CLINICAL IMMUNOLOGICAL AND IMMUNOGENETICAL ASPECTS OF NEUROLOGICAL DISEASES

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PhD Theses

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ABBREVIATIONS

αGalCer – α-galactosylceramide

AIS – acute ischemic stroke

APC – antigen presenting cell

CD – cluster of differentiation

CDR – complementarity-determining region

CI – confidence interval

CIDP – chronic inflammatory demyelinating polyneuropathy

CNS – central nervous system

CTLA-4 – cytotoxic T lymphocyte-associated antigen-4

DEG – differentially expressed genes

DN – double negative ($CD4^-CD8^-$)

EAE – experimental autoimmune encephalomyelitis

FasL – Fas ligand

GOS – Glasgow Outcome Scale

HLA – human leukocyte antigen

hsCRP – high-sensitivity C-reactive protein

IBD – inflammatory bowel disease

ICOS – inducible co-stimulatory molecule

IFN-γ – interferon-γ

Ig – immunoglobulin

IL – interleukin

iNKT cells – invariant natural killer T cells

LAR – leukocyte antisedimentation rate

MAIT – mucosal-associated invariant T cells

MHC – major histocompatibility complex

MS – multiple sclerosis

NK cells – natural killer cells

NKT-like cells – natural killer T-like cells

OR – odds ratio

PCT – procalcitonin

RRMS – relapsing-remitting multiple sclerosis

SNP – single nucleotide polymorphism

SSCP – single-strand conformation polymorphism

TCR – T cell receptor

Th – T helper

TIA – transient ischemic attack

TNF-α – tumor necrosis factor-α

UTR – untranslated region

WHO – World Health Organization

I. INTRODUCTION:

Basis of experimental work and theses

Immunity

The term *immunity* is derived from the Latin word *immunitas*, which referred to the protection from legal prosecution offered to Roman senators during their tenures in office. Historically, immunity meant protection from disease and, more specifically, infectious disease. The major function of the immune system is to recognize invading microbes, infected cells and tumors, and efficiently launch attacks that destroy them while ignoring healthy tissues – basically to preserve and maintain the body's integrity. The immune system, however, provides defense not only against bacteria, parasites, fungi and viruses that can cause infections, but against noninfectious foreign substances, macromolecules, such as proteins and polysaccharides, and small chemicals that are recognized as foreign, and also against tumor cells. The immune system consists of special organs, billions of cells, sets and subsets, molecules responsible for immunity – all organized into an elaborate and dynamic communications network. The immune system is amazingly complex – and so is the nervous system [1,2].

Defense against foreign cells and substances is mediated by the early reactions of innate immunity and the later responses of adaptive immunity, the two functional arms of the immune system. Innate immunity (also called natural or native immunity) provides the early, rapid, non-specific, first line of defense against microbes and tumor cells and activates the adaptive immunity, which provides specific immune responses against pathogens, and bears immune memory: the adaptive response that takes days to develop on first encounter with a microorganism is effective almost immediately on subsequent infection with the same organism. The principal components of innate immunity are physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces, neutrophils, macrophages, dendritic cells and natural killer (NK) cells, blood proteins, including members of the complement system and other mediators of inflammation, acute phase proteins, and cytokines that regulate and coordinate many of the activities of the cells of innate immunity [3,4].

Natural killer (NK) cells and cytotoxicity

NK cells are crucial components of the innate immune system, which are able to rapidly produce abundant cytokines, mainly interferon-gamma (IFN-γ), and lyse target cells without prior sensitization through cytotoxicity. NK cells play a key role in resistance to infections and destruction of tumor cells. They express CD56 on their surface, which is widely used as an NK cell marker to identify them, and lack the expression of antigen-specific T cell receptor complex, CD3 [5,6].

Perforin and Fas/FasL pathways are the two major mechanisms of cellular cytotoxicity [7,8]. Exocytosis of cytotoxic granules containing pre-formed perforin and serine esterase molecules leads to cell lysis by forming pores in target cell membranes. Perforin is a marker of cytotoxic cells [9]. FasL produced by effector cells induces apoptosis through Fas, its receptor on target cell surfaces. The Fas/FasL-mediated apoptotic pathway thus depends on cell-surface ligand-receptor interaction [10].

T cell receptor (TCR)

T lymphocytes are one of the major cellular components of the adaptive immune system. Lymphocytes are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants and are responsible for the two defining characteristics of the adaptive immune response, specificity and memory mediated by antigen-specific receptors.

