Functional HLA-E studies in healthy individuals and MS-patients

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Preface

Although my first day at the university seemed yesterday, my time as a student is already ending. Now five years have already past. By finishing this thesis I am closing this nice chapter of my life. Naturally, I could not have gotten this far on my own. For this reason I would like to use this page to thank them.

First of all, I would like to thank My promoter, Dr. Niels Hellings, for giving me the opportunity to perform scientific research at the Biomedical Research institute, for critically reading my thesis and giving valuable suggestions during the meetings.

I especially want to thank my supervisor, Kim Pannemans. Kim, thank you for sharing me your knowledge, for your teaching talent, for the numbers corrections of my thesis, for your expert help in the lab and for everything else. I have learned so much over past months for which I am very grateful.

I also thank Tom Broekmans for providing the patient samples and off course, I want to show appreciation to all blood donors.

Further I would like to express thanks to the students at BIOMED for our nice times together. Thanks everyone for your hearty welcome, for your help and your advices.

Finally, I would like thank my parents for giving me the opportunity to study and for always being there for me.

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List of abbreviations

α-GalCer= α-glactosylceramide

Ab= antibody

ARMS-PCR= amplification refractory mutation system-polymerase chain reaction

BBB= blood-brain-barrier

BD= Behcet's disease

bp= basepairs

CD= cluster of differentiation

CM= culture medium

CMV= cytomegalovirus

CNS= central nervous system

CSF= cerebrospinal fluid

CTLS= cytotoxic T cells

CTLA-4= cytotoxicT-lymphocyte antigen-4

DC= denditric cells

DZ= dizygotic

EAE= experimental autoimmune encephalomyelitis

FACS= fluorescence activated cell sorting

FCS= foetal calf serum

FSC= forward scatter

FITC= fluorescein isothiocynate

Foxp 3= forkhead box protein 3

GA= glatiramer acetate

gDNA= genomic DNA

GITR= glucocorticoid induced tumor necrosis factor receptor-protein

HC= healthy control

HLA= human leucocyte antigen

HSP60= heat shock protein 60

IFN= interferon

IL= interleukin

ITIM= immunoreceptor tyrosine-based inhibition motif

LPS= lipopolysacharide

MBP= myelin basic protein

MHC= major histocompatibility complex

MRI= magnetic resonance imaging

MS= multiple sclerosis

MZ= monozygotic

NK= natural killer

PBMC= peripheral blood mononuclear cells

PE= phycoerythrine

PerCP= peridin chlorophyll protein

PP MS= primary progressive multiple sclerosis

Qdm= Qa-1 determinant modifier

RR MS= relapsing-remitting multiple sclerosis

SP MS= secondary progressive multiple sclerosis

SSC= side scatter

TAP= transporter associated with antigen processing

TCR= T cell receptor

TGF= transforming growth factor

Th= T helper cells

TLR= toll like receptor

TNF= tumor necrosis factor

Tr1= type 1 regulatory T cells

Summary

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system in which autoreactive T cells are key players. In animal models, Qa-1 restricted $CD8^+$ T cells play an important role in the suppression of these autoreactive T cells. Qa-1, a non classical MHC class Ib molecule, is expressed on activated cells and recognized by a subtype of $CD8^+$ T cells which become activated and exercise their regulatory function. Information about the functional role of human leukocyte antigen-E (HLA-E), the human variant of Qa-1, is limited. Therefore the aim of this study is to gain information about the HLA-E surface expression by immunologic cells during inflammatory and regulatory processes. The first objective of this project was to investigate whether the HLA-E surface expression is modulated after activation of immune cells in healthy controls (HC) as well as in MS patients. To reach this goal, cells were isolated from blood. After activation, these cells were stained with antibodies against specific surface markers. HLA-E surface expression was monitored using fluorescence activated cell sorting (FACS). HLA-E expression in both B and T cells is upregulated after 1 day of activation. In B cells, HLA-E expression remains high troughout the activation time while in T cells HLA-E expresssion is back downregulated after 2 days of activation. These observations correspond with the Qa-1 expression profile. It could mean that, like Qa-1, HLA-E present peptides to HLA-E restricted $CD8^+$ T cells and activate their suppressive function.

Our second objective was to study the correlation of HLA-E polymorphisms with the surface expression of HLA-E before and after activation. To this extend, genomic DNA of HC was isolated and the genotype of HLA-E in these HC was determined via ARMS-PCR. In a second step, HLA-E expression after activation was studied in 3 HC for each genotype. The data suggest that the up-regulated HLA-E expression is lower in HC homozygous for the 0103 allele. It is presumable that in these individuals, the antigen presentation by HLA-E will be lower which subsequently con lead to less stimulation of HLA-E restricted $CD8^+$ T cells.

Further experiments need to be performed to investigate if the same observations are present in MS patients. In this way, this study can lead to a better understanding of the pathogenesis of MS and may contribute to the development of new and more specific therapies.

Part 1: introduction

1.Introduction

1.1 General aspects of multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disorder of the central nervous system (CNS) (1).

MS typically starts between 20 and 40 years old: 70% of MS patients are diagnosed between 20 and 40 years old, 10% in adolescence and 20% after 40 years of age (2).

MS has a high prevalence that ranges between 2 and 150 each 10^5 depending on the country or specific population (3). More than 2.5 million individuals are affected in the world (4).

MS is characterized by plaque or lesion formation in the CNS. These lesions are most commonly found in white matter areas close to the ventricles of the cerebellum, brain stem, basal ganglia and spinal cord and the optic nerve. The lesions are the result of destruction of oligodendrocytes, the cells responsible for creating and maintaining the myelin sheath, the isolating layer around the axons. Initially, the oligodendrocytes are preserved and a repair process, called remyelination, occurs. In a later phase of the disease, the damaging of the oligodendrocytes will eventually lead to axonal loss (5-6).

As a consequence, MS patients experience plasticity which is responsible for mobility and daily activity impairments. Fatigue is a second symptom commonly seen in MS patients. It is defined as a intense feeling of weakness, lack of strength and energy or total exhaustion that is perceived by the individual and interferes with usual and desired activities. Other neurological symptoms are blurred vision, bladder dysfunction and muscle weakness (7).

Several subtypes of MS, or patterns of progression, have been described. The first subtype of MS is the relapsing-remitting (RR) form of MS. This subtype is an alteration of relapses characterized by sudden intensification of inflammation followed by phases of symptomatic remission characterized by recovery. The duration of the phases of relapse and remission varies from one patient to another, lasting from several weeks to several years. This describes the initial course of 85-90% of the MS patients (Fig. 1A) (5-6).

About 1/3 of RR-MS patients develops secondary progressive (SP) MS. In the beginning of this phase of the disease, recovery still occurs but it tends to develop in a more continuous inflammation without any recovery (Fig. 1B) (5-6).

The third and last subtype of MS – primary progressive multiple sclerosis (PP-MS) - accounts for around 20 % of all MS cases. The major characteristic of this subtype is the continuous inflammatory process from the beginning with the absence of recovery (Fig. 1C) (5-6).

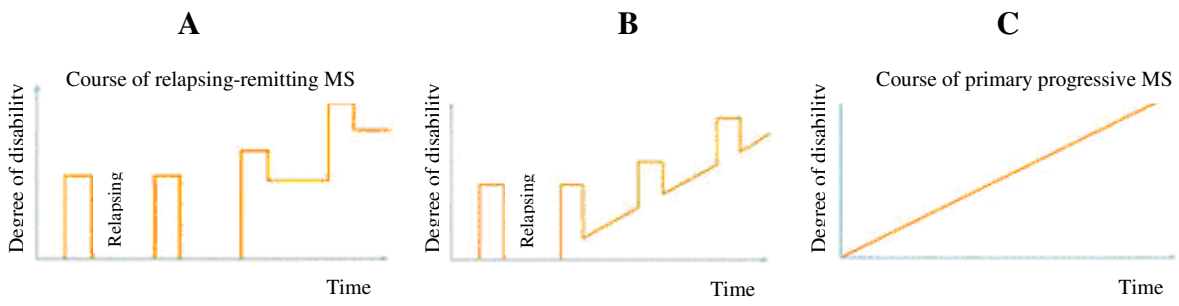


FIGURE 1 : Patterns of progression of MS

A. relapsing-remitting multiple sclerosis (RR MS) B. secondary-progressive multiple sclerosis (SP MS) C. primary-progressive multiple sclerosis (PP MS) (8).

1.2. The etiology and pathology of MS

The etiology of MS remains unclear but MS likely occurs as a result of a combination of both exogenous and genetic factors (6).

Several studies have shown that viral infections can be involved in the development of MS

There is a high rate of antibody (Ab) against some viruses in MS patients, particulary Epstein-Barr virus, Cytomegalovirus (CMV), etc. In addition, a significant number of relapses are preceded by common viral infections (9).

Some studies provide evidence supporting a genetic etiology of MS. Twin studies suggest that genetic factors influence the pathogenesis of MS. The different clinical concordance between monozygotic (MZ) (31%) ans dizygotic (DZ) twins (5%) demonstrate the genetic influences (6). Specific genes have been linked to MS such as human leucocyte antigen (HLA). Differences in these genes increase the probability of suffering from MS (10). Studies show that MS is associated with the HLA class II haplotype DRB1*15,DQB1*06. The HLA-DRB1*1501 (beta chains of HLA-DR2) molecule may explain about 50% of the genetic presdisposition MS and its role in the pathogenesis is supported by studies of transgenic mice. Also in humans, these loci bring the strongest genetic risk in the African-American population (4). Also HLA-A Class I has been implicated in MS (11). Other genes that are associated with MS are CTLA-4 (cytotoxicT-lymphocyte antigen-4), IL-17R, CD58 (12).

Besides genetic association, environmental factors can also be involved in the etiology of MS. MS is more common in people who live more distant from the equator and occurs more frequently in temperate climates (5,13). Besides all the factors, hormones may influence the relapsing-remitting course. For example, during pregnancy, the apparition of relapses seems to decrease and are more frequent during the first 3 months after the childbirth (14).

1.3 Immunopathogenesis of MS

Autoreactive T cells are thought to play a crucial role in the development of the disease. Myelin reactive T cells are activated in the periphery. These activated cells expand and migrate into the CNS. The release of pro-inflammatory cytokines and the expression of adhesion molecules by the activated T cells promote the migration through the blood-brain-barrier (BBB). In the CNS, when the migrated cells meet their specific myelin epitope presented by the resident microglia cells or perivascular macrophages, they become reactivated. The frequency of MBP reactive T cells and their state of activation is increased in MS patients. These reactivated cells will generate pro-inflammatory cytokines such as TNF, IFNγ, which are cytokines produced by Th1 $CD4^+$ T cells. These cytokines induce upregulation of adhesion molecules on endothelium of the BBB and upregulation of expression of MHC class II on astrocytes and microglia cells. This will increase the immune inflammatory response because of the amplification of the influx of B cells, T cells and macrophages. The final result is the demyelination due to the combinatorial effects of cytotoxic cells (macrophages and gamma-delta T cells), oxygen radicals, demyelinating autoantibodies and cytokines (6).

It was thought that the activation of autoreactive $CD4^+$ T cells specific for myelin antigen and differentiation to Th1 effectors were crucial for the development of the disease (15-16) but besides Th1 cells, the Th17 cells producing IL-17, driven by IL-23, play a role in initiation and maintenance of tissue inflammation in MS (16).

Experimental Auto-immune Encephalomyelitis (EAE) is the animal model most commonly used to study MS. EAE represents a lot of clinical, immunological, neuropathological aspects of MS and is valuable to study inflammatory aspects of the disease. EAE can be induced in several species like mice, rats, guinea pigs and marmosets. Three main ways are used to induce EAE in these animals: active sensitization, passive transfer of activated immunologic cells and the use of TCR transgenic mice (17).

1.4 Diagnosis and therapy of MS

The diagnosis of MS is difficult to etablish because the signs and symptoms of MS are similar to many other diseases. In 2001, an International Panel has invented diagnostic criteria. These criteria are also called the Mc Donald criteria. In order to facilitate the diagnosis, clinical investigation, imaging (MRI) and paraclinical results are included in these criteria.(5,18).

Magnetic resonance imaging (MRI) is the most common diagnostic tool to evaluate MS. Demyelinating plaques or lesions are shown by MRI of the spine or the brain. The visualized lesions are often old and asymptomatic. The young lesions, responsible for the remitting phase in process can be revealed after intravenous injection of gadolinium, a substance which shows the contrast between young and old lesions (19).

