The Role of HLA-G-expressing Regulatory T cells in Multiple Sclerosis

: A Perspective of Beneficial Inflammation in the Central Nervous System Inflammation

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Yu-Hwa, Huang

Würzburg, July 2009

To my parents and mz sister

CONTENTS

1. Zusammenfassung

Die Regulation von Effektor-T-Zellen ist ein wichtiger Mechanismus zur Kontrolle organ-spezifischer Entzündungen. Dabei sind regulatorische T-Zellen (T_{reg}) maßgeblich an der Aufrechterhaltung peripherer Immuntoleranz und parenchymaler Immunhomöostase beteiligt. Eine neue Population von humanen, natürlich vorkommenden T_{reg} Zellen wurde durch ihre konstitutive Expression des immuntolerogenen Moleküls HLA-G identifiziert.

Im ersten Teil dieser Arbeit wurden die Mechanismen, durch die $CD4^+$ HLA-G^{pos} T_{reg} Zellen ihre Zielzellen (autologe HLA-G^{neg} T-Zellen) modulieren, aufgeklärt. Unter Verwendung eines Suppressionsansatzes in Abwesenheit von antigenpräsentierenden Zellen (APC) wurden T-T-Zell-Interaktionen, die die Proliferation von HLA-G^{neg} T-Zellen hemmen, demonstriert. Diese Suppression, die durch die Stimulierung des T-Zell-Rezeptors auf HLA-G^{pos} T_{reg} Zellen verstärkt wurde, war unabhängig vom Zell-Zell-Kontakt. Die HLA-G^{neg} T-Zellen erlangten nach Entfernung der HLA-G^{pos} T_{reg} Zellen und einer erneuten Stimulierung ihrer T-Zell-Rezeptoren ihre Fähigkeit zur Proliferation wieder. Dies wies auf die Umkehrbarkeit dieser Suppression hin.

Darüber hinaus war die HLA-G^{pos} T_{reg}-vermittelte Suppression entscheidend von der IL-10-Sekretion, nicht jedoch von TGF-β abhängig. Zusammengefasst beschreibt dieser Teil der Arbeit eine detaillierte Charakterisierung der Mechanismen, wie HLA-G^{pos} T_{reg} HLA-G^{neg} T-Zellen supprimieren. Das tiefere Verständnis der Wirkmechanismen von HLA-G^{pos} T_{reg} könnte in therapeutischen Strategien verwendet werden, in denen die regulatorische Funktion der T-Zell-Suppression verstärkt oder moduliert werden soll.

Im zweiten Teil dieser Arbeit wurde die potenzielle Rolle von HLA-G^{pos} T_{reg} bei der Multiplen Sklerose (MS) untersucht, einer klassischen Autoimmunerkrankung des Zentralnervensystems (ZNS). Im Gegensatz zu Vergleichspatienten mit nicht-entzündlichen Erkrankungen konnte im Liquor von MS Patienten eine erhöhte Anzahl von HLA-G^{pos} T_{reg} gefunden werden. Diese aus dem Liquor isolierten HLA-G^{pos} T_{reg} wiesen phänotypische Merkmale von zentralen Gedächtnis-T-Zellen ($CD45RA^-$ $CD27^+$) auf, exprimierten den Aktivierungsmarker ICOS sowie deutlich höhere Level des Chemokinrezeptors (CCR) CCR5 und agierten als starke Suppressoren der autologen $CD4^+$ T-Zellproliferation. Durch Verwendung eines *in vitro* Modells der humanen Bluthirnschranke konnte demonstriert werden, dass HLA-G^{pos} T_{reg} eine starke Neigung zur Migration haben, die durch die CCR5-Liganden MIP1α und RANTES, nicht jedoch durch MIP3β (Ligand von CCR7) unterstützt wird. Diese Chemokin-induzierte Migration von HLA-G^{pos} T_{reg} war auch mit einer Steigerung der suppressiven Kapazität nach Zelltransmigration assoziiert. Im Gegensatz zu $CD4^+CD25^+$, FoxP3-exprimierenden T_{reg} zeigten HLA-G^{pos} T_{reg} von MS-Patienten keine beeinträchtigte

Funktionalität. Dies deutet auf eine selektive Rekrutierung von HLA-G^{pos} T_{reg} zu Entzündungsherden im ZNS und ihre Beteiligung an der Bekämpfung der destruktiven Entzündung hin.

Die Ergebnisse dieser Studien tragen zum weitergehenden Verständnis der Rolle und Funktion HLA-G^{pos} T_{reg} Zellen bei und stellen somit ein wichtiges pathophysiologisches Beispiel „gutartiger“ T-Zell-Entzündung während der ZNS Autoimmunität dar, das sowohl aus pathophysiologischer als auch therapeutischer Sicht interessant ist.

2. Summary of the study

Regulation of effector T cells is an important mechanism to control organ-specific inflammation. Thereby regulatory T cells (T_{reg} cells) are essential for maintaining peripheral immune tolerance and for establishing parenchyma immune homeostasis.

A novel population of natural human T_{reg} characterized by the constitutive expression of the immune-tolerogenic human HLA-G molecule has been identified.

In the first part of the study, we elucidated the mechanism(s) by which $CD4^+$ HLA-G^{pos} T_{reg} modulates their cellular targets namely autologous HLA-G negative responder T cells (HLA-G^{neg} T_{resp}). Using a suppression system free of antigen-presenting cells (APC), we demonstrate a T-T cell interaction resulting in suppression of HLA-G^{neg} T_{resp}. We could also show that this suppression was independent of cell-cell contact. Importantly, stimulus of T cell receptor (TCR) on HLA-G^{pos} T_{reg} facilitated their suppressive capacity. We also observed that removal of HLA-G^{pos} T_{reg} from the established co-cultures could restore the ability of HLA-G^{neg} T_{resp} to proliferate upon TCR re-stimulation, indicating that the suppression was reversible. Further, HLA-G^{pos} T_{reg}–mediated suppression was critically depending on the secretion of IL-10 but not TGF-β. Taken together, this part of the work provides an in-depth characterization of the mechanisms of how HLA-G^{pos} T_{reg} suppresses T responder cells in direct T-T interactions. Understanding the suppressive mechanism used by HLA-G^{pos} T_{reg} may help to develop therapeutic strategies to modulate regulatory arms of T-cell suppression.

In the second part of this study, the potential role of HLA-G^{pos} T_{reg} in the pathophysiological process of Multiple Sclerosis (MS), a prototypic autoimmune inflammatory central nervous system (CNS), has been investigated. We found that HLA-G^{pos} T_{reg} are enriched in the cerebrospinal fluid (CSF) from MS patients, but not in non-inflammatory controls. CSF-derived HLA-G^{pos} T_{reg} showed predominance of central memory ($CD45RA^-CD27^+$) phenotype, exhibited markers of activation (ICOS), and had significantly higher expression of the inflammatory chemokine receptor CCR5. Importantly, these cells demonstrated as potent suppressors to autologous $CD4^+$ T-cell proliferation. Using an *in vitro* model of human blood brain barrier, we showed that HLA-G^{pos} T_{reg} have a strong propensity to migrate, which could be facilitated by MIP1α and RANTES (ligands of CCR5) but not MIP3β (a ligand of CCR7). The HLA-G^{pos} T_{reg} migration triggered by chemokines was also associated with a gain of suppressive capacity upon cellular transmigration. In contrast to $CD4^+CD25^+$ naturally occurring FoxP3-expressing T_{reg}, HLA-G^{pos} T_{reg} from patients with MS did not exhibit impaired function, suggesting that HLA-G^{pos} T_{reg} are selectively recruited to the sites of CNS inflammation in an effort to combat destructive inflammation during MS. Our results contribute to the understanding of the role and function of HLA-G^{pos} T_{reg} and provide an

important example of “beneficial” T-cell inflammation in CNS autoimmunity- interesting both from a patho/-physiological and a therapeutically point of view.

3. Introduction

3.1 The tolerogenic non-classical MHC class I molecule HLA-G

3.1.1. Classical major histocompatibility complex class I molecules

The major histocompatibility complex (MHC) is an essential element in immune function and combines two classes of different functions: class I antigens (-A, -B and -C) which can present peptides from inside the cell (including viral peptides if present); and class II antigens (DR, DP, & DQ), which can present phagocytozed antigens from outside of the cell to T-lymphocytes (Davis and Bjorkman, 1988). In short, the biological function of MHC is in the process to help the T- lymphocytes to identify non-self, infected, and tumor cells via surface antigens.

The MHC is a group of genes that, in humans, code for a complex of cell surface proteins called the human leukocyte antigens (HLA). It is located on chromosome 6 and has evolved to form two structurally related but different groups of molecules: class Ia antigens (-A, -B, and -C) and class Ib antigens (also known as non-classical HLA-E, -F, and -G). To establish the diversity of the immune responses, the polymorphism of HLA class Ia antigens is very important (Ojcius et al., 1994); On the other hand, two main characteristics that differentiate class Ib from class Ia antigens are: limited polymorphism and lower cell surface expression. These distinguishing features suggest possible generalizations regarding the evolution and function of this class (Rodgers and Cook, 2005). Furthermore, class Ib antigens tend to have shorter cytoplasmic domains or in some cases may be secreted or may substitute a lipid anchor for the transmembrane domain (Rodgers and Cook, 2005). Besides, unlike the ubiquitous distribution of HLA class Ia antigens, HLA class Ib antigens have a tissue-restricted expression (Shawar et al., 1994) suggesting their specialized immune functions.

3.1.2 Non-classical HLA class Ib molecule: HLA-G

Among other members of the non-classical HLA class Ib, the HLA-G gene is located within the HLA at 6p21.3, one of the most polymorphic regions in the human genome. In spite of their close proximity located within the chromosome, HLA-G has been called “non-classical” because of its limited polymorphism by showing only ~20 nucleotide alleles encoding <10 different protein sequences (Apps et al., 2008). The gene structure of HLA-G primary transcript generates 7 alternative mRNAs that encode membrane-bound (HLA-G1, G2, G3, G4) and soluble (HLA-G5, G6, G7) protein isoforms. HLA-G has a stop codon in exon 7, resulting in a truncated cytoplasmic tail; this premature termination leads to the retention of HLA-G in the endoplasmic reticulum (ER) until a high-affinity peptide is bound, and an

extended surface half-life. (Onno et al., 1994) (Also see schematic diagram of overview of HLA-G, Figure 3.1.2A and B)

3.1.3 HLA-G and its firstly described biological role in pregnancy

Human placental trophoblasts lie at the maternal-fetal interface, a position in which they could play an important role in maternal tolerance of the fetal tissue. The extravillous trophoblasts are the cells from the placental tissue of fetal origin, which can invade the maternal decidua during the implantation of the embryo. These extravillous trophoblasts do not express polymorphic HLA class Ia and the HLA class II antigens (Faulk and Temple, 1976; Goodfellow et al., 1976), whereas, they express polymorphic HLA-G (McIntyre, 1992; McMaster et al., 1995). It has been proposed that the selective expression of HLA-G at this site may protect the semiallogeneic fetal tissues from maternal immune rejection by preventing the activation of maternal alloreactive T and NK cells resident in the decidua (reviewed by (Hunt and Orr, 1992)). Alternatively, thymic epithelial cells also express HLA-G (Crisa et al., 1997). In murine fetal thymus, the population of γ/δ T cells are positively selected (Lafaille et al., 1989); In concord with this, oligoclonal murine decidual γ/δ lineage T cells are found (Itohara et al., 1990), and they are restricted by nonpolymorphic MHC class Ib antigens (Balk et al., 1991). In view of these studies, one may suggest that selection events occurring in the thymus may influence the repertoire of T cell immune responses at the maternal-fetal interface. Thereby, thymic selection restricted by HLA-G may be proposed as other mechanism utilized by the maternal immune system adapted to the acceptance of the fetal tissue, which presents the same HLA antigens.

3.1.4 Receptors for HLA-G

HLA-G possesses the capability to bind to inhibitory receptors (Hofmeister and Weiss, 2003). To date, three HLA-G receptors have been described: ILT2 (Ig-like Transcript 2)/CD85j/LILR (Leukocyte Ig-like receptor) B1, ILT4 (Ig-like Transcript 4)/ CD85d/LILR (Leukocyte Ig-like receptor) B2, and KIR2DL4 (killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4, KIR2DL4)/CD158d (Colonna et al., 1997; Colonna et al., 1998; Rajagopalan and Long, 1999). B cells, some T cells, some NK cells, and all monocytes/dendritic cells express ILT2 (Colonna et al., 1997), ILT4 is myeloid-specific and only expressed by monocytes/dendritic cells (Colonna et al., 1998). With regard to KIR2DL4, its expression is mainly restricted to the CD56-bright subsets of Natural Killer (NK) cells (Goodridge et al., 2003), which constitute a minority of peripheral NK cells, but a majority of uterine NK cells (Cooper et al., 2001). Nevertheless, through these differentially expressed

receptors, HLA-G can interact with B cells, T cells, NK cells, and antigen-presenting cells (APCs).

Several differences in these three HLA-G receptors have been considered. Since, there is a single immunoreceptor tyrosine-based inhibitory motif located within the KIR2DL4 cytoplasmic tail, where the transmembrane region bears a positively charged arginine, KIR2DL4 seems to be able to deliver the inhibitory as well as activatory signals (Selvakumar et al., 1996; Yusa et al., 2002). On the contrary, ILT2 and ILT4 are both receptors that deliver inhibitory signals.

Another difference between the ILTs and KIR2DL4 is that ILT2 and ILT4 bind classic HLA molecules (Colonna et al., 1997; Colonna et al., 1998), whereas HLA-G is the sole ligand of KIR2DL4 (Rajagopalan and Long, 1999). Moreover, ILT2 and ILT4 differ at the level of the HLA-G structures they recognize: ILT2 is a receptor for HLA-G associated with β_2-microglobulin, whereas ILT4 also recognizes HLA-G free heavy chains (Gonen-Gross et al., 2005; Shiroishi et al., 2006). (Also see schematic diagram of overview of HLA-G, Figure 3.1.2C).

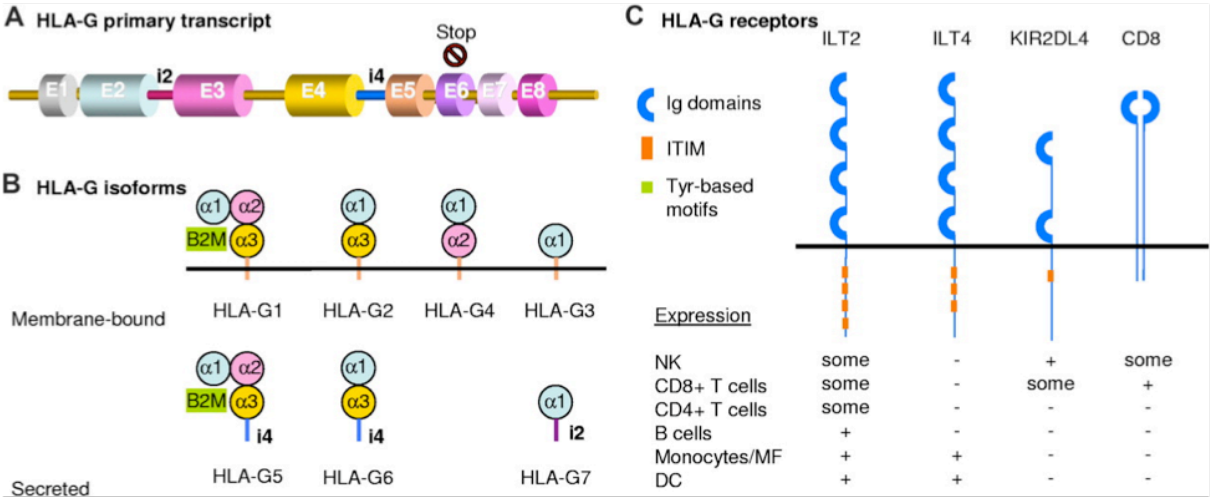

Figure 3.1.2 Schematic diagram of overview of HLA-G proteins, isoforms, and receptors

(A) This HLA-G primary transcript is homologous to that of classic HLA class I molecules but contains a stop codon in exon 6, shortly after the coding sequence for the transmembrane domain. (B) Alternative splicing of the primary transcript yields 7 protein isoforms: truncated isoforms are generated by excision of one or 2 exons encoding globular domains, whereas translation of intron 4 or intron 2 yields soluble isoforms that lack the transmembrane domain. (C) HLA-G is well known to act through binding of inhibitory receptors, such as immunoglobulin-like transcript 2 (ILT2), ILT4, and killer-cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail, 4 (KIR2DL4), that are differentially expressed by immune cells, but binding to CD8 has also been reported. ***(Taken from LeMaoult et al. BLOOD. 2008).***

3.1.5 HLA-G and T-cell responses

Within the framework concept of immune-inhibitory signals provided by HLA-G, there are numerous studies on the biological events mediated by HLA-G. For example, soluble HLA-G could induce antigen-specific T cell apoptosis, which involves p56lck, calcium calmodulin kinase II, and calcium- independent protein kinase C signaling pathways and NF-KB and NF-AT nuclear translocation (Contini et al., 2005). HLA-G5 inhibits the cell cycle progression of alloreactive T cells by decreasing the expression of cyclins and by upregulating the expression of the inhibitor of cyclin-dependent kinase activity, p27kip (Bahri et al., 2006). HLA-G controls the maturation and migration of dendritic cells (DCs) via ILTs by downregulating both antigen presentation to naıve T cells and expression of chemokine receptors that govern trafficking of DCs (Liang et al., 2006). (See also Table 3.1.5).

Table 3.1.5 Involvement of HLA-G in regulating T-cell responses

HLA-G on Effector T cells	Receptors involved	Cellular or molecular events involved	Reference
$CD8^+$ T cells			
Inhibition of cytotoxic function	-	Increases p56lck/ calcium calmodulin kinase II/ calciumindependent protein kinase C signaling pathways / NF-KB and NF-AT nuclear translocation	(Contini et al., 2005; Contini et al., 2003)
Inhibition of proliferation	ILT2	Increases Cyclins/ p27kip	(Bahri et al., 2006)
Generation of CD8low regulatory T cells	-	Increases CD45RA, HLA-DR; reduces CD62L	(Naji et al., 2007)
Apoptosis	CD8	Fas ligand/Fas	(Contini et al., 2005)
$CD4^+$ T cells			
Inhibition of alloreactivity	ILT2, ILT4	Reduces cyclin D2, E, A, and B, accumulates p27ki	(Riteau et al., 1999), (Bahri et al., 2006), (LeMaoult et al., 2004)
Inhibition of proliferation	ILT2	-	(Bahri et al., 2006), (LeMaoult et al., 2007)
Up-regulation of inhibitory receptors	-	Increases ILT2, ILT3, ILT4, and KIR2DL4 expression on NK, T anc APC	(LeMaoult et al., 2005)
Generation of CD4low regulatory T cells	-	Increases CD45RA, HLA-DR; reduces CD62L	(Naji et al., 2007)
-: not identified yet; APC: antigen-presenting cells			

3.1.6 HLA-G expression under pathological conditions

Expression of HLA-G is very restricted under physiologic conditions (Wiendl, 2007), however, under certain pathological conditions, HLA-G molecules can be induced after allograft transplantation (Crispim et al., 2008; Le Rond et al., 2006; Sheshgiri et al., 2008) and during mixed lymphocyte reaction as well (Lila et al., 2001). Moreover, the induction of

HLA-G may be one of the mechanisms used by tumor cells to escape immune surveillance (Paul et al., 1998).

There is accumulating evidence demonstrating that HLA-G is induced in the course of inflammatory pathologies, such as myositic lesions, psoriatic skin lesions (Aractingi et al., 2001), or in atopic dermatitis (Khosrotehrani et al., 2001). This also seems to have important functions at immunologically privileged sites, such as the thymus (Crisa et al., 1997), or the cornea (Higa et al., 2006). Therefore, HLA-G has been proposed as a mechanism to protect target tissues from autoaggressive inflammation and to serve as a fundamental mechanism of immune surveillance (Carosella et al., 2001; Carosella et al., 2008a; Carosella et al., 2008b; Wiendl, 2007; Wiendl et al., 2003). During pregnancy, transplantation or autoimmune diseases, HLA-G turns to be beneficial in terms of the ability to turn down immune reactions against the fetus, allograft, or self-components. However, this ability might become deleterious in cancer and viral infections by permitting evasion of malignant or virus-infected cells from anti-tumor or anti-viral responses.

3.2 Multiple sclerosis

3.2.1 Pathology

Multiple sclerosis (MS) affects approximately one million people worldwide, mostly in North America and North Europe. Women are affected two times more frequently than men. The disease often begins in young adulthood with recurrent inflammatory attacks against the white matter of the brain, involving much neurological impairment, for example blindness, loss of sensation, lack of coordination, bowel and bladder incontinence, and difficulty in walking. About one third of patients who start out with the relapsing–remitting form of MS progress to a more chronic form with more widespread disability (Noseworthy et al., 2000).

The hallmark pathology in MS is the plaque, which is an area of myelin that has been damaged by inflammation and by non-neural cells in the brain, including bone-marrow-derived microglia and brain-derived astroglia. The cause of MS is enigmatic. First there is the injury, then the process of repair; the repair process itself, in response to the wound, evokes a cascade of immunological activity. However, most people believed that it is the immune attack against white matter that initials the disease, resulting in degeneration of axons and myelin. But the possibility that the immune response is itself a reaction to some initiating neurodegenerative process must also be considered (Hafler et al., 2005; Hauser et al., 1986; Noseworthy et al., 2000; Traugott et al., 1983). Nevertheless, MS is generally considered as a complex genetic disease associated with inflammation in the CNS white matter, which thought to be mediated by autoreactive T cells. Clonal expansion of B cells, their antibody

products, and T cells, hallmarks of inflammation in the CNS, are found in MS (Hafler, 2004). The inflammatory cell profile of active lesions is characterized by perivascular infiltration of oligoclonal T cells (Wucherpfennig et al., 1992) consisting of $CD4^+$/$CD8^+$ α/β (Traugott et al., 1983) (Hauser et al., 1986) and γ/δ T cells (Wucherpfennig et al., 1992) and monocytes with occasional B cells and infrequent plasma cells (Prineas and Wright, 1978).

Moreover, chemokines and chemokine receptors have been described to be directly involved in the pathogenesis of MS (reviewed in (Szczuciński and Losy, 2007)). The immune cell trafficking across the blood-brain barrier (BBB) can be facilitated by the expression of distinct sets of adhesion molecules, chemokines and their receptors (Campbell et al., 1998). The elevated levels of chemokine receptors have been previously detected in actively demyelinating lesions and in the CSF of MS patients (Pashenkov et al., 2003). MIP-1α and RANTES have been described as a pair of chemokines important for T cell entry into the CNS (Andjelkovic and Pachter, 2000; Glabinski et al., 2003). MIP-1α was detected at high levels in the CSF of patients with MS and other inflammatory disorders, but not in samples from healthy control subjects (Miyagishi et al., 1995). Stimulated by pro-inflammatory cytokines, brain endothelial cells as well as astrocytes and microglia have been shown to be the main producers of MIP-1a and RANTES chemokines, which both serve as ligands for CCR5 (Glabinski et al., 1997; Hvas et al., 1997; McManus et al., 1998; Miyagishi et al., 1997; Simpson et al., 1998; Sorensen et al., 2003). Moreover, a remarkably consistent relationship between the expression of chemokines in the brain and clinical disease activity has been suggested in MS (Sorensen et al., 1999). (See also Figure 3.2.1 Schematic diagram of the inflammatory phase of multiple sclerosis).