T cell receptors comprise an essentially unlimited repertoire of variants, each variant expressed on a different cell, that can altogether recognize virtually any molecule. Versatility of T cell receptors and thus of antigen recognition is primarily a result of the rearrangement of antigen receptor coding genes. The diversity of the lymphocyte repertoire is further enhanced by randomly adding and removing nucleotides during the joining of different gene segments and spontaneous mutations also occur. The TCR is a heterodimer consisting of two transmembrane polypeptide chains, designated α and β, covalently linked to each other by a disulfide bridge. Each α-chain and β-chain consists of one immunoglobulin (Ig)-like N-terminal variable (V) domain, one Ig-like constant (C) domain, a hydrophobic transmembrane region, and a short cytoplasmic region. The V regions of the TCR α and β chains contain short stretches of amino acids where the variability between different TCRs is concentrated, and these form the hypervariable or complementarity-determining regions (CDRs). Three CDRs in the α chain are juxtaposed to three similar regions in the β chain to form the part of the TCR that specifically recognizes peptide-MHC complexes. In the α and β chains of the TCR,

the third hypervariable regions are composed of sequences encoded by V and J (joining) gene segments (in the α chain) or V, D (diversity), and J segments (in the β chain) [1-4] (***Figure 1***).

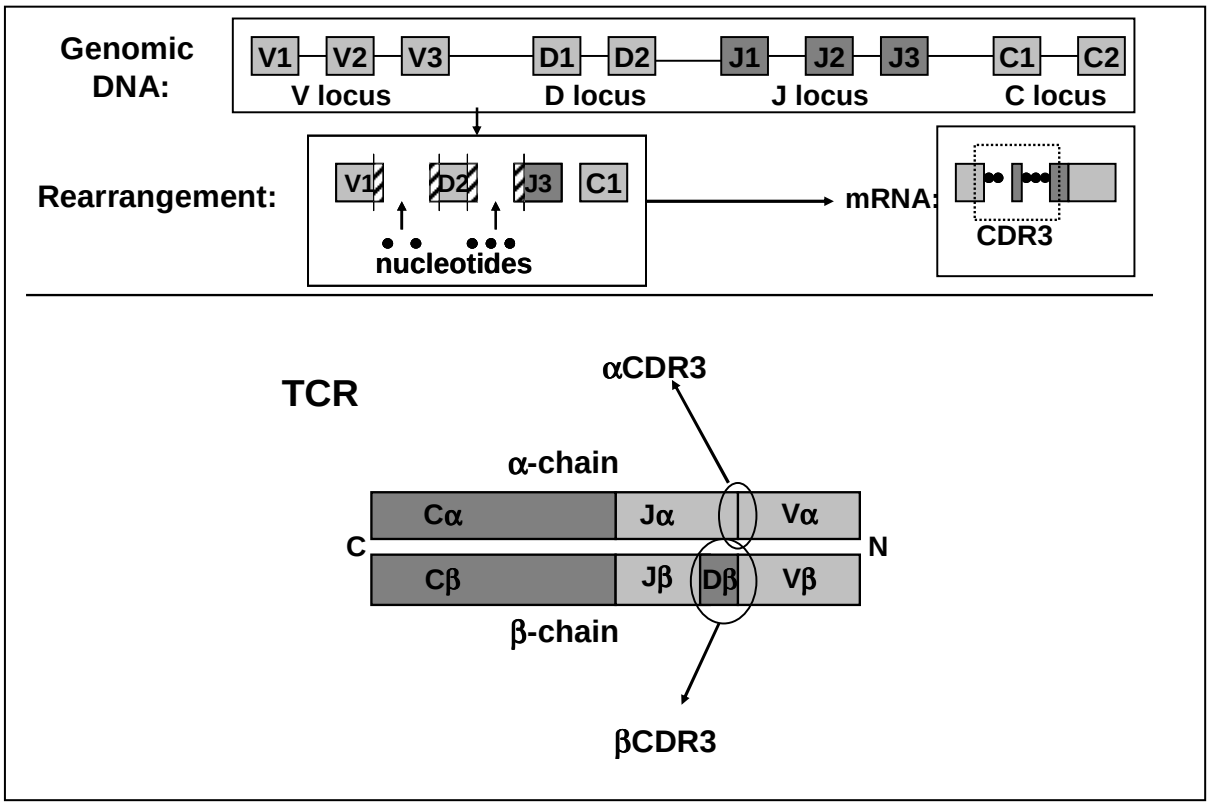

Figure 1. Rearrangement and structure of the T cell receptor

Cytotoxic CD8$^+$, Thelper CD4$^+$ cells, and co-stimulation (CTLA-4)