Cerebrospinal fluid (CSF) analysis which is obtained by a lumbar puncture, is used to support the diagnosis but is not indispensable. In this CSF, oligoclonal bands are investigated because they reflect the inflammation in MS patients. Oligoclonal bands are found in 75-85% of individuals with MS (5,20).

Up to now, there is still no treatment that cures MS. Current therapies are aimed at reducing inflammation and suppressing ongoing disease processes. Interferon β 1b (IFNβ) and Glatiramer acetate (GA) are immunomodulatory agents and are used for RR-MS patients. They have demonstrated to be efficiency in MS: the evolution of the disability is lower, the inflammation measured by MRI is reduced and the relapses are less severe and become scarce (21-22). IFNbeta has an inhibitory effect on T cells through its antiproliferative action. IFNbeta also inhibits the costimulatory pathway through downregulation of costimulatory molecules such as CD80/CD28 and CD40/CD40L. In addition, the cytokine profile is changed. For example, the secretion of the anti-inflammatory cytokine IL-10 is increased and the production of the pro-inflammatory IL-12 is decreased. IFN gamma is also a target of the IFNbeta therapy (5,21-22).

GA, a synthetic molecule, is an antagonist of the T cell receptor (TCR) and by binding the TCR it decreases the T-cell responses. Furthermore, GA induces a lymphocyte tolerance in relation to myelin by decreasing myelin-reactive T cells. GA also provokes induction of Th2 cells. Additionally, GA treatment stimulates the production of neurotrophins such as BDNF, NT-3, and NT-4 which lead to neuroprotective effects (22).

Mitoxantrone, an immunosuppressor, is used to treat secondary progressive and progressive relapsing patients. Mitoxantrone seems to ameliorate the disease course by inhibiting B-cell, T-cell and macrophage proliferation (23).

An other immunosuppressor, Natalizumab is used to treat an agressive forms of MS and when the IFNβ treatment fails. Natalizumab is a monoclonal Antibody against alpha4-integrin which is expressed by leucocytes (24).

1.5 General aspects of immunoregulation

In order to prevent autoimmunity, the immune system must distinguish between self and non-self antigens (25). In the thymus, during the lymphopoiesis, thymocytes expressing a T cell receptor (TCR) capable of efficient binding to self major histocompatibility complex (MHC) are positively selected while thymocytes bearing high-affinity receptors for self-antigens are eliminated by apoptosis (negative selection) (26-27). However, some self-reactive T cells escape thymic negative selection and head towards the periphery where they are, in response to autoantigen, activated, proliferate, and differentiate into potentially pathogenic effector cells (27). If the elimination or the inactivation of these autoreactive cells fails, an autoimmune disease might arise. To prevent the expansion of these "escaped" pathogenic effector cells, regulatory mechanisms are considered to be very important (25). First, several homeostatic feedback mechanisms intrinsic to antigen activation of ($CD4^+$) T cells regulate the peripheral immune response. Apoptosis, induction of anergy and differentiation into Th subsets are important to prevent the outgrowth of possible pathogenic effector cells.

Secondly, different subsets of regulatory $CD4^+$ (25) and $CD8^+$ T (28) cells as well as NK T cells (29) possibly suppress the pathogenic antigen-reactive T cells and thus these suppressor cells are necessary for the control of autoimmunity. Recent studies even suggest that MS is due to defects in this regulatory T-cell function (30).

1.6 CD4+ regulatory T cells

One population which plays a role in the prevention of autoimmune diseases are $CD4^+$ T cells expressing CD25 (IL-2 receptor). They represent 5-10% of the peripheral lymphocytes. The markers expressed by these cells, in addition to CD25, are FoxP3, CTLA-4, GITR (glucocorticoid induced tumor necrosis factor receptor–protein) and lymphoid homing receptors such as CD103, CD62L. FoxP3 controls the positive selection of $CD4^+$ $CD25^+$ cells in the thymus. Studies showed that they suppress the function of effector $CD4^+$ CD25 T cells, cytotoxic $CD8^+$ T cells, NK cells and B cells. This suppression is cell contact dependent. Therefore, they are key regulators in the control of autoimmunity.

An other type of suppressive $CD4^+$ T cells are type 1 regulatory T cells (Tr1) . They have a cytokine profile expression different from Th1 or Th2 cells and produce high levels of IL-10 and TGF-β. The immunosuppressive mechanism used by these cells seems to be IL-10 dependent (31).

1.7 NK T cells

Natural killer T cells (NK T cells) express a TCR but also share some markers chracteristic to NK cells such as CD161 or NKR-P1. The TCR interacts with glycolipids presented by CD1d, a non classical antigen-presenting molecule present on various antigen presenting cells.

NKT cells are stimulated by glycolipids such as ganglioside GD3 and some forms of β-glactosylceramide and more efficiently, by a synthetic glycolipid know as α-glactosylceramide (α-GalCer). These glycolipids bind to CD1d which then is turn can bind to the NK TCR leading to activation of these cells.

When activated, NKT cells release Th1-type and Th2-type cytokines including IFN-γ, TNF and IL-4 and IL-13 respectively. They can generate a Th1 immune response and enhance cell-mediated immunity via production of Th1-type cytokines while in other systems, they cause a Th2 immune response and are immunosuppressive by producing Th2-type cytokines like IL-10. Some studies with autoimmune disease models suggested that NKT cells influence autoimmunity. The results acquired from EAE studies on the other hand are contradictory. The activation of NKT cells via α-GalCer either prevents disease outcome or has no effect or accelarates the disease. In most of the cases, α-GalCer prevents EAE by shifting the balance from a pathogenic Th1 towards an anti-inflammatory Th2 response to CNS antigens (29).

1.8 CD8+ regulatory T cells

MS has long been considered as a $CD4^+$ T cell-mediated disease. Only recently, the importance of $CD8^+$ T cells in MS has emerged. A number of studies report that the proportion of autoimmune $CD8^+$ T cells is increasing in MS patients and $CD8^+$ T cells demonstrate oligoclonal expansion in the MS brain and CSF, supporting their implication in autoimmune processes (32).

In contrast, EAE experiments suggest a regulatory function of CD8+ T cells. First, it has been demonstrated in EAE, that a subpopulation of $CD8^+$ T cells, $CD8^+$ $CD28^-$ T cells, show a supressive capacity. These cells play an important role in preventing EAE in $CD28^{-/-}$ mice. The $CD8^{-/-}$ $CD28^{-/-}$ double knockout mice are highly suceptible to EAE. In addition, adoptive transfer of $CD8^+CD28^-$ into $CD8^{-/-}$ could impair the development of EAE (31).

A second subpopulation of suppressive $CD8^+$ T cells are Qa-1 restricted $CD8^+$ T cells. In EAE the importance of these cells became clear as the absence of the MHC class I locus Qa-1 lead to a higher susceptibility for developing EAE. This suggested that the regulatory function of these $CD8^+$ cells in MS is Qa-1 dependent (30).

1.8.1 Qa-1 restricted CD8+ cells

The murine Qa-1 is a member of the non classical MHC class 1b family. Qa-1 has a molecular weight of 48 kDa and presents a 9 amino acid signature peptide (AMAPRTLLL) derived from the signal sequence of classical MHC class I proteins, named Qa-1 determinant modifier (Qdm). The surface expression of Qa-1 is restricted to activated hematopoietic cells, T cells, DCs, macrophages and B cells. On the surface of the cells, this glycoprotein is associated with beta2-microglobulin (33). The repertoire of peptides presented by Qa-1 is small and includes Qdm, peptides derived from heat shock protein 60 (HSP60), insulin, salmonella GroEL and TCR Vβ chains. Qdm peptide is processed and presented via a TAP-dependent pathway similar to that of the MHC class Ia molecule while the other peptides are processed and presented in a TAP-independent manner (1).

Qa-1 is a ligand for the inhibitory CD94/NKG2A receptor and for the stimulating CD94/NKG2C receptor expressed on NK cells and a subset of $CD8^+$ T cells (33). CD94 is a type II glycoprotein that is expressed on most NK cells and a subset of T cells. It forms a heterodimer with the NKG2A and NKG2C which contain C-type lectin-like ectodomains that bind to Qa-1 (34-37). The outcome of this binding is respectively an inhibition of the activity of NK or cytotoxic T cells (CTLS) and a stimulation of the cytotoxicity of these cells (33). The different outcomes of NKG2A versus NKG2C can be explained by the immunoreceptor tyrosine-based inhibition motif (ITIM) present on NKG2A cytoplasmic domain. NKG2C receptors do not have this motif (1).

Recent studies have shown that a subpopulation of $CD8^+$ cells might also suppress the response of activated $CD4^+$ T cells through an interaction via Qa-1 on target cells and the TCR on the $CD8^+$ T cells. This interaction leads to amplification and differentiation of the $CD8^+$ Tregs and subsequent inhibition of $CD4^+$ T-cell activation (Fig. 2). The mechanisms of interaction and suppression remain unclear. However, studies demonstrated the regulatory potential of these cells. The group of Cantor showed that Qa-1 deficient mice developed exaggerated secondary CD4 responses after viral infection. Immunization with foreign and self peptides because the $CD4^+$ cells became resistant to the suppressive activity of the $CD8^+$ cells. Consequently, the suceptibility of EAE has increased (26).

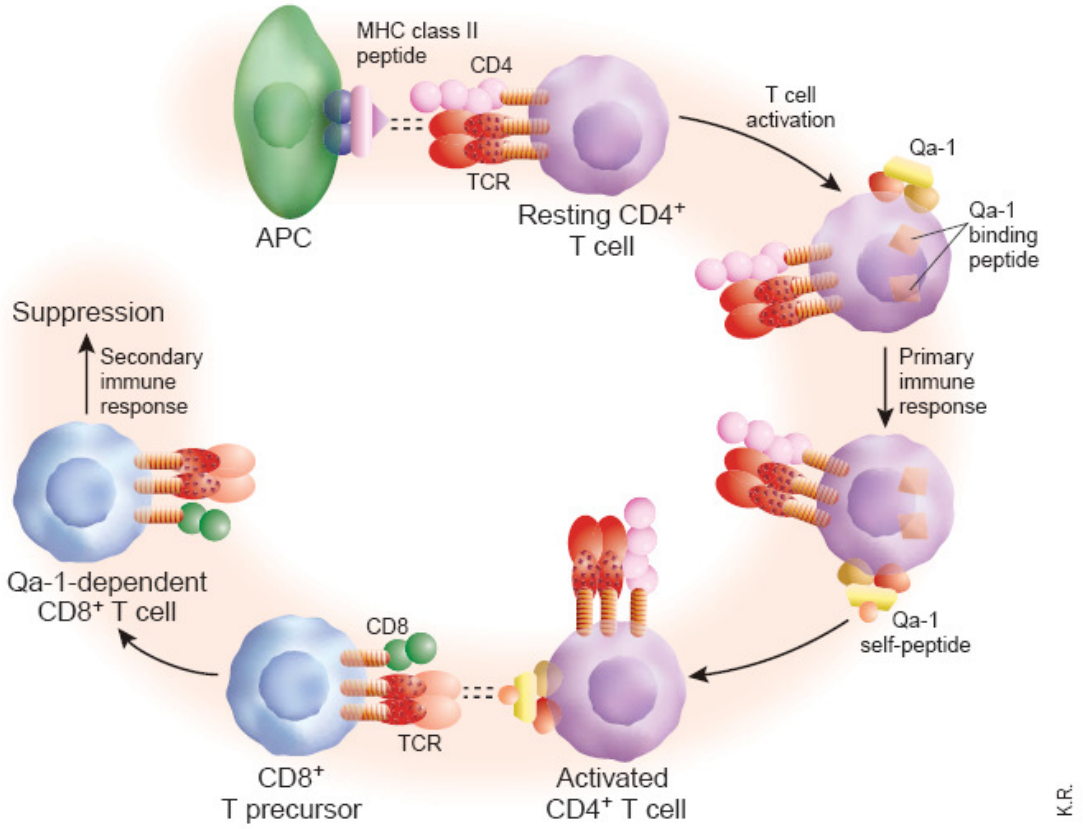


FIGURE 2: Regulation of CD4+ T cells responses by CD8+ Tregs

Activated $CD4^+$ T cells presents Qa-1 peptide complexe at $CD8^+$ T cells. This interaction leads to activation and differentiation of Qa-1 restricted $CD8^+$ T cells wich inhibits the activation of the CD4+ T cells expressing Qa-1 peptide complexe (38).

