and herpes simplex virus (HSV) reactivation from latency [33] suggests a possible implication of estradiol high levels in HHV-6A infection. In fact, we observed a correlation between the levels of estradiol and the presence of HHV-6A infection ($p = 0.02$; r^2: 0.84; Spearman correlation). The other clinical parameters did not present differences in the two cohorts (Table 3). When we considered the immunological characteristics, we observed a difference in endometrial (e)NK cell immune-phenotype and cytokine levels in the uterine environment. We found a lower $CD56^{pos}CD16^{neg}$ eNK cell number in HHV-6A positive infertile women (65.1 ± 12.1) in comparison with HHV-6A negative infertile women (192.1 ± 28.4) ($p = 0.001$) (Table 4). The distribution of $CD56^{bright}$ and $CD56^{dim}$ eNK cells was different between HHV-6A positive and negative infertile women, with lower $CD56^{bright}$ and $CD56^{dim}$ eNK cells number in HHV-6A positive infertile women (30.0± 15.1; 35.8 ± 10.2) in comparison with HHV-6A negative infertile women (110.3 ± 2.1; 82.1 ± 6.4) ($p = 0.001$) (Table 4). $CD3^+$ lymphocytes were not detected in both cohorts, while CD14+ monocytes presented no differences in cell number ($p = 0.51$). The uterine flushing levels of cytokines showed a different

Table 3. Infertile women subdivided on the basis of the presence/absence of HHV-6A infection. Demographical and clinical parameters of infertile and control women.

Parameters (median;mean±SD)	HHV-6A positive	HHV-6 negative	p value*	Control (36)
Age (yrs)	34.3(32.3±2.3)	34.1(33.1±1.5)	0.97	34.4(32.5±3.1)
Duration of infertility (yrs)	3.3(3.1±1.2)	2.1(2.3±2.1)	0.56	2.1(2.2±1.6)
Length of menstrual cycle (days)	3.9(3.8±2.1)	4.4(4.1±1.3)	0.45	4.2(3.9±2.1)
FSH (mUI/mL) (day 3)	9.7(8.9±2.1)	7.0(7.0±3.5)	0.32	7.2(7.1±3.1)
LH (mUI/mL) (day 3)	7.5(7.1±1.3)	6.3(6.1±2.1)	0.45	6.9(6.5±2.)
Estradiol (pg/mL) (day 3)	103.9(99.2±45.2)	72.3(69.6±39.4)	**0.045****	66.3(56.2±45.2)
TSH (uUI/mL)	3.2(2.6±2.8)	2.5(2.3±1.4)	0.76	2.5(2.4±2.1)
FT4 (pg/mL)	2.7(2.1±2.5)	2.4(2.2±2.8)	0.89	2.5(2.2±1.5)
Progesterone (pg/mL) (day 21)	17.7(15.2±3.1)	12.0(11.1±4.5)	0.34	14.0(13.6±6.7)
Smoke habits (%)	22	21.3	0.86	19.3
Day (mestrual cycle) of sample collection	14.0(13.1±2.1)	12.9(12.3±1.3)	0.85	13.8(13.2±1.8)

*Student T test

** Significant p value

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pattern in HHV-6A positive and negative infertile women. We observed a significant increase in IL-10 and a decrease in IFN-gamma levels in HHV-6A positive in comparison with HHV-6A negative infertile women (p = 0.014, p = 0.012, respectively) (Table 4). The other cytokines, TNF-alpha, IL-12 and IL-22, were similarly expressed in the uterine flushing samples of the two cohorts (Table 4). When we compared infertile and control women, we observed no differences in clinical parameters except for estradiol levels, that are higher in both HHV-6A negative and positive infertile women in comparison with controls (p = 0.042; 0.046, respectively) (Table 3). We found a similar frequency of eNK cell subtypes and cytokine levels with HHV-6A negative infertile women and controls (Table 4), while HHV-6A positive infertile women presented lower $CD56^{bright}$ and $CD56^{dim}$ eNK cell number, higher IL-10 and lower IFN-gamma levels in uterine flushing samples (Table 4). Peripheral blood NK cell subtypes and cytokine levels did not differ between the three cohorts (S1 Table).

Table 4. Infertile women subdivided on the basis of the presence/absence of HHV-6A infection. Immunological parameters in endometrial samples.

Immune cells (mean±SD)	HHV-6A positive	HHV-6 negative	p value*	Control
NK $CD56^{pos}CD16^{neg}$ (N)	65.1 ± 12.1	192.1 ± 28.4	**0.001****	199.8 ± 21.3
NK $CD56^{bright}CD16^{neg}$ (N)	30.0 ± 15.1	110.3 ± 2.1	**0.001****	120.1 ± 15.8
NK $CD56^{dim}CD16^{-}$ (N)	35.8 ± 10.2	82.1 ± 6.4	**0.02****	79.6 ± 5.9
$CD3^{+}$ (N)	0	0	NA	0
$CD14^{+}$ (N)	3.7 ± 4.8	4.2 ± 5.1	0.51	3.9 ± 5.7
Cytokines(median)	**HHV-6A positive**	**HHV-6 negative**	**p value***	**Control**
IL-10 (pg/ml)	311.9	171.4	**0.014****	151.4
IFN-gamma (pg/ml)	65.0	221.5	**0.012****	214.4
TNF-alpha (pg/ml)	7.3	5.6	0.31	6.5
IL-22 (pg/ml)	46.9	33.9	0.22	39.8
IL-12 (pg/ml)	210.6	194.4	0.37	198.7

*Student T test

** Significant p value

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HHV-6A Modifies Endometrial NK Cell Functions

Immunological memory is classically regarded as an attribute of antigen-specific T and B lymphocytes of the adaptive immune system. Cells of the innate immune system, including NK cells, are assumed to be short-lived cytolytic cells that can rapidly respond against pathogens in an antigen-independent manner and then die off. However, NK cells have recently been described to possess traits of adaptive immunity, such as clonal expansion after viral antigen exposure to generate long-lived memory cells [34]. We evaluated the possible differences between eNK cells response to HHV-6A infection in HHV-6A positive and negative infertile women. As a challenge, we used HeLa, human epithelioid cervix carcinoma, infected with HHV-6A laboratory strain. We observed the expression of HHV-6 proteins on the surface of HeLa cells 3 days post infection (Fig 3A). eNK cells purified from endometrial biopsies were challenged with HHV-6A HeLa infected cells and the activation status was evaluated. We observed the highest activation in eNK cells from HHV-6A positive infertile women toward HHV-6A HeLa infected cells (Fig 3B). On the contrary, eNK cells from HHV-6A negative infertile women presented a lower activation towards HHV-6A HeLa infected cells (Fig 3B), similar to eNK cells from women with a previous successful pregnancy (Fig 3B). The same eNK cells were challenged with other stimuli: LPS, CpG-ODN and HSV-1 HeLa infected cells. LPS and CpG-ODN stimulation induced a similar activation in eNK cells from the three cohorts (Fig 3C). HSV-1 HeLa infected cells induced a slight increase in CD107a expression in all the three cohorts (Fig 3C).