T cells with their TCRs recognize MHC (major histocompatibility complex) molecules complexed with different (fragments of) antigens. CD8$^+$ cytotoxic T cells recognize peptide antigens generated in the cytosol from viruses, or cytosolic bacteria, and presented by MHC class I molecules on the surface of infected cells: this activates the cytotoxic T cell to kill the infected cell, limiting the replication of the infecting agent. MHC class I molecules are expressed on almost all cells. CD4$^+$ T helper (Th) cells, which activate other cells of the immune system, recognize peptide fragments presented by MHC class II molecules, and are thereby activated to secrete cytokines and activate other immune cells, which kill the microorganisms or secrete antibodies [1,2].

The antigen-specific stimulation through the TCR is not enough for the full activation of T cells, a second signal called co-stimulation is necessary. Both antigen presenting cells (APC) and T cells will be activated during co-stimulation, a sequence of ligand-receptor interactions on the surface of both cells. Such interactions are required also for the termination

of activation, a mechanism important in autoimmune tolerance. This termination is mainly mediated by CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) expressed on the surface of T cells upon activation [3]. Another molecule, called inducible co-stimulatory molecule (ICOS) may also be important in autoimmune tolerance due to its role in Th2-mediated anti-inflammatory responses [11] (***Figure 2***).

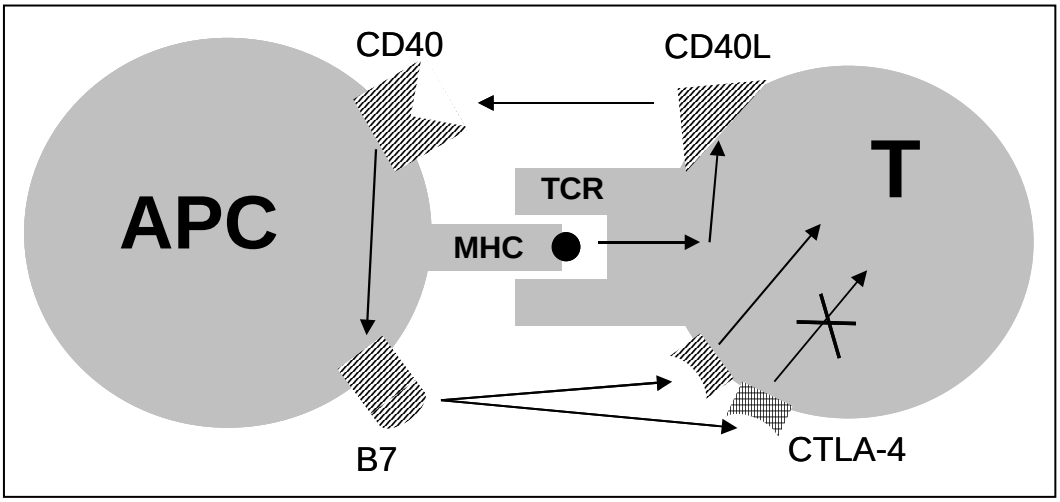

Figure 2. Antigen-specific and co-stimulatory signal, CTLA-4

Cytokines and Thelper subsets

Cytokines are proteins secreted by the cells of innate and adaptive immunity that mediate many functions of these cells (***Figure 3***). The activation of B cells and phagocytes by $CD4^+$ T helper cells depends on secretion of cytokines by the T cells that not only activate the antigen presenting cell but can bias its response. T cells can direct the type of antibody secreted in the case of a B cell, and the other immune cells recruited in the case of a macrophage, depending upon the particular cytokines produced and thus shape the nature of the inflammatory response. Pro-inflammatory (Th1) cytokines – IFN-γ, interleukin (IL)-12 – produced by Th1 cells enhance cellular immune responses, cell-mediated cytotoxicity, and activate macrophages. Th2 cytokines called anti-inflammatory cytokines – IL-4, IL-5, IL-6, IL-10, IL-13 – produced by Th2 cells generate humoral immune response, and antibody production. Previous data emphasized the importance of IL-12 driving differentiation of naïve T cells into IFN-γ producing Th1 cells essential for cell-mediated immunity [12]. However, mice deficient in components of the IFN-γ pathway remain susceptible or develop more severe autoimmune diseases, suggesting that these factors are not always necessary in the

development or can even regulate such disorders. Recently, other pro-inflammatory cytokines, IL-23 and IL-17 have been indicated to play an important role in the establishment of autoimmune diseases and T cells producing IL-17 have been dubbed Th17 cells. One of the Th17 differentiation pathways is IL-23-dependent and IL-23 is necessary for the survival and expansion of Th17 cells; this effect is mediated through the IL-23 receptor (IL-23R) signaling pathway [13-15].