1.8.2 HLA-E restricted CD8+ T cells

Human classical MHC Ia molecules include three members know as HLA-A, -B, -C. These molecules are expressed at the surface of all nuclear cells and their structure consist of one α chain, one β2-microglobulin chain and one peptide cavity. Nonclassical MHC Ib molecules share some characteristics with classical MHC Ia molecules but are defined by limited polymorphisms, low cell surface expression, and more restricted tissue distribution. Nonclassical class MHC I molecules include HLA-G, -F and -E (39). In contrast to HLA-G, the expression and the function of HLA-E remains unclear.

HLA-E is expressed by B and T lymphocytes, NK cells, macrophages and is mainly expressed by endothelial cells (34). The repertoire of peptides that binds to HLA-E is restricted in contrast to class MHC Ia molecules, which present a highly diverse repertoire of peptides. HLA-E can bind nonameric peptides derived from the leader sequence of certain HLA class I such as

HLA-A, -B, -C and -G but also CMV, TCR and HSP60 derived peptides which is comparable to Qa-1 (34,37,40).

Like Qa-1, HLA-E complexed to nonameric peptide is a ligand for the CD94/NKG2A and CD94/NKG2C receptors. Again, the interaction of HLA-E with CD94/NKG2A leads to inhibition of cytotoxic T lymphocyte (CTL)-dependent lysis while the interaction with CD94/NKG2C leads to activation of CTLS. In addition to serving as a ligand for CD94/NKG2 receptors, HLA-E can also interact with αβ and γδ TCR on $CD8^+$ T cells. The consequence of this interaction has not yet been described (34).

In contrast to classical MHC I molecules which exhibit a high level of polymorphisms, previous studies demonstrated that HLA-E harbors only two polymorphic sites. HLA-E^R or HLA-E*0101 encodes an adenin (**A**GG) at codon 107 while HLA-E^G or HLA-E*0103 encodes a guanin (**G**GG) (35,41). This results respectively in a arginin or glycine at amino level. It has been shown that the surface expression of the HLA-E*0103 molecule is higher than that of HLA-E*0101. Different levels of surface expression of HLA-E*0101 versus HLA-E*0103 could alter its ability to protect cells from NK lysis by NKT cells (35).

Secondly, a silent substitution is present at codon 77 dividing HLA-E*0103 into two variants HLA-E*010301 (AA**C**) and HLA-E*010302 (AA**T**) (41). The functionnal consequence of this substitution is not known.

Studies on HLA-E allele in Behcet's disease (BD), an auto-immune inflammatory disorder characterized by recurrent attacks of oral and genital aphtous ulcers showed that the HLA-E*0101 allele was associated with a reduced risk of BD (42). In BD patients, the frequency of HLA-E*010302 allele is highly increased, whereas the frequency of HLA-E*0101 allele is low. Lower expression of HLA-E*0101 molecules leads to an ineffective inhibitory signal to the NK and/or CTL cells. In addition, Lajoie *et al.* showed that, compared with women who are homozygous for the HLA-E^G allele, the women who are heterozygous or homozygous for the HLA-E^R have a 4-fold increased risk of HIV-1 infection (43).

1.9 Study aim

HLA-E expression and function remains unclear in light of atoimmunity since relatively few functional studies of HLA-E have been conducted in HC and MS patients. For this purpose, the aim of this study is to investigate whether the HLA-E surface expression is differentially modulated after activation of T-, B- cells and monocytes in healthy controls (HC) compared to MS patients.

To reach this aim, PBMCs of HC and MS patients will be isolated from blood. T cells will be activated with α-CD3, B cells with CpG and monocytes with LPS. HLA-E surface expression will be monitored during 5 days using fluorescence activated cell sorting (FACS).

The second objective is to study the correlation of HLA-E haplotypes with the surface expression of HLA-E. To this extend, genomic DNA of HC was isolated and the genotype of HLA-E in these HC will be determined via ARMS-PCR. Next, the different immunologic cell subsets of the same HC will be activated to investigate the correlation between HLA-E expression and genotype.

The results of these experiments could contribute to a better understanding of the pathogenesis of MS and more specific the role of HLA-E surface expression by immunologic cells during inflammatory processes.

Part 2: Materials and methods

2. Materials and methods

2.1 Healthy controls and MS patients

To perform the experiments, peripheral blood mononuclear cells (PBMCs) were isolated from blood collected from 38 healthy controls and 3 MS patients. The donors are presented in table 1.

TABLE 1: Healthy controls and MS patients

Donor	Sex (M/F)	Age	Type MS	Duration	Treatment
Healthy controls					
PEV	F	27	/	/	/
RUI	F	48	/	/	/
BAK	M	28	/	/	/
TIS	F	24	/	/	/
HEJ	M	32	/	/	/
GAB	F	29	/	/	/
VEV	F	28	/	/	/
PAK	F	26	/	/	/
FRJ	F	25	/	/	/
VAK	F	28	/	/	/
WAK	F	38	/	/	/
HEN	M	36	/	/	/
MIM	F	46	/	/	/
VAA	F	24	/	/	/
VAAn	F	22	/	/	/
BER	F	51	/	/	/
BRT	M	26	/	/	/
GOC	F	28	/	/	/
ROE	M	51	/	/	/
SMN	M	25	/	/	/
SWQ	F	30	/	/	/
BRB	F	24	/	/	/
BROB	M	32	/	/	/
DAR	F	34	/	/	/
DEB	M	27	/	/	/
DIM	F	47	/	/	/
FAJ	M	54	/	/	/
LEE	F	28	/	/	/
MAJ	M	45	/	/	/
MIL	M	51	/	/	/
RAA	F	24	/	/	/
SLL	F	28	/	/	/
SOV	F	37	/	/	/
SWN	F	22	/	/	/
THK	M	22	/	/	/
DEK	F	28	/	/	/
SWA	F	29	/	/	/
GIB	F	49	/	/	/

MS patients					
KUA	F	43	RR	9 years	glucocorticoïds
CAA	F	60	PP	10 years	no medication
VAL	M	54	SP	10 years	Tysabri

2.2 Media and stimuli

Cells were cultured in culture medium (CM) which consists of RPMI-1640 (Gibco) supplemented with sodium pyruvate (1%, Gibco), non essential amino acids (1%, Gibco), penicillin-streptomycin (Pen/Strep, 0,5%, Invitrogen) and foetal calf serum (FCS, 10%, Hyclone). To activate the cells, different stimuli were used. Monocytes were activated with lipopolysacharide (LPS, 10ng/ml, Calbiochem, VWR), T cells with anti-CD3 (2 µg/ml, BIOMED, clone 2G3) and B cells with CpG (2µg/ml, Invivogen) and interleukine-2 (IL-2, 100U/ml). Cells were incubated at 37°C and 5% of CO_2 which mimics the human physiological conditions.

2.3 Isolation of PBMCs

2.3.1 Ficoll separation

PBMCs were isolated by Ficoll density centrifugation. The blood diluted with blanco was brought onto the ficoll (Sigma-Aldrich). Via centrifugation, the contents of the blood are separated according to their density (Fig. 3). The red blood cells, which have the highest density, are in the bottom of the tube, then a layer of ficoll while the plasma, which has the lowest density, is above the ring of PBMCs. The PBMC ring is taken off and brought into a new tube and washed 3 times. Finally, the cells were resuspended in culture medium and counted. Therefore, the cells were stained with trypan blue and brought onto a counting chamber. Trypan blue (Sigma) is a substance only taken up by dead cells. As a consequence, viable PBMCs appear as bright cells.

2.3.2 Thawing of PBMCs

The cells freezed in cryovials at -196°C are defrosted in the water bath and resuspended in cold thaw-medium (20% FCS). When cells are thawed, a high level of apoptosis occurs. Therefore, after washing the cells, DNAse (100 µl/ml, Macherey-Nagel) was added and cells were incubated at 37°C. The released DNA during apoptosis which cloths the cells will be broken down by DNAse. Finally, the excess of DNAse was washed after which the cells were resuspended in culture medium and counted in the way described above.

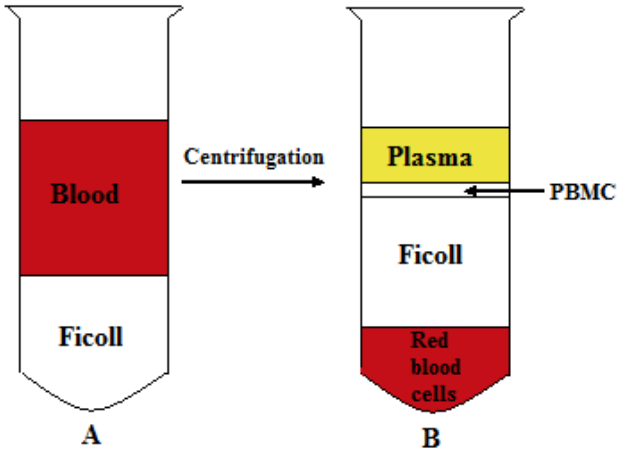


FIGURE 3: Representation of Ficoll separation

A: The blood is brought onto the ficoll and two layers are formed.

B: After centrifugation, a layer of PBMC appears between the ficoll and the plasma. The red blood cells are in the bottom of the tube because of their highest density.

2.4 Isolation of monocytes by positive selection

PBMCs were isolated by ficoll centrifugation. Then, the monocytes were isolated using a CD14 Microbead kit (Milteny Biotec). This kit is used for positive selection of human monocytes and macrophages out of the PBMC population. PBMCs were resuspended in magnetic activated cell sorting (MACS) buffer (PBS, 0,5% BSA and 2mM EDTA by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing Solution) and magnetically labeled with CD14 Microbeads. The cells were incubated at 2-8°C and finally resuspended in buffer (500 µl up to 10^8 cells). After rinsing the column with buffer, the cell suspension was loaded onto an LS MACS Column (Milteny Biotec) which is placed in the magnetic field of an MACS Separator (Milteny Biotec). The magnetically labeled $CD14^+$ cells are retained within the column. The unlabeled cells run through it; this cell fraction is thus depleted of $CD14^+$ cells. After removing the column from the magnetic field, the magnetically retained $CD14^+$ cells can be eluted as the positively selected fraction (Fig. 4). At last, the purity of the magnetically labeled $CD14^+$ cells was determined by flow cytometry.

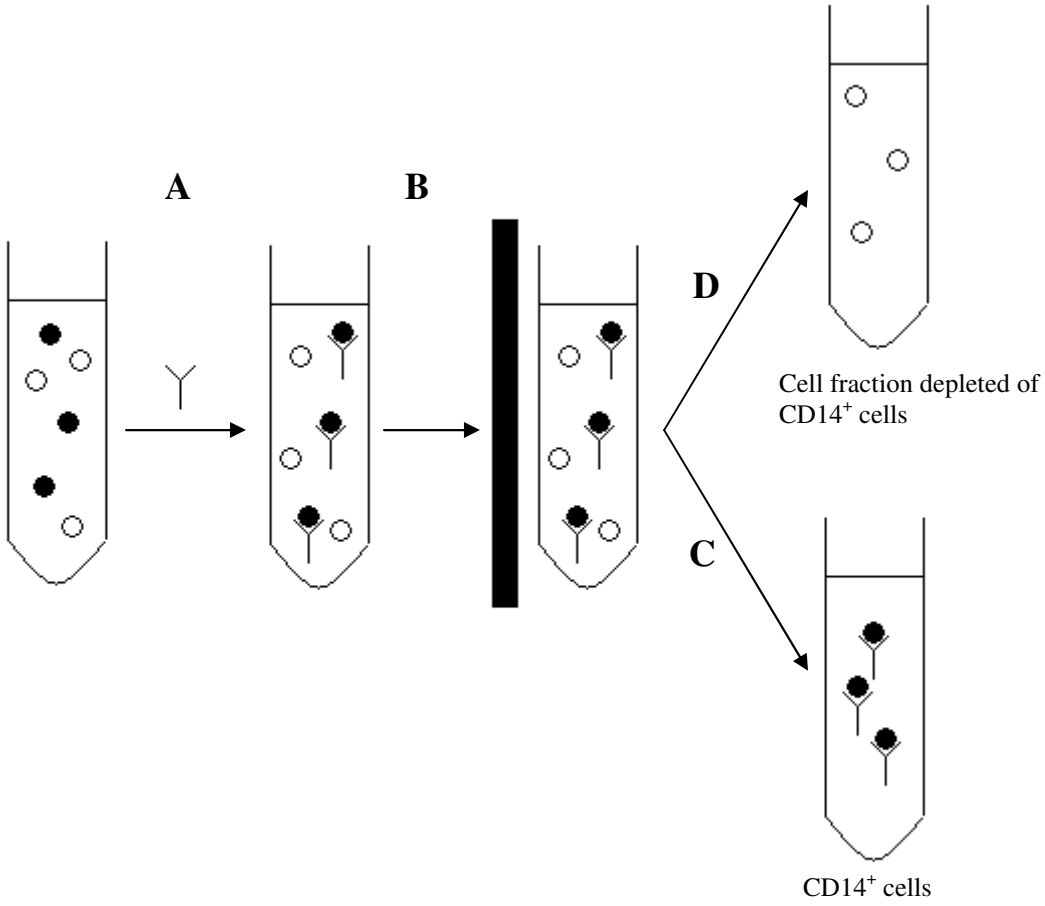


FIGURE 4 : Isolation of monocytes

Antibodies against CD14 expressed by monocytes (black) are added to the PBMC population (A). Cells are placed onto a column and a magnetic field is applied (B). $CD14^+$ cells are retained (C) while the unlabeled cells run through the column (D).