Similarly, peripheral blood pNK cells from the three cohorts were challenged with HHV-6A HeLa infected cells, LPS, CpG-ODN and HSV-1 HeLa infected cells. We observed no activation towards HHV-6A HeLa infected cells (Fig 3D) and a slight and comparable increase in the activation of pNK cells from the three cohorts (Fig 3E). UV-inactivated viral preparations induced no eNK and pNK cell activation in all the three cohorts (S1A and S1B Fig).

Discussion

Viral infections have been considered as possible environmental factors in human infertility [35]. In particular, herpesviruses have been implicated in male infertility [36], but no specific virus has yet been conclusively identified as associated with female infertility.

In our report, 43% of endometrial epithelial cells from women with unexplained infertility were found positive for HHV-6A DNA, whereas no control women (with at least one previous successful pregnancy) harbored the virus. Furthermore, endometrial epithelial cells from women with unexplained infertility harbored significant viral loads (approximately 4 copies of viral DNA per cell), but no HHV-6A infection was detected in stromal cells and PBMC, excluding the presence of chromosomally integrated HHV-6 DNA [37]. Interestingly, the percentage of HHV-6B positive PBMC in both cohorts was similar to the common population [32]. These observation strengthen the notion that HHV-6A and B have different biological behavior and pathological associations [11]. This different behavior could reflect the fact that HHV-6 variants use different cell receptors. In fact, HHV-6A uses CD46, an ubiquitous molecule present on all cell types [38], while HHV-6B uses CD134, that is expressed mainly on activated Treg T lymphocytes [39]. Furthermore, our results indicate that the uterus of infertile women may constitute a site of active HHV-6A infection/replication. Considering that these women have no HHV-6A in their PBMC, unidentified micro-environmental factors are probably required to allow HHV-6A infection of the uterine epithelial cells. Possibly, the estradiol high levels found in infertile women could act as a co-factor allowing HHV-6 infection of endometrium [33]. Permissiveness to HHV-6A infection of uterine epithelial cells was confirmed by our *ex vivo* experiments showing HHV-6A protein expression during the secretory

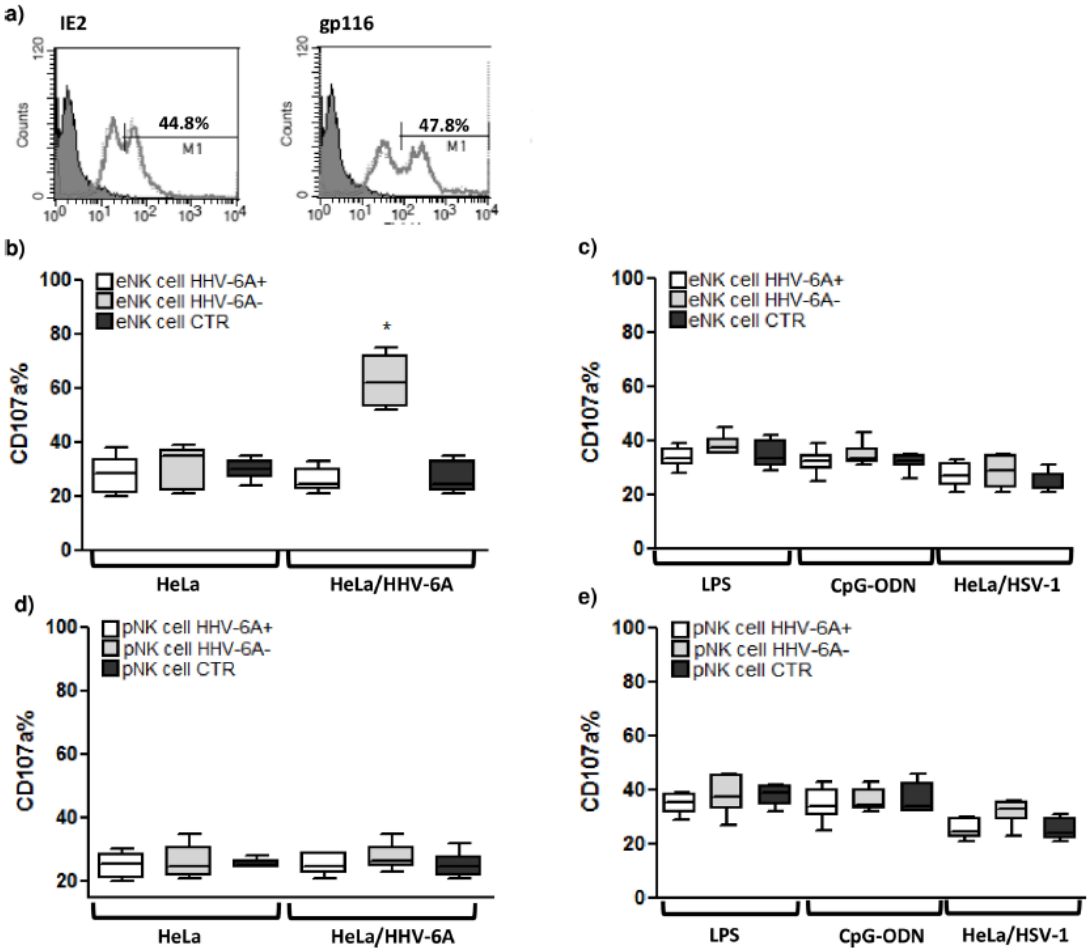

Fig 3. NK cell status. a) HeLa cells were infected with HHV-6A and virus antigen expression was analyzed by flow cytometry using a mouse mAb directed against HHV-6 IE2 early and gp116 late antigens. HeLa HHV-6A infected cells were co-cultured for 4 hours with NK cells, purified from endometrial biopsies and peripheral blood. NK cell activation status was evaluated after CD107a staining by flow cytometry. Percentage of CD107a NK cells are reported after co-culture with: b, d) HHV-6A infected HeLa cells; c, e) LPS, CpG-ODN or HSV-1 infected HeLa cells. Results are expressed in percentage and represent the mean copy number ± SD referred to duplicates of 2 independent assays. eNK cells: endometrial NK cells; pNK cells: peripheral NK cells; HHV-6A+: HHV-6A positive infertile women; HHV-6A-: HHV-6A negative infertile women; CTR: women with a previous successful pregnancy. * significant p value obtained by Student T test.