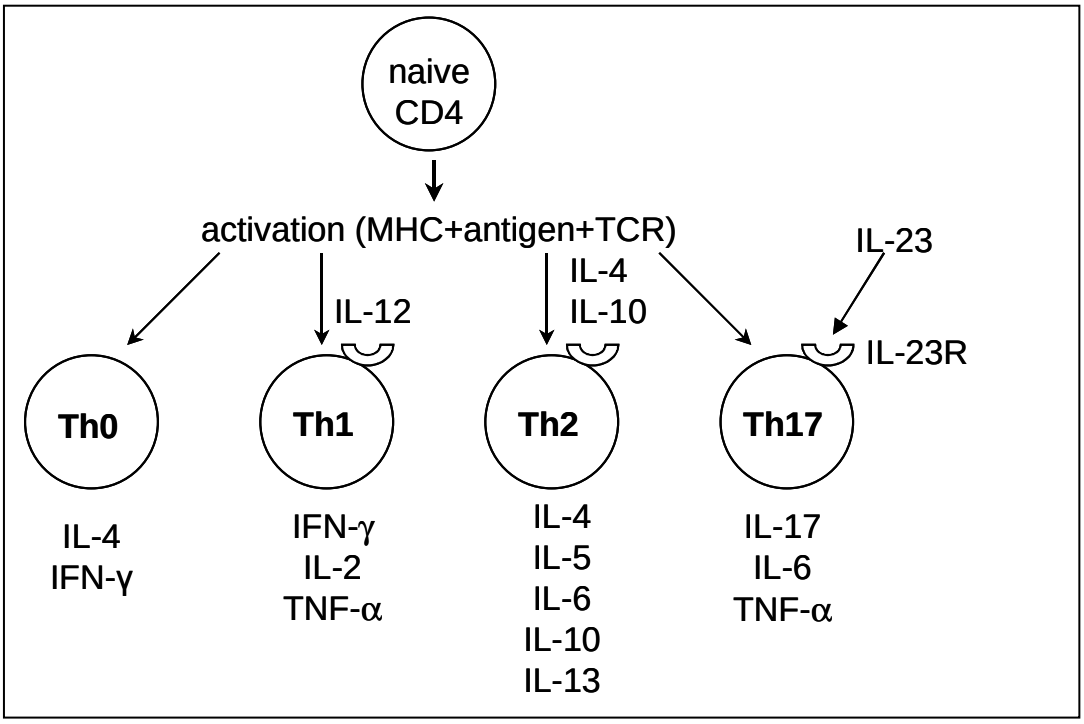

Figure 3. Thelper subsets

γδT cells

The vast majority of T cells have an αβTCR and thereby called αβT cells. A small subset of T cells called γδT cells have γδTCRs and are αβ-negative. γδT cells express a TCR consisting of a γ-chain combined with a δ-chain, composing a distinct T cell population from αβT cells. γδT cells are proposed to bridge the innate and adaptive immune responses. They are able to produce great amounts of IFN-γ in a short period of time, which is characteristic to cells of the innate immune system [16,17]. γδT cells are important regulators of immune responses, play a necessary role in protective immune responses against certain pathogens and in tumor immunity through provision of an early source of IFN-γ [18]. The Vδ2 subset,

expressing a δ2TCR variable chain, represents the majority of adult γδT cells mainly with a cytotoxic property [17,19].

iNKT, $CD3^+CD56^+$ NKT-like T and MAIT cells

In the last decades, novel T cell populations have been described, which may play an important role in protection against autoimmunity (***Table 1***).

Table 1. T cell subsets expressing an invariant αCDR3

	Vβ	Vα	Jβ	CD4	CD8	DN[a]	Selecting molecule	Ligand	Selection	Th
NKT cell	Vβ11	Vα24	JαQ	+	+	++	CD1d	αGalCer[b]	Thymus	Th1/Th2
MAIT cell	Vβ13 Vβ2	Vα7.2	Jα33	-/+	+	++	MR1	?	Gut?	Th2

[a]double negative ($CD4^-CD8^-$), [b]non-natural ligand

A small percentage of αβTcells express molecules specific to NK cells, such as CD56 in addition to the TCR. Therefore these T cells have been historically termed natural killer T, *ie.* NKT cells. This population bridges the innate and the adaptive immune responses. NKT-like cells represent a small lymphocyte subpopulation that has important immunoregulatory functions [20,21].