2.5 Measuring HLA-E expression before and after activation of immunological cells using fluorescence-activated cell sorting (FACS)

PBMCs were isolated via ficoll density centrifugation. Monocytes were isolated as described (2.4). PBMCs were cultured in a 24 well plate at density of $1x10^6$ cells/well while monocytes were plated into a flat bottom 96 well plate at a density of $5x10^4$ cells/well. B cells were activated with CpG and IL-2 (Roche). CpG is a short DNA sequence which is recognized by the toll like receptor 9 (TLR9) expressed by numerous cells of the immune system such as dendritic cells, B lymphocytes and natural killer (NK) cells. This interaction leads to activation of B cells (44). It has also been demonstrated that IL-2 is necessary to prevent apoptosis of B cells after an extended activation. CpG has no effect on T cells. T cells were activated with anti-CD3. CD3 is a protein complex and is composed of four distinct chains. These chains associate with the TCR. Anti-CD3 can't bind to the B cells surface and thus, B cells can't be

activated by anti-CD3. To activate monocytes, LPS was used. LPS is found in the outer membrane of Gram-negative bacteria, acts as endotoxins and elicits strong immune responses (45). Monocytes cultured in culture medium without any stimulus was used as a negative control. During 5 days, HLA-E surface expression was monitored every days by flow cytometry. First, cells are centrifuged and resuspended in 100 µl of FACS buffer (10% 10x PBS, 2% FCS, MQ). In a second step, all immunological cell subsets were stained with an antibody against HLA-E. Hence, to stain $CD4^+$ and $CD8^+$ T cells and B-cells, anti-CD4 PerCP, anti-CD8 PerCP and CD19 PerCP (BD Biosciences) were respectively added. Anti-CD14 PerCP (BD Biosciences) antibody was used to stain monocytes. To determine the activation level of B and T cells an anti-CD25 FITC (BD Biosciences) antibody was used while anti-CD80 FITC (BD Biosciences) allows to study the activation of monocytes. Cells were incubated with these antibodies at 4°C during 30 minutes. The stainings are shown in table 2.

TABLE 2: Antibodies used to stain immunological subsets

Stainings	FITC	PE	PerCP
PBMCs			
1	CD25	HLA-E	CD4
2	CD25	HLA-E	CD8
3	CD25	HLA-E	CD19
Monocytes			
1	CD80	HLA-E	CD14

Finally, the cells were analysed by the Facs callibur. To this extend, cells are irradiated via a laser one by one. A number of detectors are aimed at the point where the stream of cells passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. FSC correlates with the cell volume and SSC depends on the granularity of the cells. Simultaneous utilization of these parameters allows to dinstingish monocytes, lymphocytes and granulocytes from each other. Fluorescent detectors registrate the lights emited by the excited fluorochromes attached to antibodies. These fluorochromes (FITC, PerCP, PE) correspond to different wavelengths, that allow to detect them simultaneously. In this way, one cell can be stained with maximum 3 different fluorescently labeled antibodies.

On the basis of the SSC and FSC, lymphocytes and monocytes were gated. A second gate was placed on $CD4^+$, $CD8^+$ or $CD19^+$ positive cells for studying different cell types in the lymphocyte population and $CD14^+$ cells for studying the monocytes (Fig. 5). Finally, HLA-E expression was studied on the different gated cell types.

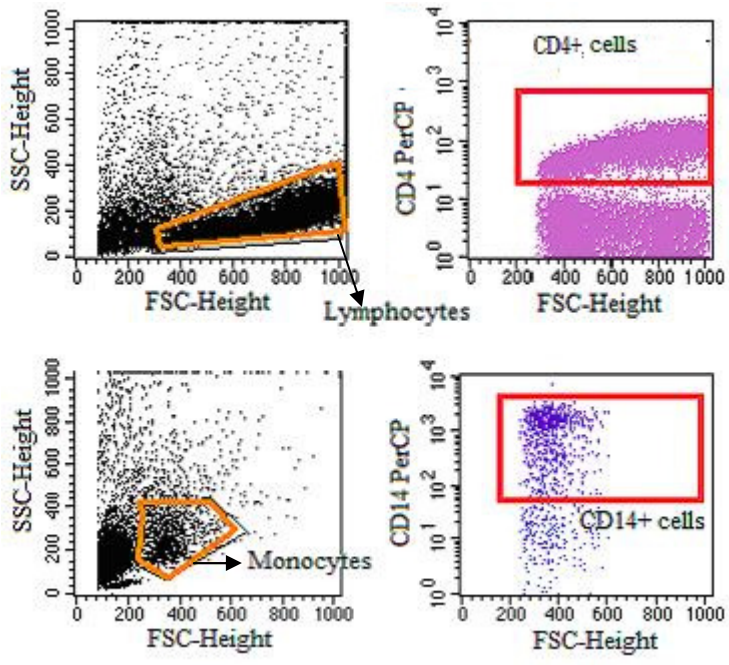


FIGURE 5: Analysing HLA-E expression on different immunological subsets

Lymphocyte and monocyte populations were gated on the basis of SSC and FSC (orange outlines). To study HLA-E expression in different cell types, a second gate was placed on $CD4^+$ (shown here), $CD8^+$, $CD19^+$ or $CD14^+$ cells (red outlines).

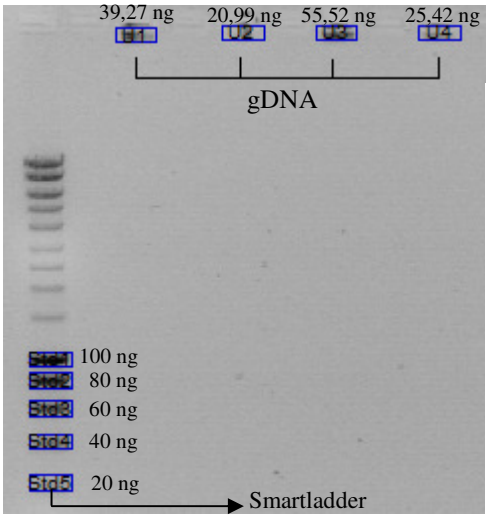
2.6 Determination of HLA-E polymorphims

To determine HLA-E polymorphisms, PBMCs were again isolated by ficoll density centrifugation. Then, genomic DNA was isolated and its concentration was determined using a smart ladder. Finally, Polymorphisms were identified via ARMS-PCR.

2.6.1 Isolation of genomic DNA(gDNA) and determination of gDNA concentration

The pellet of white blood cells was lysed in lysis buffer. 20% SDS and proteinase K were added and overnight incubated at 37°C. During this incubation, proteinase K will destroy the proteins. After incubation, proteins were removed with 6M NaCl and water-satured chloroform. DNA was collected out of the water phase without disturbing the protein layer.

Ethanol was added to precipitate the DNA after which the pellet was dried and dissolved in water. To determine the concentration of gDNA, gel electrophoresis was used. A smart ladder (Eurogentec) was added to define the concentration of gDNA. In this technique the ladder is runned at 130V. As a consequence of the electric charge, the ladder is separated into band pattern with a different density. The density of the gDNA is then compared to the density of the bands of the ladder which matches a defined concentration. In this way, the unknow concentration of the gDNA can be determined (Fig. 6).



Index	Name	Volume INT*mm2	Adj. Vol. INT*mm2	% Adj. Vol.	Concentration
1	Std1	4427.929688	4427.929688	24.99	100.0000000000
2	Std2	4133.496094	4133.496094	23.33	80.00000000000
3	Std3	3641.210938	3641.210938	20.55	60.00000000000
4	Std4	3065.820313	3065.820313	17.31	40.00000000000
5	Std5	2447.558594	2447.558594	13.82	20.00000000000
6	U1	3013.671875	3013.671875	13.26	39.27149671029
7	U2	2546.777344	2546.777344	11.21	20.99490807277
8	U3	3428.808594	3428.808594	15.09	55.52202553232
9	U4	2659.765625	2659.765625	11.71	25.41783487754

FIGURE 6: Determination of the concentration of gDNA

The concentration of gDNA is determined using a smartladder. Via a computer program, standard concentrations are adjugded to the smartladder bands. On the basis of these standards, the concentration of gDNA can be defined.

2.6.2 Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR)

HLA-E polymorphims were detected via ARMS-PCR (Fig. 7). A standard PCR contains 3 main steps. In a first step, gDNA is denaturated and the strands are separated. The second step is the annealing step which means that the primers bind to single-stranded DNA. The last step, is the elongation step. During this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand.

ARMS-PCR requires a single PCR reaction but instead of two primers, four primers are used: two primers correspond to the polymorphic site and are complementary with the 3'-terminal nucleotides. The other two primers are external to the first set and target nonpolymorphic sites. Thus, the polymorphism is detected on the basis of the lengths of the PCR products (46). The primer sequences are described in table 3.

TABLE 3: Primer sequences

Polymorphism	Primer sequence	Products lengths
Codon 77	5'-TGAAGTATTTCCACACTTCCGTGTCCCGGCC-3'	control: 346
(C --> T)	5'-CAGGGACACCGCACAGATTTTCCGAGTCGA**C**-3'	C: 159
	5'-ATTGTAGTAGCCGCGCAGCGTCCGCGG**A**-3'	T: 245
	5'-AATCTGGGACCCGAAGATTCGAGGGGACCC-3'	
Codon 107	5'-CAAAATGCCCACAGGGTGGTGGCGACGGG-3'	control: 381
(A --> G)	5'-ATGCATGGCTGCGAGCTGGGGCCCGAA**A**-3'	A: 290
	5'-GAACTGTTCATACCCGCGGAGGAAGCGAC**C**-3'	G: 158
	5'-GGAGATGGGAGAGTAGCCCTGTGGACCCTC-3'	

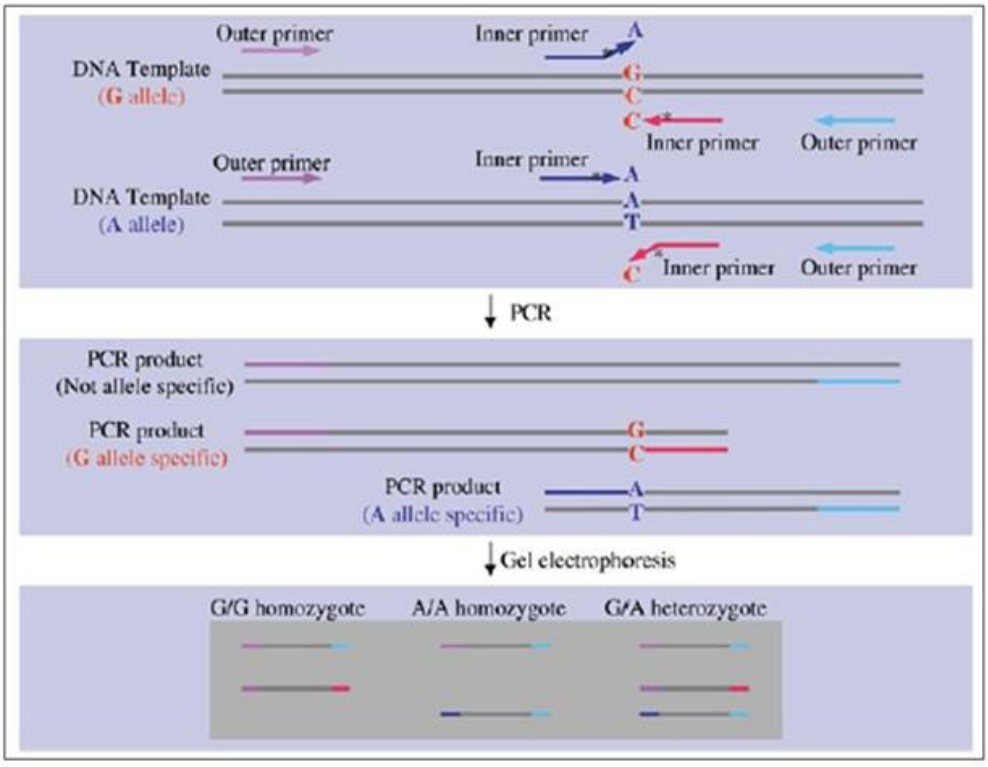


FIGURE 7: ARMS-PCR

ARMS-PCR is a PCR which requires four primers. The two outer primers, in purple and light-blue, amplificate a bigger part of the HLA-E allele. The two inner primers, in dark-blue and in pink, correspond to the plymorphyc sites. This technique allows to define if individuals are homozygous or heterezygous for the alleles found at codon 107 and 77.