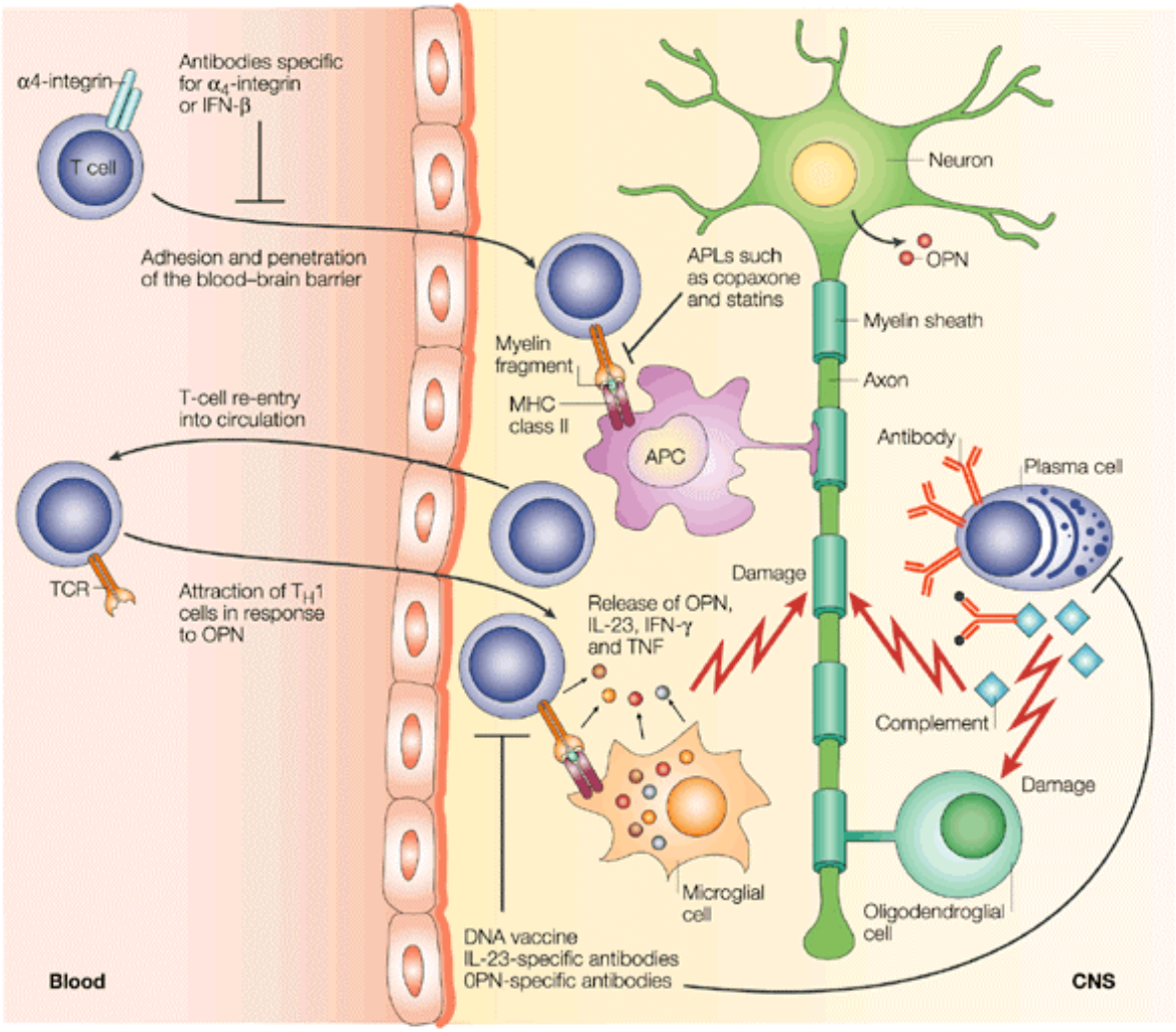

Figure 3.2.1 Schematic diagram of the inflammatory phase of multiple sclerosis

T cells, B cells and antigen-presenting cells (APCs), including macrophages, enter the central nervous system (CNS), where they secrete certain chemicals known as cytokines that damage the oligodendroglial cells. These cells manufacture the myelin that insulates the neuronal axon. The injured myelin cannot conduct electrical impulses normally, just as a tear in the insulation of a wire leads to a short circuit. Lymphocytes diapedese into the CNS through use of a surface receptor known as 4-integrin. This step is impeded by antibodies specific for 4-integrin or by interferon- (IFN-). Once the blood brain barrier is breached, other inflammatory cells accumulate in the white matter. Inside the brain, T cells and accompanying macrophages and microglial cells release osteopontin (OPN), interleukin-23 (IL-23), IFN- and tumor necrosis factor (TNF), all of which damage the myelin sheath. Also, the presence of OPN might lead to the attraction of T helper 1 (T_H1) cells. T-cell activation can be blocked by altered peptide ligands (APLs), such as copaxone, or by statins. Concomitantly, B cells (plasma cells) produce myelin-specific antibodies, which interact with the terminal complex in the complement cascade to produce membrane-attack complexes that further damage oligodendroglial cells. DNA vaccination can be used to tolerize T- and B-cell responses to myelin. ***(Taken from Steinman, and Zamvil, Nat Rev Immunol. 2003).***

3.2.2 HLA-G in MS

HLA-G, although nearly absent under normal conditions in the CNS parenchyma, is abundantly expressed in MS lesions and in the periplaque white matter (Wiendl et al., 2005). In MS, HLA-G was found up-regulated on monocytes in the cerebrospinal fluid (CSF) relative to peripheral blood, and MS patients exhibit significantly higher CSF levels of soluble HLA-G compared to other neurologic diseases, emphasizing the immunobiologic relevance of this pathway for neuroinflammation during CNS immunoregulation (Wiendl et al., 2005). In accordance with this, soluble HLA-G was also found to be present in MS patients and suggested to play a protective role during MS as an anti-inflammatory molecule to down-regulate MS inflammatory responses (Fainardi et al., 2008; Wiendl et al., 2005). These previous observations are in good correlation with new data on functional significance of sHLA-G in CNS immune-regulation.

3.3 Regulatory T cells

3.3.1 Immunological tolerance

Immunological tolerance ensures proper discrimination of self from non-self antigens by the immune system. Several mechanisms are responsible for the maintenance of the normal state of tolerance to itself. The basis of immune tolerance is associated with the ability of the immune system to purge autoreactive T cells in the thymus (central tolerance) and impose unresponsiveness in the periphery (peripheral tolerance) (Bluestone and Abbas, 2003; Sakaguchi, 2005; Schwartz, 2005). Autoantigens that are presented as a very low level or located at the immunologically privileged sites (i.e testis, uterus, eyes, and brain) are ignorant to the immune system (Gay et al., 1993; Lang et al., 1997). Clonal deletion of lymphocytes in the thymus and clonal anergy (Goodnow et al., 1988), or receptor editing (McGargill et al., 2000) are established for maintaining tolerance. However, in some cases, self-destructive cells do escape into the periphery, therefore, potentiating the peripheral regulatory mechanisms. One such mechanism is the suppressive action of a particular lineage of regulatory T cells (T_{reg}) (Nishizuka and Sakakura, 1969) (Hori et al., 2003; Sakaguchi et al., 1995). These cells are mandatory to control and maintain immunotolerance. This is the mechanism of tolerance that involve T-cell-T-cell interactions, also known as immune deviation or immune suppression

3.3.2 Distinct subsets of regulatory T cells and their mechanisms of suppression

A number of subsets of T_{reg} distinguished by their unique suppressive mechanisms (Sojka et

al., 2008) (See also Figure 3.3.2), and phenotypes (See Table 3.3.2, adapted from (Zozulya and Wiendl, 2008) have been proposed. Naturally occurring $CD4^+CD25^+$ FoxP3-expressing T_{reg} (nT_{reg}) were identified as key contributors in controlling auto-reactive T cells and in maintaining peripheral tolerance to self and non-self antigens (Sakaguchi, 2005; Sakaguchi et al., 1995). nT_{reg}–mediated suppression requires direct cell-cell interaction or acts on responder T cells through antigen presenting cells (APC) (Mahnke et al., 2007; Sojka et al., 2008). Various phenotypically distinct inducible T_{reg} (iT_{reg}) were identified in humans (Bluestone and Abbas, 2003). In contrast to nT_{reg}, these cells may or may not express FoxP3 but are characterized by the secretion of certain cytokines, e.g. IL-10 (Tr1) or TGF-beta (Th3). These cytokines serve as key factors of their suppressive function (Aractingi et al., 2001; Groux et al., 1997; Roncarolo et al., 2001; Walker et al., 2003). A new type of nT_{reg} identified by the expression of non-classical MHC I molecule, HLA-G, was described by our group (Feger et al., 2007b). These cells were found in small but appreciable quantities in the peripheral blood of healthy individuals. They seem to be of thymic origin, hypo-proliferative, negative for CD25, FoxP3 expression, and exhibit potent suppressive properties that are partially mediated by HLA-G.

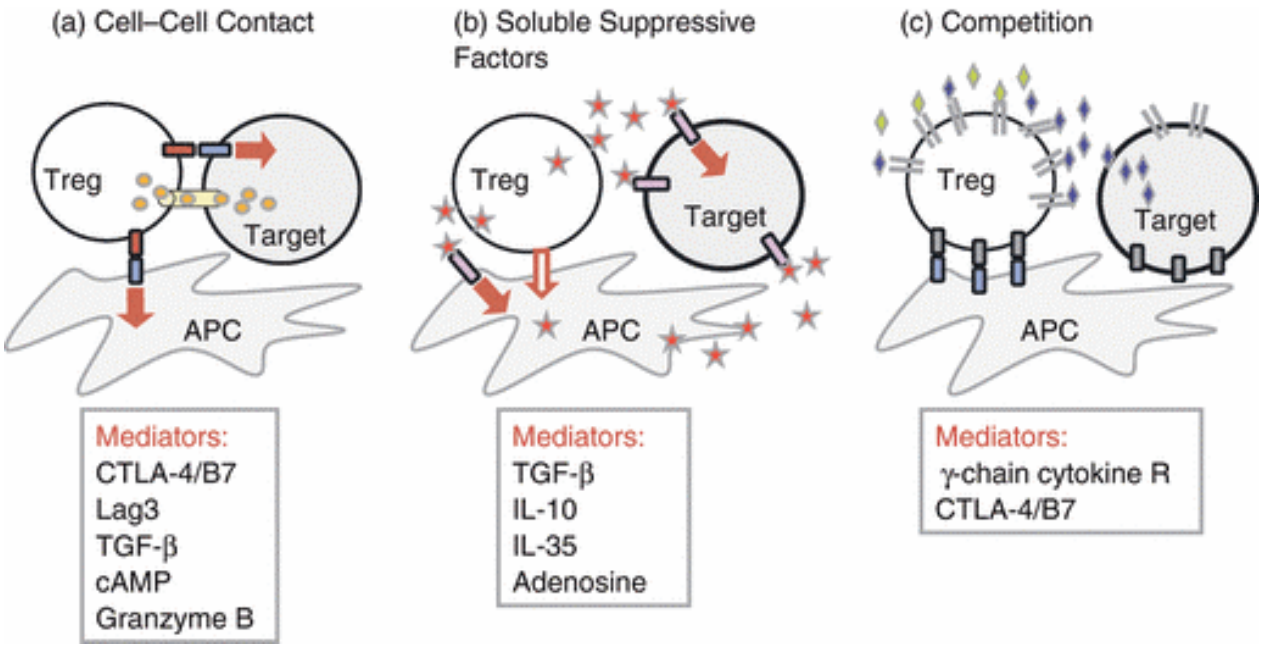

Figure 3.2.2 Schematic diagram of mechanisms of regulatory T-cell (Treg) suppression

(A) Cell–cell contact. Tregs may suppress target cells via direct interaction of receptor–ligand pairs on Tregs and target cells; delivery of suppressive factors via gap junctions including cyclic adenosine monophosphate (cAMP); direct cytolysis; membrane-bound suppressive cytokines such as transforming growth factor-β (TGF-β); and/or indirectly via modulating the antigen-presenting cell (APC) through cell–cell contact, possibly through reverse signalling via Treg–cytotoxic T-lymphocyte antigen-4 (CTLA-4) engagement of B7 on dendritic cells. (B) Soluble suppressive factors. Tregs can

directly secrete interleukin-10 (IL-10), TGF-β and IL-35 or induce APCs to secrete such factors. Expression of CD73/CD39 by Tregs facilitates the local generation of adenosine that can down-modulate immune function. (C) Competition. Tregs may compete for some cytokines that signal via receptors that contain the common γ-chain (IL-2, IL-4 and IL-7). Additionally they may compete for APC costimulation via constitutive expression of CTLA-4. Red arrow indicates an inhibitory signal. ***(Taken from from Sojka, Immunol Rev, 2008)***

Table 3.3.2 Major subsets of human regulatory T cells. (*Taken from Zozulya and Wiendl, 2008)*

Characteristic	**Natural regulatory T cells**		**Inducible regulatory T cells**		
	$CD4^+CD25^{high}$	**$HLA\text{-}G^+$**	**T-regulatory 1**	**T-helper 3**	**$CD8^+CD28^-$**
Origin	Thymus-derived	Seems to be thymus-derived	Induced in periphery by IL-10 and immature dendritic cells	Induced in periphery by IL-2 and TGF-beta	Induced in periphery by IL-10, TGF-beta and plasmacytoid dendritic cells
Cytokine secretion	IL-10^+, TGF-$beta^+$	soluble HLA-G^{++}	IL-10^{+++}, TGF-$beta^+$, IL-4^+, IL-5^+	TGF-$beta^{+++}$, IL-4^+, IL-10^+	IL-10^{++} or TGF-$beta^{++}$
Cell markers	$CD25^{+++}$, $CTLA4^{+++}$, $GITR^{++}$, $CD39^+$, $FOXP3^{++}$, galectin-1^+	$FOXP3^-$, HLA-G^{+++}, CD4 or CD8	$CD25^+$, $CTLA4^{++}$, $FOXP3^{+/-}$(?)	$CD25^+$, $CTLA4^{++}$, $FOXP3^{+/-}$(?)	$CTLA4^+$, $GITR^+$, $FOXP3^+$
Antigen specificity	Self antigens in thymus	Not known	Tissue-specific and foreign antigen	Self or foreign antigens	Self or foreign antigens
Costimulation requirements for cell activation	Depends on costimulatory interaction between CD28 and CD80 and/or CD86	Not known	Costimulation-independent	Costimulation-independent	Costimulation-independent
Mechanism of suppression	Depends on T cell–T cell or T cell–antigen-presenting-cell contact	Cell–cell-contact-independent	Cell–cell-contact-independent, cytokine-dependent	Cytokine-dependent	Depends on T cell–T cell or T cell–antigen-presenting-cell contact
In vitro expansion	Expansion protocols (e.g. use of sirolimus [rapamycin]) established, albeit difficult	Not yet established	Established protocols involve TCR signaling, IL-10, TGF-, vitamin D_3 and steroids	Depends on TCR signaling, IL-10 and TGF-	TCR signaling

Abbreviations: CTLA4; cytotoxic T-lymphocyte-associated antigen 4; FOXP3, forkhead box protein P3; GITR, glucocorticoid-induced tumor-necrosis factor receptor-related protein; HLA-G, human leukocyte antigen G; IL-, interleukin; TCR, T-cell receptor; TGF-, transforming growth factor; +++, very high expression; ++, high expression; +, moderate expression; -, low or no expression.

3.3.3 Regulatory T cells in multiple sclerosis

The etiology of MS is unknown, but most work on MS has focused on a central role of the immune system in causing the disease, either through autoimmune mechanisms or in response to some chronic infection. Knowledge on the cellular and molecular identification of T_{reg} has greatly stimulated many concepts of adaptive immunity, including mechanisms controlling autoimmunity. Targeted deletion of T_{reg} causes spontaneous autoimmune disease in mice, whereas its augmentation can prevent the development of EAE, the commonly used animal model of MS (Baecher-Allan and Hafler, 2004). There has been an increase of studies that have investigated the role of naturally occurring $CD4^+CD25^+$ FoxP3-expressing T_{reg} (nT_{reg}) in patients with MS. Putheti et al. first analyzed $CD4^+$ cells from peripheral blood of MS patients compared to healthy controls. Some patients were untreated; others were treated with IFN-β, glatiramer acetate (GA), or both. Few differences were noted between the groups. Subsequently a larger study yielded similar results concerning the frequency of T_{reg} does not differ between patients with MS and healthy controls (Putheti et al., 2004; Putheti et al., 2003).

However, it is logical to speculate, even if normal in number, nT_{reg} in MS patients may not be normal in function. Indeed, several groups have shown that nT_{reg} are functionally impaired or have deficits in their maturation or in their thymic emigration in patients with MS. (Baecher-Allan et al., 2004; Feger et al., 2007a; Haas et al., 2005; Hug et al., 2003; Kumar et al., 2006). Understanding the role and features of T_{reg} is interesting both from an immuno-pathogenic as well as a therapeutic view. The dysfunction of T_{reg} populations might influence disease susceptibility, disease course, or might be associated with the relapsing-remitting nature of MS.

To elucidate the mechanism underlying the influence of T_{reg} on the inflammatory process in MS, it is essential to understand how peripheral tolerance to self-reactive components is maintained, and how local immune regulation in the central nerve system is referred to. (Reviewed by (Zozulya and Wiendl, 2008), see also Table 3.3.3). T_{reg} putatively influence the immunopathology of MS at different levels (De Jager and Hafler, 2007; Hafler et al., 2005; Zozulya and Wiendl, 2008). T_{reg} show a propensity to migrate to sites of inflammation, both in experimental models (Belkaid et al., 2002; Green et al., 2002; Herman et al., 2004; Hori et al., 2002; Mottet et al., 2003; Suffia et al., 2005; Suvas et al., 2004; Wysocki et al., 2005) and in human studies (Feger et al., 2007a). The specific localization of human T_{reg} is essential for their ability to control ongoing inflammatory conditions, ranging from autoimmune diseases to allergic disorders (Cao et al., 2003; Lécart et al., 2001; Maul et al., 2005; Sugiyama et al., 2005; van Amelsfort et al., 2004). Like with other leukocytes, the

migration and infiltration of T_{reg} into inflamed tissues is mainly governed by the expression of specific chemokine receptors (CCRs). Different patterns of homing receptors such as CCR4, CCR8, CCR5, and CCR6 (Iellem et al., 2001; Kleinewietfeld, 2005; Oswald-Richter et al., 2004) have been described on human T_{reg}, suggesting their role in targeting the suppressor cells to the sites of inflammation in response to a variety of pathological conditions. Therefore, the hypothesis has been put forth that T_{reg} are specifically recruited to the CNS in order to counterbalance autoimmune inflammatory activity. For instance, a small subpopulation of $CCR6^+$ T_{reg} with effector memory phenotype accumulates in the CNS of mice with experimental autoimmune encephalomyelitis (EAE), indicating that this may control the inflammatory response (Kleinewietfeld, 2005).

Table 3.3.3 Studies that have investigated the role of regulatory T-cell in human multiple sclerosis. (*Adapted from Zozulya and Wiendl, 2008*)

Key aspects	Main findings	References
Frequency of T_{reg} in peripheral blood	Frequency of $CD4^+CD25^{high}$ T cells in blood is similar between patients with MS and healthy controls, and among patients with different MS subtypes	(Haas et al., 2005; Putheti et al., 2004); (Feger et al., 2007a; Viglietta et al., 2004)
	First study indicating reduced levels of FOXP3 on peripheral $CD4^+CD25^{high}$ T cells in patients with MS	(Huan et al., 2005)
	Reduced numbers of $CD39^+$ T_{reg} in blood of patients with MS compared with healthy controls; degree of reduction correlated with disease state and could be normalized to control levels by clinical treatments	(Borsellino et al., 2007)
Frequency of T_{reg} in CSF	CSF and peripheral blood from patients with MS show equivalent proportions of $CD4^+CD25^{high}$ T cells within the $CD4^+$ T-cell population	(Haas et al., 2005)
	Frequency of $CD4^+CD25^{high}FOXP3^+$ T cells is elevated in CSF compared with blood in patients with MS; these cells might be important for regulation of immune responses in the CNS	(Feger et al., 2007a)
	Frequency of $CD4^+HLA\text{-}G^+$ T_{reg} is elevated in the CSF of patients with MS in the acute relapse phase	(Feger et al., 2007a)
Functional analysis of T_{reg}	First evidence that $CD4^+CD25^{high}$ T_{reg} display impaired suppressive function in patients with MS	(Viglietta et al., 2004)
	Ability of $CD4^+CD25^{high}$ T_{reg} to suppress effector-T-cell-mediated immune responses specific for recombinant human myelin	(Haas et al., 2005)

	Ability of $CD4^{+}CD25^{high}$ T_{reg} to suppress effector-T-cell-mediated immune responses specific for recombinant human myelin oligodendrocyte glycoprotein is altered in patients with MS	(Haas et al., 2005)
	MBP-induced suppression of T_{reg} is lost in 55% of MS patients; suppressive activity of T_{reg} is lost to a higher degree in cultures induced by MBP than in cells that undergo mitogen or polyclonal stimulation	(Kumar et al., 2006)
	Inducible T-regulatory 1 cells have impaired suppressive function and lack IL-10 expression in patients with MS; functional impairment is attributable to CD46 costimulation	(Astier et al., 2006)
Effect of therapeutic interventions on T_{reg} in patients with MS	First study to demonstrate that IFN-1a enhances T_{reg}-cell frequency and function in patients with MS; combination of *ex vivo* and *in vitro* data	(de Andres et al., 2007)
	Glatiramer acetate therapy upregulates suppressive ability of	(Tennakoon et al., 2006)

4. Aims of the study

The immune system has to distinguish self from non-self structures, but also between harmful and innocuous foreign antigens to prevent non-essential and self-destructive immune responses. Regulatory T cells (T_{reg}) play a key role in the immune-regulatory processes of autoimmune disorders (Baecher-Allan and Hafler, 2004; Shevach, 2004) leading to prospects of future therapeutic strategies aiming at the reconstitution of immune tolerance in autoimmunity (Bluestone, 2005; Bluestone and Tang, 2005; Bluestone et al., 2007).

In Multiple sclerosis (MS) $CD4^+$ T cells are believed to play a major role in driving the immune response within the central nervous system (CNS). The dysregulation of inflammatory responses and immune self-tolerance is considered to be a key element in the autoreactive immune response in MS.

The role of regulatory T cells (T_{reg}) in controlling parenchymal autoimmune inflammation in humans is a subject of intensive discussion. Conceptually, T_{reg} within the CNS parenchyma could combat destructive inflammatory cell components, thereby providing anti-inflammatory or protective properties. Understanding the role and function of different subsets of T_{reg} is important to control parenchyma immune homeostasis. However, up to now, human studies to clarify the distribution and function of T_{reg} were addressed exclusively in the periphery, whereas the role of T_{reg} at the site of inflammation in the CNS was not properly studied. Nevertheless, some studies report dysfunctional states of $CD4^+CD25^+$ naturally occurring FoxP3-expressing T_{reg} and certain inducible T_{reg} in MS.

We have previously described a new type of naturally occurring $CD4^+$ regulatory T cells constitutively expressing the immune-tolerogenic histocompatibility leukocyte antigen G (HLA-G) (HLA-G^{pos} T_{reg}) in humans, classified according to their surface phenotype and cytokine secretion profile. Thus, HLA-G^{pos} T_{reg} seem to be naturally occurring T_{reg} (Feger et al., 2007b). This T_{reg} represents a distinct cell population that is capable of maintaining the quality of immune response, and these cells could be a novel target for the treatment of autoimmune diseases, including MS. The goals of this study were to focus on the cellular and molecular identification of HLA-G^{pos} T_{reg}, and further, the beneficial/pathological role of HLA-G^{pos} T_{reg} in MS.

Specific aims of the study:

(1) *To characterize the suppressive mechanism of action mediated by HLA-G^{pos} T_{reg}.*

We previously demonstrated that HLA-G^{pos} T_{reg} strongly suppress the proliferation of polyclonaly stimulated (soluble anti-CD3, OKT3) T cells in the presence of irradiated allogeneic feeder cells. One of the major goals of this study was to understand how $CD4^+$

HLA-G^{pos} T_{reg} influence autologous HLA-G negative responder T cell (HLA-G^{neg} T_{resp}) proliferation. A more simple *in vitro* setup free of other allogeneic cells was required to dissect the question which cellular mechanism(s) is (are) involved in HLA-G^{pos} T_{reg}-mediated suppression. To understand mechanisms used by HLA-G^{pos} T_{reg} to regulate effector T-cell function might have a high relevance in finding therapeutic molecules for various autoimmune disorders.

(2). *To understand the patho/physiological role of HLA-G^{pos} T_{reg} in MS patients.*

T_{reg} putatively influence the immunopathology of MS at different levels. T_{reg} suppress immune responses against foreign and self-antigens and play a key role in the mechanisms of autoimmunity. We here studied the role and function of HLA-G^{pos} T_{reg} in MS patients, with a particular interest in the immunomodulatory roles of HLA-G^{pos} T_{reg} in CNS.