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menstrual cycle phase, suggesting a hormonal implication in HHV-6A sub-clinical reactivation. It is known that steroids cause HHV-6 to replicate disproportionately [40] and reactivate HHV-6 in transplant patients [41]. Here we provide evidence indicating that HHV-6A may induce a modification in eNK cell immune-phenotype and cytokine levels. We observed a decrease in $CD56^{bright}CD16^{neg}$ eNK cells in HHV-6A positive infertile women, an increase of

Th2 IL-10 cytokine and a decrease of Th1 IFN-gamma cytokine, with an increase of the Th1/Th2 ratio. These results are in agreement with the correlation of Th1/Th2 ratio increase and female infertility condition [42]. Moreover, it is known that HHV-6 infection increases IL-10 expression by monocytes [43] and reduces the release of IFN-gamma [44] by T lymphocytes. Interesting, HHV-6A negative infertile women presented a similar distribution of eNK cell subtypes and cytokine levels in uterine flushing samples in comparison with control women. These data suggest the presence of different immunological components implicated in the infertile condition of the two cohorts of HHV-6A positive and negative women with unexplained infertility. Intriguingly, enhanced HHV-6A-specific eNK cell responses were observed in HHV-6A positive women with unexplained infertility, with a marked increase in the number of eNK cells activating towards HHV-6A infected cells. On the contrary, eNK cells have a similar activation pattern to other stimuli (LPS, CpG-ODN, HSV-1 infection) in comparison with eNK cells from HHV-6A negative women with unexplained infertility and control women. Peripheral blood NK cells from all the three cohorts present no activation towards HHV-6A infected cells and a slight but comparable activation towards LPS, CpG-ODN and HSV-1 infection. These findings are consistent with an abnormal, probably persistent, immune response towards HHV-6A antigens in a subgroup of women with unexplained infertility, possibly favored by the local cytokine and eNK cell environment induced by HHV-6A infection. These HHV-6A-specific responses elicit an over-activation of the eNK cell compartment, as suggested by the increased responses of eNK cells to HHV-6A infection and a different immune-phenotype of eNK cells. In fact, we observed a decrease in $CD56^{bright}CD16^{neg}$ eNK cells in HHV-6A positive infertile women, that modifies the eNK cell subset at the uterine environment. These results are in agreement with our previous data on HHV-6 infection in Hashimoto thyroiditis patients, where we observed an increased sensitization of NK cells to this virus [30].

Interestingly, previous observations support our results on the possible adverse effect of HHV-6A on pregnancy: i) Gervasi and coauthors [45] observed the presence of HHV-6 infection in amniotic fluid of a patient that developed gestational hypertension at term and of a patient who delivered at 33 weeks for premature rupture of membranes; ii) Revest and coauthors [46] reported a maternal–fetal HHV-6 infection leading to abortion following HHV-6 seroconversion during pregnancy; iii) Ando and coauthors [47] and Drago and coauthors [48] found a correlation between miscarriages and increased HHV-6 antibody levels and reactivation; Gibson and coauthors [49] observed the association of HHV-6 (and HHV-7, Varicella zoster) newborn infection with preterm pregnancies and pregnancy-induced hypertensive disorders.

However, further studies are required to confirm the association of HHV-6A infection as a trigger of female primary unexplained infertility. Indeed, there are several potential mechanisms by which HHV-6 might induce female infertility. Viral infections might trigger eNK cell functional modifications that could also induce aberrant expression of cytokines thereby promoting a dysfunctional uterine environment.

Overall, our study indicates that HHV-6A infection might be an important factor in female primary unexplained infertility.

Supporting Information

S1 Fig. UV-inactivated viral preparations effect on NK cell status. HeLa cells were infected with UV-inactivated HHV-6A or HSV-1 and co-cultured for 4 hours with NK cells, purified from endometrial biopsies and peripheral blood. NK cell activation status was evaluated after CD107a staining by flow cytometry. After co-culture with UV-inactivated HHV-6A (HeLa/

HHV-6A UV) or UV-inactivated HSV-1 (HeLa/HSV-1 UV) infected HeLa cells, we evaluated the percentage of CD107a positive a) endometrial (e)NK and b) peripheral (p)NK cells. Results are expressed in percentage and represent the mean copy number ± SD referred to duplicates of 2 independent assays. eNK cells: endometrial NK cells; pNK cells: peripheral NK cells; HHV-6A-: HHV-6A negative infertile women; CTR: women with a previous successful pregnancy.
(TIF)

S1 Table. Immunological parameters of peripheral blood samples.
(DOCX)

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Author Contributions

Conceived and designed the experiments: RR RM DDL. Performed the experiments: VG DB EC SB. Analyzed the data: AR RR. Contributed reagents/materials/analysis tools: GLM. Wrote the paper: RR RM DDL.