The PCR mixture consists of 10 X PCR buffer (Roche), 0,2mM dNTP (Roche), 2 µl of each primer, 0,9M of TAQ polymerase (Roche), 100 ng/ml of gDNA and AD. PCR programs are shown in table 4. PCR products were visualized by electrophoresis. Amplificated DNA and a 100 basepairladder (invitrogen) were added on a 2% agarose gel. This ladder allows to define the length oh the PCR products. For codon 107, products which express a length of 290 basepairs have an adenin on DNA level while the one which have a length of 158 express a guanin. For codon 77, DNA with 159 basepairs has a cytosine and DNA with 245 basepairs expresses a thymidine.

TABLE 4: PCR program

Cycles	PCR steps	Temperature (°C)	Time (min.)
1x	Predenaturation	94	5
35x	Denaturation	94	1
	codon 77 (hybridization)	58	1
	codon 107 (hybridization)	68	1
	Elongation	72	1
1x	Elongation	72	10

Part 3: Results

3. Results

The aim of the study is to investigate HLA-E expression after activation on T cells, B cells and monocytes. To this extend, monocyte isolation procedures were first optimised. Positive and negative selection techniques were tested to determine which one gains the highest purity and yield. Next, the influence of activation on HLA-E expression of immune cells was studied in HC and MS patients. On the other hand the influence of the HLA-E genotype on HLA-E surface expression after activation was examined. To reach this aim of the study, HLA-E polymorphisms were first determined in HC via ARMS-PCR. Secondly, the correlation between HLA-E polymorphisms and HLA-E surface expression after activation was studied in 3 HC for each genotype.

3.1 Optimising monocyte isolation: negative and positive selection procedures

Monocytes can be isolated via positive and negative isolation. To test which isolation technique leads to the highest purity, the maximal yield and best activation conditions both isolation procedures were tested for one donor. In negative selection, non-monocytes, such as T cells, NK cells, B cells, dendritic cells, and basophils, are indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies. Unlabeled monocytes are obtained by depletion of the magnetically labeled cells. In positive isolation, monocytes are magnetically labeled with CD14 Microbeads. The labeled cells are placed in a column and subjected to a magnetic field. The magnetically labeled monocytes are retained in the column while the other cells pass through it.

First, after isolation both techniques were compared at the purity level. The amount of cells obtained by both techniques is the same and the purity of positive selection (91%) is slightly higher compared to the purity obtained after negative selection procedure (87%).

Secondly, best activation conditions were determined. Monocytes obtained by both techniques were plated in a 24 well plate at a density of $0{,}5x10^6$ cells/well or in a flat or round bottom 96 well plate at a density of $10x10^4$ and $5x10^4$ cells/well. Monocytes were activated with LPS during 96h. At different time points activation level of monocytes was studied. To this purpose, cells were stained with CD14PE and CD80 Fitc. The results are shown in figure 8. In monocytes obtained by positive selection and plated in a flat bottom 96 well plate at a concentration of $5x10^4$ cells/well, CD80 expression is upregulated after stimulation with LPS

but not in the control conditions (CM) especially after 24h and 48h of activation. On the other hand, all other conditions also led to activation of monocytes cultured without any stimuli. Therefore, the conditions in which monocytes are obtained by positive selection and plated in a flat bottom 96 well plate at a density of $5x10^4$ cells/well give the best results. Therefore, in future experiments, monocytes will be isolated by positive selection and plated in a flat bottom 96 well plate at a density of $5x10^4$ cells/well.

3.2 The influence of activation on HLA-E expression of immune cells

Animal models have demonstrated the importance of Qa-1 restricted $CD8^+$ T cells in suppressing autoreactive T cells. Qa-1 is a non classical MHC class Ib molecule to which, the human variant, HLA-E, is structurally equal. Little information is available about the expression profile of HLA-E on immunological cells in HC and MS patients. For this reason HLA-E surface expression was investigated before and after activation of immunological subsets. PBMCs and monocytes were isolated from peripheral blood of HC (n=15) and MS patients (n=3). B cells were activated with CpG and IL-2, T cells with anti-CD3 and monocytes with LPS. Figure 9 shows an example of an analysis report of HLA-E expression on the activated T cells (A,B), B cells (C) and monocytes (D). First, the lymphocyte and the monocyte population was gated based on there FSC and SSC. A second gate was then placed on $CD4^+$ (A), $CD8^+$ (B) or $CD19^+$ (C) positive cells for the lymphocytes and $CD14^+$ (D) cells for the monocytes. CD25 expression for T and B cells is defined as a percentage of the gated cell fraction. CD25 is the alpha chain of the IL-2 receptor and is present on activated T cells and activated B cells. The interaction between IL-2 and its receptor leads to B and T cells proliferation. CD80, on the other hand is expressed here as the mean fluorescence intensity (MFI) and is a protein found on activated monocytes. CD80 binds to CD28, which is involved in T cell activation. HLA-E surface levels are also expressed as the mean fluorescence intensity as shown in the right part of figure 9.

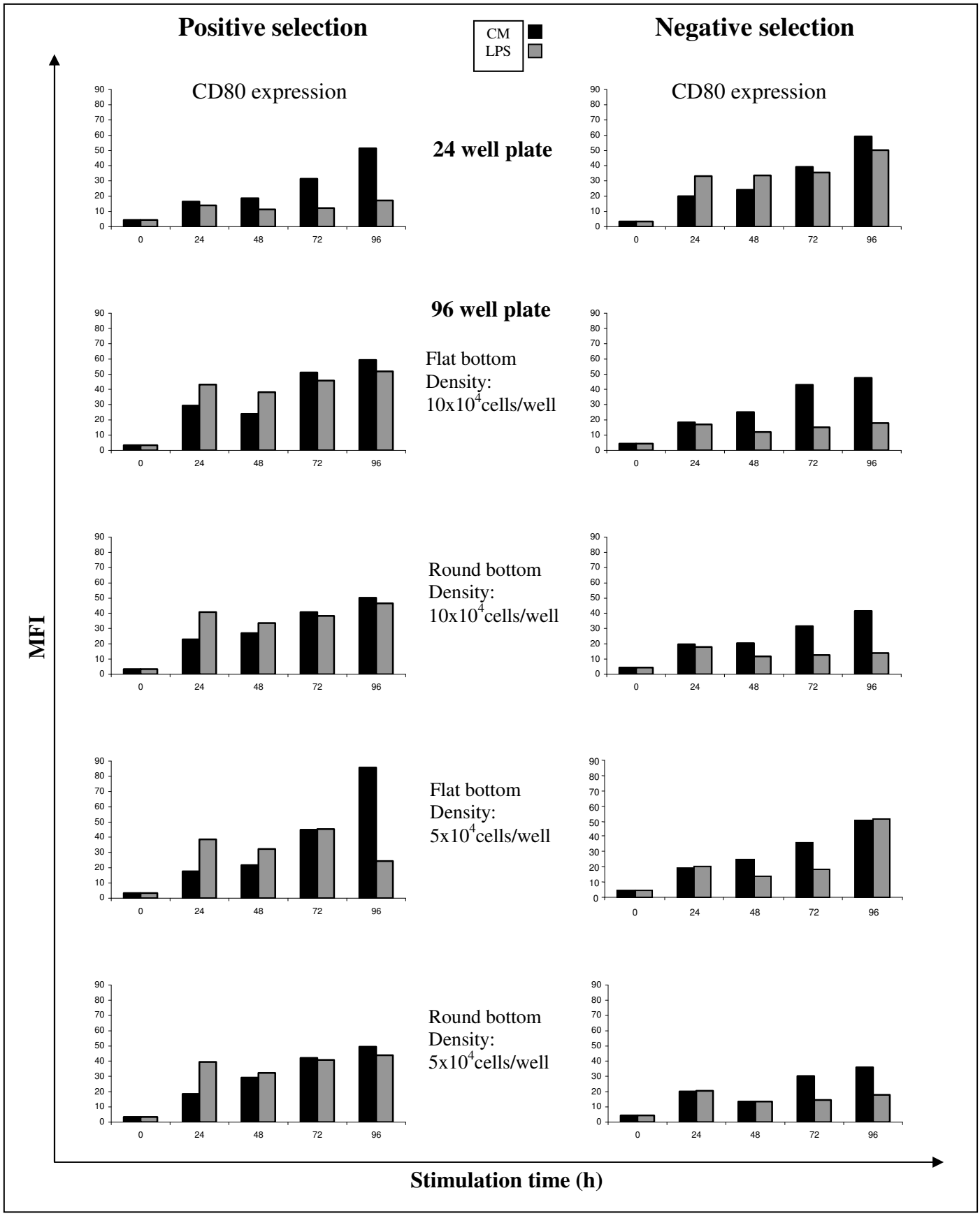


FIGURE 8: Optimising monocyte isolation

After using negative selection CD80 expression is higher in monocytes cultured in culture medium compaired to monocytes activated with LPS. Monocytes isolated via positive selection plated in a flat bottom 96 well plate at density of $5x10^4$ cells/well show an upregulation of CD80 expression after stimulation with LPS.

The influence of activation on HLA-E expression was measured in 15 HC (Fig. 10). Part A of the figure demonstrates that HLA-E expression is upregulated after 1 day of stimulation on both $CD4^+$ and $CD8^+$ T cells and back downregulated after 2 days, while CD25 expression remains high during the entire activation period. Stimulation of B cells with CpG also leads to the upregulation of HLA-E expression after 1 day. This expression level remains high throughout the activation time similar to CD25.

Activation of monocytes with LPS leads to an upregulation of CD80 which increases throughout the stimulation time (Fig. 10 B). CD80 expression is also upregulated in the negative control (CM) but this upregulation tends to be lower than the one observed in activated monocytes. No differences in HLA-E expression was detected between non activated and activated monocytes. However, high constitutive levels of HLA-E expression on non activated monocytes is observed compare to non activated T cells and B cells.

To test whether HLA-E expression is also upregulated in MS patients, the same experiment as described above was performed with PBMC of 3 MS patients (Fig. 11). No evident differences between HC and MS patients could be detected. However, this study group is to small to draw any conclusions and expanding the amount of MS samples is necessary to confirm these observations.