Kronenberg and Gapin [20] categorized murine NKT cells into several distinct subsets based on their TCR repertoire, expression of antigen-presenting co-receptor molecules, and their anatomical compartmentalization in the host. Type I NKT cells exhibit a rearrangement of the Vα14-Jα18 variable region of the TCR and are either $CD4^+$ or $CD4^-$, $CD8^-$ (double negative, DN). The responsiveness of these NKT cells is CD1d, a non-conventional MHC class I molecule-restricted, and these cells are primarily located in the thymus, liver, spleen, and bone marrow. The reactivity of this subset appears restricted to α-galactosylceramide (αGalCer) and represents a major component of the overall NKT cell population. This NKT subset coexpresses Ly49 receptors that are also found on NK cells.

The human analogue of the murine Vα14-Jα18 type I NKT cells expresses an invariant Vα24-JαQ TCR α-chain paired with TCR β-chain using a biased V gene segment (Vβ11). These cells are referred to as invariant NKT (iNKT) or CD1d-restricted NKT cells, and reside mostly in the $CD4^-CD8^-$ (DN) and $CD4^+$ cell subsets [22-24]. Emerging data

indicate the functional diversity of human iNKT cells and their involvement in tumor immunity and autoimmunity [25-29].

Besides iNKT cells, the $CD3^+CD56^+$ T cell subset contains large granular lymphocytes with conventional, diverse αβTCR. Recently, these cells have been termed NKT-like cells. The role of NKT-like $CD3^+CD56^+$ cells has been suggested and intensively investigated in various immune responses, including response to infectious agents, tumor rejection and autoimmunity [30-32].

Along with the identification of Vα24 iNKT cells, another DN T cell population expressing an invariant Vα7.2-Jα33 TCR has been described, and named mucosal-associated invariant T (MAIT) cells relating to their preferential location, the gut lamina propria [22,33]. Similarly to CD1d, a non-classical MHC class Ib molecule, MR1 presents ligands to MAIT cells, possibly glycolipids similarly to iNKT cells [34]. In addition to the conserved CDR3α, MAIT cells express a restricted Vβ2 and Vβ13 driven by the selecting antigen [35]. So far, data about function of human MAIT cells are very limited, partly due to the absence of clonotypic antibodies. Their anti-inflammatory role has been proposed for the invariant Vα7.2-Jα33 TCR of MAIT cells was found to be present in autoimmune lesions of both the central and peripheral nervous system, which correlated with the expression of IL-4 [28,36].

In addition to iNKT and MAIT cells, two other T cell subsets have been suggested expressing an invariant α-chain: Vα4-Jα29 and Vα19-Jα48 TCR, respectively [37].

Deficiency of human iNKT cells has been described in MS, indicated by a decreased frequency in the peripheral blood and absence of the invariant Vα24-JαQ TCR in MS plaques despite the presence of Vα24 TCR. Such deficiencies are not characteristic to the chronic autoimmun demyelinating disease of the peripheral nervous system, CIDP. Other T cell subsets with invariant TCR, *ie.* MAIT, Vα4-Jα29 and Vα19-Jα48 T cells have not been affected either in MS [26,29,36].

Multiple sclerosis

Multiple sclerosis (MS) is a chronic, progressive disease of the central nervous system, which predominantly affects young adults – most people experience their first symptoms of MS between the ages of 20 and 40. Pathologically, MS is characterized by the presence of areas of demyelination and T cell predominant perivascular inflammation resulting in varying neurological symptoms that can range from relatively benign to somewhat disabling to

devastating. There are about 2-3 million people suffering from MS in the world, in Hungary the number of MS patients is approximately 6,000-10,000 [38].