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Research Article

Study of Soluble HLA-G in Congenital Human Cytomegalovirus Infection

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Human leukocyte antigen-G (HLA-G) is a nonclassical HLA class I antigen that is expressed during pregnancy contributing to maternal-fetal tolerance. HLA-G can be expressed as membrane-bound and soluble forms. HLA-G expression increases strongly during viral infections such as congenital human cytomegalovirus (HCMV) infections, with functional consequences in immunoregulation. In this work we investigated the expression of soluble (s)HLA-G and beta-2 microglobulin (component of HLA) molecules in correlation with the risk of transmission and severity of congenital HCMV infection. We analyzed 182 blood samples from 130 pregnant women and 52 nonpregnant women and 56 amniotic fluid samples from women experiencing primary HCMV infection. The median levels of sHLA-G in maternal serum of women with primary HCMV infection were higher in comparison with nonprimary and uninfected pregnant women ($p < 0.001$). AF from HCMV symptomatic fetuses presented higher sHLA-G levels in comparison with infected asymptomatic fetuses ($p < 0.001$), presence of HLA-G free-heavy chain, and a concentration gradient from amniotic fluid to maternal blood. No significant statistical difference of beta-2 microglobulin median levels was observed between all different groups. Our results suggest the determination of sHLA-G molecules in both maternal blood and amniotic fluid as a promising biomarker of diagnosis of maternal HCMV primary infection and fetal HCMV disease.

1. Introduction

Human cytomegalovirus (HCMV) is the most common cause of intrauterine infection, occurring in 0.3% to 2.3% of births [1]. HCMV intrauterine transmission is more common after primary infection (30–40% of probability) than after nonprimary infection (1%) [2, 3]. Nevertheless, it was estimated that, for all population seroprevalences, nonprimary maternal infections are responsible for the majority of congenital CMV infections [4].

Ten to fifteen percent of congenitally infected infants of primarily infected women will have symptoms at birth and around 10% of them will not survive. Moreover, 70–80% of surviving babies will suffer delayed sequelae such as sensorineural hearing loss, delay of psychomotor development, and visual impairment [5]. Most congenital infected infants

(85–90%) have no symptoms at birth, but 8% to 15% of them will develop delayed injury [3, 5].

The fetal compartment can be studied by invasive (amniocentesis) and noninvasive (ultrasound examination) techniques [6]. Ultrasonographic findings are helpful but not diagnostic findings since HCMV has features in common with other intrauterine infections and its sensitivity is poor [7]. HCMV detection in amniotic fluid with virus isolation and/or Real-Time PCR is useful for prenatal diagnosis of fetal infection, due to its high sensitivity and specificity [8–10]. There is still a need for reliable prognostic factors for the outcome of HCMV fetal infection.

HCMV can modulate the expression and/or function of human leukocyte antigens (HLA), by encoding proteins to detain and destroy the expression of HLA molecules on the surface of infected cells, or selectively upregulate specific HLA class I molecules binding to immune cell inhibitory receptors [11]. In this scenario, there is an interesting nonclassical HLA class I antigen, HLA-G, characterized by low allelic polymorphism, restricted tissue distribution, and alternative mRNA splicing which creates different isoforms, 4 membrane-bound (HLA-G1–G4) and 3 soluble (HLA-G5–G7) [12]. In addition, the HLA-G1 isoform can produce a soluble form called sHLA-G1, derived from membrane proteolytic shedding [13]. HLA-G is expressed at the maternal-fetal interface, on surface of trophoblasts [12], and the concentrations of soluble (s)HLA-G increase in the plasma samples of pregnant women during the first trimester of pregnancy [14]. HCMV infection modifies HLA-G expression in tissues and immune cells, with a downmodulation in infected cytotrophoblasts [15] and upregulation in infected peripheral blood cells [16]. Specific HCMV proteins modify HLA-G expression interacting with the HLA-G promoter, and affecting mRNA stability, protein translation, and the secretory pathway [17–19]. The increase in HLA-G expression is suggested as a mechanism for virus immune escape, due to the immune-inhibitory functions of HLA-G. Finally, it has been observed that another important component of HLA class I molecules, beta-2 microglobulin (b2M), has a diagnostic efficacy for differentiating symptomatic from asymptomatic HCMV congenital infection [20].

In order to explore the possible role of HLA-G molecules in congenital HCMV infection, we analyzed maternal and fetal sHLA-G and b2M expression in correlation with the risk of transmission and severity of HCMV infection.

2. Materials and Methods

2.1. Subjects. The study analyzed the serum samples of a cohort of 130 pregnant women who were referred to the Maternal-Fetal Medicine Unit, St. Orsola-Malpighi University Hospital, Bologna, between 2006 and 2011 for suspected primary maternal HCMV infection. Maternal primary HCMV infection was assessed at the Virology Unit of the same University Hospital. Written informed consent for the studies was obtained from all patients according to the protocol approved by the Scientific Ethical Committee of the Ferrara and Bologna Universities.

The women, aged between 18 and 40 years, were in the first or second trimester of pregnancy. They presented no previous autoimmune and inflammatory diseases and they were not on any anti-inflammatory or immune-modulatory drugs or hyperimmune globulin.

Primary infection was diagnosed based on clinical and laboratory history and HCMV IgM-positive and low/moderate HCMV IgG avidity results as well as positive DNAemia and/or seroconversion for HCMV. Nonprimary maternal HCMV infection was diagnosed, within the first 16 weeks of gestation, according to blot-confirmed IgM-positivity with high avidity anti-HCMV IgG and presence of DNA-HCMV in blood and/or urine and/or saliva.

HCMV-seronegative women (for both IgG and IgM) were defined as uninfected.

Fifty-two nonpregnant women with HCMV past infection (IgG positive and IgM negative) were recruited as healthy controls.

Moreover, 56 amniotic fluid samples were collected during amniocentesis (20-21 weeks of gestation) from those pregnant women with primary HCMV infection arising before the 14th week of gestation; HCMV detection on amniotic fluid samples was performed with virus isolation and real-time PCR.

2.2. Diagnosis of Congenital HCMV Infection in Fetuses and Infants. Infection status of the aborted fetuses was classified on the basis of histological and immunohistochemical tissue examination, whereas the infection status of infants was classified on the basis of viral isolation and real-time PCR from urine within the first 2 weeks of life.

Fetal symptomatic infection was defined as the presence of ultrasound abnormalities and histological and immunohistochemical findings in fetal organs with particular attention to the brain [21]. CMV disease in infected newborns was investigated through clinical, instrumental, and laboratory examination in the neonatal and subsequently monitored up to 6 years of age [22].

2.3. Anti-HCMV IgM and IgG Detection and IgG Avidity. Maternal serum samples were tested using the Enzygnost® HCMV IgM and Enzygnost HCMV IgG assays (Siemens Healthcare Diagnostics) and an in-house immunoblot for detection of HCMV-specific IgM [23]. HCMV IgG avidity was tested with the Radim® Cytomegalovirus IgG Avidity EIA WELL assay (Radim).