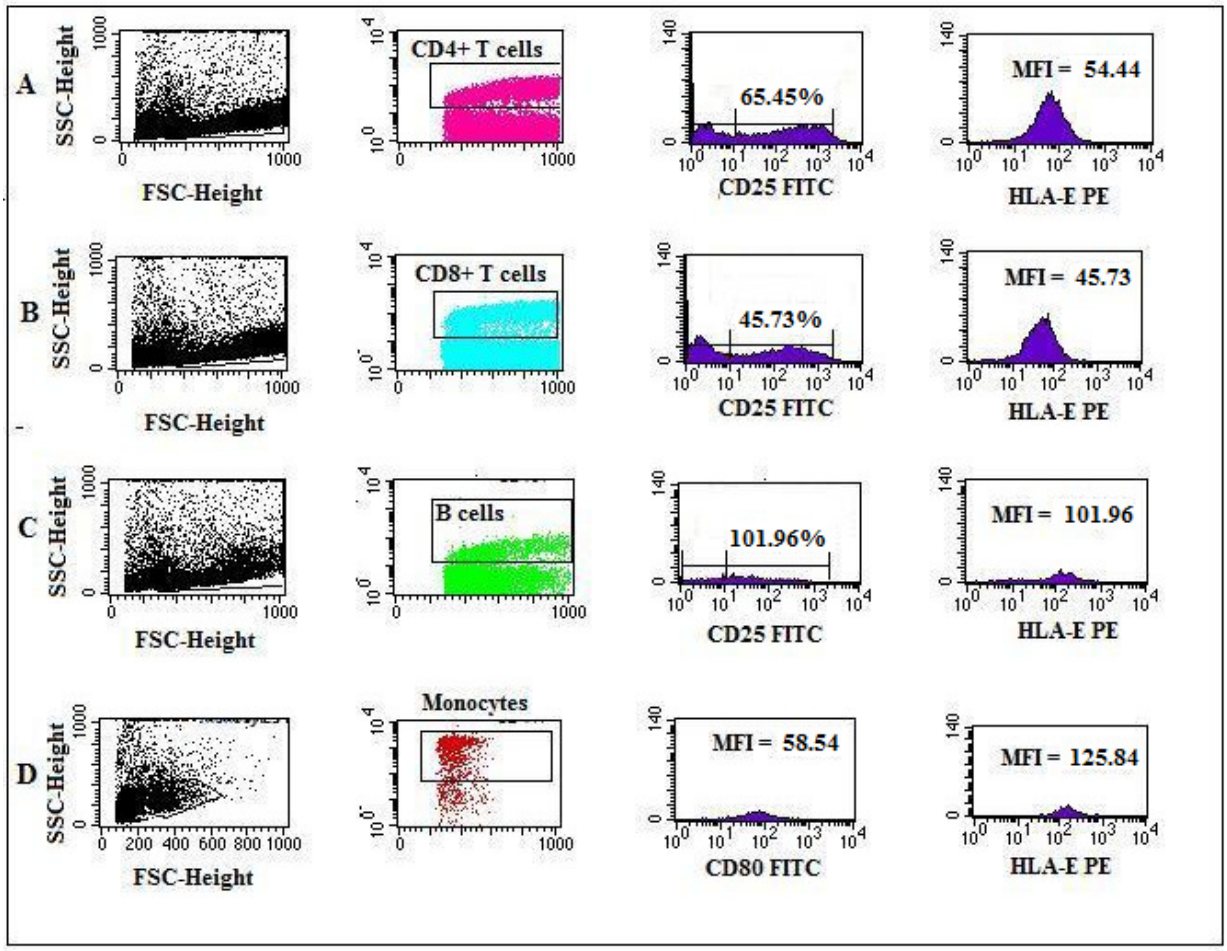


FIGURE 9 : Analysis of CD25, CD80 and HLA-E expression on $CD4^+$ and $CD8^+$ T cells, B cells and monocytes

T cells, B cells and monocytes were activated during 5 days with respectively anti-CD3, CpG and LPS. Lymphocyte and monocyte populations were gated. A second gate was placed on the $CD4^+$ (A), $CD8^+$ (B), $CD19^+$ (C) and $CD14^+$ (D) cells. CD25, CD80 and HLA-E expression was studied in these immunological subsets. CD25 expression is expressed as a percentage of the gated cells while CD80 and HLA-E expression are expressed as the mean fluorescence intensity (MFI).

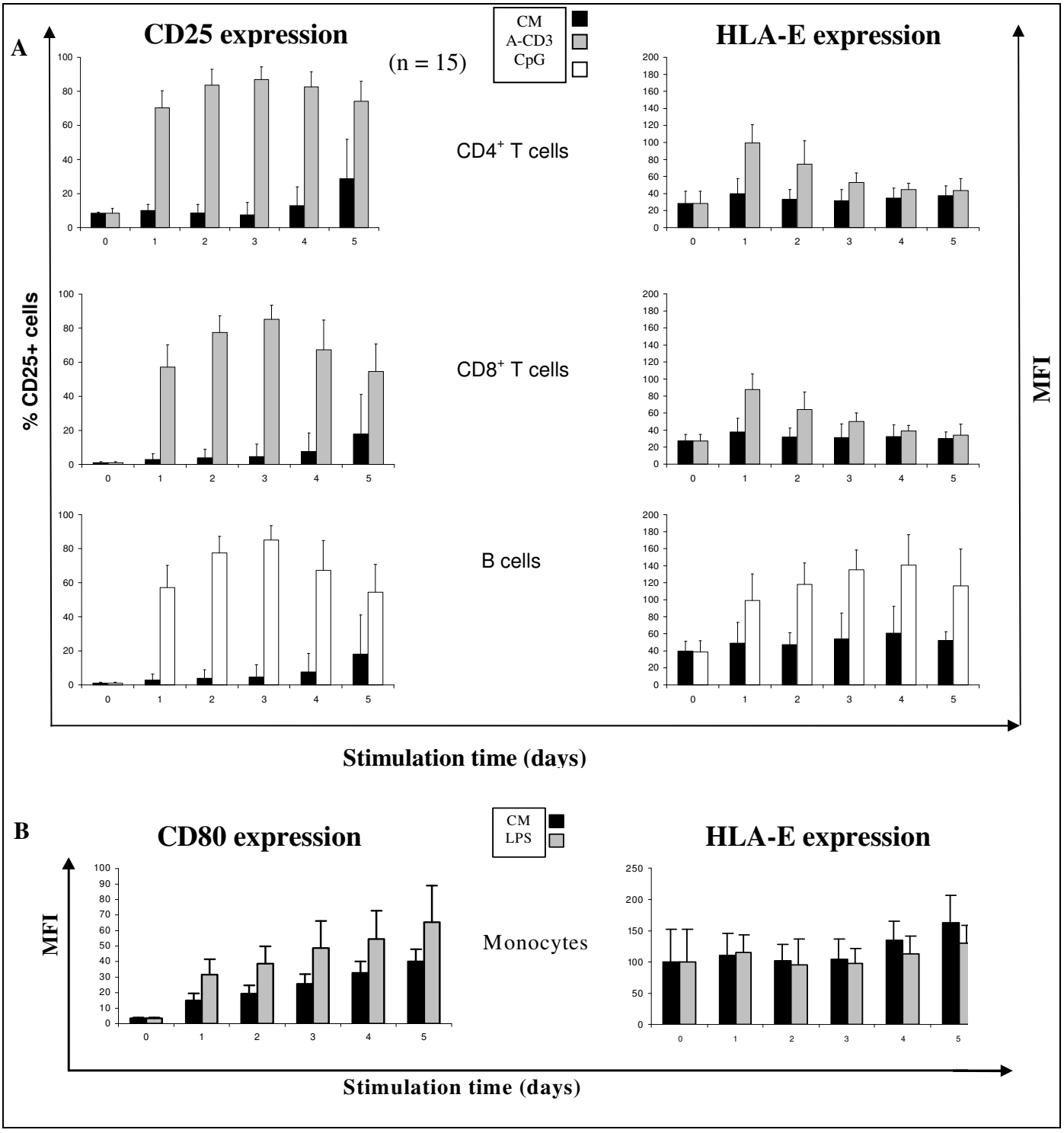


FIGURE 10 : Measuring HLA-E expression after activation of T cells, B cells and monocytes

A. Cells were activated during 5 days. Culture medium (CM) without any stimulus is used as a negative control. Anti-CD3 and CpG were used to respectively activate T cells B cells. The surface expression of CD25 and HLA-E were determined on $CD4^+$, $CD8^+$ T cells and B cells. In T cells HLA-E expression is upregulated after 1 day of activation and back downregulated while CD25 expression remains high during the stimulation time. In B cells HLA-E expression is upregulated after 1 day of activation and remains high during the 5 days of activation.

B. Monocytes were activated during 5 days with LPS. CM is again used as a the negative control. Although CD80 expression is also upreagulated in CM, this upregulation is lower compaired to the CD80 upregulation on activated monocytes. For HLA-E expression, no differences between CM and activated monocytes can be observed.

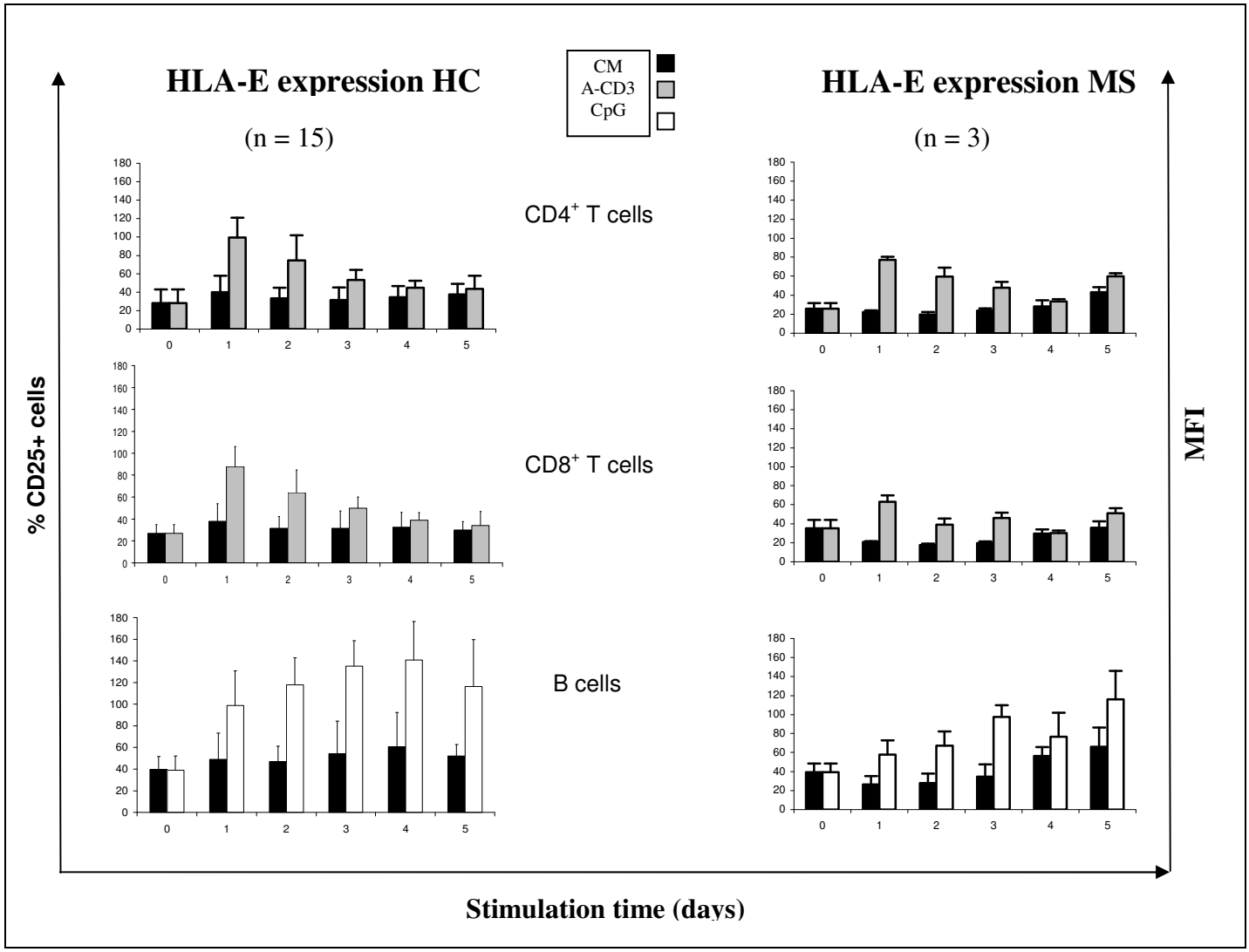


FIGURE 11: Comparing HLA-E expression after activation between HC and MS patients

Again, T and B cells of 3 MS patients were activated during 5 days with anti-CD3 and CpG respectively. Like in HC, HLA-E expression in T cells is upregulated after 1 day of activation and back downregulated. Also in B cells, HLA-E expression is upregulated after 1 day of activation and remains high throughout the activation time.

3.3 Studying the functional aspect of HLA-E polymorphisms

HLA-E harbors only two polymorphic sites. At codon 107, HLA-E*0101 (A) codes for the aminoacid arginin while HLA-E*0103 (G) codes for a glycine. On the other hand, at codon 77, HLA-E*0103 is dividing in HLA-E*010301 (T) and HLA-E*010302 (C). This is a silent substitution. In this part of the study, HLA-E genotypes were first determined in HC. Secondly, the influence of each HLA-E genotype on HLA-E surface expression was investigated before and after activation.