5. Material and methods

5.1 Material

5.1.1 Reagent kits

Kit	Company
QCM™ 24-well Invasion Assay	Chemicon International, Inc. Germany
Human soluble HLA-G ELISA kit	BioVendor Laboratrory Medicine, Inc. Germany
IL-10 ELISA kit	R&D system, Germany
IFN-gamma ELISA kit	R&D system, Germany
Human CD4 T cells isolation kit	Miltenyi Biotech, Germany
Human CD3 T cells isolation kit	Miltenyi Biotech, Germany
BD CytoFix/CytoPerm intracellular staining kit	eBioscience, Germany

5.1.2 Primary antibody

Anti-human monoclonal antibody	Clone	Company
ILT-2/CD85j	292319	R&D Systems, Germany
IL-10R/CD210	3F9	BioLegend, Germany
TGF-βR1 kinase inhibitor	SD-208	Kindly provided by Michael Weller
PLP		Biozol, Germany
CD3	UCHT1	Serotec, UK
HLA-G	MEM-G/9 (specific for the membrane HLA-G1 and soluble HLA-G5 isoform in its beta2-microglobulin associated form)	Exbio, Belgium
HLA-G	4H84 (specific for the beta2m free HLA-G isoform)	Exbio, Belgium
HLA-G	16G1 (specific for the soluble HLA-G5	Kindly provided by Joel

	isoform)	LeMaoult
CD3	HIT3a/ OKT3	eBioscience, Germany
CD4	RPA-T4	eBioscience, Germany
CD8	RPA-T8	eBioscience, Germany
CD14	M5E2	eBioscience, Germany
CD19	HIB19	eBioscience, Germany
CD25	M-A251	eBioscience, Germany
CD27	340425	eBioscience, Germany
CD28	CD28.2	eBioscience, Germany
CD69	FN50	eBioscience, Germany
CD27	LT27	eBioscience, Germany
CCR5	2D7	eBioscience, Germany
CCR7	2H4	eBioscience, Germany
ICOS	2C7	eBioscience, Germany

5.1.3 Secondary antibody

Antibody	Company
Streptavidin-APC	BD Biosciences, San Jose, CA
Cy-2- and Cy-3-labelled antibodies	Jackson ImmunoResearch Laboratories, Inc. USA

5.1.4 Chemical reagents

Reagents	Company
α-CD3/CD28 beads	Dynal, Oslo, Norway
Biotin-avidin technique	Sigma, St. Louis, USA
β-mercaptoethanol	Gibco, Eggenstein, Germany
Bovine Serum Albumin (BSA)	Roth, Karlsruhe, Germany
5-(and-6)-carboxyfluorescein diacetate Succinimidyl ester (CFSE)	Molecular, Karlsruhe, Germany
Collagen type II	Sigma-Aldrich Chemie GmbH, Germany
Dulbecco's Phosphate Buffered Saline (dPBS)	PAA Laboratories, Linz, Austria
Dimethyl sulfoxide (DMSO)	Sigma, Taufkirchen, Germany
EDTA	AppliChem GmbH, Darmstadt, Germany

Ethanol	AppliChem GmbH, Darmstadt, Germany
Fibronectin	Sigma-Aldrich Chemie GmbH, Germany
HEPES	AppliChem GmbH, Darmstadt, Germany
Hydrocortisone	Sigma-Aldrich Chemie GmbH, Germany
Recombinant human interleukin-2 (rhIL-2)	Chiron, Novartis, USA
Ionomycin	Sigma, Deisenhofen, Germany
Lymphocyte separation medium	PAA Laboratories, Linz, Austria
MIP1-alpha	R&D Systems, MN, USA
MIP3-beta	R&D Systems, MN, USA
NaN3 (Sodium Azide)	Merck, Darmstadt, Germany
4- amino- 5- (4-chlorophenyl)- 7- (t-butyl) pyrazolo [3,4-d] pyrimidine (PP2)	Calbiochem, Germany
Hanks balanced salt solution (HBSS)	Sigma Chemical Co., St. Louis, MO
RANTES	R&D Systems, MN, USA
Sodium Chloride	Roth, Karlsruhe, Germany
Trypan blue	Sigma, Germany
Tween 20	Sigma, Germany

5.1.5 Media, Buffers and Solutions:

Media	Company
Gentamicin	Invitrogen, Germany
DMEM (Dulbecco's Modified Eagle´s Medium) Pyruvate, without HEPES (#41966-029)	PAA Laboratories, Linz, Austria
RPMI 1640	BioWhittaker, Verviers, Belgium
HEPES	Gibco, Invitrogen GmbH, Germany
Human AB serum	PAA Laboratories, Linz, Austria
Penicillin-Sstreptomycin	Biochrom, Berlin, Germany
L-glutamine	PAA Laboratories, Linz, Austria
Dulbecco's Modified Eagle's Medium (DME)	PAA Laboratories, Linz, Austria
Ham's F-12 Nutrient Mixture	PAA Laboratories, Linz, Austria

PBS (Phosphate buffered saline):

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Dissolve in 800 ml dH_2O, adjust pH to 7.4 with HCl

Volume adjust to 1 L, autoclave and store at room temperature

Standard complete RPMI 1640 culture medium:

10% human AB serum

(Heat inactivated at 56°C for 45 min)

20 µg/mL gentamicin

2 mM L-glutamine

100 µg/mL Penicillin-Streptomycin (10000U/ml)

25mM HEPES

RPMI 1640 culture/ processing medium:

10% FCS (Foetal calf serum)

(Heat inactivated at 56°C for 45 min)

2 mM L-glutamine

100 µg/mL Penicillin-Streptomycin (10000U/ml)

Transmigration medium:

DMEM/F12 1:1

100 µg/mL Penicillin-Streptomycin (10000U/ml)

1mg/ml Hydrocortisone

HBMEC Coating medium:

10 µg/mL Fibronectin

4% Collagen type II

Trypan Blue Solution:

0.4% (w/v) Trypan Blue in PBS

Dilute 1:10 in PBS for live cell counting

FACS-buffer:

PBS

0.1% BSA

0.01% Azide

ELISA Coating buffer (0.1 M Sodium Carbonate, pH 9.5)

8.40 g $NaHCO_3$

3.56 g Na_2CO_3

Volume adjusted to 1L with dH_2O

ELISA Assay diluent

PBS with 10% Foetal Bovine Serum, pH 7.0

ELISA Wash buffer

PBS with 0.05% Tween-20

Substrate solution for ELISA

Tetramethylbenzidine (TMB)

Hydrogen peroxide (H_2O_2)

ELISA Stop solution

2 N H_2SO_4

MACS buffer, pH 7.3

PBS

2% FCS

2 mM EDTA

FACS buffer, pH 8.0

PBS

2% BSA

0.03% NaN_3

20 mM EDTA

5.1.6 Consumables

Products	Company
96, 48, 24, 12, 6 well plate	Greiner, Germany
96 well plate for ELISA	NUNC, Germany
1.5ml Eppendorf centrifuge tube	Sarsted, Germany
15 ml, 50 ml centrifuge tube	Greiner, Germany
Cell strainer	BD Falcon, Germany
FACS tube	Greiner, Germany
MACS separation LS/LD/MS column	Miltenti Biotech, Germany
Pipette tips (yellow, blue)	Roth, Germany

Sterile filter (0.45 or 0.22μm)	Millipore, Germany
5ml single-use syringe	Melsungen, Germany
T-25, T-75 & T-250 tissue culture flasks	NUNC, Germany
Transwells® 3-μm pore-size	Corning, NY, USA
Transwells® 0.4-μm pore-size	Corning, NY, USA

5.1.7 Instruments and Software

96-well plate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany)
FACS-Calibur™ using Cell Quest™ (Becton Dickinson, Heidelberg, Germany)
FlowJo (Tristar, USA) software.

5.1.8 Human Subjects

5.1.8.1 Healthy volunteers

Seventy-five healthy volunteers (male: female = 11: 20, mean age, 31.5 years; range, 20- 51 years) were included into this study. None of the volunteers had a chronical illness nor was under any chronic medication.

5.1.8.2 Patients

For flow cytometry analysis

MS patients

Sixty-four patients from the Department of Neurology (University of Wuerzburg, male: female = 21: 43, mean age, 37 years; range, 27- 60 years) with different types of MS were included into this study. Patients were diagnosed according to the revised McDonald criteria (Polman et al., 2005) and were without corticosteroids for at least 2 months. Patient details including the therapy at the time point of inclusion in our study are given in Table 5.1.8.1.

Table 5.1.8.1 Clinical characteristics of MS individuals include in this study.

Patient	Sex	Age, y	Sample studied	Diagnosis	DD, y	EDSS	Therapy
MS1	M	47	Blood and CSF	RRMS, stable	2	0	-
MS2	F	42	Blood and CSF	RRMS, relapse	8	2	-
MS3	F	37	Blood and CSF	RRMS, relapse	9	5.5	Mitoxantrone
MS4	M	20	Blood and CSF	RRMS, relapse	1	0	-
MS5	F	43	Blood and CSF	RRMS, relapse	1	0	-

MS6	M	47	Blood and CSF	RRMS, stable	12	4.5	Interferon beta
MS7	M	35	Blood and CSF	RRMS, relapse	1	0	-
MS8	M	25	Blood and CSF	RRMS, stable	7	0	Azathioprin
MS9	F	51	Blood and CSF	SPMS, stable	13	4.5	Interferon beta
MS10	F	26	Blood and CSF	RRMS, relapse	1	0	-
MS11	F	39	Blood and CSF	RRMS, stable	4	2	Interferon beta
MS12	F	33	Blood and CSF	RRMS, stable	11	0	Interferon beta
MS13	F	45	Blood and CSF	SPMS, stable	12	5	-
MS14	M	36	Blood and CSF	SPMS, stable	14	6.5	-
MS15	F	22	Blood and CSF	RRMS, relapse	4.5	4	-
MS16	M	55	Blood and CSF	SPMS, stable	4	6.5	-
MS17	F	41	Blood and CSF	RRMS, relapse	1	0	-
MS18	F	29	Blood and CSF	RRMS, relapse	13	3.5	-
MS19	F	27	Blood and CSF	RRMS, stable	3	0	-
MS20	F	45	Blood and CSF	SPMS, stable	10	5	Mitoxantrone
MS21	F	55	Blood and CSF	SPMS, stable	25	8	-
MS22	M	45	Blood and CSF	PPMS, stable	5	3	Mitoxantrone
MS23	F	35	Blood and CSF	RRMS, relapse	13	3.5	Interferon beta
MS24	M	33	Blood and CSF	RRMS, relapse	10	3	Natalizumab
MS25	F	46	Blood and CSF	RRMS, stable	5	1.5	-
MS26	F	41	Blood and CSF	RRMS, stable	6	3	Mitoxantrone
MS27	M	26	Blood and CSF	RRMS, relapse	8	4	Interferon beta
MS28	F	40	Blood and CSF	RRMS, stable	3	3	Interferon beta
MS29	M	31	Blood and CSF	RRMS, stable	4	2	Natalizumab
MS30	F	46	Blood and CSF	RRMS, stable	2	2	Interferon beta
MS31	M	35	Blood and CSF	RRMS, stable	4	2	-
MS32	F	25	Blood and CSF	RRMS, stable	7	4	Interferon beta
MS33	F	24	Blood and CSF	RRMS, relapse	1	2	Rituximab
MS34	M	55	Blood and CSF	SPMS, stable	8	4.5	Interferon beta
MS35	M	36	Blood	RRMS, stable	7	1	-
MS36	M	22	Blood	RRMS, stable	1	2	-
MS37	F	29	Blood	RRMS, stable	1	2	-
MS38	F	43	Blood	RRMS, stable	2	0	-
MS39	F	65	Blood	RRMS, stable	19	5.5	-
MS40	F	33	Blood	RRMS, stable	1	1.5	-
MS41	F	27	Blood	RRMS, stable	1	0	-
MS42	M	33	Blood	RRMS, stable	1	1	-
MS43	F	33	Blood	RRMS, stable	1	1	-
MS44	F	67	Blood	RRMS, stable	46	2.5	-
MS45	M	31	Blood	RRMS, stable	6	2	-
MS46	F	39	Blood	RRMS, stable	2	1	-
MS47	F	38	Blood	RRMS, stable	10	2.5	-

MS48	F	31	Blood	RRMS, stable	2	1	-
MS49	F	27	Blood	RRMS, stable	6	2	-
MS50	F	50	Blood	RRMS, stable	22	3.5	-
MS51	F	41	Blood	RRMS, stable	5	2	-
MS52	F	30	Blood	RRMS, stable	7	0	-
MS53	F	39	Blood	RRMS, stable	12	2.5	-
MS54	F	23	Blood	RRMS, stable	1	1	-
MS55	F	20	Blood	RRMS, stable	1	1.5	-
MS56	F	63	Blood	RRMS, relapse	14	3	-
MS57	F	41	Blood	RRMS, relapse	3	2	-
MS58	F	41	Blood	RRMS, relapse	1	1.5	-
MS59	M	25	Blood	RRMS, relapse	1	2	-
MS60	M	35	Blood	RRMS, relapse	14	6	-
MS61	F	38	Blood	SPMS, stable	12	5	-
MS62	F	47	Blood	SPMS, stable	10	5.5	-
MS63	F	48	Blood	SPMS, stable	16	7.5	-
MS64	F	42	Blood	SPMS, stable	21	5	-

MS, multiple sclerosis; F, female; M, male; CNS, central nervous system; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; -, none; DD, Disease duration y, years

OND patients

9 patients with other neurological diseases (OND, male: female =4: 5, mean age, 48 years; range, 19- 79 years) were included in Table 5.1.8.2

Table 5.1.8.2 Clinical characteristics of OND individuals include in this study.

Patient	**Sex**	**Age, y**	**Sample studied**	**Diagnosis**
OND1	M	79	Blood and CSF	Dementia syndrome
OND2	F	54	Blood and CSF	Vertigo and cerebral metastasis of marrarian cancer
OND3	M	73	Blood and CSF	Diabetic amyotrophy with paresis of leg
OND4	M	57	Blood and CSF	Tetraparesis, possible motoneuron-disease
OND5	F	46	Blood and CSF	Leukencephalopathy, non inflammtory
OND6	F	35	Blood and CSF	Hemihypaestehsia of unknown origin, no inflammatory CNS disorder
OND7	F	31	Blood and CSF	Other neurological disease, non inflammatory
OND8	F	19	Blood and CSF	No neurological disorder, non-inflammatory
OND9	M	39	Blood and CSF	No neurological disorder, non-inflammatory

OND, other neurological disease; F, female; M, male; CNS, central nervous system, CSF, cerebrospinal fluid; -, none; y, years

Immunohistochemical studies of CNS samples

(This part of work has been performed in cooperation with Wolfgang Brück, University of Göttingen)

MS patients

For detection of HLA-G expressing T cells directly in the inflamed brains of MS patients, five additional untreated MS patients were included. All lesions fulfilled the generally accepted criteria for the diagnosis of multiple sclerosis (Bruck et al., 1995). Lesion activity was classified as inactive in 3 cases, late active in 1 case and, in the last case, as early active with a typical inflammatory pathology pattern according to "immunopattern II" as described in (Bruck et al., 1995). All patients had given written informed consent in accordance with the Declaration of Helsinki and the research protocol was approved by the Ethics Committee of the University of Würzburg.

Control patients

As for controls for this, we included (1) a case of CNS vasculitis, (2) a case of an infiltration zone from a glioblastoma in which inflammation was present, and (3) cortex infiltrated by a glioblastoma with no apparent inflammation.

5.2 Methods

5.2.1 Immunohistochemical studies of CNS samples

(This part of work has been performed in cooperation with Wolfgang Brück, University of Göttingen)

Five MS cases were chosen in which formalin-fixed paraffin embedded as well as cryo-fixed brain tissue was available. The lesions fulfilled the morphological criteria of an inflammatory demyelinting process consistent with MS when stained with H&E, LFB-PAS myelin stain and Bielschowsky´s silver impregnation for axons. The lesions were classified according to their demyelinating activity and included one early active, one late active and three inactive lesions. Further immunohistochemical stainings included proteolipidprotein (PLP) and CD3 T-cell staining.

HLA-G expression was investigated in cryo-fixed brain biopsy tissue from the same 5 patients and the three control cases. Slides of 6µm thickness were stained with primary antibodies directed against HLA-G. Two different antibodies for HLA-G immunoreaction were used: 4H84 (specific for the beta2m free HLA-G isoform) and 16G1 (specific for the soluble HLA-G5 isoform). Staining was performed with a biotin-avidin technique.
Double immunofluorescence was performed with primary antibodies directed against HLA-G and CD3. Secondary antibodies were Cy-2- and Cy-3-labelled.

5.2.2 Cell culture

5.2.2.1 Primary cells

Isolation of human peripheral blood mononuclear cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood obtained from healthy volunteers or generated from buffy coat products (obtained from the Dept. of Transfusion Medicine, University of Würzburg, Würzburg, Germany, from healthy donors after informed consent was given) by density gradient centrifugation using lymphocyte separation medium after centrifugation at 180 g for 20 min at room temperature. Cells were collected and washed three times in Hanks balanced salt solution (HBSS) for heparinized blood or Dulbecco's Phosphate Buffered Saline (DPBS) for isolation from buffy coats. In addition, PBMC within the buffy coats were counted and rested for 2 hours in standard culture completed RPMI 1640 medium used in this study prior to experiments at cell densities of 1 x 10^6 cells/ml.

Preparation of cells from CSF samples

CSF (8-10 ml) was obtained by lumbar puncture and 10 ml of peripheral blood by venous puncture. After collection of CSF, cells were obtained by centrifugation at 200 g for 20 min at 4^{o}C. The supernatant was removed, and the CSF cells were washed once in completed RPMI medium.

5.2.2.2 Cell lines

Table 5.2.2.2 Overview of cells and media

LCL 721.221 (LCL) cell line	Kindly provided by Joel LeMaoult (Institut Universitaire d'Hematologie, Hopital Saint Louis, Paris, France)

LCL 721.221 cells transfected with a genomic construction of HLA-G1 (LCL-HLA-G1)	Kindly provided by Joel LeMaoult (Institut Universitaire d'Hematologie, Hopital Saint Louis, Paris, France)
HeLa-cells transfected with a genomic construction of HLA-G5 (Hela-HLA-G5)	kindly provided by Joel LeMaoult (Institut Universitaire d'Hematologie, Hopital Saint Louis, Paris, France).
Human brain microvascular endothelial cell (HBMEC).	Originally obtained from ScienceCell Research Laboratories (San Diego, CA).

All cell lines were cultivated in an incubator at 37°C, 5% CO_2 and saturated water atmosphere. Cells were trypsin-treated (0.25% trypsin in PBS) for passaging. A fraction of the resulting suspension was seeded into new culture flasks and fresh medium was added.

5.2.3 Cell purification

5.2.3.1 FACS sorting

Total $CD4^+$ T cells were stained for anti-CD4, and ant-mHLA-G surface molecules antibodies. Live lymphocytes were gated, and $CD4^+$ HLA-G^- $CD4^+$ HLA-G^+ cells were electronically sorted on a fluorescence-activated cell sorter MoFlo (Dyko).

5.2.3.2 Preparation of HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp}

$CD4^+$ T cells were purified from PBMC by negative selection using human CD4 T cells isolation kit and stained with 5 µg/mL of anti-mHLA-G mAb coupled with PE or IgG isotype control to define HLA-G-positive and negative populations. $CD4^+$ HLA-$G^{pos/neg}$ T cells were then purified either by FACS sorting using the fluorescence-activated cell sorter (also see section 5.2.3.1) or following incubation with anti-PE–coupled microbeads by positive selection.

5.2.3.3 Freezing and thawing of cultured cells

For storage, cells were kept at -80°C. Long term storage was performed in liquid nitrogen.

Freezing

Cells were resuspended (and trypsinized, if necessary) in appropriate medium. After centrifugation (300 x g for 5 min at 4°C) cells were resuspended in freezing medium (90% RPMI-1640 medium, 10% DMSO), divided into cryotube aliquots (1mL) of approximately 5

x 10^6 cells and frozen at -80°C.

Thawing

Cryotubes were incubated in a water bath at 37°C until about half part of ice thawed. Then the cell suspension was immediately transferred into a Falcon tube with 15 ml pre-warmed medium. To remove the cytotoxic DMSO, cells were subsequently centrifuged (300 x g for 5 min at RT), resuspended in fresh medium and seeded into appropriate culture flasks.

Manual counting of cells

After shaking of cell suspension, 50 µl were taken and mixed 1:1 in a 96-well microtiter plate together with trypan blue solution. Dilutions of 1:10 to 1:50 were prepared for analysis in a Neubauer counting chamber and covered with a slip. Vital lymphocytes - as characterized by pale grey color - were counted manually per microscope in the four large squares at the corners of the chamber grid. Cell numbers were calculated using the following formula:

Cells/ml = n/4 x dilution x 10^4 x volume [ml] of total suspension

n = counted cells in four squares

5.2.4 CFSE labeling

For analysis of cell division purified responder cells (1 x 10^7cells/ml) were washed twice in PBS and labeled with CFSE at a final concentration of 10µM in warm PBS for 10 min at RT. Cells were washed twice in RPMI medium supplemented with 15% FCS and subsequently quenched with medium containing 15% FCS for another 20 min, and thereafter cells were washed twice in RPMI medium supplemented with 10% FCS and cell number were then determined.

5.2.5 Analysis of T cell suppression

Classical suppression assays

Responder cells (HLA-G^{neg} T_{resp}, 1x10^6) were incubated in 500µL PBS containing 10µM CFDA-SE. To access the suppressive nature of HLA-G^{pos} T_{reg}, CFSE-labelled HLA-G^{neg} T_{resp} (1x 10^5 cells per well) were cultured with autologous HLA-G^{pos} T_{reg} (1x10^5) in the presence of α-CD3/CD28 beads at a ratio of 25 to 1 (cells to beads) in a standard complete RPMI 1640 culture medium prior to experiments. After 4 days *in vitro*, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™, using FlowJo software (7.2). The ratio of suppression was determined after normalization of the values to a maximum given by the proliferation of α-CD3/28 beads-stimulated HLA-G^{neg} T_{resp} alone.

Suppression assay in direct co-culture experiments

For cell-cell contact in direct co-culture experiments, 10^5 CFDA-SE-labeled HLA-G^{neg} T_{resp} were cultured with 10^5 autologous HLA-G^{pos} T_{reg} in the presence of α-CD3/CD28 beads (Dynal, Oslo, Norway) at a ratio of 25 to 1 (cells to beads). After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

Suppression assay in the Transwell® system

For cell-cell contact independent co-cultures, the Transwell® system was used. For this, $5x10^5$ HLA-G^{neg} T_{resp} were co-cultured with 10^5 autologous HLA-G^{pos} T_{reg} either directly in the lower chamber or separated by a Transwell® membrane. Both chambers of each Transwell® received α-CD3/CD28 beads stimulation. After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

TCR-engagement on HLA-G^{pos} T_{reg} facilitates suppression experiments

For stimulating HLA-G^{pos} T_{reg} separately, HLA-G^{pos} T_{reg} were pre-stimulated with soluble α-CD3/TCR (1µg/mL, OKT3) for 30 minutes at 37°C, 5% CO_2 incubator, thereafter, plated in the upper chamber with the same concentration of soluble α-CD3/TCR stimulation during the co-cultures. HLA-G^{neg} T_{resp} plated in the lower chamber always received α-CD3/CD28 beads stimulation. After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

Inhibition of TCR signalling on HLA-G^{pos} T_{reg} experiments

For inhibition of TCR signalling experiments, HLA-G^{pos} T_{reg} were pre-treated with 10 µM of TCR blocking reagent, 4- amino- 5- (4-chlorophenyl)- 7- (t-butyl) pyrazolo [3,4-d] pyrimidine (PP2) together with α-CD3/TCR stimulation for 30 minutes at 37°C, 5% CO_2 incubator. After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

Suppression assay in ILT-2, IL-10R, and TGF-βR1 blocking experiments

For blocking experiments, 10 µg/mL of α-ILT-2/CD85j mAb, or 40 µg/ml of α-IL-10R/CD210 mAb or TGF-βR1 kinase inhibitor (SD-208), and representative IgGs were added into lower chambers of the Transwell® system. After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

Suppression assays with CSF cells

For functional studies, peripheral HLA-G^{neg} T cells were CFSE-labeled and co-cultured with either autologous peripheral blood- or CSF-derived HLA-G^{pos} T cells at ratio of 10 to 1 under α-CD3/CD28 beads stimulation (cells: beads: 25:1). After 7 days, cells were collected, and

their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

Suppression assays with transmigrated cells

HLA-G^{pos} T_{reg} were collected from the basolateral compartments of the migration chamber and suppression assays were then performed. Migrated cells from twelve wells were pooled for analysis. After 4 days, cells were collected, and their proliferative responses were detected based on CSFE division by FACS-Calibur™ using FlowJo software (version 7.2).

5.2.6 Flow cytometric analysis

Cells were resuspended in ice-cold PBS containing 1% bovine serum albumin (BSA) and 0.5% sodium azide and stained with fluorescence-labeled mAbs at 4°C for 30 minutes. Cells then were analyzed with FACS-Calibur™ using Cell Quest™ and FlowJo software.

5.2.7 Detection of intracellular proteins

To determine intracellular soluble (s)HLA-G, ILT-2, and IL-10, the cells were treated with brefeldin A for 4 hours, and stained afterwards for surface CD4. Cells were fixed, permeabilized, and then stained for sHLA-G by HLA-G5-biotinylated mAb followed by Streptavidin- APC, ILT-2 and IL-10 by a-IL-10-APC mAb. sHLA-G and cytokines (IL-10 and IFN-γ) in the supernatants were determined by ELISA following the manufacturer's instructions (Exbio for sHLA-G; R&D system for IL-10 and IFN-γ).