2.4. Virological Examinations. HCMV isolation from amniotic fluid was performed by shell-vial procedure as described elsewhere [24]. DNA was extracted from amniotic fluid and saliva with the NucliSens easyMAG System (bioMerieux) and from blood and urine with the QIAsymphony SP/AS System (QIAGEN).

HCMV-DNA was quantified with a real-time PCR assay (HCMV ELITe MGB kit, ELITechGroup) using the ELITe MGB technology. Amplification, detection, and analysis were performed with the ABI PRISM 7300 platform (PE Applied Biosystems). The detection limit was 11 copies/reaction and

viral load was reported as number of copies/mL for all body fluids examined.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) for Soluble HLA-G. sHLA-G levels in serum and amniotic fluid samples were assayed in triplicate as previously reported [25, 26] using the monoclonal antibody (MoAb) MEM-G9 (Exbio), which recognizes the HLA-G molecules, in b2M associated form. The intra-assay coefficient of variation (CV) was 1.4% and the interassay CV was 4.0%. The limit of sensitivity was 1.0 ng/mL.

2.6. ELISA for Soluble Beta-2 Microglobulin and Albumin. b2M concentration was determined in triplicate using a commercial human beta-2 microglobulin ELISA Kit (Abcam) with a detection limit <6 pg/mL.

Albumin concentration was determined in triplicate with a 1 : 200 dilution using the commercial human albumin ELISA Kit (Alpha Diagnostic International) with intra-assay CV of 6.8 to 11.4% and interassay CV of 3.5 to 6.4%.

2.7. Determination of sHLA-G Index. Fetal production of HLA-G was calculated with the following formula [27]:

$$\text{sHLA-G Index} = \frac{\text{amniotic fluid : serum sHLA-G}}{\text{amniotic fluid : serum albumin}}, \quad (1)$$

where the ratio between amniotic fluid and serum albumin concentrations represents the status of placental barrier.

2.8. Western Blot Analysis. Serum samples and amniotic fluids were biotinylated with 0.2 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in pH 8.0 PBS 1x for 30 min at 4°C [26]. Samples were then immunoprecipitated for 2 hrs at room temperature with anti-HLA-G MoAb (MEM-G1, specific for HLA-G free-heavy chain, or MEM-G9, specific for b2M conjugated HLA-G, Exbio), washed twice in PBS 1x, and incubated overnight with protein G-Sepharose beads (Santa Cruz) at 4°C. The samples were washed twice and resuspended in 20 μL Laemmli Buffer (BioRad). We quantified protein concentration in immunoprecipitates by the Bradford assay (Bio-Rad Laboratories) using bovine albumin (Sigma-Aldrich) as standard. Total protein was denatured at 100°C for 5 min. Proteins were loaded with native or reducing buffers in 10% TGX-Precast gel (Biorad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore) [28]. The membrane was incubated with a horseradish peroxidase- (HRP-) conjugated antimouse antibody (1 : 5000; Amersham Biosciences) and developed with the ECL kit (Amersham Biosciences). The images were acquired by Geliance 600 (Perkin Elmer).

2.9. Statistics. Statistical analysis was performed with Stat View software package (SAS Institute Inc). Given that the data, screened by Kolmogorov-Smirnov test, presented a normal distribution, statistical analyses were performed using Student's t-test. Frequencies of positive samples for a specific variable were compared by Fisher exact test. A logistic regression analysis was performed to evaluate the effect of different variables. The relationship between sHLA-G presence and HCMV infection status was investigated by the Receiver Operating Characteristic (ROC) curve analysis (JROCFIT software, John Hopkins University).

3. Results

3.1. sHLA-G and Beta-2 Microglobulin Levels in Maternal Serum Samples. We evaluated sHLA-G and b2M levels in sera of 130 pregnant women, 30 uninfected, 56 with primary HCMV infection, and 44 with nonprimary HCMV infection, and 52 nonpregnant women with HCMV past infection.

Detectable serum levels of sHLA-G were significantly more frequent in pregnant (130/130; 100%) than in nonpregnant women (31/52; 59.6%) ($p < 0.0001$). b2M molecules presented only slightly higher positive samples in pregnant women (65/100; 50%) than in nonpregnant women (20/52; 38.5%) ($p = 0.14$) (data not shown).

Regarding the median levels of sHLA-G, pregnant women showed higher levels of molecules in comparison with nonpregnant women (49 versus 21 ng/mL; range: 37.4–76.5 ng/mL versus 0.0–20.5 ng/mL) irrespective of HCMV infection ($p < 0.001$) (Figure 1(a)). In addition, we observed no statistical differences in sHLA-G serum median levels between actively HCMV infected (56 with primary and 44 with nonprimary infection) and 30 uninfected pregnant women (47 versus 50 ng/mL; range: 36.2–69.8 versus 37.4–76.5 ng/mL; $p = 0.43$) (data not shown).

Interestingly, subdividing the subjects according to the maternal HCMV infection status, primary infected pregnant women presented higher levels of sHLA-G median concentrations (62 ng/mL; 46.9–69.8 ng/mL) than nonprimary infected women (44 ng/mL; 36.2–57.2 ng/mL) ($p < 0.001$). Moreover, primary infected pregnant women presented higher sHLA-G median concentrations than uninfected women (50 ng/mL; 37.4–76.5 ng/mL) ($p = 0.006$) (Figure 1(a)).

The median levels of b2M were only slightly higher in actively HCMV infected (primary and nonprimary) than in uninfected pregnant women (1.8 versus 1.3 μg/mL; $p = 0.14$), in primary than in nonprimary HCMV infected (1.9 versus 1.4 μg/mL; $p = 0.12$), and, finally, in primary HCMV infected than in uninfected women (1.9 versus 1.3 μg/mL; $p = 0.06$) (Figure 1(b)).

3.2. sHLA-G and Beta-2 Microglobulin Levels in Amniotic Fluid. We evaluated sHLA-G and b2M levels in 56 amniotic fluid samples from women who were primarily HCMV infected before week 14 of gestation and accepted amniocentesis.