3.3.1 Annealing patterns of the primers used in the ARMS-PCR

HLA-E polymorphisms was determined via ARMS-PCR. In this technic, the gDNA is amplificated with a PCR reaction and then separated via electrophoresis. The length of the gDNA fragments is determined with the help of a 100 basepair ladder. Figure 12 shows a part of the gDNA sequence of HLA-E with the two polymorphic sites. The different HLA-E sequences were aligned via clustalW which allows to highlight the polymorphics sites and shows the annealing sites of the used primers. At codon 77, the polymorphism is indicated in pink and at codon 107 it is indicated in blue. The annealing sites of the primers are showed in the same colors. The outer primers bind the DNA independently of the present polymorphism. The inner primers are specific to the polymorphic sites and binds exactly at this site.

```
                          5′    Outer primer 1          3′
0101     CCCCAGGCTCCCACTCCTTGAAGTATTTCCACACTTCCGTGTCCCGGCCC 3050
010301   CCCCAGGCTCCCACTCCTTGAAGTATTTCCACACTTCCGTGTCCCGGCCC 3050
010302   CCCCAGGCTCCCACTCCTTGAAGTATTTCCACACTTCCGTGTCCCGGCCC 3050
         **************************************************

0101     GGCCGCGGGGAGCCCCGCTTCATCTCTGTGGGCTACGTGGACGACACCCA 3100
010301   GGCCGCGGGGAGCCCCGCTTCATCTCTGTGGGCTACGTGGACGACACCCA 3100
0103012  GGCCGCGGGGAGCCCCGCTTCATCTCTGTGGGCTACGTGGACGACACCCA 3100
         **************************************************

0101     GTTCGTGCGCTTCGACAACGACGCCGCGAGTCCGAGGATGGTGCCGCGGG 3150
010301   GTTCGTGCGCTTCGACAACGACGCCGCGAGTCCGAGGATGGTGCCGCGGG 3150
010302   GTTCGTGCGCTTCGACAACGACGCCGCGAGTCCGAGGATGGTGCCGCGGG 3150
         **************************************************

0101     CGCCGTGGATGGAGCAGGAGGGGTCAGAGTATTGGGACCGGGAGACACGG 3200
010301   CGCCGTGGATGGAGCAGGAGGGGTCAGAGTATTGGGACCGGGAGACACGG 3200
010302   CGCCGTGGATGGAGCAGGAGGGGTCAGAGTATTGGGACCGGGAGACACGG 3200
         **************************************************
               5′      inner primer 1       3′         inner
0101     AGCGCCAGGGACACCGCACAGATTTTCCGAGTGAACCTGCGGACGCTGCG 3250
010301   AGCGCCAGGGACACCGCACAGATTTTCCGAGTGAACCTGCGGACGCTGCG 3250
010302   AGCGCCAGGGACACCGCACAGATTTTCCGAGTGAATCTGCGGACGCTGCG 3250
         ********************************** **************
          primer 2   5′
0101     CGGCTACTACAATCAGAGCGAGGCCGGTGAGTGACCCCGGCCAGGGGAGC 3300
010301   CGGCTACTACAATCAGAGCGAGGCCGGTGAGTGACCCCGGCCAGGGGAGC 3300
```


010302 CGGCTACTACAATCAGAGCGAGGCCGGTGAGTGACCCCGGCCAGGGGAGC 3300
5′ outer
0101 AGGTCACGACCCCTCCCCATCCCCCACGGACGGCGCGGGTCCCCTCGAAT 3350
010301 AGGTCACGACCCCTCCCCATCCCCCACGGACGGCGCGGGTCCCCTCGAAT 3350
010302 AGGTCACGACCCCTCCCCATCCCCCACGGACGGCGCGGGTCCCCTCGAAT 3350
primer 2 3′
0101 CTTCGGGTCCCAGATTCACCCCAAGGCTGCGGAACCCGCCCAGACCCTAG 3400
010301 CTTCGGGTCCCAGATTCACCCCAAGGCTGCGGAACCCGCCCAGACCCTAG 3400
010302 CTTCGGGTCCCAGATTCACCCCAAGGCTGCGGAACCCGCCCAGACCCTAG 3400
5′
0101 ACCGGGGAGAGTCTCAGGCGCCTTTACCCGGTTCTTTTTCAGTTTAGGCC 3450
010301 ACCGGGGAGAGTCTCAGGCGCCTTTACCCGGTTCTTTTTCAGTTTAGGCC 3450
010302 ACCGGGGAGAGTCTCAGGCGCCTTTACCCGGTTCTTTTTCAGTTTAGGCC 3450
outer primer 1 3′
0101 AAAATGCCCACAGGGTGGTGGCGACGGGGGCGGGGCTTGGTGGGCGGGAC 3500
010301 AAAATGCCCACAGGGTGGTGGCGACGGGGGCGGGGCTTGGTGGGCGGGAC 3500
010302 AAAATGCCCACAGGGTGGTGGCGACGGGGGCGGGGCTTGGTGGGCGGGAC 3500
5′ inner
0101 TGACTAAGGGGCGGGGCCAGGGTCTCACACCCTGCAGTGGATGCATGGCT 3550
010301 TGACTAAGGGGCGGGGCCAGGGTCTCACACCCTGCAGTGGATGCATGGCT 3550
010302 TGACTAAGGGGCGGGGCCAGGGTCTCACACCCTGCAGTGGATGCATGGCT 3550
primer 1 3′ inner primer 2 5′
0101 GCGAGCTGGGGCCCGACAGGCGCTTCCTCCGCGGGTATGAACAGTTCGCC 3600
010301 GCGAGCTGGGGCCCGACGGGCGCTTCCTCCGCGGGTATGAACAGTTCGCC 3600
010302 GCGAGCTGGGGCCCGACGGGCGCTTCCTCCGCGGGTATGAACAGTTCGCC 3600
0101 TACGACGGCAAGGATTATCTCACCCTGAATGAGGACCTGCGCTCCTGGAC 3650
010301 TACGACGGCAAGGATTATCTCACCCTGAATGAGGACCTGCGCTCCTGGAC 3650
010302 TACGACGGCAAGGATTATCTCACCCTGAATGAGGACCTGCGCTCCTGGAC 3650
0101 CGCGGTGGACACGGCGGCTCAGATCTCCGAGCAAAAGTCAAATGATGCCT 3700

FIGURE 12 : gDNA sequence of the different HLA-E polymorphisms

gDNA sequence of the different HLA-E polymorphisms have been obtained via NCBI and ClustalW was used to test whether the primers bind to the specific polymorphic sites. In pink the primers are shown which amplificate codon 77 and in blue codon 107.

3.3.2 Determination of HLA-E polymorphisms in healthy controls

ARMS-PCR was performed for gDNA isolated out of PBMC of 39 HC. For each PCR reaction, one positive and negative control was included. The negative control is water. The positive control is gDNA of a healthy control which expresses all the polymorphisms. Figure 13 shows an example for 2 HC. For codon 107 (left part of the gel), a PCR product of 290 basepairs (bp) corresponds to the HLA-E*0101 polymorphism (A) while the band of 158 bp represents the HLA-E*0103 polymorphism (G). The 381 bp band is present in all HC and serves as an intern control for each performed PCR reaction. PCR products of 290 bp and 158 bp are present in both HC1 and HC2. Therefore, HC1 and HC2 express both alleles. For codon 77 (right part of the gel), HLA-E*010301 polymorphism is represented by 159 bp bands while the 245 bp band corresponds to HLA-E*010302 polymorphism. As for codon 107, an intern control represented by 346 bp is present in all HC. In HC1, both HLA-E*010301 and HLA-E*010302 are amplificated while in HC2 only HLA-E*0103101 polymorphism can be detected. On the basis of these results, we can conclude that the genotype of HC1 is 0101/010302 and the genotype of HC2 is 0101/010301.

The genotypes determined in 39 HC are given in table 5. Of all HC, 7,69% express the 0101/010301 (AT/CC) genotype, 25,64% 0101 /0101 (AA/CC), 43,59% 0101/010302 (AG/CT), 10,25% 010302/010302 (GG/TT), 12,82% 010301/010302 (GG/CT). No HC express the 010301/010301 genotype.

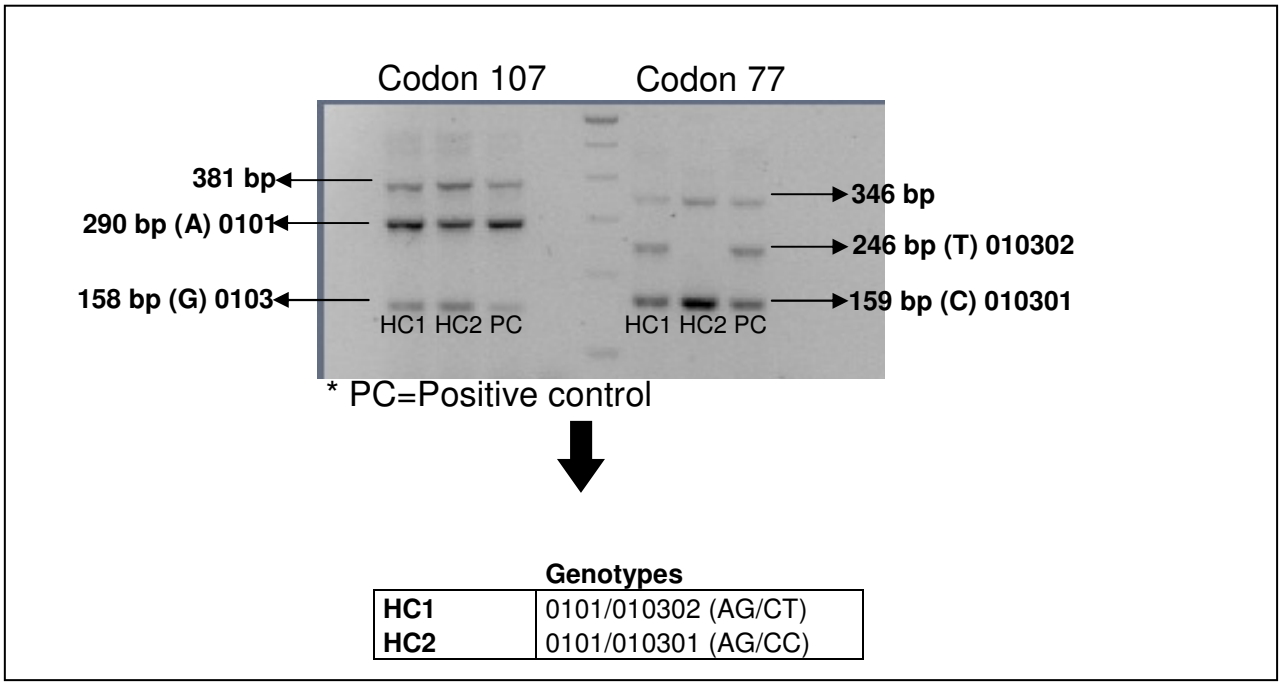


FIGURE 13 : Example of a performed ARMS-PCR (n = 39)

gDNA was isolated and the genotype of HLA-E was determined by ARMS-PCR. For codon 107, in both HC alleles 0101 and 0103 are present. At codon 77, HC1 has 010301 and 010302 while HC2 has only 010301. Therefore, the genotype of HC1 is 0101/010302 and the genotype of HC2 is 0101/010301.

TABLE 5: Genotypes of the HC

Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 5	Gentoype 6
0101/010301 (AG/CC)	**0101/0101 (AA/CC)**	**0101/010302 (AG/CT)**	**010302/010302 (GG/TT)**	**010301/010302 (GG/CT)**	**010301/010301 (GG/CC)**
7.69%	25.64%	43.59%	10.25%	12.82%	0%

3.3.3 Correlation study between HLA-E haplotypes and HLA-E surface expression kinetics after activation

The correlation between HLA-E haplotypes and HLA-E surface expression after activation was studied in 3 HC for each genotype except for the 010301/010301 genotype which could not been found in any HC (table 7). Again, HLA-E expression on T cells is upregulated after 1 day of activation and back downregulated after 2 days of activation. In B cells, HLA-E expression

is also upregulated after 1 day of activation and remains high throughout the activation time. However, the upregulation of HLA-E expression on the surface of B and T cells is significantly lower for HC homozygous for 0103 (G) ($p<0,05$) (Fig. 14). Future experiments need to elucidate the correlation between HLA-E genotypes and HLA-E expression on monocytes. In addition, the functional aspect of HLA-E will also be evaluated in MS patients.