5.2.8 Transmigration assay

Transmigration assays were performed with HBMEC cultured on the apical side of a filter membrane of Transwell ® till confluence. 2.5 x 10^5 of purified T cells in 0.1 ml of pre-warmed RPMI medium were added to the top of the HBMEC monolayers and 0.6 mL of media supplemented with 10 ng/ml of MIP-1α or 100 ng/ml of RANTES was added in the outer chamber of the inserts. The cells were allowed to migrate for 18 h in a humidified cell culture incubator at 37 °C and 5% CO_2.

5.2.9 Chemotaxis assays

T-cell migration across in vitro HBMEC monolayers was analyzed using QCM™ 24-well Invasion Assay. After 18 hours, T cells were removed by Cell Detachment Solution from the lower chambers, and incubated with CyQuant GR nucleic acids Dye. Fluorescent intensity was proportional to the number of migrated T cells. Fluorescent intensity at 480/520 nm was

analyzed with a 96-well plate reader. The migration index was calculated as a percentage of migrated T cells from the whole cell population.

In addition, the percentage of HLA-G^{pos} T_{reg} was determined for transmigrated cells from total $CD4^+$ T cells from each healthy donor in each assay by flow cytometry. After 18 h of transmigration, the percentage of HLA-G-expressing cells in total $CD4^+$ T cells and the percentage of CCR5-expressing cells within migrated HLA-G^{neg} T or HLA-G^{pos} T_{reg} were determined. Migrated cells from two to four wells were pooled for analysis.

5.2.10 Statistics

Significance was determined by Student's t test. Paired t tests were used for statistical comparisons of blood and CSF cell distribution and for the comparison of cell distribution in acute versus stable forms of disease. The unpaired t test was conducted for $CD4^+$ HLA-G^{pos} T-cell frequencies in MS and OND individuals. The p values were calculated as 2-tailed and considered statistically significant if p was less than 0.05. If p was less than 0.01, then the results are considered as statistically very significant.

6. Results

6.1 Suppressive mechanism mediated by naturally occurring HLA-G expressing regulatory CD4$^+$ T cells

6.1.1 Identification of naturally occurring HLA-G-expressing regulatory CD4$^+$ T cells in human peripheral blood

CD4$^+$ regulatory T cells expressing the HLA-G (HLA-G^{pos} T_{reg}) exist in small but appreciable quantities in the peripheral blood of healthy individuals (Feger et al., 2007b); they are hypo-proliferative, negative for CD25, and FoxP3 expression, and importantly, they can be found in the thymus (Feger et al., 2007b), suggesting a natural origin of two populations.

We first analyzed whether human peripheral blood T cells express HLA-G under physiological conditions. Peripheral blood from healthy volunteers (age 21 to 65 years) was accessed for HLA-G1 expression on CD4 T cells by flow cytometry using the monoclonal antibody (mAb) MEM-G/9, HLA-G^{pos} T_{reg} and HLA-G-negative responder T cells (HLA-G^{neg} T_{resp}) were purified by flow cytometry sorting (FACS) based on the membrane HLA-G expression (as shown in Figure 6.1.1.1A, upper, right panel). Using this, we characterized an average of 3.5% of CD4 T cells express HLA-G (Figure 6.1.1.1B).

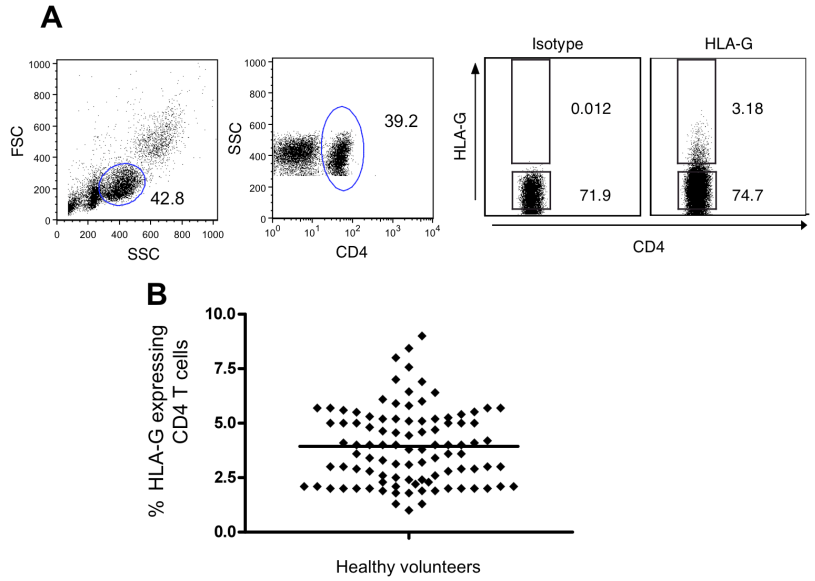

Figure 6.1.1.1 Frequencies and phenotype of CD4$^+$ HLA-G^{pos} T_{reg}

(A) The cell population of interest was gated on the lymphocytes population expressing CD4; CD4$^+$ cells were stained with isotype control and HLA-G and analyzed by flow cytometry (from left to right, upper panels). The numbers in the upper boxes indicate the percentage of CD4$^+$HLA-G^{pos} T_{reg}. (B) Percentages of CD4$^+$HLA-G^{pos} T_{reg} in the CD4$^+$subpopulations of healthy volunteers (n= 98, mean

and SD).

These isolated $CD4^+HLA\text{-}G^{pos}$ and $HLA\text{-}G^{neg}$ T cell subsets were cultured *ex vivo*. Percentages of HLA-G–positive T cells were normalized to HLA-G expression of the ex vivo population (set to 1). Levels of HLA-G expression on $HLA\text{-}G^{pos}$ T_{reg} did not change significantly over 72 hours in culture (Figure 6.1.1.2). Noteworthy, a drop of HLA-G expression by $HLA\text{-}G^{pos}$ T_{reg} at 24 hours in culture can be self-explained by the internalization of the HLA-G antibody which was used for isolation. Therefore we strongly believe that cell surface HLA-G is rather a stable phenotype than an inducible phenomenon.

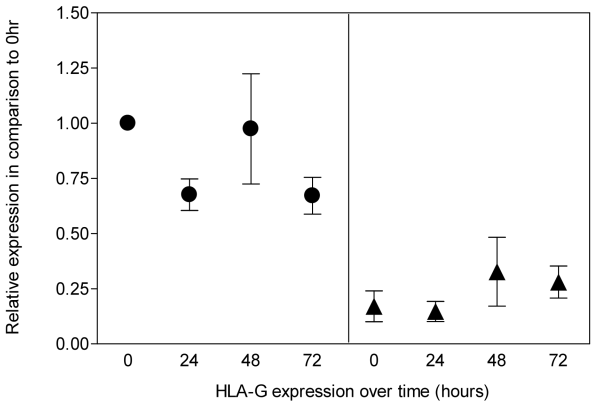

Figure 6.1.1.2 Stability of membrane-bound HLA-G expression by $CD4^+$ $HLA\text{-}G^{pos}$ T_{reg}
MACS-isolated $CD4^+HLA\text{-}G^{neg}$ T_{resp} and $HLA\text{-}G^{pos}$ T_{reg} were cultured *in vitro* for 72 hours to assess membrane HLA-G expression over time. *Ex vivo* expression of $CD4^+$ $HLA\text{-}G^{pos}$ T cells was set as reference. Graph shows summary of 3 experiments.

Next we studied the soluble factors produced by $HLA\text{-}G^{pos}$ T_{reg} in response to T-cell receptor (TCR) stimulation. We investigated the production of the soluble isoform HLA-G5. Purified $HLA\text{-}G^{pos}$ T_{reg} and $HLA\text{-}G^{neg}$ T cells were stimulated for 4 days with α-CD3/CD28 beads and after fixation stained intracellularly with the HLA-G5–specific antibody 5A6G7. Almost all (98%) of the $HLA\text{-}G^{pos}$ T_{reg} were positive for HLA-G5. Of note, 26% of the T cells lacking membrane HLA-G (HLA-G1) on the surface also expressed HLA-G5 intracellularly after stimulation (Figure 6.1.1.3A). To further ensure production of soluble HLA-G, we also tested the supernatants of the stimulated cells by enzyme-linked immunosorbent assay (ELISA). $HLA\text{-}G^{pos}$ T_{reg} showed production of soluble HLA-G, whereas $HLA\text{-}G^{neg}$ T cells had only little sHLA-G in their supernatants, suggesting that HLA-G5 is retained intracellularly in cells not bearing the membrane-bound form of HLA-G at the same time (Figure 6.1.1.3B). As a

positive control, we used LCL-HLA-G1 and Hela-HLA-G5 transfectants. These experiments could demonstrate the presence of soluble HLA-G in HLA-G^{pos} T_{reg} but not in the HLA-G^{neg} T cells counterparts and thus provide an additional method to demonstrate the characteristics of HLA-G^{pos} T_{reg}.

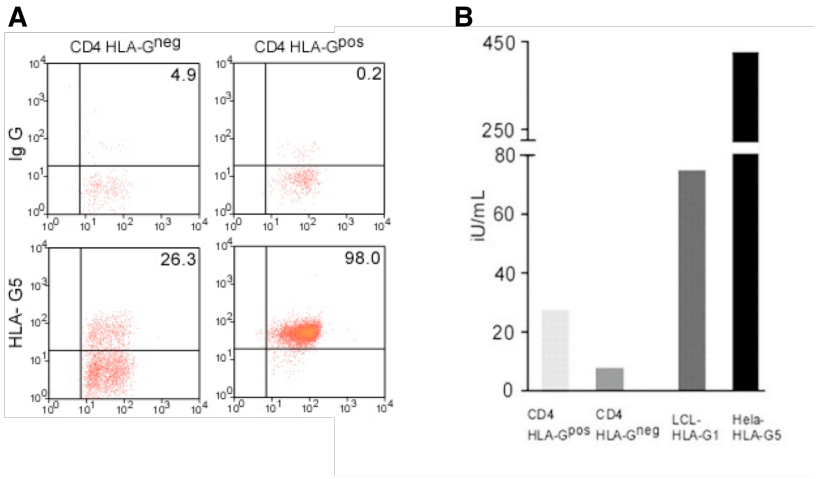

Figure 6.1.1.3 $CD4^+$ HLA-G^{pos} T_{reg} produce soluble HLA-G

(A). Intracellular expression of HLA-G5 by intracellular staining for the soluble isoform HLA-G5 in sorted $CD4^+$HLA-G^{neg} and HLA-G^{pos} T cells was performed after 4 days of stimulation with α-CD3/CD28 beads using monoclonal antibody 5A6G7. HLA-G5 expression (lower panel) was assessed in comparison with the control isotype staining (upper panel). A representative staining is shown. Values indicate the percentage of cells in gates. (B). Production of soluble HLA-G. Supernatants of α-CD3/CD28-stimulated $CD4^+$HLA-G^{neg} and HLA-G^{pos} T cells were tested by ELISA for soluble HLA-G. Cell lines transfected with either HLA-G1 or HLA-G5 were used as positive controls. One representative experiment is shown.

6.1.2 HLA-G^{pos} T_{reg}–mediated suppression is independent of cell-cell contact and is T-T interaction

To dissect the question which cellular mechanism(s) is (are) involved in HLA-G^{pos} T_{reg}-mediated suppression, we established a suppression assay system free of other allogeneic cells. Similar to commonly used applying of FoxP3-expressing nT_{reg} (Baecher-Allan et al., 2001; Ng et al., 2001), HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} were purified by flow cytometry sorting based on the membrane HLA-G expression (as depicted in Figure 6.1.1.1). The CFSE-labeled autologous HLA-G^{neg} T_{resp} were co-cultured in the absence or presence of HLA-G^{pos} T_{reg} at different ratios, where the soluble α-CD3/CD28 beads were used as the stimulus in the co-culture. We compared the suppression under direct cell-cell contacts

(Figure 6.1.2.1A) or through soluble factors using Transwell® membrane separating HLA-G^{pos} T_{reg} from HLA-G^{neg} T cells (Figure 6.1.2.1B). HLA-G^{pos} T_{reg}–mediated suppression was dependent on the suppressor-to-responder ratio and independent of cell-cell contact. Therefore, we suggest a T-T cell direct inhibition mediated by HLA-G^{pos} T_{reg}-secreted soluble factors/cytokines rather than direct cell-cell interactions.

To further investigate whether suppressive capacity of HLA-G^{pos} T_{reg} is dependent of soluble factors, we collected supernatants from suppression assays and transferred them to new proliferation assays of CFSE-labeled HLA-G^{neg} T cells. Supernatants from assays performed in the presence of HLA-G^{pos} T_{reg} clearly suppressed T-cell proliferation (mean = 71.0% ± 17.40%), while control supernatants even enhanced proliferation compared with fresh medium only (Figure 6.1.1.2C).

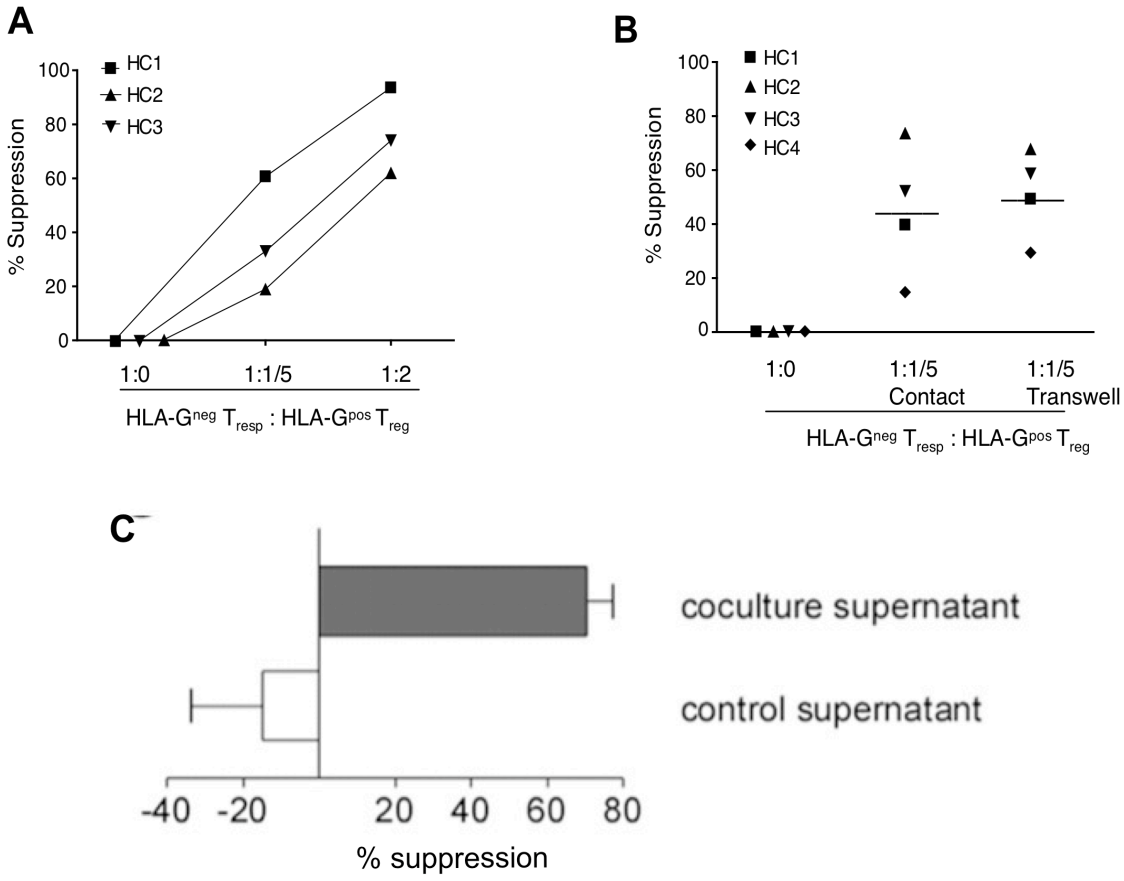

Figure 6.1.2.1 HLA-G^{pos} T_{reg}–mediated suppression through T-T interaction

(A). Cell-cell suppression depends on ratio of suppressor to responder cells. CFSE-labeled HLA-G^{neg} T_{resp} were co-cultured with autologous HLA-G^{pos} T_{reg} at different ratios (1:0, 1:1/5, and 1:2), in the presence of α-CD3/CD28 beads for 4 days in vitro. Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of HLA-G^{neg} T_{resp} alone. Independent experiments from three healthy controls (HC) are shown. (B). Cell-cell suppression is independent of cell-cell contact. HLA-G^{pos} T_{reg} were co-cultured with HLAG^{neg} T_{resp} at 1:1/5 ratio and in the presence of α-CD3/CD28 beads in a two-chamber system (Transwell®) separated by a

permeable filter membrane. Independent experiments from four HC are shown. (C). Supernatants of suppression assays with a HLA-G^{neg}/HLA-G^{pos} ratio of 1:1 (coculture; gray bar) and 1:0 (control; white bar) were transferred to new proliferation assays of CFSE–labeled HLA-G^{neg} T_{resp}. Suppressive activity of supernatant was measured after 4 days by FACS analysis. Graph shows summary of 6 independent experiments. Error bars indicate standard deviation.

6.1.3 TCR-engagement facilitates HLA-G^{pos} T_{reg}–mediated suppression

$CD4^+CD25^+$ nT_{reg}-mediated suppression was shown to inversely correlate with the strength of TCR stimulation (Thornton and Shevach, 2000). In some cases, enhancing T cell signals might abrogate $CD4^+CD25^+$ nT_{reg}-mediated suppression, whereas a weaker TCR stimulus might sustain a longer suppression (Baecher-Allan et al., 2002). We therefore challenged the influence of TCR-involvement on the suppressive action of $HLAG^{pos}$ T_{reg}. The activation of HLA-G^{pos} T_{reg} with experimentally defined optimal concentration of α-CD3 (1µg/mL, Figure 6.1.3.1A) was used. TCR stimulated (α-CD3) or non-stimulated HLA-G^{pos} T_{reg} were separated from CFSE-labeled HLA-G^{neg} T_{resp} by a permeable filter insert allowing for exchange of soluble factors (Figure 6.1.3.1B, upper panel). The activation of HLA-G^{pos} T_{reg} (α-CD3, 1µg/mL, Figure 6.1.3.1A) resulted in 55.62 ± 5.86 % suppression, whereas non-activated HLA-G^{pos} T_{reg} suppressed the responders in a range of 27.06 ± 6.79 % ($p < 0.05$, Figure 6.1.3.1B, lower panel). Activation of T cells by TCR engagement stimulates Src family tyrosine kinases (SFK) to phosphorylate downstream targets (Lysechko and Ostergaard, 2005). The PP2 inhibitor was firstly discovered to selectively hamper TCR signaling by inhibiting SFK in human T lymphocytes (Hanke et al., 1996). We assessed the effect of PP2 inhibition pathway to address $HLAG^{pos}$ T_{reg}–mediated suppression under impaired TCR signaling. Blocking TCR signaling on HLA-G^{pos} T_{reg} by PP2 clearly decreased suppressive capacity of HLA-G^{pos} T_{reg} (Figure 6.1.3.1C), demonstrating the relevance of TCR engagement for the facilitation of suppression. We could show that TCR engagement on HLA-G^{pos} T_{reg} behaves quite similar to that on $CD4^+CD25^+$ nT_{reg} and requires TCR activation to become more suppressive (Jonuleit et al., 2002; Piccirillo and Shevach, 2001; Thornton and Shevach, 2000). Similar to nT_{reg}, once HLA-G^{pos} T_{reg} are activated, their suppressor effector function is antigen-nonspecific and can be fulfilled in the absence of APC (Takahashi et al., 1998).

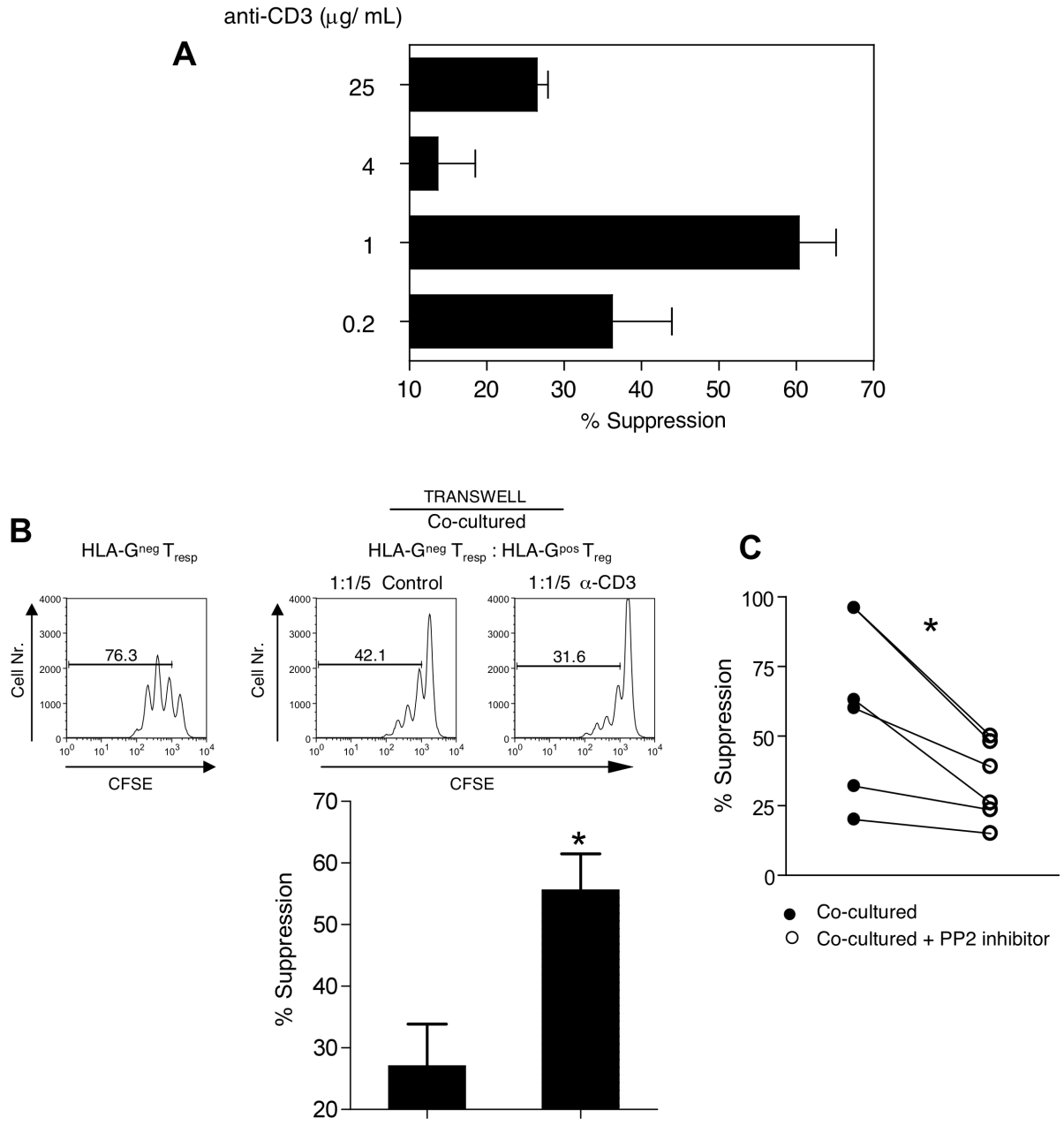

Figure 6.1.3.1 TCR engagement facilitates HLA-G^{pos} T$_{reg}$–mediated suppression.

(A). CFSE-labeled HLA-G^{neg} T$_{resp}$ were stimulated with α-CD3/CD28 beads and placed into the lower chamber, while HLA-G^{pos} T$_{reg}$ treated with different concentrations (0.2-, 1-, 4, -25-µg/mL) of α-CD3 were added to the upper chamber at a ratio of 1:1/5 (HLA-G^{neg} T$_{resp}$: HLA-G^{pos} T$_{reg}$). Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of HLA-G^{neg} T$_{resp}$ alone. Means± SEM are indicated as bar graph (n=3). (B). CFSE-labeled HLA-G^{neg} T$_{resp}$ were stimulated with α-CD3/CD28 beads and placed into the lower chamber (upper left panel), while HLA-G^{pos} T$_{reg}$ were added to the upper chamber without (upper middle panel) or with optimal α-CD3/TCR stimulation (upper right panel), at the ratio of 1:1/5 (HLA-G^{neg} T$_{resp}$: HLA-G^{pos} T$_{reg}$). One representative experiment is shown. Means± SEM are indicated as bar graph (lower panel, n=5, *, $p < 0.05$). (C). The disruption of TCR signaling on HLA-G^{pos} T$_{reg}$ reveals a decrease in suppression.

HLA-G^{pos} T_{reg} were cultured in the presence of 10 μM of PP2 (open circle), or vehicle (DMSO) control (closed circle) alone with α-CD3/TCR stimulation before they were used in the suppression assay. (N=6, *, p=0.02).