Out of the 56 amniotic fluids, 39 samples were HCMV negative with PCR and virus isolation and no congenitally infected newborns were found in this group.

Out of 17 amniotic fluids from mothers who transmitted the virus to their fetuses/babies, two were negative for both virus isolation and PCR. Despite these negative results, the 2 babies were congenitally infected, but asymptomatic at birth and during the follow-up period, as already described in the literature [9, 10]. The remaining 15 amniotic samples were positive for both virological tests (6×10^5 copies/mL median

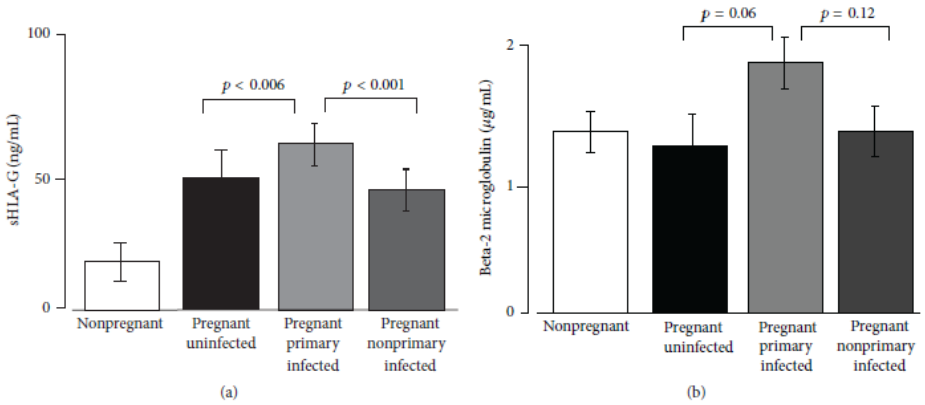

FIGURE 1: Maternal serum samples expression of (a) sHLA-G and (b) b2M molecules in nonpregnant and pregnant women. Pregnant women are classified as uninfected and primarily and nonprimarily HCMV infected. p values obtained by Student's t-test and mean ± standard deviation are reported.

viral load) except for one case where only HCMV-DNA was detected (10^3 copies/mL).

Overall, out of the 17 fetuses/babies infected with congenital HCMV, 5 newborns were asymptomatic at birth and during subsequent monitoring and 11 fetuses and 1 newborn were symptomatic.

The brains of all symptomatic fetuses were HCMV positive with severe histological brain damage and cerebral necrosis; 4 of the fetuses also showed pathological neurosonographic findings (periventricular hyperechogenicity and ventriculomegaly).

Moreover, the only symptomatic newborn had hepatosplenomegaly, thrombocytopenia (platelet count: <100.000/mm^3), and alanine aminotransferase elevation (>80 U/L) at birth and developed sequelae with sensorineural hearing loss and mild psychomotor retardation.

No statically significant difference in amniotic fluid HCMV load was observed between asymptomatic and symptomatic fetuses/newborns (p = 0.88, 95% CI: −3583021 to 3110188).

All the 56 amniotic fluid samples were positive for sHLA-G and b2M molecules (data not shown).

Median detectable levels of sHLA-G were significantly higher in amniotic fluids from infected symptomatic fetuses (73 ng/mL; 69–79 ng/mL) than in infected asymptomatic fetuses (32 ng/mL; 28–42 ng/mL) (p < 0.001) and in uninfected fetuses (31 ng/mL; 29–40.2 ng/mL) (p < 0.001) (Figure 2(a)).

b2M presented slightly higher median levels in amniotic fluids from infected symptomatic fetuses (4.5 μg/mL) than in infected asymptomatic fetuses (3.6 μg/mL) (p = 0.039) and uninfected fetuses (3.9 μg/mL) (p = 0.042) (Figure 2(b)).

When we considered maternal serum levels according to fetus infection status, we observed that sHLA-G concentrations were slightly higher in serum from women with symptomatic fetuses (51.2 ng/mL, 45–57 ng/mL) and in women with infected asymptomatic fetuses (49 ng/mL, 42–55 ng/mL) in comparison with uninfected fetuses (46 ng/mL, 44–52 ng/mL) (p = 0.045, p = 0.042, resp.) (Figure 2(c)).

b2M presented no differences in serum samples from women with infected symptomatic fetuses, infected asymptomatic fetuses, and uninfected fetuses (Figure 2(d)).

3.3. sHLA-G Concentration Gradient between Maternal Serum and Amniotic Fluid. The sHLA-G increase in amniotic fluids of infected symptomatic fetuses prompted the question of whether sHLA-G was produced locally in amniotic compartment or derived from maternal blood. Fetal and maternal compartments are mutually interconnected and several molecules are exchanged through the amniotic and chorionic membrane. This molecular interchange could be hypothesized also for HLA molecules. Therefore we evaluated the concentration gradient between serum samples from primary infected women and the corresponding amniotic fluids.

A sHLA-G concentration gradient from the amniotic fluid to the maternal serum was observed only in infected symptomatic fetuses, while uninfected and infected asymptomatic fetuses presented an inverse sHLA-G gradient (Figure 3(a)). These results suggest a local fetal production of sHLA-G, increased only in fetuses with symptomatic HCMV infection.

3.4. sHLA-G Index. The association between fetal HCMV infection and increased sHLA-G expression in amniotic fluid was confirmed calculating the sHLA-G index in comparison with albumin. Albumin is the most prevalent serum protein which surrounds the embryo and is detected in amniotic fluids. Fainardi et al. [27] reported the use of cerebrospinal fluid and serum albumin content to evaluate sHLA-G brain production. Since both blood-brain interface and placenta are considered selective barriers, we applied the same concept to quantify the fetal compartment production of sHLA-G,