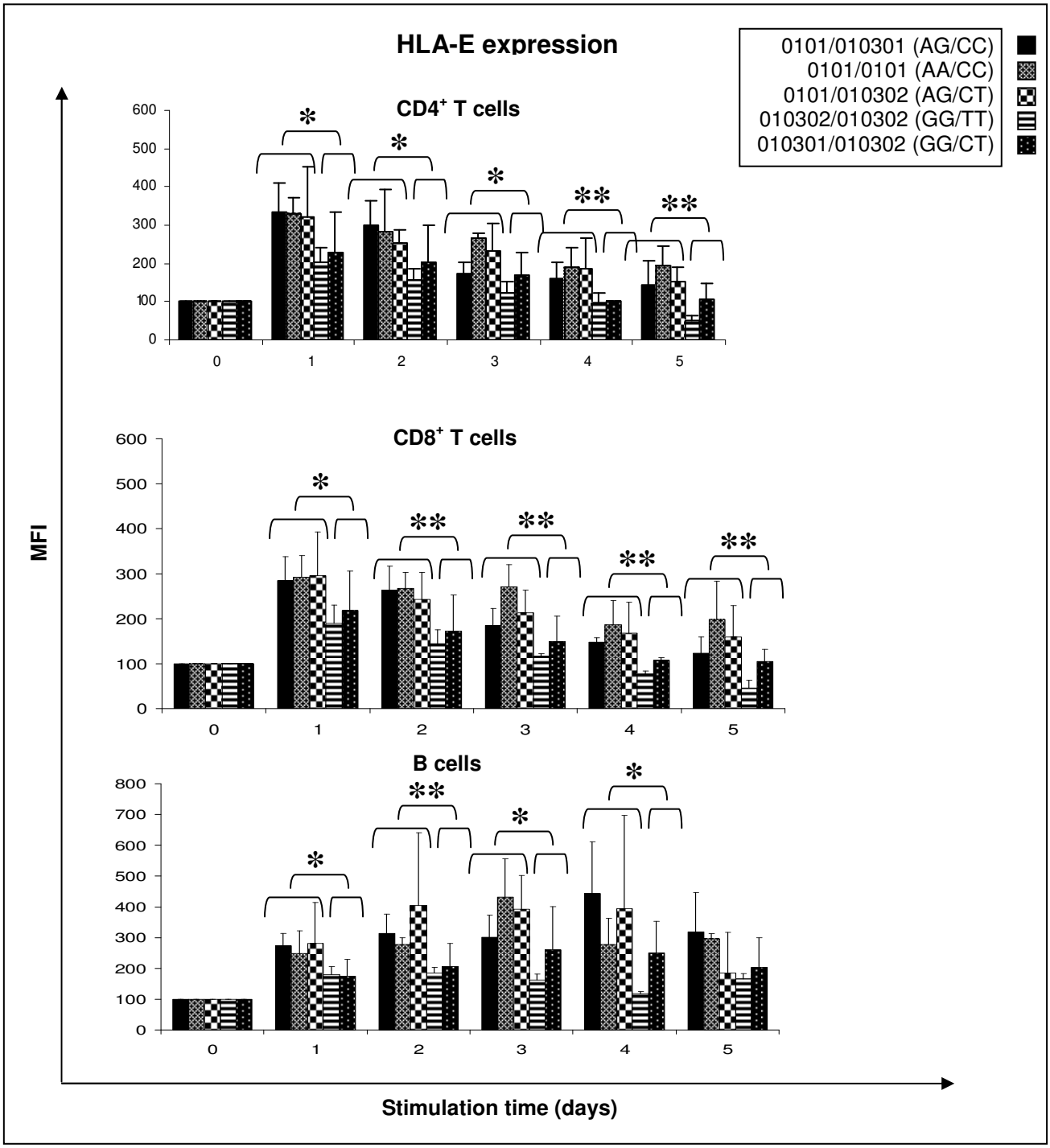


Figure 14: Correlation study between HLA-E surface expression and HLA-E genotypes

HLA-E expression was determined in 3 HC for each genotype. Upregulation of HLA-E expression after activation of B and T cells is lower in HC homozygous for the 0103 (G) allele. * = $p<0.05$ ** = $p<0.01$

Part 4: Discussion

4. Discussion

In MS, there is increasing evidence suggesting that the autoreactive T cell responses to myelin antigens may play an important role in the disease process. These autoreactive cells are present in MS patients as well as in HC but in MS patients, they have a more activated status (47). The presence of these cells and activation in MS patients could be due to deficiencies in self-tolerance mechanisms. One of these mechanisms are Treg cells. It is known that they are involved in the suppression of autoreactive T cells in autoimmunity. However, in MS, the number of Tregs cells is reduced (48-49). A population of naturally occuring regulatory T cells are $CD4^+$ CD25 cells. Animal models show that they are protective regulators in MS by suppressing the function of effector $CD4^+$ $CD25^+$ T cells, cytotoxic $CD8^+$ T cells, NK cells and B cells (48). It's only recently that the concept of regulatory $CD8^+$ T cells in MS has emerged. A number of studies report that $CD8^+$ T cells in EAE, the animal model for MS, have a regulatory function (32). It has been proven that the transfer of $CD8^+$ $CD28^-$ T cells into $CD8^-$ deficient mice suppress EAE. Therefore these cells behave as regulatory T cells. A second type of regulatory $CD8^+$ T cells is found in EAE. These cells are called Qa-1 restricted $CD8^+$ cells. Recent studies show that Qa-1 restricted $CD8^+$ T cells suppress autoreactive T cells (31). Activated $CD4^+$ T cells expressing Qa-1 interact with the TCR of $CD8^+$ T cells (26). This interaction leads to activation of the suppressive activity of the Qa-1 restricted $CD8^+$ T cells. It has recently been discovered that the human homologue, HLA-E, has the same structure as Qa-1 but less information about its function and its expression is available at the moment (50). For that reason, HLA-E surface expression after activation of immunological subsets in HC and MS patients was investigated in this project. The results obtained from HC and MS patients experiments show that HLA-E is expressed on the surface of resting T cells. This HLA-E expression is upregulated after activation but this upregulation is short term. These results are in line with the expression profile of Qa-1 which is also briefly expressed on activated cells (50-51). Studies have showen that Qa-1 is expressed at low levels on resting T cells but that the expression is increased following activation (51). This is not unexpected since both Qa-1 and HLA-E form a heterodimer with β2-microglobulin at the surface of the cells and presents similar peptides. Qdm peptide, for example, is processed and presented via a TAP-dependent pathway while the other peptides as hsp60 are presented in a TAP-indenpendent manner (1,33,39). These peptides can bind to and presented by both Qa-1 and HLA-E. On the other hand, animal models demonstrate that Qa-1 restricted $CD8^+$ T cells have a suppressive function. During the primary immune response, the TCR of autoreactive $CD4^+$ T cells interacts with the

MHC/peptide complex presented by APC which leads to the activation of autoreactive $CD4^+$ T cells and the upregualtion of Qa-1. This subsequently will lead to the activation of Qa-1 restricted $CD8^+$ T cells (38). Since HLA-E is also upregulated after activation, it is presumable, like Qa-1, that HLA-E can present peptides to HLA-E restricted $CD8^+$ T cells and activate the suppressive function of these cells. Co-culture experiments with HLA-E restricted $CD8^+$ T cells and HLA-E expressing APC need to further clarify this hypothesis.

Regarding B cells, studies reported that Qa-1 expression on the surface of activated B cells could stimulate $CD8^+$ T cell suppressive activity and therefore B cells are potent APCs for self-antigens (33). In the performed experiments, HLA-E surface expression on B cells is also upregulated after activation and this upregulation remains high troughout the activation time. Taken together, these data raise the possibility that B cells are better antigen presenting cells in activating HLA-E restricted $CD8^+$ T cells because of the longer HLA-E surface expression. That is consistent with classical immunology in which B cells are professional APCs. T cell activation requires the interaction of the T cell receptor with a MHC/peptide complex. To reach an efficient activation, co-stimulatory signals are necessary. Indeed, the interaction between CD28 expressed on T cells and B7 family members expressed on professional APC like B cells is essential.

The results obtained for the monocytes are not conclusive. Monocytes cultured in culture medium without any stimulus added were also activated. This can be due to a non-efficient isolation procedure because granulocytes (CD14 low +) were still present after magnetic separation (+/- 30%). The present granulocytes can be phagocytosed by the monocytes which leads to their activation even without any stimulus. In the future monocytes will be sorted using a facs aria to generate a more pure monocyte population (>95%).

In MS patients the same results were observed but since the experiment was performed in only 3 MS patients no definite conclusions may be drawn. To further elucidate the functional role of HLA-E in MS patients that is to say its possible suppresive activity, it is necessary to expand this sample group.

According to the literature, HLA-E*0101 ($HLA\text{-}E^R$) and HLA-E*0103 ($HLA\text{-}E^G$) were preserved during the evolution and the distribution of these two alleles in various population groups is nearly equal (HLA-E5). To explain this distribution, a balancing selection was proposed indicating that a functional difference may exist between both alleles (35). The results obtained by the determination of the genotype in HC experiment is in line with the literature.

The homozygous states fro 0101 and 0103 alleles are equeal. Therefore these alleles are preserved during the evolution.

Studies on Behcet's disease (BD) show that the T allele at codon 77 (010302) is associated with a higher risk of this disease while HLA-E*0101 is associated with a reduced risk of BD. This can be explained by the observation that HLA-E*0103 allele is more thermally stable than HLA-E*0101 which means that its surface expression is higher. In addition, CD94/NKG2 is more efficiently recognized by HLA-E*0103. The presentation of the leader peptide from class Ia by HLA-E*0103 results in an inhibition of NK cells while HLA-E*0101 doesn't inhibit NK cells (42). Studies on sickle cell anemia which is a blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape, suggest that, in contrast to BD, HLA-E*0101/0101 is more prevalent in patients with severe bacterial infections. One of the hypotheses proposed to explain this was an inefficient or absent presentation of bacterial peptides due to the poor expression of HLA-E*0101 on the cell surface. This impaired presentation may lead to the alteration of an efficient T cell response. Furthermore, it has been show that HLA-E 0101/0103 was more prevalent in individuals without infections. Therefore, this genotype has a protective effect (52).

Recent studies performed at BIOMED show that there is no significant association between HLA-E polymorphisms and MS, though the C allele is overrepresented in MS patients. Therefore, in this part of the research functional differences were studied, more specific the correlation between HLA-E genotypes and HLA-E surface expression in HC. The results show that in HC homozygous for 0103 allele, the upregulation of HLA-E after activation is lower compared to the others genotypes. This could mean that, in HC homozygous for 0103 allele, presentation of peptides by HLA-E is decreased because of the lower surface expression. This in turn could lead to an inefficient activation of HLA-E restricted $CD8^+$ T cells wich as a consequence may not exert their regulatory function. On the contrary, in HC homozygous for 0103 allele, peptides could bind less well to HLA-E which leads to a lower surface expression because previous studies showed that HLA-E surface expression is only stable when a peptide is bound in the HLA-E groove (35). However, this is not in line with the litterature in which it has been proven that the surface expression of the HLA-E*0103 molecule is more stable than the HLA-E*0101 molecule.

In conclusion, this study demonstrates that, like Qa-1, HLA-E is expressed on resting B and T cells and upregulated after activation. On T cells, upregulation of HLA-E expression is short

term while in B cells, this expression remains high. Furthermore, we show that this upregulation is lower in HC homozygous for 0103.

Future research in necessary to further unravel the role of HLA-E in HC and MS patients. Therefore HLA-E expression will be further investigated in MS patients to see whether it is differenting modulated after activation. In addition, correlation between HLA-E genotypes and surface expression will again be determined. Finally to complete this study, the influence of a pro- and anti-inflammatory environment on HLA-E surface expression will be investigated to gain more information about its role in the pathological processes involved in MS. These experiments will also lead to a better understanding of the contribution of HLA-E in the activation of suppressive $CD8^+$ T cells and the suppressive function of the HLA-E restricted $CD8^+$ T cells. This in turn may gain more insight into the role of these cells in HC and MS patients which can contribute to the development of new and more specific therapies in MS.

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