6.1.4 HLA-G^{pos} T_{reg}–mediated suppression starts from day two of co-culture and is reversible

To test whether HLA-G^{pos} T_{reg} act on a single cell level or require a "regulatory milieu", we followed their ability to suppress the responder cells at different time points of co-culture. We observed an increased efficiency of HLA-G^{pos} T_{reg} to suppress the responder cells starting after 2 days of *in vitro* (DIV2) co-culture and gradually increasing from DIV1 to DIV4 (Figure 6.1.4). Unexpectedly, HLA-G^{neg} T_{resp} deprived of HLA-G^{pos} T_{reg} on DIV4 regained their proliferation upon TCR re-stimulation, which could be observed after additional two days of culture (DIV6) (Figure 6.1.4.1), suggesting that the inhibitory events require close cellular proximity. These experiments suggest that in contrast to $CD4^+CD25^+$ nT_{reg} (Qiao et al., 2007; Sukiennicki and Fowell, 2006), HLA-G^{pos} T_{reg} -mediated suppression is reversible. A co-culture of HLA-G^{neg} T_{resp} and HLA-G^{pos} T_{reg} for DIV6 resulted in a failure of responder cells to escape HLA-G^{pos} T_{reg}-mediated suppression. These results suggest that the inhibitory events require close cellular proximity. In contrast to $CD4^+$ $CD25^+$ FoxP3-expressing nT_{reg} (Qiao et al., 2007; Sukiennicki and Fowell, 2006), HLA-G^{pos} T_{reg}-mediated suppression is therefore reversible.

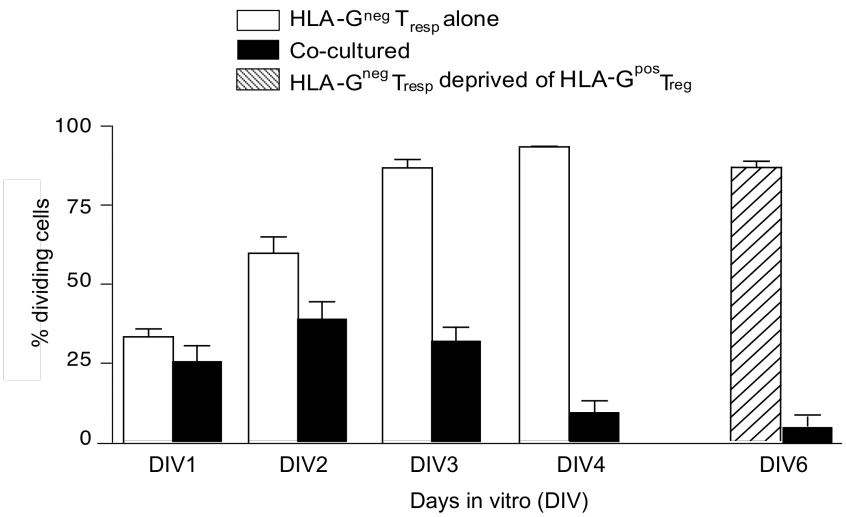

Figure 6.1.4.1 Kinetic of HLA-G^{pos} T_{reg}–mediated suppression.

CFSE-labeled and αCD3/28 beads-stimulated HLA-G^{neg} T_{resp} were either cultured alone (white bar) or co-cultured with α-CD3/TCR stimulated HLA-G^{pos} T_{reg} (black bar) in Transwell®. The percentage of

dividing cells (CSFE dilution) given by flow cytometry analysis is shown for days of in vitro (DIV) 1, 2, 3, 4, and 6 of co-cultures. Means± SEM is indicated (n=3). At DIV4, HLA-G^{pos} T_{reg} were removed from the established co-culture and the proliferation of HLA-G^{neg} T_{resp} remained in the lower chamber of Transwell® was followed for another 2 days of *in vitro* culture (DIV6) after α-CD3/TCR re-stimulation (striped bar). Means± SEM of proliferating cells is indicated (n=3).

6.1.5 The HLA-G^{pos} T_{reg}-mediated suppression does not entirely depend on sHLA-G-ILT-2 interaction

It has been demonstrated that the modulating function of HLA-G is partly mediated by its inhibitory receptors like immunoglobulin-like transcript 2 (ILT-2) (Navarro et al., 1999). It was therefore tempting to hypothesize the involvement of soluble (s) HLA-G-ILT-2 pathway in $HLAG^{pos}$ T_{reg}–mediated suppression. The ILT-2 might provide a distinct signal to the $HLAG^{neg}$ T_{resp}, rendering their responsiveness to its ligand, sHLA-G, which has been known to be mainly produced by HLA-G^{pos} T_{reg} (Feger et al., 2007b). While a small fraction of T cells expressing ILT-2 on the cell surface was identified, the majority of all T cells ($CD4^+$ and $CD8^+$) revealed ILT-2 in the cytoplasm (Saverino et al., 2000). Therefore, we analyzed the intracellular expression of ILT-2 on isolated $CD4^+$ T cells in the HLA-G^{pos} and HLA-G^{neg} T cell populations *ex vivo* (Figure 6.1.5.1). No differences in the expression of intracellular ILT-2 between HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} were noted.

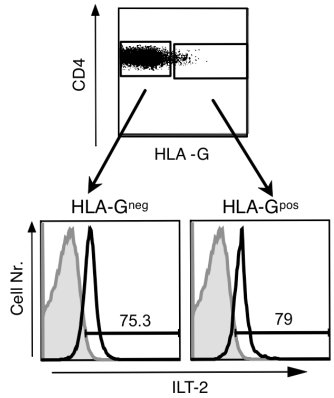

Figure 6.1.5.1 ILT-2 expression by HLA-G^{pos} T_{reg}

Intracellular expression of ILT-2 on ex vivo isolated $CD4^+$ T cells in the HLA-G^{pos} and $HLAG^{neg}$ T cell populations (upper panel). ILT-2 positive staining was normalized with IgG isotype control and shown for HLA-G^{pos} T_{reg} (lower right panel) and HLA-G^{neg} T_{resp} (lower left panel).

Next we blocked the suppression of HLA-G^{neg} T_{resp} using a neutralizing antibody against ILT-2. For this, HLA-G^{neg} T_{resp} was cultured either alone (I) or upon α-CD3/CD28 stimulation (II).

HLA-G^{pos} T_{reg} applied to the upper part of Transwell® filters were inserted into the wells with HLA-G^{neg} T_{resp} establishing a co-culture of these cells before (III) and after ILT-2 neutralization (IV) (Figure 6.1.5.2A, upper panel). ILT-2 blocking only partially antagonized the suppressive effect leading to a 20% reduction of suppressive capacity of HLA-G^{pos} T_{reg} (Figure 6.1.5.2A, lower panel). This effect was not sufficient for explaining suppression. In addition, we found no cell surface expression of ILT-2 on HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} that have been co-cultured for 4 days *in vitro* (Figure 6.1.5.2B).

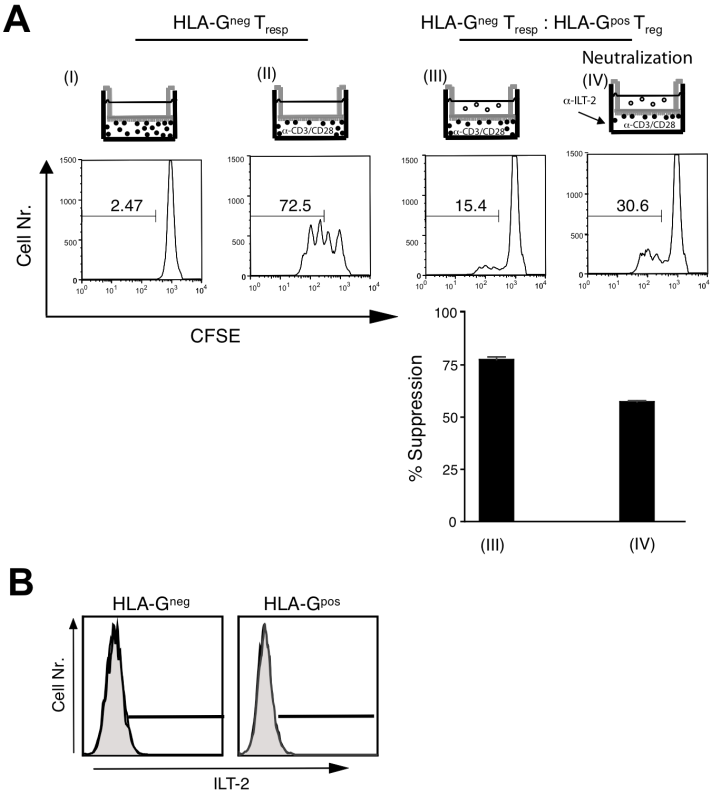

Figure 6.1.5.2 HLA-G^{pos} T_{reg}- mediated suppression does not entirely depend on sHLAG-ILT-2 interaction.

(A) CFSE-labeled HLA-G^{neg} T_{resp} were grown either without (I) or with α-CD3/CD28 beads stimulation (II-IV), while HLA-G^{pos} T_{reg} were α-CD3/TCR stimulated (III-IV, bar graph). α-ILT-2 neutralizing antibody was applied to HLA-G^{neg} T_{resp} (IV). Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of HLA-G^{neg} T_{resp} alone. (B) Surface expression of ILT-2 on HLA-G^{pos} and HLAG^{neg} T_{resp} populations after co-cultures for 4 days *in vitro*. ILT-2 positive staining was normalized with IgG isotype control and shown for HLA-G^{neg} T_{resp} (left panel) and HLA-G^{pos} T_{reg} (right panel).

As mentioned above, it has been suggested that the suppressive capacity of HLA-G^{pos} T_{reg} might be mediated by sHLA-G (Feger et al., 2007b). Although an accumulating amount of

sHLA-G was detected in the cell supernatants collected from co-cultured HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} after DIV4 and gradually increasing from DIV4 to DIV10 by ELISA (Figure 6.5.1.3, black bar), there was no significant upregulation of sHLA-G measured by intracellular flow within the co-cultured cells (Figure 6.5.1.3, white bar). Moreover, HLA-G can be cleaved from the membrane by metalloproteinases (Park et al., 2004). Hence, the accumulated sHLA-G in the supernatants of co-cultured cells might be the result of constantly shedding sHLA-G molecules from HLA-G^{pos} T_{reg} leading to increased levels of HLA-G with time. Besides, as mentioned above, HLA-G^{pos} T_{reg} were suppressive already on day 2 of co-culture with $HLAG^{neg}$ T_{resp} (Figure 6.1.4.1). However, our preliminary data revealed that the secretion of sHLA-G could not be detected until DIV4 (under detectable level). Therefore, it is rather likely that alternative pathways apart from HLA-G–ILT-2 are operative.

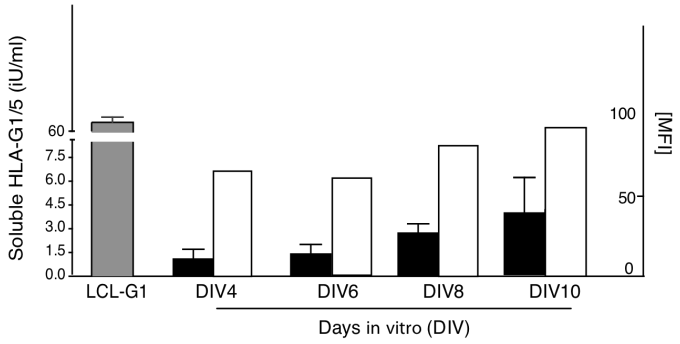

Figure 6.1.5.3 HLA-G^{pos} T_{reg}- mediated suppression does not entirely depend on sHLAG production

sHLA-G secretion in cell supernatants collected from co-cultures on DIV 4, 6, 8, and 10 measured by ELISA (black bars) and flow cytometry (white bars). The transfectant cell line LCL-HLA-G1 (gray bar) was used as a positive control. Means± SEM of HLA-G-secreting cells detected by ELISA are indicated (n=3).

6.1.6 HLA-G^{pos} T_{reg}-mediated suppression critically depends on IL-10

Since HLA-G^{pos} T_{reg} act in a cell contact independent manner, it was tempting to presume that HLA-G^{pos} T_{reg} might be able to directly act on HLA-G^{neg} cells creating a suppressive milieu down-regulating pro-inflammatory and up-regulating anti-inflammatory cytokines. The role of IL-10 as an anti-inflammatory cytokine beneficially contributing to iT_{reg} (Tr1)-mediated suppression has been described for different models of experimental diseases (Astier and Hafler, 2007; Astier et al., 2006; Kasama et al., 1995; Rosenbaum and Angell, 1995;

Woiciechowsky et al., 1998). Our previous data show that HLA-G^{pos} T_{reg} do not produce elevated mRNA levels of IL-10 and TGF-β and they are weak producers of IFN-γ mRNA (Feger et al., 2007b).

Here, we demonstrated a higher IL-10 cytokine secretion by HLA-G^{pos} T_{reg} under αCD3/TCR activation after 4-days of culture *in vitro* detected by both intracellular flow and ELISA (Figure 6.1.6.1A and B, respectively).

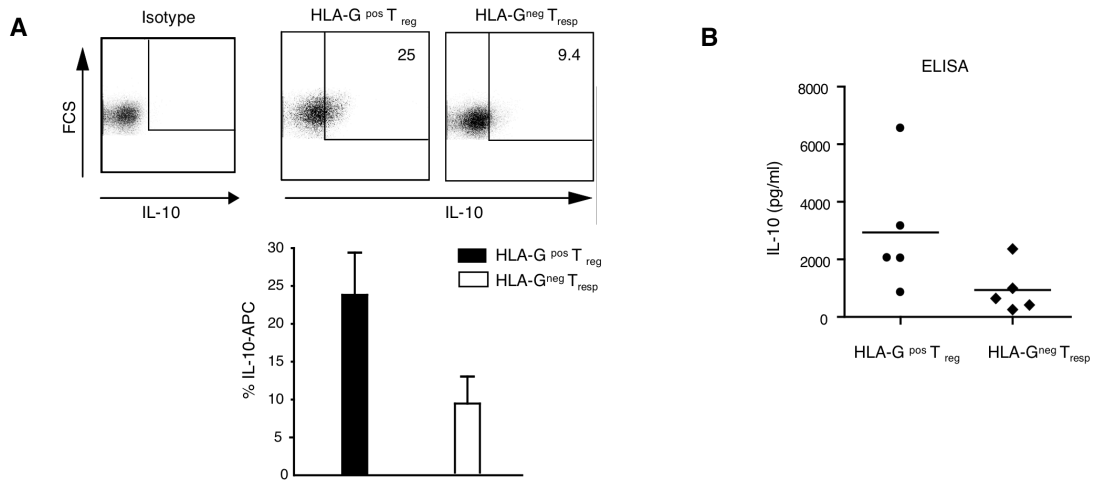

Figure 6.1.6.1 HLA-G^{pos} T_{reg} produce IL-10

HLA-G^{pos} T_{reg} or HLA-G^{neg} T_{resp} were stimulated with soluble α-CD3/TCR for 4 days *in vitro*. (A) The level of IL-10 expression was measured by flow cytometry (upper panel) and the percentage of IL-10-producing cells was subtracted from isotype control staining (lower panel). (B) IL-10 production by HLA-G^{pos} T_{reg} (circle) and HLA-G^{neg} T_{resp} (diamond) was determined by ELISA.

Moreover, α-CD3/TCR stimulation facilitated HLA-G^{pos} T_{reg}-driven shift of cytokine production from pro-inflammatory (IFN-γ) to IL-10 enriched regulatory environment under co-culture conditions (Figure 6.1.6.2). In accordance with these data, a delayed suppressive action of HLAG^{pos} T_{reg} starting from 2-days of co-culture *in vitro* (Figure 6.1.4.1), suggests a necessity of IL-10 accumulation for the generation of regulatory milieu. This could also explain why we previously did not observe the up-regulation of IL-10 after 1-day of HLAG^{pos} T_{reg} culture *in vitro* (Feger et al., 2007b), but could detect the up-regulation of IL-10 after 4 days of co-cultures *in vitro* (Figure 6.1.6.1A and B).

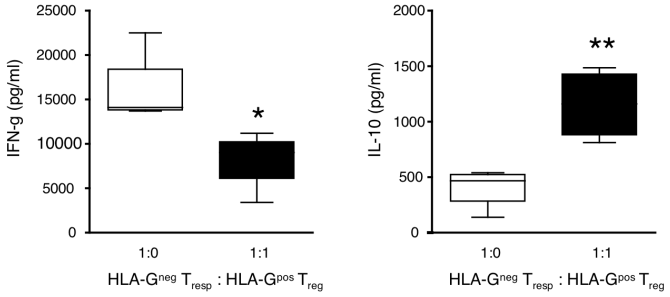

Figure 6.1.6.2 HLA-G^{pos} T_{reg} produces IL-10

The production of IFN-γ and IL-10 from HLA-G^{neg} T_{resp} cultured either alone (white bar) or co-cultured with HLA-G^{pos} T_{reg} (black bar) was determined by ELISA. Means± SEM are indicated (*, p=0.03 for IFN-γ and **, p=0.007 for IL-10).

Suppressive cytokines IL-10 and TGF-β have been suggested as implicative players of T_{reg}–mediated suppressive function (Asseman et al., 1999; Powrie et al., 1996). Next, we challenged the involvement of these cytokines in HLA-G^{pos} T_{reg}–mediated suppression. We used neutralizing monoclonal antibodies (mAb) during the suppression assays. IL-10R neutralization significantly reversed HLA-G^{pos} T_{reg}–mediated suppression in direct co-cultures (Figure 6.1.6.3A). We have also performed the experiments using Transwell membrane and separating HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} by a filter. Under this experimental condition, we also observed HLA-G^{pos} T_{reg}-mediated suppression of responder T-cell proliferation (reduction of 25%, Figure 6.1.6.3B). In contrast, neutralization of TGF-βR1 with a kinase inhibitor (Figure 6.1.6.3C) had no effect on the suppression.

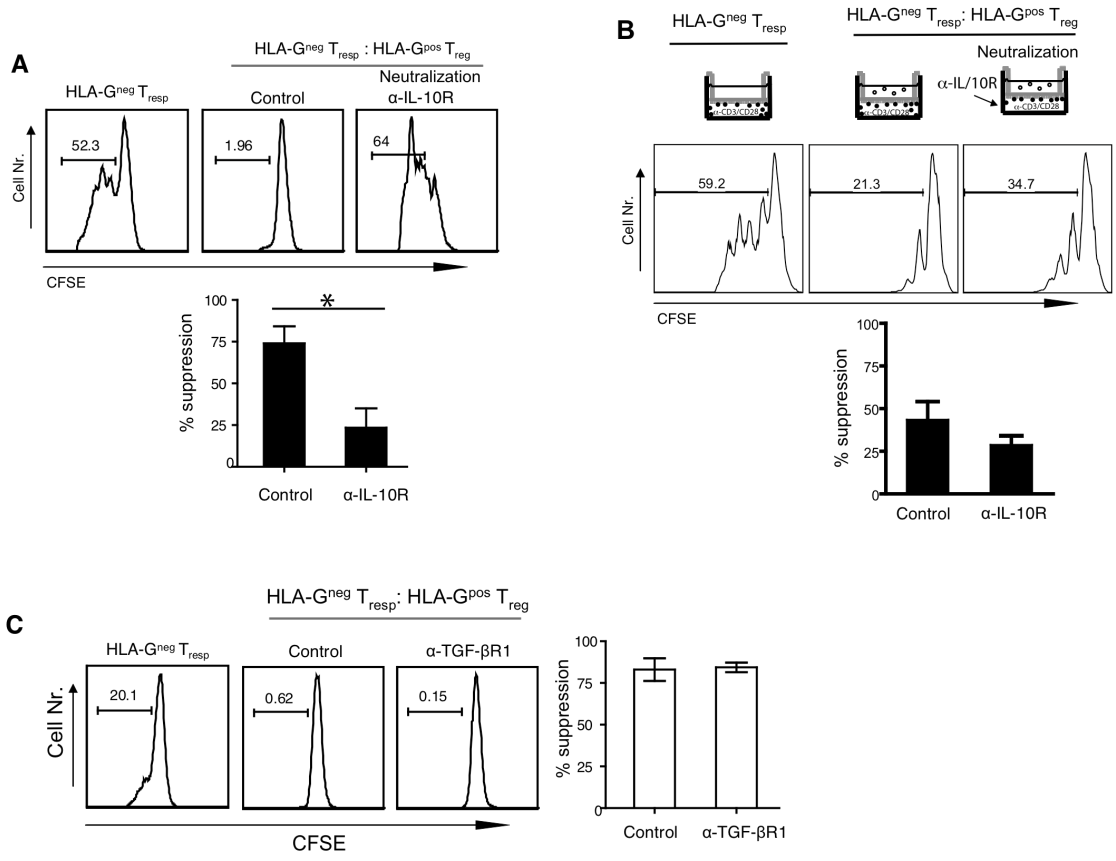

Figure 6.1.6.3 IL-10 but not TGF-β is essential for HLA-G^{pos} T$_{reg}$–mediated suppression.

(A-B) From left to right (upper panels): α-CD3/28 beads-stimulated and CFSE-labeled HLA-Gneg Tresp cultured alone or co-cultured with α-CD3/TCR-stimulated HLA-G^{pos} T$_{reg}$ in the presence of IgG control or specific mAb against IL-10R, when the cells were directly co-cultured (A, lower panel, n=3, Means± SEM indicated, $p<0.05$, *, significant) or when the Transwell were applied (B, lower panel, n=4, Means± SEM indicated, $p=0.26$, n.s, non significant). Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of HLA-G^{neg} T$_{resp}$ alone. C, From left to right: α-CD3/28 beads-stimulated and CFSE-labeled HLA-G^{neg} T$_{resp}$ cultured alone or co-cultured with α-CD3/TCR-stimulated HLA-Gpos Treg in the presence of IgG control or specific mAb against TGF-βR1 kinase. The plots show one representative experiment out of three performed (left panel). Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of HLA-G^{neg} T$_{resp}$ alone (right panel). Means± SEM indicated (n=3).

6.2 Specific CNS recruitment and suppressive function of HLA-G-expressing regulatory T cells in MS patients

6.2.1 HLA-G^{pos} T_{reg} are enriched in the cerebrospinal fluid (CSF) and found in brain lesions from MS patients

To evaluate the potential role of HLA-G^{pos} T_{reg} in CNS inflammation, we first characterized the distribution of HLA-G^{pos} T_{reg} in MS patients compared to patients with other non-inflammatory neurological disorders (OND). The frequency of HLA-G^{pos} T_{reg} in the CSF was compared with peripheral blood. A significantly higher frequency of HLA-G^{pos} T_{reg} was detectable in the CSF (5.9± 0.8% of all CD4$^+$ T cells) in comparison to the blood (2.7± 0.3%, p = 0.0002) from MS patients, while no significant differences in the frequency of HLA-G^{pos} T_{reg} in the CSF (2.7± 0.4%) were found in comparison to the blood (2.4± 0.5%) from OND patients (Figure 6.2.1.1).

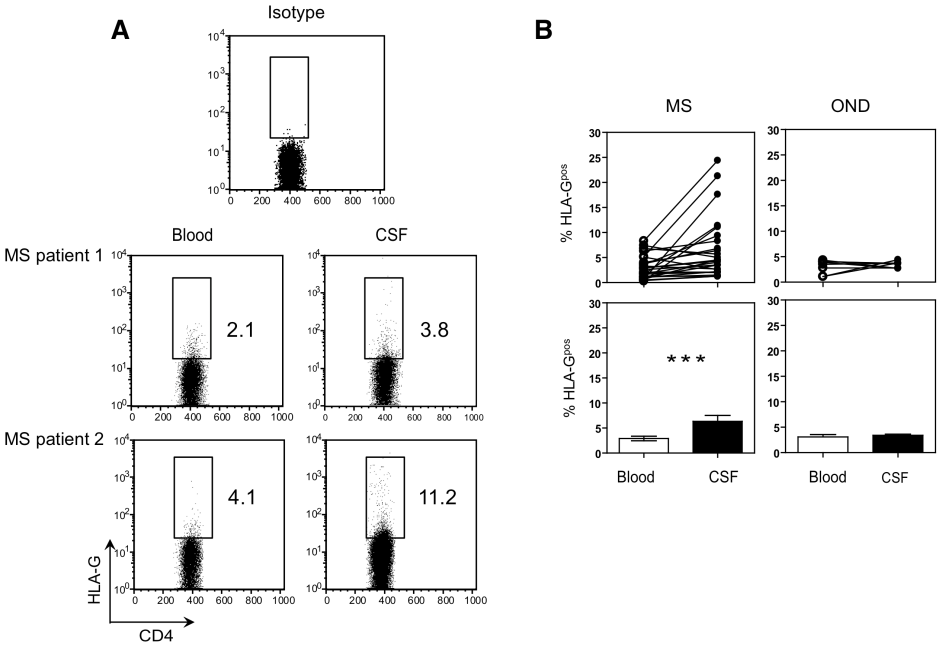

Figure 6.2.1.1 HLA-G^{pos} T_{reg} are enriched locally within CSF from MS patients

(A) Mononuclear cells from peripheral blood (left panel) and CSF (right panel) were obtained from the same MS patients and stained with antibodies to CD4 and HLA-G. The numbers in the boxes indicate the percentages of HLA-G^{pos} T cells within CD4$^+$ population accessed in comparison to the isotype control (upper panel) staining by flow cytometry. The representative staining of two MS patients is shown. (B) Single pairing of blood- and CSF-derived samples of HLA-G^{pos} T cells for each patient (upper panel) and a summary of paired blood- and CSF-derived samples from all patients

(lower panel) with MS (left panel, n=27; ***p=0.0002) and other neurologic disease (OND, right panel, n=9) are shown. (Mean ± SEM for each patient group).