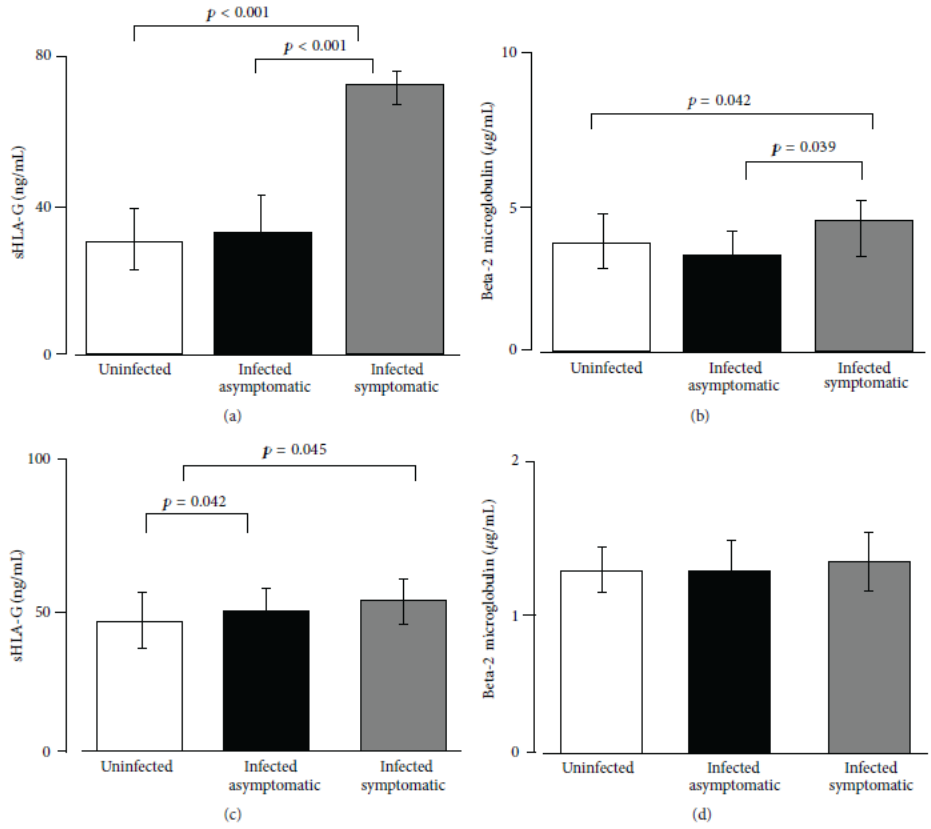

FIGURE 2: Amniotic fluid samples expression of (a) sHLA-G and (b) b2M molecules according to the fetal/neonatal outcome. Maternal serum samples expression of (c) sHLA-G and (d) b2M molecules according to the fetal/neonatal outcome. p values obtained by Student's t-test and mean ± standard deviation are reported.

evaluating the relative amount of amniotic sHLA-G and albumin compared with maternal serum levels. Any increase in the index could be ascribed to sHLA-G production in the fetal compartment. The highest sHLA-G indexes were detected in infected symptomatic fetuses (19.5%) compared to infected asymptomatic fetuses (6%) and uninfected (5.1%) ($p < 0.001$, $p < 0.001$, resp.) (Figure 3(b)).

3.5. HLA-G Free-Heavy Chain Analysis. HLA-G can be expressed as b2M associated or free-heavy chain. Previous studies documented a different distribution of these two conformations at the maternal-fetus interface [29]. We evaluated the presence of HLA-G free-heavy chain in both sera and in amniotic fluids from primary HCMV infected pregnant women with asymptomatic or symptomatic fetus. HLA-G free-heavy chain was detected with a tendency to be more frequent in amniotic fluids from symptomatic fetuses ($p = 0.074$). On the contrary, maternal sera did not present HLA-G free-heavy chain (Table 1).

Representative examples of maternal serum and AF reactivity to antigens on the Western blot are shown in Figure 3(c). In lines 1, 2, and 3, we reported HLA-G positive samples analyzed for b2M associated form (MEM-G9 detection), while the same samples were analyzed for HLA-G free-heavy chain (MEM-G1 detection) in lines 4, 5, and 6.

3.6. sHLA-G Predictive Efficacy. We analyzed serum and amniotic fluid samples for sHLA-G and selected different cut-offs to be used as differentiation values. ROC analysis showed serum values above 50 ng/mL and amniotic values above 30 ng/mL with the highest sensitivity and specificity and an Area Under an ROC Curve of 0.83 and 0.86, respectively

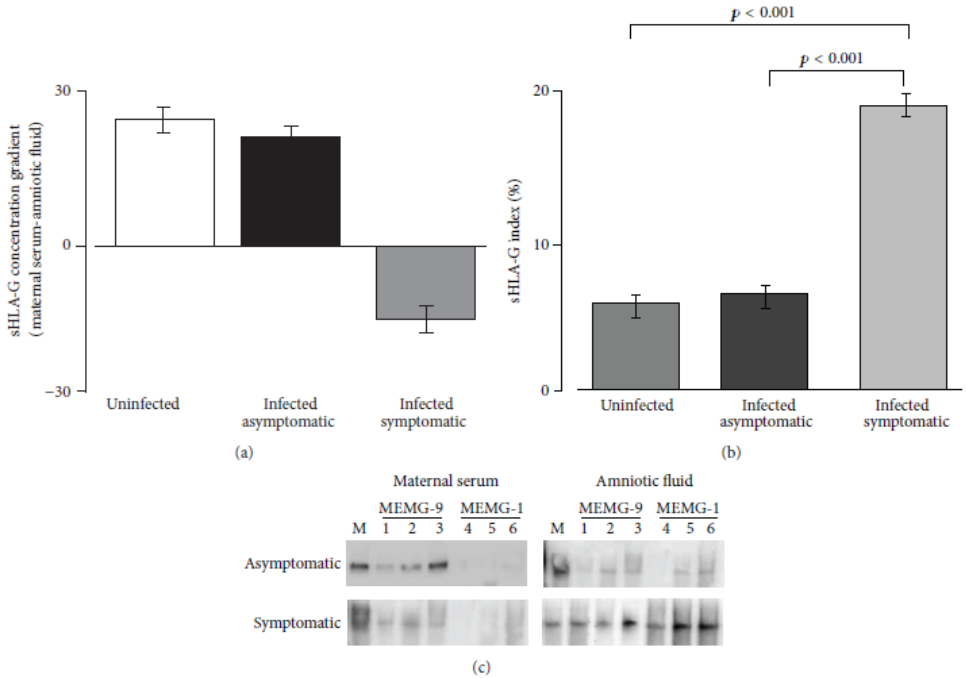

FIGURE 3: (a) sHLA-G concentration gradients according to the fetal/neonatal outcome. (b) sHLA-G indexes (%) according to the fetal/neonatal outcome. (c) Representative Western blot analysis of maternal serum and amniotic fluids samples from HCMV primary infected pregnancy subdivided according to the fetal/neonatal outcome. The analysis were performed after immunoprecipitation with anti-b2M associated HLA-G moAb (MEM-G9, Exbio) (lines 1 to 3) and anti-free HLA-G HC moAb (MEMG-1, Exbio) (lines 4 to 6). JEG3 cell line supernatants were used as positive control (M) and the positivity for HLA-G molecule was evidenced at 39 kD.