To further substantiate our data, we aimed at demonstrating the presence of HLA-G^{pos} T_{reg} at sites of acute inflammatory demyelination. Therefore, we analyzed brain tissue specimens from MS patients who had a diagnostic biopsy showing an inflammatory demyelinating process consistent with multiple sclerosis. The brain biopsy in these cases was performed to exclude other treatable diseases such as acute disseminated encephalomyelitis (ADEM), vasculitis or lymphoma. The demyelinating lesions studied were infiltrated by CD3-positive T cells diffusely within the parenchyma and in the perivascular space ((Figure 6.2.1.2 A and B). In all lesions of the five MS cases investigated, single T cells co-expressing CD3 and HLA-G were identified. They were preferentially seen in the perivascular space (Figure 6.2.1.2 C-F). The HLA-G-positive cells were morphologically identified as T cells and this was confirmed by double immunofluorescence staining. The quantification of HLA-G-positive cells in MS lesions revealed the presence of 1.1-7.1 HLA-G-positive cells per mm^2 with either one of two antibodies used. Single HLA-G-positive cells were observed in the control CNS vasculitis case and the inflamed glioblastoma infiltration zone. They were absent in the infiltrated cortex of the second glioblastoma case.

Taken together, these data demonstrate that HLA-G^{pos} T_{reg} are enriched in the CSF of MS patients and are also present in the brain parenchyma. Accumulation of HLA-G^{pos} T_{reg} in the CSF and their presence in inflammatory demyelinating brain lesions might both indicate a role for HLA-G^{pos} T_{reg} in intraparenchymal regulation of CNS inflammation.

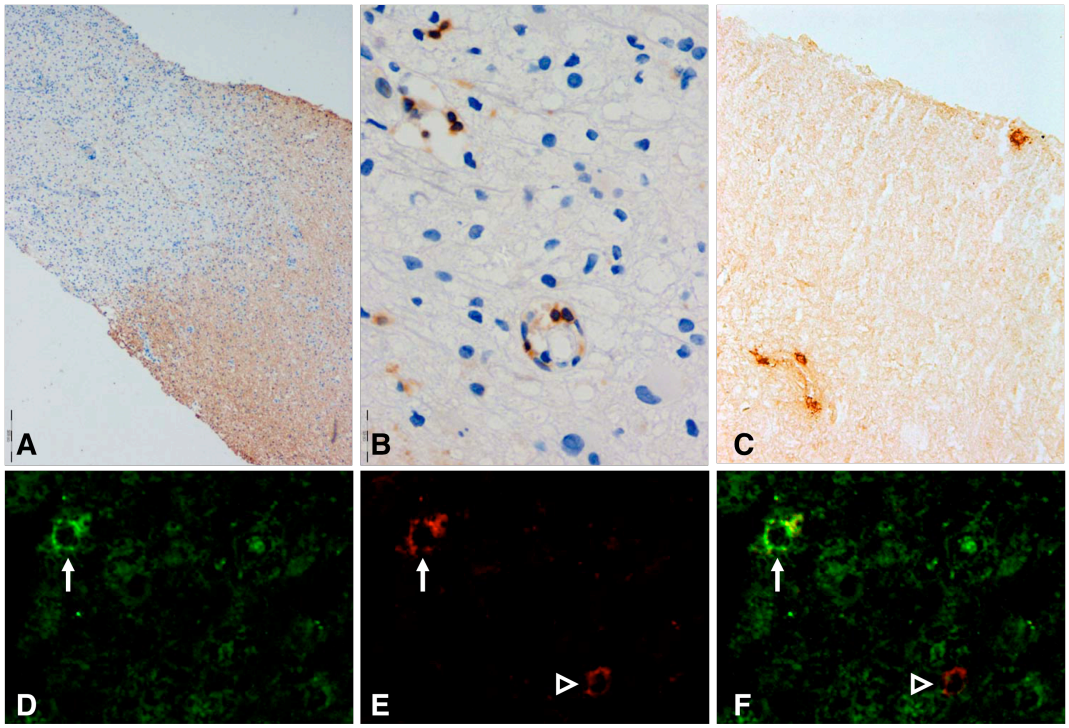

Figure 6.2.1.2 HLA-G^{pos} T_{reg} are found in MS lesions.

HLA-G^{pos} T_{reg} are localized in the depicted inflammatory demyelinating brain lesion (A and B) A formalin-fixed, paraffin-embedded section from an MS brain biopsy specimen was used. (A) The PLP myelin staining shows an inactive demyelinated brain lesion with adjacent normal appearing white matter (original magnification (OM) 40x). (B) Perivascular and parenchymal CD3$^+$ T cells are present within the lesion (OM 400x). (C) Cryo section from the same lesion as shown in A and B reveals mainly perivascular HLA-G-expressing cells with the morphology of T cells (OM 200x) within the lesion. (D, E and F) Double immunofluorescence staining in the lesion demonstrates a co-localization of HLA-G (D, green) and CD3 (E, red) staining (arrows). The section is representative of similarly looking brain lesions in the other 4 patients. Arrowheads point to another CD3 positive T-cell, which does not express HLA-G (OM 40x).

6.2.2 Phenotypes of CSF-derived HLA-G^{pos} T_{reg} in MS patients

We next evaluated the phenotype of CSF-derived HLA-G^{pos} T_{reg}. To delineate the naïve and effector T cell populations, we used CD45RA and CD27 as differentiating cell surface markers (Kivisakk et al., 2003) (Figure 6.2.2.1A, left panel). Effector memory T cells (Tem) were found as a small, distinct subpopulation within CSF- and blood-derived HLAG^{pos} T_{reg}. Blood-derived HLA-G^{pos} T_{reg} consisted of a mixture of all lymphocyte sub-populations, whereas CSF-derived HLA-G^{pos} T_{reg} exhibited a clear predominance of the central memory (Tcm) phenotype (69.8± 6.8%) (Figure 6.2.2.1A and B). A similar distribution of cellular phenotype was also observed within HLA-G^{neg} T cells (Figure 6.2.2.2). Together, these data

are consistent with the reported data that most CSF-derived T cells represent the $CD4^+$ Tcm sub-population. We further investigated the expression of inducible co-stimulator (ICOS) on these cells, which was shown to be upregulated on locally activated T cells that have migrated (Herman et al., 2004; Hutloff et al., 1999). The mean fluorescence intensity (MFI) for ICOS expression on HLA-G^{pos} T_{reg} was elevated in direct comparison to HLA-G^{neg} T cells (Figure 6.2.2.1C).

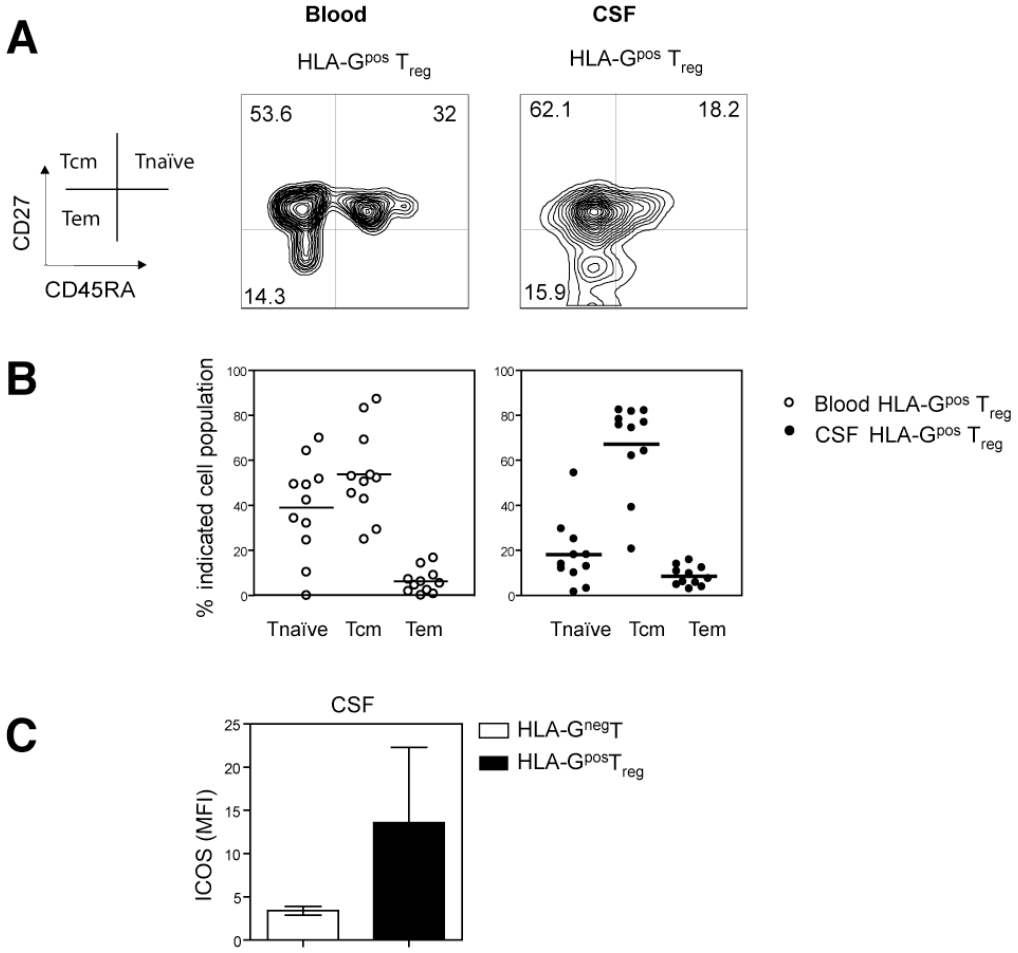

Figure 6.2.2.1 Phenotypes for effector/memory markers reveals a predominance of central-memory phenotype within CSF-derived HLA-G^{pos} T_{reg}

(A). One representative FACS analysis of blood- (left panel) and CSF-derived (right panel) HLA-Gpos Treg from the same MS patient. Naïve (CD4+CD45RA+CD27+), central memory (Tcm) (CD4+CD45RA-CD27+), and effector memory (Tem) (CD4+CD45RA-CD27-) T cells could be identified. (B). The percentage of sub-populations within HLA-G^{pos} T_{reg} from blood- (opened circle) and from CSF (closed circle) samples of MS patients (n=11, mean± SEM). (C). Mean fluorescence intensity (MFI) of ICOS expression on CSF-derived HLAG^{pos} T_{reg} (black bar) and HLA-G^{neg} T cells (white bar) from MS patients (n=4, mean ± SEM).

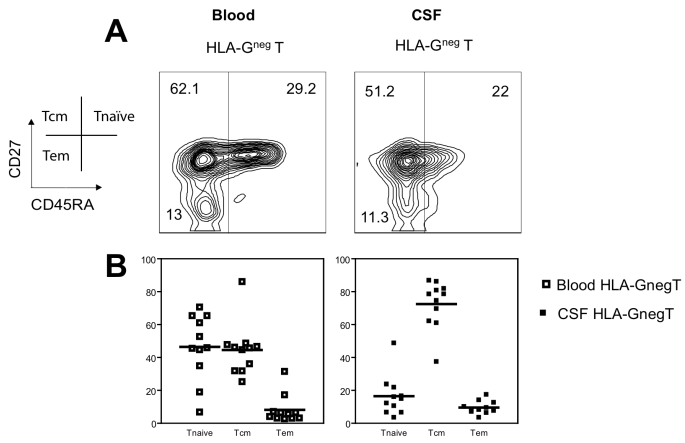

Figure 6.2.2.2 Phenotypes for effector/memory markers reveals a predominance of central-memory phenotype within CSF-derived HLA-G^{neg} T cells

(A). One representative FACS analysis of blood- (left panel) and CSF-derived (right panel) HLA-G^{neg} T cells from the same MS patient. Naïve ($CD4^+CD45RA^+CD27^+$), central memory (Tcm) ($CD4^+CD45RA^-CD27^+$), and effector memory (Tem) ($CD4^+CD45RA^-CD27^-$) T cells could be identified. (B). The percentage of sub-populations within HLA-G^{neg} T cells from blood- (opened square) and from CSF (closed square) samples of MS patients (n=11, mean± SEM).

6.2.3 Phenotypes of CSF-derived HLA-G^{pos} T_{reg} in MS patients reflect their ability to migrate

Similar to other leukocytes, the accumulation of HLA-G^{pos} T_{reg} in the CNS is likely to be determined by the expression of tissue-specific chemokine receptors (CCRs) (Siegmund et al., 2005). Therefore, we looked for the expression of chemokine receptors on CSF-derived HLA-G^{pos} $T_{reg.}$ The vast majority of CD4+ T cells in the CSF express CCR7, an indicator of the T cell ability to return to secondary lymphoid organs (Debes et al., 2005). The frequency of CCR7-expressing CSF-derived HLAG^{pos} T_{reg} was similar to those found in the blood, and there was no difference in the percentage of CCR7-expressing HLA-G^{neg} T cells between MS and OND patients (Figure 6.2.3.1A, B and C). No correlation of CCR7 expression in HLA-G^{pos} T_{reg} between blood and CSF was observed for these patients (Figure 6.2.3.1D). Furthermore, no differences were found in the expression of CCR4 and CXCR1 on HLA-G^{pos} T_{reg} and HLA-G^{neg} isolated from blood or CSF samples (data not shown).

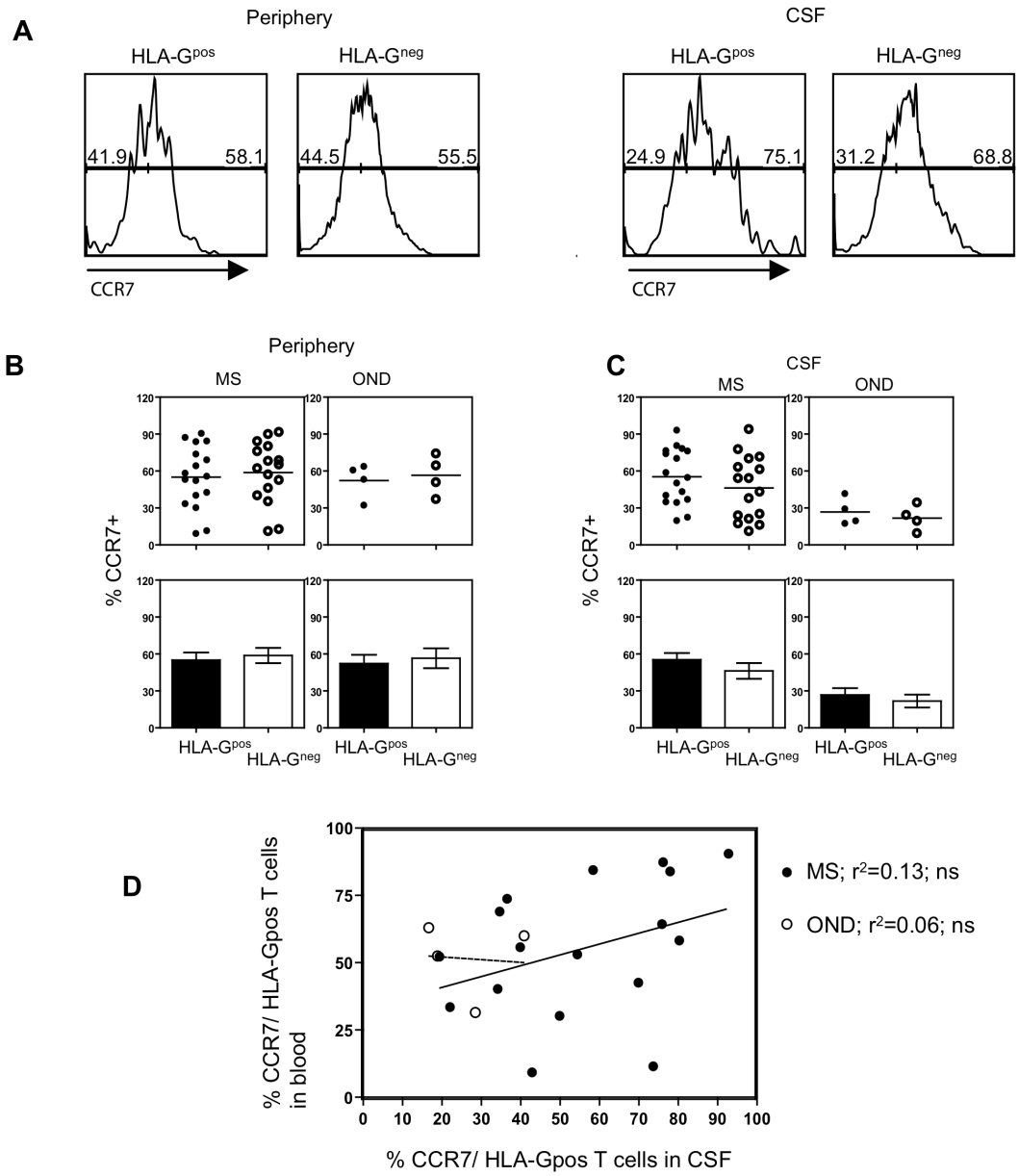

Figure 6.2.3.1 Similar expression of CCR7 by CSF-derived HLA-G^{pos} T_{reg} and HLA-G^{neg} T cells

(A). HLA-G^{pos} T_{reg} and HLA-G^{neg} T cells in paired periphery- CSF sample used flow cytometry to characterize CCR7 expression. Shown are the histograms of the CCR7 expression on gated HLA-G^{pos} T_{reg} or HLA-G^{neg} T cells. (B, C). CCR7 expression (mean fluorescence intensity; MFI) by HLA-G^{pos} T_{reg} (upper panel: closed circle, and lower panel: black bar) and HLA-G^{neg} T cells (upper panel: opened circle, and lower panel: white bar) in paired periphery (B)-and-CSF (C) samples from patients with MS (left panel) or with OND (right panel). Single pairing of periphery-and-CSF samples for each patient (upper panel) and a summary of paired periphery-and-CSF samples from patients (lower panel). (D) Linear regression analysis demonstrated no relationship between levels of HLA-G^{pos} T_{reg} in periphery and CSF from individual patients with MS (closed circle) or with OND (opened circle). Indicated r and p values were measured using Pearson's correlation tests.

However, we observed a dominant CCR5 expression on CSF-derived HLA-G^{pos} T_{reg} in comparison to HLA-G^{neg} cells, which was not observed in the blood-derived samples (Figure 6.2.3.2A, B and C). High expression of CCR5 on CSF-derived HLAG^{pos} T_{reg} was found both in MS and in OND samples (Figure 6.2.3.2C). Yet, a significant correlation in the frequency of CCR5 expression between blood- and CSF-derived HLA-G^{pos} T_{reg} was only observed in patients with MS but not in OND samples (Figure 6.2.3.2 D).

These findings suggest that CCR5 expression is a critical component in the recruitment of HLA-G^{pos} T_{reg} from the periphery to the CSF compartment.

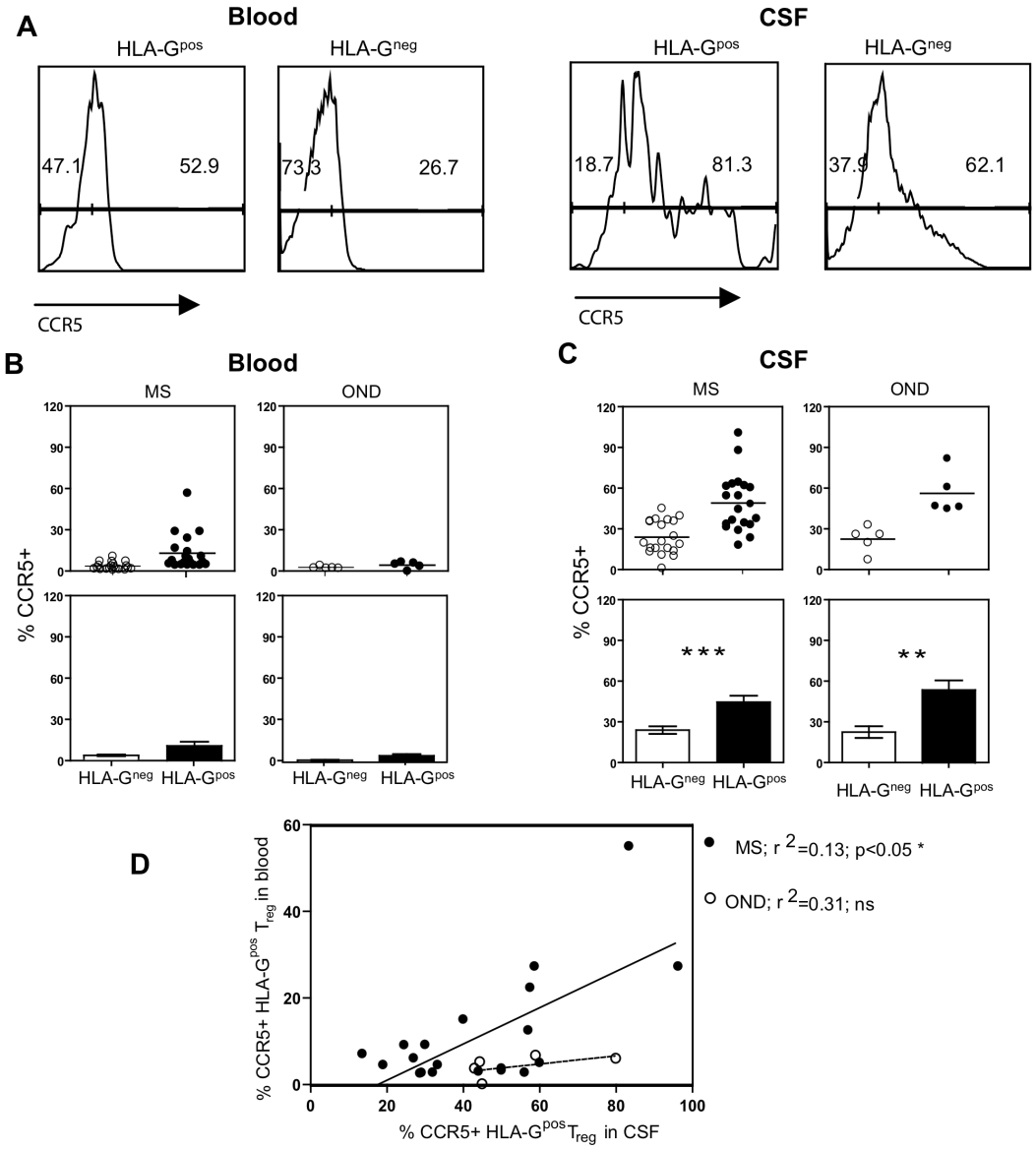

Figure 6.2.3.2 Elevated CCR5 expression on CSF-derived HLA-G^{pos} T_{reg}

(A). One representative histogram of sorted HLA-G^{pos} and HLA-G^{neg} T cells from the blood (left panel) or CSF (right panel) of MS samples analyzed for CCR5 expression is shown. (B and C). Graphs show the frequency of CCR5 expression on gated HLA-G^{pos} T_{reg} and HLA-G^{neg} T cells (n=20, mean ±

SEM). Individual analysis (upper panel) and summary values (lower panel) for the frequency of CCR5-expressing cells within HLA-G^{pos} (filled circles) and HLA-G^{neg} T cells (open circles) derived from the blood (B) and CSF (C) samples of MS (n=20, mean ± SEM ***$p<0.001$ HLA-G^{pos} compared to $HLAG^{neg}$, left panel) or OND (n=5, mean ± SEM **$p<0.01$ HLA-G^{pos} compared to $HLAG^{neg}$, right panel) patients are shown. (D) Linear regression analysis between levels of frequency of CCR5-expressing cells within HLA-G^{pos} T cells in blood versus CSF samples from individual patients with MS (filled circles) or OND (open circles). r and p values were measured using Pearson's rank correlation tests.

6.2.4 *HLA-G^{pos} T_{reg} preferentially express CCR5*

To validate the CCR5 expression on HLA-G^{pos} T_{reg}, we performed flow cytometry analysis on total $CD4^+$ T cells from resting or TCR-stimulated HLA-G^{pos} T_{reg} or HLA-G^{neg} T cells (Figure 6.2.4.1). In freshly isolated, unstimulated $CD4^+$ T cells, a similar level of CCR5 expression was observed in both counterparts. By 16 hrs post TCR stimulation, CCR5 expression on HLA-G^{pos} T_{reg} was ~2.5-fold higher than on HLA-G^{neg} T cells and even higher (~4-fold increase) after 28 hrs post TCR engagement. Therefore, although detectable on both HLA-G^{pos} T_{reg} and HLA-G^{neg} T cells in the absence of activation, the expression of CCR5 can be significantly increased on HLA-G^{pos} T_{reg} upon TCR stimulation.

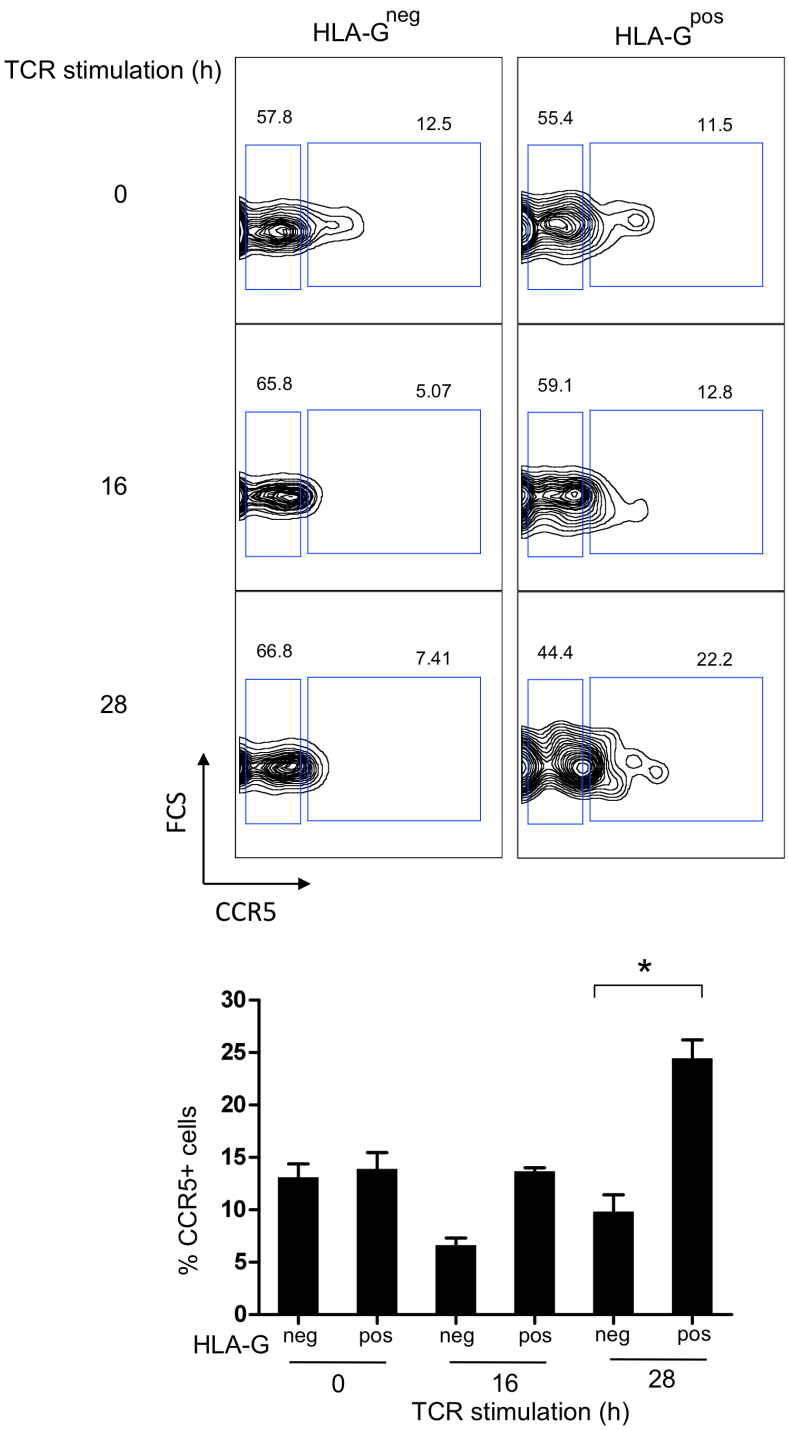

Figure 6.2.4.1 HLA-G^{pos} T$_{reg}$ cells preferentially express CCR5 upon TCR stimulation

(A and B) Freshly isolated CD4^{+} T cells were analyzed before (upper panel) and after anti-CD3 (16h, middle and 28h, lower panels) stimulation. A, The plots represent a flow cytometry analysis of CCR5 expression performed on CD4^{+} HLA-G^{neg} (left) and HLA-G^{pos} (right panel) T-cell sub-populations. One representative experiment out of three performed is shown. B, The graph depicts quantification of the percentage of CCR5 expression on CD4^{+} HLA-G^{neg} and HLA-G^{pos} T cells calculated from three independent experiments, each with three or more patient samples (*, $p < 0.05$).

6.2.5 *Inflammatory chemokines facilitate HLA-G^{pos} T_{reg} trafficking in a model of the human blood-brain barrier*

Elevated expression of CCR5 on migrating cells was previously shown to be the main attribution of their recruitment into the CSF in MS patients (Pashenkov et al., 2002; Sorensen et al., 2003). The significantly increased frequency of CCR5-expressing T_{reg} in the CSF of MS patients supports the notion that HLA-G^{pos} T_{reg} might be instrumental in the migration process. Several studies have previously reported an enrichment of CCR5 ligands, such as MIP-1α and RANTES in the CSF from relapsing-remitting MS patients (Bartosik-Psujek and Stelmasiak, 2005; Miyagishi et al., 1995; Sorensen et al., 1999) and in MS plaques (McManus et al., 1998; Simpson et al., 1998). In experimental settings, MIP-1α and RANTES were shown to play critical roles in cell migration, and these chemokines were shown to be upregulated in the CNS under inflammatory conditions (Cardona et al., 2003; Hofmann et al., 2002).

Therefore, we set out to define the mechanism underlying HLA-G^{pos} T_{reg} recruitment using an *in vitro* model of the human BBB, a two-chamber system (Transwell®) (Figure 6.2.5.1A). When HLA-G^{pos} T_{reg} migration was followed across human brain microvascular endothelial cells (HBMEC) (Reese and Karnovsky, 1967), both chemokines, MIP-1α and RANTES, significantly facilitated migration of HLA-G^{pos} T_{reg} (Figure 6.2.5.1B and C). HLA-G^{pos} T_{reg} were found to be the predominant population recruited by MIP1α and RANTES (Figure 6.2.5.1B and C), but not to MIP-3β, the ligand for CCR7 (not shown). This suggests a specific role of CCR5 binding chemokines for HLA-G^{pos} T_{reg} migration. Additionally, we found that the expression of CCR5 within HLA-G^{pos} T_{reg}, but not within HLA-G^{neg} T cells favors MIP-1α and RANTES-driven migration (Figure 6.2.5.1D), suggesting CCR5-dependent recruitment of HLA-G^{pos} T_{reg} to the inflamed CNS.

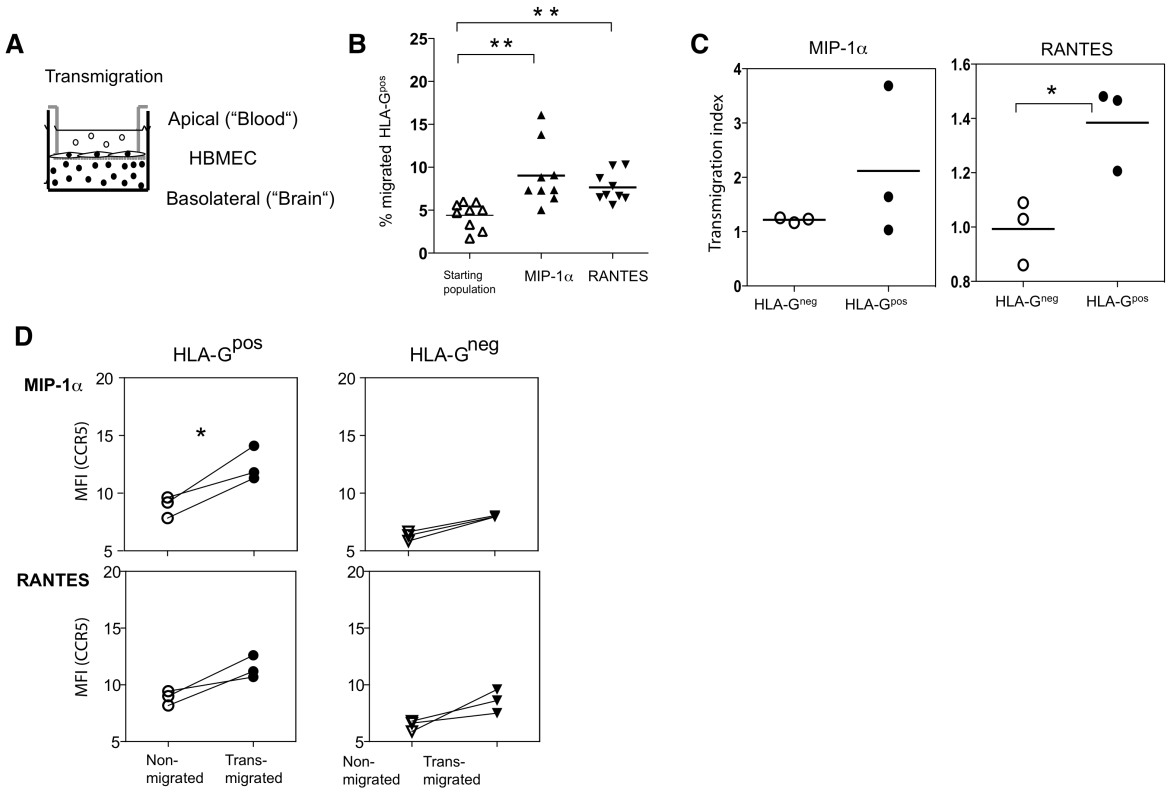

Figure 6.2.5.1 HLA-G^{pos} T_{reg} have a higher migratory capacity across HBMEC monolayer toward inflammatory chemokines *in vitro* than HLA-G^{neg} T_{reg}

The chemotactic response of HLA-G^{pos} T_{reg} to MIP-1α and RANTES was analyzed in an *ex vivo* transmigration assay. (A) The transmigration chambers consisted of a permeable filter coated with a confluent monolayer of HBMEC, and a basolateral side supplemented with either MIP-1α or RANTES chemokines. The T cells were added on the apical part of the chamber. (B) The percentage of transmigrated HLA-G^{pos} T_{reg} chemoattracted by MIP-1α (triangles) and RANTES (inverted triangles) out of the total population of transmigrated CD4$^+$ T cells was compared to the percentage of HLA-G^{pos} within all CD4$^+$ T cells before migration (open triangles, starting population). (n= 9, **p<0.01). (C) The migration index was calculated as a percentage of transmigrated HLA-G^{neg} (open circles) and HLA-G^{pos} (filled circles) T cells from the total T-cell population (2.5 x 10^5) added to the upper compartment of the migrating chambers. MIP-1α- (left panel) and RANTES (right panel)-driven transmigration of HLA-G^{pos} and HLA-G^{neg} T cells is shown. Each data point was run in triplicate (n=3, *p<0.01). (D) MFI of CCR5 expression on non-transmigrated (open symbols) versus transmigrated (filled symbols) HLA-G^{pos} (left panel) and HLA-G^{neg} (right panel) T cells, driven by MIP-1α (upper panel) and RANTES (lower panel). Data presented are the mean ± SEM of three independent experiments using healthy volunteer samples. (n=3, * p<0.05).

6.2.6 *Inflammatory chemokine-mediated trafficking across the blood-brain barrier increases the suppressive capacity of HLA-G^{pos} T$_{reg}$*

To address whether the chemokine-induced HLA-G^{pos} T$_{reg}$ *ex vivo* transmigration across HBMEC alters their function, we compared the suppressive properties of transmigrated versus non-transmigrated cells. Since RANTES had a stronger effect on HLA-G^{pos} T$_{reg}$ migration (Figure 6.2.5.1C), we performed suppression assays with RANTES-driven HLA-G^{pos} T$_{reg}$ following their transmigration. HLA-G^{pos} T$_{reg}$-mediated suppression upon transmigration appeared significantly stronger than suppression triggered by non-migrated HLA-G^{pos} T$_{reg}$ (Figure 6.2.6.1A). To elucidate whether the functional impact depended on the cell trafficking or secretion of soluble factors, we tested the influence of RANTES chemokine on the suppression activity of HLA-G^{pos} T$_{reg}$ in the absence of their migration. The application of different concentrations of RANTES alone did not alter the suppressive activity of HLA-G^{pos} T$_{reg}$, if the cells were not allowed to migrate (Figure 6.2.6.1B). In addition, we also observed no influence of RANTES on CCR5 expression by HLA-G^{pos} T$_{reg}$ (not shown).

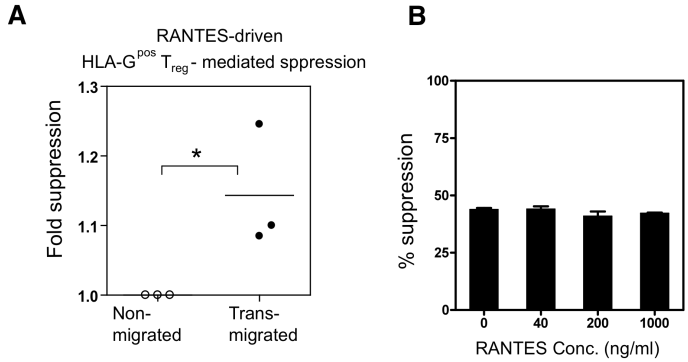

Figure 6.2.6.1 Inflammatory chemokine-mediated trafficking across the blood-brain barrier increases the suppressive capacity of HLA-G^{pos} T$_{reg}$

(A) Suppressive capacity mediated by HLA-G^{pos} T$_{reg}$ collected from transmigration assay in comparison of non-transmigrated (open circles, set as 1) versus transmigrated (filled circles; expressed as "fold suppression") populations trafficking across HBMEC toward RANTES *in vitro*. The fold of suppression was calculated by subtracting the percentage of suppression mediated by transmigrated to non-migrated HLA-G^{pos} T$_{reg}$ and set as one. Data presented is the mean of three independent experiments from different donors (n=3, * $p < 0.05$). (B) Suppressive capacity mediated by HLA-G^{pos} T$_{reg}$ under different concentrations of RANTES in the absence of migration chamber. Data presented is the mean of independent experiments from three different donors.

6.2.7 *HLA-G^{pos} T_{reg} are functionally active in the periphery from MS patients*

Some autoimmune disorders including MS have been associated with altered frequency and/or dysfunction of certain T_{reg} populations (Astier et al., 2006; Viglietta et al., 2004; Zozulya and Wiendl, 2008). We therefore assessed the frequency and suppressive capacity of HLA-G^{pos} T_{reg} *ex vivo* in MS patients in comparison to matched healthy individuals (Figure 6.2.7.1A). Different clinical stages of the disease were investigated (stable relapsing-remitting disease course (RR-MS) versus acute relapse in RR-MS) to delineate possible correlations of HLA-G^{pos} T_{reg} frequency and functional capacity with disease course or status. We also assessed the suppressive capacity of HLA-G^{pos} T_{reg} from MS patients in comparison to HD (Figure 6.2.7.1B). A group of patients with secondary progressive MS (SP-MS) has been included in this study. HLA-G^{pos} T_{reg} revealed a similar capacity to suppress responder cells in healthy volunteers as in various subgroups of MS (Figure 6.2.7.1B). Of note, we used CD4$^+$CD25$^+$FoxP3$^+$CD127^{dim} T_{reg} as control T_{reg} as it was demonstrated by several groups including ours, these cells are likely to be dysfunctional (Feger et al., 2007a; Haas et al., 2005; Kumar et al., 2006; Viglietta et al., 2004). Thus, peripheral blood HLA-G^{pos} T_{reg} appear to be functionally competent in MS patients, in contrast to other T_{reg} populations.

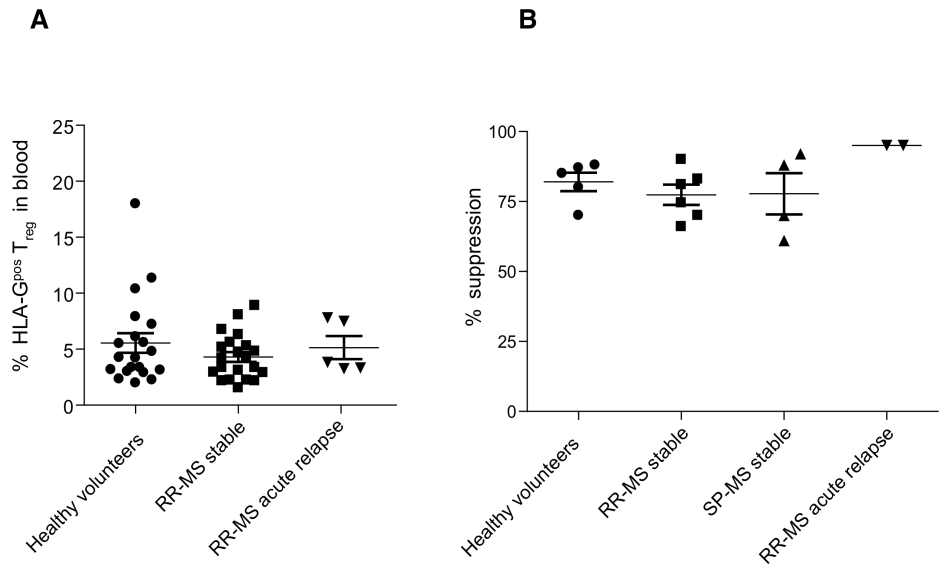

Figure 6.2.7.1 The frequency and function of HLA-G^{pos} T_{reg} at different stages of disease in MS patients.

(A) The frequency of HLA-G^{pos} T_{reg} within total CD4$^+$ T-cell population obtained from the blood of healthy volunteers (n=20, filled circles), RR-MS stable (n=21, filled squares) and RR-MS, acute relapse (n=5, inverted triangles) was calculated based on surface expression of HLA-G. (B) The percentage of suppression mediated by HLA-G^{pos} T_{reg} isolated from healthy volunteers (n=5, filled circles), RR-MS stable (n=6, filled squares), SP-MS (n=4, filled triangles) and RR-MS, acute relapse

(n=2, inverted triangles) was calculated after normalization of the values to a maximum given by the proliferation of CFSE-labeled HLA-G^{neg} T cells alone (mean ± SEM are shown).

6.2.8 *HLA-G^{pos} T_{reg} are functionally competent to suppress autologous T-cell activation at sites of inflammation in MS*

Next, we determined whether CSF-derived HLA-G^{pos} T_{reg} were functional regulatory T cells (Feger et al., 2007b), which would strongly suggest their potential suppressive role *in vivo*. We used an *in-vitro* culture system to test their suppressive capacity when isolated directly from the CSF. HLA-G^{pos} T_{reg} and their counterparts (HLA-G^{neg} T cells) were sorted by flow cytometry immediately after spinal fluid and peripheral blood samples have been taken. Sorting was performed on the basis of cell surface expression of membrane-bound HLA-G (Figure 6.2.8.1A). Suppression assays were performed as described in materials and methods. Around 800 to 6000 $CD4^+$ HLA-G^{pos} T_{reg} could be isolated from the CSF. Vigorous proliferation of blood-derived HLA-G^{neg} T cells in response to polyclonal stimulation by anti-CD3/28 was observed (Figure 6.2.8.1B, left panel, and 6.2.8.1C) and could be inhibited by both blood- (Figure 6.2.8.1B, middle) and CSF-derived (Figure 6.2.8.1B, right panel, and 6.2.8.1C) HLA-G^{pos} T_{reg} from MS patients. In contrast, CSF-derived HLA-G^{pos} T_{reg} (similarly to the blood-derived HLA-G^{pos} T_{reg}) when cultured alone proliferated poorly after activation with monoclonal anti-CD3-CD28 antibodies (data not shown). Thus, HLA-G^{pos} T_{reg} are functional regulatory T cells. The demonstration of suppressive activity of CSF-derived HLA-G^{pos} T_{reg} from MS patients suggests their functional relevance in regulating CNS inflammatory responses.

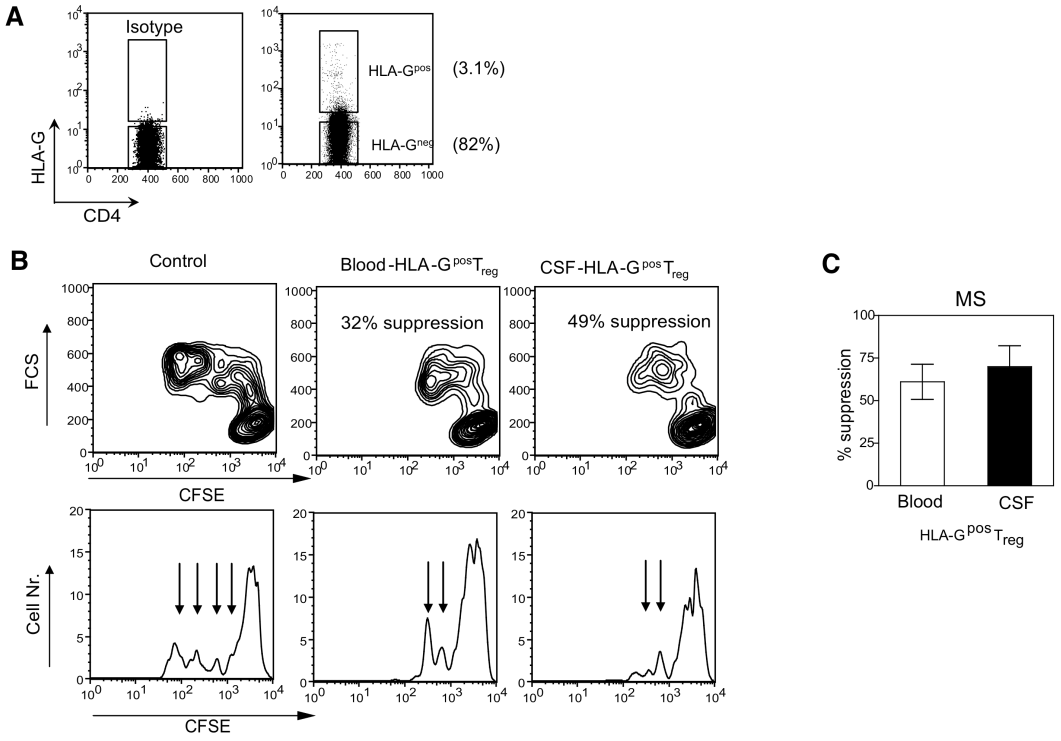

Figure 6.2.8.1 CSF-derived HLA-G^{pos} T_{reg} are functionally suppressive.

(A) Purified CD4^{+} T cells were stained with isotype control (left panel) or fluorophor-labelled HLA-G (right panel) to separate HLA-G^{pos} from HLA-G^{neg} T cells by FACS with a MoFlo sorter. (B) Suppression assays were performed using CFSE-labeled HLA-G^{neg} T cells grown either alone (left panel) or co-cultured with autologous blood- (middle panel) or CSF-derived (right panel) HLA-G^{pos} T_{reg} from the same MS patient. The co-cultures were set up at a ratio of 10 to 1 (HLA-G^{neg} T cells: HLA-G^{pos} T_{reg}) for 7 days *in vitro* under polyclonal (anti-CD3/CD28) stimulation. Four patients with MS were investigated (3 relapsing-remitting type, 1 secondary-progressive type; 1 patient had an acute relapse, others were stable). One representative MS patient out of four is shown. (C) A summary of percentages of suppression mediated by blood- or CSF-derived HLA-G^{pos} T_{reg} from MS patients is shown (n=4, mean± SEM). Percentage of suppression was calculated after normalization of the values to a maximum given by the proliferation of CFSE-labeled HLA-G^{neg} T alone.

7. Discussion

7.1 The mechanism of suppression mediated by naturally occurring HLA-G expressing regulatory CD4$^+$ T cells

Extensive efforts have been made to understand the cellular and molecular mechanisms underlying T cell-mediated immune regulation. Several molecular candidates and effector molecules including a variety of secreted soluble mediators have been proposed to be involved in or to directly execute the suppression cascade (Vignali, 2008; von Boehmer, 2005). In the first part of this study, we aimed at characterizing the mechanism of suppressive action triggered by HLA-G^{pos} T_{reg}.