TABLE 1: Prognostic HLA-G biomarker of symptomatic congenital HCMV infection.

Parameters	Cutoff		Fetuses		Diagnostic accuracy 95% CI				AUA
			Sympt	Asympt	Sens%	Spec%	PPV%	NPV%	
Maternal serum samples									
HLA-G free-heavy chain		Presence	0	0	0	100	0	29.4	0.5
		Absence	12	5	0–26.7	47.9–100		10.4–55.9	
sHLA-G	50 ng/mL	Above	10	0	83.3	100	100	71.4	0.83
		Below	2	5	51.6–97.4	47.9–100	68.9–100	29.3–95.5	
Amniotic fluid samples									
HLA-G free-heavy chain		Presence	12	3	100	40	80	100	0.79
		Absence	0	2	73.3–100	6.4–84.6	51.9–95.4	19.2–100	
sHLA-G	30 ng/mL	Above	11	0	91.7	100	100	83.3	0.86
		Below	1	5	61.5–98.6	47.9–100	71.3–100	36.1–97.2	

Sens: sensitivity; Spec: specificity; PPV: positive predictive value; NPV: negative predictive value; AUA: Area Under an ROC Curve; Sympt: symptomatic; Asympt: asymptomatic.

(Table 1), in differentiating symptomatic from asymptomatic congenital infections. Similarly, the presence of HLA-G free-heavy chain in amniotic fluids shows a high sensitivity and specificity and an Area Under an ROC Curve of 0.79, in identifying symptomatic congenital infections. Logistic regression analysis excluded the presence of confounding variables.

4. Discussion

The prenatal diagnosis provides the optimal means for diagnosing HCMV fetal infection. The specificity is very good and the sensitivity depends on the kind of samples used (amniotic fluid > fetal blood), the technique used (Real-Time PCR-Polymerase Chain Reaction > viral culture), and the timing of the procedure with respect to the onset of maternal infection and the gestational age.

All literature data report that the amniotic fluid is the most appropriate material for the diagnosis of fetal HCMV infection. Positive results in amniotic fluid identify all HCMV infected fetuses (positive predictive value = 100%) but do not identify the infants who will have symptoms at birth [6].

Although the highest median values of HCMV-DNA in amniotic fluid tend to indicate an increased risk of severe infection, high viral loads may be associated with symptomatic or asymptomatic congenital infections. Indeed, a correlation between the high HCMV load in amniotic fluid and fetal/neonatal outcome has not been demonstrated [6].

More recently, some studies have evoked the prognostic value of fetal viremia/viral load and/or level of specific IgM; however this remains controversial. It has been proposed that platelet count gives a better indication. New data has demonstrated that the determination of multiple markers (haematological, biochemical, and virological markers) in fetal blood following virus detection in amniotic fluid is predictive of perinatal outcome in fetuses with HCMV infection [21]. Further studies in a larger number of symptomatic cases should be performed to verify the prognostic efficacy of determination of multiple parameters in fetal blood.

In this work, we analyzed the levels of sHLA-G and b2M molecules in the maternal serum samples and showed that sHLA-G median levels were significantly higher in maternal serum from pregnant women with primary HCMV infection than in nonprimary and uninfected, respectively ($p < 0.001$ and $p < 0.006$). On the contrary, beta-2 microglobulin levels were only slightly higher in maternal serum from pregnant women with primary HCMV infection than in nonprimary and uninfected, respectively ($p = 0.12$ and $p = 0.06$). The differences in immune competence towards HCMV in primary infected and in nonprimary infected mothers could explain the different production of sHLA-G that can act as viral immune escape mechanism or as a tentative reduction of immune activation in primary HCMV infection, which is not induced in nonprimary HCMV infected women.

Furthermore, we evaluated whether sHLA-G molecules could be considered prognostic biomarkers of symptomatic congenital HCMV infection.

We observed that sHLA-G levels in both maternal serum and amniotic fluid samples are significantly related to symptomatic HCMV fetal infections as supported by ROC analysis (Table 1). In fact, sHLA-G levels above 50 ng/mL in maternal blood and 30 ng/mL in amniotic fluid are correlated with symptomatic HCMV congenital infections. High levels of sHLA-G in maternal and fetal compartments show a specificity of 100% for symptomatic congenital infection and a sensitivity ranging between 83 and 92%.

The evidence that sHLA-G levels increase only in the presence of symptomatic fetuses suggests a specific fetal production of these molecules. We obtained confirmation of this hypothesis through (i) the evaluation of the concentration gradient, which is higher in the amniotic fluid versus the maternal blood in case of symptomatic infection; (ii) the sHLA-G indexes calculation, which support a fetal production; (iii) the HLA-G free-heavy chian, which is commonly expressed by distal trophoblasts [29], which is present only in amniotic fluid.

The increase of fetal HLA-G expression could be caused by HCMV-encoded proteins that are known to interact with HLA mRNAs and proteins, modifying their stability and secretory pathways [15–19]. The increase of HLA-G expression could enhance HCMV immune escape, increasing the risk of congenital infections and symptomatic sequelae. Our results suggest that serum and amniotic fluid sHLA-G might be an additional biomarker of congenital HCMV infection that could be considered in combination with currently used viral and biological markers [20, 30], providing the best key to the reliable identification of fetuses at risk of congenital disease as well as of fetuses with a favorable outcome.

5. Conclusions

To the best of our knowledge, this is the first observation that considers the possible use of serum and amniotic fluid sHLA-G as a biomarker to discriminate between symptomatic and asymptomatic HCMV congenital infection. However, future studies with larger cohort of fetuses should be performed in order to verify whether the addition of serum sHLA-G determination to virologic markers may be crucial in identifying fetuses at highest risk of severe pathologies.

Competing Interests

All authors reported no competing interests.

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