We could provide a detailed cellular and molecular description of how HLA-G-expressing CD4$^+$T cells, a novel population of human regulatory T cells, modulate the function of autologous T cells (Huang et al., 2009a). HLA-G^{pos} T_{reg} are present in thymus and peripheral blood and display a characteristic regulatory phenotype suppressing the polyclonal activation of T cells under allogeneic (Feger et al., 2007b) and autologous (Figure 6.1.2.1) co-culture conditions. Suppression of T-cells is contact independent and can be fullfilled in the absence of APC. HLA-G-mediated suppression can also be facilitated by TCR stimulation and reversed upon Src-kinase inhibitor treatment. Although HLA-G^{pos} T_{reg} produce soluble HLA-G in the short-term culture (Feger et al., 2007b), and the soluble HLA-G-ILT2 pathway is not entirely responsible for suppressive ability of HLA-G^{pos} T_{reg} in the long-term culture. Importantly, an involvement of IL-10 in the mechanisms of HLA-G^{pos} T_{reg}-mediated suppression was noticeable: HLA-G^{pos} T_{reg} expressed higher amounts of IL-10 than HLA-G^{neg} T_{resp}; the presence of HLA-G^{pos} T_{reg} in a co-culture with responder cells was associated with a shift from IFN-γ to IL-10-producing cells, and a specific blocking antibody to the IL-10 receptor significantly reversed HLA-G^{pos} T_{reg} -mediated suppression.

Among human CD4$^+$ T cells, three most prevalently studied regulatory T cells are known: 1) naturally occurring CD4$^+$CD25$^+$ FoxP3-expressing T_{reg}, 2) inducible T_{reg} (Tr1) cells, 3) inducible Th3 (TGF-β) cells. Naturally occurring CD4$^+$CD25$^+$ FoxP3-expressing T_{reg} require physical cell contact and are believed to perform their suppressive function independently of cytokine production (Baecher-Allan et al., 2001; Baecher-Allan et al., 2002). In contrast, inducible Tr1 cells inhibit T-cell proliferation mainly via IL-10 and TGF-β secretion, whereas Th3 cells appear to reduce T-cell activity through TGF-β production, acting on responder T cells independently from cell-cell contacts (Weiner, 2001). Our previous data show that HLA-G^{pos} T_{reg} do not produce elevated mRNA levels of IL-10 and TGF-β and they are weak producers of IFN-γ mRNA (Feger et al., 2007b). Here, we demonstrate a higher IL-10

cytokine secretion by HLA-G^{pos} T_{reg} grown alone or in a co-culture with HLA-G^{neg} T_{resp} detected by both flow cytometry and ELISA. More, polyclonal TCR stimulation facilitated HLA-G^{pos} T_{reg}- driven shift of cytokine production from Th1 (high level of IFN- γ) to IL-10 enriched regulatory environment under co-culture conditions. Generally, optimal $CD4^+$ T cell activation requires two signals, TCR recognition of MHC class II complex and interaction with APC via co-stimulatory molecules (Varma, 2008). Using an APC-free system, we could demonstrate that HLA-G^{pos} T_{reg} -mediated suppression is independent from the co-stimulatory system on APC. Since the potential of HLA-G^{pos} T_{reg} to suppress the proliferation of responder cells was not affected by the absence of APC, we suggest a T-T cell direct inhibition mediated by other HLA-G^{pos} T_{reg} -secreted factors.

It has been assumed that suppressive action of T_{reg} depends on their activation stage. Such activated T_{reg} can act either directly or indirectly (via dendritic cells) and exert their suppressive effect on effector T cells. This, for example, might help to control immune activation at sites of parenchymal inflammation (Zozulya and Wiendl, 2008). Thus, a moderate level of TCR stimulation is required for exertion of suppressive effects by T_{reg} cells (Viglietta et al., 2004). Similar to the $CD4^+CD25^+$ FoxP3-expressing T_{reg} (Viglietta et al., 2004), HLA-G^{pos} T_{reg} showed a dependency of their ability to effectively suppress the responders on optimal TCR stimulation. Thus, enhancing T-cell signals via CD3 stimulation, we could show an improved suppression when compared to un-stimulated HLA-G^{pos} T_{reg} (Figure 6.1.3.1) In line with this, interference with TCR-signal transduction reduced suppressive capacity, which we found crucial for the normal suppressive activity of HLA-G^{pos} T_{reg} under polyclonal conditions. Collectively, our data imply that TCR engagement on HLA-G^{pos} T_{reg} react with faster kinetics and/or a higher suppressive capacity than freshly isolated HLA-G^{pos} T_{reg}. Although a low number of HLA-G^{pos} T_{reg} are found in the peripheral blood of healthy donors, their presence increased at sites of inflammation, such as in the central nervous system of patients with neuroinflammatory disorders and in muscle tissue in patients with idiopathic myositis (Feger et al., 2007b; Huang et al., 2009b). We also observed that the amplified TCR signal on HLA-G^{pos} T_{reg} may convey the activated- HLA-G^{pos} T_{reg} into close contact with the target cells by up-regulation of chemokine receptor(s) (e.g. CCR5) expression resulting in more potent suppressive capacity (Figure 7.2 Discussion, Figure 6.2.6.1A). These findings make a strong point towards the potential pathophysiological relevance of these cells: a facilitated HLA-G^{pos} T_{reg}- mediated suppression upon TCR stimulation would explain why these cells might be relevant in regulation of tissue inflammation at the target organ, suggesting the role in suppression of unwanted T cell proliferation to counterbalance the ongoing inflammatory responses (also reviewed in

(Zozulya and Wiendl, 2008)).

IL-10 has been recognized as a major mediator of immune regulation and regulatory T cell activity, specifically for Tr1 cells. Previous observations also suggest that the suppression effect of IL-10 secreting cells is not entirely due to IL-10 cytokine production (Sundstedt et al., 2003). Indeed, in some models, IL-10 administration can exacerbate the course of diseases such as graft-versus-host-disease (Blazar et al., 1995). Nevertheless, the role of IL-10 as an anti-inflammatory cytokine beneficially contributing to Tr1-mediated suppression has been described for different models of experimental diseases (Astier and Hafler, 2007; Astier et al., 2006; Kasama et al., 1995; Rosenbaum and Angell, 1995; Woiciechowsky et al., 1998). In our system, we found an elevated IL-10 secretion by HLA-G^{pos} T_{reg} in comparison to HLA-G^{neg} T_{resp} cells under TCR facilitated conditions, as well as maintained levels of IL-10 under co-culture conditions (Figure 6.1.6.2). An altered suppression mediated by HLA-G^{pos} T_{reg} upon IL-10R mAb treatment indicates the importance of IL-10 during HLA-G^{pos} T_{reg}-mediated suppression (Figure 6.1.6.3A and B).

The kinetics and mechanism of T_{reg} suppression include three categories of action: 1) establishment of cell–cell contacts, 2) local secretion of inhibitory cytokines, and 3) local competition for growth factors (Scheffold et al., 2007). For all three of them multiple examples of non-exclusive inhibitory pathways exist (Sojka et al., 2008). In our system, the stable HLA-G^{pos} T_{reg} interactions with effector T cells promote the condition under which the immune response depends both on soluble factors and cell-cell interaction. Therefore, in a direct co-culture setup blocking of IL-10 signaling significantly reversed HLA-G^{pos} T_{reg}-mediated suppression (Figure 6.1.6.3A), whereas blocking of IL-10 signaling on responder cells separated from the suppressors by the transpermeable membrane had a milder effect decreasing this reversibility (Figure 6.1.6.3B). Interestingly, once HLA-G^{pos} T_{reg} start proliferating, they lose their suppressive capacity, indirectly pointing to an involvement of IL-10 secretion in HLA-G^{pos} T_{reg}-mediated suppression. To summarize, we would like to emphasize that these observations indicate that IL-10 signalling has an effect on the suppressive capacity of HLA-G^{pos} T_{reg}, representing, an important factor for the suppressive function of HLA-G^{pos} T_{reg}.

Despite sharing the relevance of IL-10 secretion in exerting their suppressive function, HLA-G^{pos} T_{reg} and Tr1 regulatory subsets are clearly two distinct populations. Unlike Tr1, which acquire their suppressive function through the activation by immature dendritic cells (Wakkach et al., 2003), we would like to highlight that HLA-G^{pos} T_{reg} do not require the presence of other cell types to exert their regulatory function.

Taken together, the first part of presented data from this study provides detailed information

on the function of HLA-G^{pos} T_{reg}, especially the mechanisms how HLA-G^{pos} T_{reg}– mediated suppression of autologous T cells is exerted. This finding might help to understand the significance of this novel human regulatory T cell population under physiological and pathological conditions.

7.2 Specific CNS recruitment and suppressive function of HLA-G-expressing regulatory T cells in MS patients

In the second part of this study, we challenged the patho/physiological role of HLA-G^{pos} T_{reg} in MS patients. Using MS as a paradigmatic autoimmune disorder of the CNS, we tested the hypothesis that the natural HLA-G^{pos} T_{reg} population actively contributes to the balance of parenchymal inflammation, which is in line with the concept of beneficial inflammation in humans. The role of T_{reg} in controlling parenchymal autoimmune inflammation in humans is currently subject of intensive discussion. Conceptually, T_{reg} cells within the parenchyma could combat destructive inflammatory cytotoxic effector cell components, thereby providing anti-inflammatory or protective properties. Indeed, high numbers of nT_{reg} are found during the recovery phase of experimental autoimmune encephalomyelitis (EAE)(McGeachy et al., 2005), an established model for human CNS autoimmunity. Further, elevated frequencies of $CD4^+CD25^+$ FoxP3-expressing T_{reg} in the CSF of MS patients (Feger et al., 2007a) point to a targeted recruitment of n T_{reg} to the site of inflammation. These findings, however, are opposed by studies documenting dysfunctional states of $CD4^+CD25^+$ FoxP3-expressing T_{reg} and certain inducible T_{reg} in human MS (reviewed in (Zozulya and Wiendl, 2008)).

In accordance with our previous findings (Mitsdoerffer et al., 2005), our data indicate that HLA-G-expressing T cells can be detected in elevated frequencies at the sites of inflammation during acute neuroinflammation and also in MS lesions in brain biopsies. This suggests a selective or at least preferential attraction of HLA-G^{pos} T_{reg} to inflamed CNS tissue in an effort to reduce destructive inflammation. The phenotypical and functional characterization identified CCR5 as an important chemokine receptor, which was overexpressed on CSF-, but not on blood-derived HLA-G^{pos} T_{reg}. A close correlation between the increased frequency of CCR5-expressing HLA-G^{pos} T_{reg} derived from the CSF and the decreased number of blood-derived $CCR5^+$ HLA-G^{pos} T_{reg} was found in all individuals analyzed. However, this correlation between levels of $CCR5^+$ HLA-G^{pos} T_{reg} was only observed in MS but not in OND patients suggesting a unique property of $CCR5^+$HLA-G^{pos} T_{reg} to migrate between periphery and CSF resulting in an enrichment in the CSF during the course of autoimmune inflammation (Sorensen et al., 1999).

Most lymphocytes in CSF represent CD4 central memory T cells (Kivisakk et al., 2003) and

the expression of CD45RO, CD27, CCR7 on their cell surface characterize them as previously activated memory T cells, which are necessary for antigen engagement and cell trafficking between the secondary lymphoid organs and home tissue (Butcher and Picker, 1996). Similarly, we observed a clear predominant population of central memory HLA-G^{pos} T_{reg} found within the CSF compartment. These cells were also uniformly expressing CCR7, suggesting that central memory HLA-G^{pos} T_{reg} that might patrol the subarachnoid space. This could represent a mechanism of CNS immune surveillance. Moreover, to characterize the phenotype of the CSF-infiltrating regulatory versus effector cells, we have analyzed the expression of cell surface markers associated with activation status of T cells. Flow cytometry analysis showed that CD25, CD69 expressions did not differ between HLA-G^{pos} T_{reg} and HLA-G^{neg} T_{resp} cells. In contrast, another activation maker, ICOS expression was elevated on the HLA-G^{pos} T_{reg} subset. It may imply that ICOS up-regulated on these transmigrated HLA-G^{pos} T_{reg} after local activation (Herman et al., 2004; Hutloff et al., 1999). They thus may function directly in the target organ to mediate regulation.

Chemokines and chemokine receptors have been described to be directly involved in the pathogenesis of MS (reviewed in (Szczuciński and Losy, 2007)). The immune cell trafficking across the blood-brain barrier can be facilitated by the expression of distinct sets of adhesion molecules, chemokines and their receptors (Campbell et al., 1998). The elevated levels of chemokine receptors have been previously detected in actively demyelinating lesions and in the CSF of MS patients (Putheti et al., 2003). MIP-1α and RANTES have been described as a pair of chemokines important for T-cell entry into the CNS (Andjelkovic and Pachter, 2000; Glabinski et al., 2003). MIP-1α was detected at high levels in the CSF of patients with MS and other inflammatory disorders, but not in samples from healthy control subjects (Miyagishi et al., 1995). Stimulated by pro-inflammatory cytokines, brain endothelial cells as well as astrocytes and microglia have been shown to be the main producers of MIP-1α and RANTES chemokines, which both serve as ligands for CCR5 (Glabinski et al., 1997; Hvas et al., 1997; McManus et al., 1998; Miyagishi et al., 1997; Simpson et al., 1998). Moreover, a remarkably consistent relationship between the expression of chemokines in the brain and clinical disease activity has been suggested in MS (Sorensen et al., 1999). Using human brain microvascular endothelial cells (HBMEC) as an *in vitro* model of the human BBB, we have shown that HLA-G^{pos} T_{reg} predominantly migrated across HBMEC in response to MIP-1α and RANTES, but not to MIP-3β *in vitro*. CCR5 was strongly upregulated on HLA-G^{pos} T_{reg} upon transmigration across HBMEC in response to chemokine stimulus (Figure 6.2.5.2), further strengthening the relevance of CCR5 expression for T_{reg} trafficking.

Using an *in vitro* model of the blood brain barrier we showed that transmigration and chemo-attraction upregulated the suppressive capacity of HLA-G-expressing T_{reg}. This effect was not explained by the action of chemo-attraction factors alone and only observed when cells underwent transmigration (Figure 6.2.6.1). Albeit the true biological significance of this finding *in vivo* remains open, these data support the assumption that there is a correlation between the chemokine-derived transmigration and suppressive capacity of infiltrating cells. Further, there seems to be a correlation of a higher suppressive potency of transmigrated HLA-G^{pos} T_{reg} cells with a higher expression of CCR5 on these cells. It appears that in other models $CCR4^+$ T_{reg} were also accumulated in the inflamed skin following local antigen inoculation (Sather et al., 2007). Likewise, $CCR6^+$ T_{reg} were shown to be elevated in inflamed joints in an animal model of rheumatoid arthritis (Hirota et al., 2007). Our observations of CCR5 upregulation on transmigrated HLA-G^{pos} T_{reg} simply reflect that similar but not overlapping homing mechanisms exist. In addition, CCR5 may represent a marker dissecting naturally occurring HLA-G^{pos} T_{reg} into a $CCR5^-$ and $CCR5^+$ T_{reg} subpopulation. Based on the differential expression of homing receptors on $CD4^+CD25^+$ nT_{reg} (Haas et al., 2007; Huehn and Hamann, 2005), heterogeneic subpopulations have been described in these cells. Some of these have specific migratory properties (Iellem et al., 2001) which in our study would represent the CCR5-expressing and HLA-G-expressing $T_{reg.}$. It is tempting to speculate that presence and/or upregulation of CCR5 could be decisive in guiding these cells to specific anatomic sites depending on the specificity of the respective immune responses, which might be of functional significance for their suppressive capacity. Moreover, selective migration of HLA-G^{pos} T_{reg} through the expression of CCR5 may offer therapeutic strategies through activating their ability to effectively control T-cell activation and differentiation.

This even holds true for the highly attractive concept of "beneficial inflammation", where - up to now - no convincing functional data in humans have been provided (Hohlfeld et al., 2007). Our data clearly demonstrate, that HLA-G^{pos} T_{reg} are suppressive when directly isolated from the CSF. This compartment is considered – at least in large parts – to mirror the immune-pathological activity associated with inflammation in the CNS, and it is indeed the only CNS compartment that can be assessed in a larger number of patients. We therefore propose that our results can be regarded as a first proof-of-concept supporting the relevance of T_{reg} in human CNS immune-regulation.

Nonetheless, a number of questions remain to be answered, such as the exact source and the antigen specificity of CNS-derived HLA-G^{pos} T_{reg}. Our data do not provide information on the origin of HLA-G^{pos} T_{reg} in the CSF and CNS. The initial description suggested a thymic origin of HLA-G^{pos} T_{reg} (Feger et al., 2007a), which would classify them as "natural" T_{reg} and thus a

constitutive part of the immune system. Like the natural $CD4^+CD25^+$ T_{reg}, these cells would be autoreactive and have self-renewing capability, which allows the long-term regulation of autoreactivity (Feger et al., 2007b; Shevach, 2002). However, it cannot be excluded that HLA-G-expressing T_{reg} might also be induced at sites of inflammation (Carosella et al., 2008a; Carosella et al., 2008b). Albeit our data are mainly derived from MS patients, we do not want to make the point that this regulatory mechanism of HLA-G T_{reg} is specifically linked to MS. We rather assume that this may be shared by other CNS inflammatory conditions (Feger et al., 2007b; Huang et al., 2009b).

In the second part of this study we emphasized the ability of HLA-G^{pos} T_{reg} to actively migrate from the peripheral to the CSF and CNS compartments during autoimmune CNS inflammation. Thus supports their putative beneficial role for the outcome of ongoing disease, and is believed to provide a first example of beneficial T-cell inflammation in human CNS autoimmunity.

7.3 Final assessment

The immune system has to distinguish self from non-self structures, but also between harmful and innocuous foreign antigens to prevent non-essential and self-destructive immune responses. Active regulation of self-reactivity by regulatory T cells (T_{reg}) is assumed to play a key role in central nervous system (CNS) autoimmunity. Specifically, the relevance of T_{reg} in controlling parenchymal inflammation and immune homeostasis has been postulated. Knowledge of the role and features of T_{reg} is interesting both from an immuno-pathogenic as well as a therapeutic view. A novel population of natural human T_{reg} characterized by the constitutive expression of the immuno-tolerogenic human HLA-G molecule has been recently identified by us. The cellular and molecular identification of T_{reg} has greatly stimulated many concepts of adaptive immunity, including mechanisms controlling autoimmunity. In the first part of this study, an in-depth characterization of the mechanism of how HLA-G^{pos} T_{reg} suppress T responder cells in direct T-T interactions has been provided. Understanding the suppressive tools used by HLA-G^{pos} T_{reg} may help to develop therapeutic strategies to modulate regulatory arms of T cell suppression. Therefore, this finding is relevant for both basic research as well as clinic applications. In the second part of this study, the potential role of HLA-G^{pos} T_{reg} in Multiple Sclerosis (MS), a prototypic autoimmune inflammatory CNS disorder, has been characterized. The most striking and new data are certainly the migratory capacity of these cells into the CNS and their functional significance in MS patients, including the suppressive properties of CNS-derived HLA-G^{pos} T_{reg}. As a concept, HLA-G^{pos} T_{reg} may be selectively attracted to

migrate to the sites of CNS inflammation in an effort to combat destructive inflammation during MS. This contributes to the understanding of HLA-G^{pos} T_{reg} and may provide an important pathophysiological example of “beneficial” T cell inflammation in CNS autoimmunity, interesting both from a pathophysiological and a therapeutical point of view.

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9. Appendix

9.1 Abbreviations

Antigen-presenting cells	APC
Antibody	Ab
Blood- brain- barrier	BBB
Central nervous system	CNS
Cerebrospinal fluid	CSF
CC chemokine ligand	CCL
CC chemokine receptor	CCR
Cluster of differentiation	CD
Dimethylsulfoxide	DMSO
Experimental autoimmune encephalomyelitis	EAE
Enzyme-linked immunosorbent assay	ELISA
Factor forkhead box P3	FOXP3
Fluorescence- activated cell sorting	FACS
Fetal calf serum	FCS
Fluorescence isothiocyanate	FITC
Forward scatter in FACS, indicates particle size	FSC
Human histocompatibility leukocyte antigens G	HLA-G
Immunoglobulin	Ig
Interleukin	IL
IL-10 receptor	IL-10R
Macrophage inflammatory protein-1α	MIP1α
Major histocompatibility complex	MHC
Magnetic adsorption cell sorting	MACS
Regulated upon activation, normal T cell expressed and secreted	RANTES
Rheumatoid arthritis	RA

Side scatter in FACS, indicates particle granularity	SSC
T cell receptor	TCR

9.2 Publication List

The role of leukemia-derived B7-H1 (PD-L1) in tumor-T-cell interactions in humans
Salih HR, Wintterle S, Krusch M, Kroner A, **Huang YH**, Chen L, Wiendl H.
Exp Hematol. 2006 Jul; 34 (7): 888-94.

Postpartum-activation of multiple sclerosis is associated with down-regulation of tolerogenic HLA-G
Airas L, Nikula T, **Huang YH**, Lahesmaa R, Wiendl H.
J Neuroimmunol. 2007 Jul; 187 (1-2): 205-11.

HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation
Feger U*, Tolosa E*, **Huang YH*,** Waschbisch A, Biedermann T, Melms A, Wiendl H.
*These authors contributed equally to this work.
Blood. 2007 Jul 15; 110 (2): 568-77.

Immunoregulatory factors in multiple sclerosis patients during and after pregnancy: relevance of natural killer cells
Airas L, Saraste M, Rinta S, Elovaara I, **Huang YH**, Wiendl H; Finnish Multiple Sclerosis and Pregnancy Study Group.
Clin Exp Immunol. 2008 Feb; 151 (2): 235-43.

Mechanisms of autologous T cell suppression by natural HLA-G-expressing T regulatory cells: relevance of Interleukin-10 and Immunoglobulin like transcript-2
Huang YH, Zozulya A.L, Weidenfeller C, Schwab N, Wiendl H.
J Leukoc Biol. 2009 (86)

Specific CNS recruitment and suppressive function of HLA-G expressing regulatory T cells in the target organ of patients with multiple sclerosis
Huang YH, Weidenfeller C, Zozulya A.L, Metz I, Buck D, Toyka K.V, Brück W, Wiendl H.
Ann Neurol 2009

9.3 Performances related to this study

Scholarship:

03/2005-04/2009 University of Würzburg

Awards:

Young Investigator for the World Congress on Treatment and Research in Multiple Sclerosis, the first joint meeting of ACTRIMS, ECTRIMS and LACTRIMS.

Conference attendance related to this work

2006: 4th International conference on HLA-G. Paris. France (*Oral presentation)
Feger U*, Tolosa E, **Huang YH,** Waschbisch A, Biedermann T, Melms A, Wiendl H.
HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation.

2007: 37th Annual Meeting of the German Society for Immunology. Heidelberg. Germany (Poster)
Huang YH, Tolosa E, Zozulya A, Wiendl H.
Suppressive action of HLA-G expressing CD4 regulatory T cells

2008/09: *World Congress on Treatment and Research in Multiple Sclerosis. ACTRIMS-ECTRIMS-LACTRIMS. Palais des congrès de Montréal. (Platform presentation selected)
*This attendance will be supported by Young Investigator award.
Huang YH, Weidenfeller C, Zozulya A.L, Metz I, Buck D, Toyka K.V, Brück W, Wiendl H.
Specific CNS recruitment and suppressive function of HLA-G expressing regulatory T cells in the target organ of patients with multiple sclerosis

2008/10: 9th International congress of neuroimmunology, Texas (Platform presentation selected).
Huang YH, Weidenfeller C, Zozulya A.L, Metz I, Buck D, Toyka K.V, Brück W, Wiendl H.
Specific CNS recruitment and suppressive function of HLA-G expressing regulatory T cells in the target organ of patients with multiple sclerosis

2009: ARSEP-Frence-Germany meeting on Multiple Sclerosis, Paris, France (Poster)

Huang YH, Weidenfeller C, Zozulya A.L, Metz I, Buck D, Toyka K.V, Brück W, Wiendl H.

Specific CNS recruitment and suppressive function of HLA-G expressing regulatory T cells in the target organ of patients with multiple sclerosis

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給我最摯愛的爸爸媽媽以及姊姊-

This dissertation is dedicated to my family

9.5 Curriculum Vitae

Personal data

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09/1997 – 07/2001	Bachelor of Biology, National Taiwan University, College of life science, Department of Biochemical Science and Technology
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03/2005-present	Department of Neurology, Bayerische Julius-Maximilians-Universität Würzburg, Germany Topic: CNS inflammation and regulatory mechanism Supervisor: Prof. Dr. H. Wiendl

Hobby:

Photography. Writing. Reading. Movies. Music. Traveling. Swimming.

Würzburg, den ……. ……..

Yu-Hwa, Huang