Despite a large body of research, the exact etiology of MS remains unclear. The current hypothesis for the autoimmune mechanism of MS is that the demyelination of plaques is mediated by mainly autoimmune $CD4^+$ Th1 cells recognizing central nervous system (CNS) autoantigens due to delayed-type or type IV hypersensitivity. The primary autoimmune attack and demyelination result in secondary axonal degeneration responsible for progressive disability [39]. In contrast to this theory, another unifying concept has implied that oligodendrocyte apoptosis is the very first pathological event in all CNS plaques [40]. In this case, autoimmunity may be a secondary event triggered by antigens of dying oligodendrocytes and disrupted myelin sheath. The heterogeneous nature of MS pathogenesis is reflected by pathological studies suggesting that four different pathological subtypes of MS exist depending on the presence of cellular or humoral factors mediating immune responses and depletion of oligodendrocytes [41].

MS is a complex genetic disease mediated by interaction of several genes and environmental factors. Family studies revealed that family members of MS patients were at a significantly higher risk of developing the disease. In identical twins this risk is 250-fold, in siblings 30-fold and in half-brothers and half-sisters 10 times higher than that of the average population. Based on different genomic coupler analyses and association studies, it is assumed today that a tremendous number of different genes play a role in the risk of catching the disease (polygenetic inheritance). High-risk genes could not only be responsible for the regulation of the immune response but also, for example, for regulation of composition and stability of myelin [42].

Stroke

The World Health Organization's (WHO) standard definition of stroke is „a focal (or at times global) neurological impairment of sudden onset, and lasting more than 24 hours (or leading to death) and of presumed vascular origin".

Approximately 80% of cases are ischemic, 20% are hemorrhagic. In addition to stroke being the third leading cause of death, many survivors of stroke have to adjust to a life with varying degrees of disability [43]. Stroke has a high incidence worldwide – in the United States 700,000 people are affected each year leading to over 150,000 deaths [44]. In Hungary approximately 40,000 patients are admitted to hospital with the diagnosis of stroke and stroke mortality is extremely high in Central-Eastern European countries [45].

While direct neurological deficits cause early deaths, infectious complications prevail in the postacute phase of stroke contributing to the poor outcome. 18-28% of patients developed a poststroke infection in different studies, the incidence of pneumonia and urinary tract infections was the highest [46,47]. The increased susceptibility to infections after stroke may suggest early alteration of immune responses. Immunodepression induced by stroke has been proposed contributing to the increased susceptibility to infections and thus to the negative outcome [48-50]. Impaired T and NK cell responses, particularly a reduced IFN-γ production were described in a mouse model of focal cerebral ischemia [51]. Moreover stroke led to splenic atrophy characterized by a reduction in organ size and a drastic loss of splenocyte number in animal models [52]. Very few human immunological studies have been performed in stroke patients so far. Very recently, Vogelgesang et al. [53] revealed reduced lymphocyte counts and percentages after ischemic stroke, there was a dramatic loss of T cells in the peripheral blood of patients with acute ischemic stroke, within 12 hours from onset of stroke symptoms, which gradually normalized. No change was detected in IL-10 serum levels, while increased IL-6 levels were measured on admission that continued to rise in the following 14 days [53].

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II. AIMS OF THE STUDIES

Here, we investigated molecular bases of neurological diseases focusing mainly on molecules and mechanisms involved in regulating autoimmune tolerance *ie.* protecting against autoimmune diseases. We were particularly interested in the role of innate T cells in immune responses within the CNS and the neurogenetical background of CNS autoimmune diseases, particularly multiple sclerosis.

A. The role of innate T cells in diseases affecting the central nervous system

In the recent years, phenotypically and functionally similar iNKT and MAIT cells have been suggested to participate in immunoregulation of autoimmunity and immune surveillance of tumors.

We have recently found that Vα24-JαQ iNKT cells were absent in CNS plaques of MS, while conventional T cells expressing non-invariant Vα24$^+$ TCR were present. It was not clear whether this selective absence of iNKT cells was related to the disease or the special immunoregulation and antigens within the CNS. Therefore, we addressed:

1. Do iNKT cells infiltrate tumors within the CNS similarly to tumors outside the CNS?
2. Can we detect other invariant T cells in tumors with a special emphasis on MAIT cells, which may possess similar functions to iNKT cells?
3. If MAIT cells are present in tumors, what is the inflammatory environment? MAIT cells have been suggested to produce Th2 cytokines, but the similarity to iNKT cells and recent murine data suggested that the MAIT subset may be functionally heterogeneous as well.
4. What is the relation of MAIT and NKT-like cells in tumors? Do MAIT cells express CD56?
5. Do CD56$^+$ MAIT cells participate in anti-tumor immune responses?

Atherosclerosis and acute ischemic stroke are accompanied by immune responses. We may suspect that early changes in immune functions may be related to post-stroke infections resulting in poor outcome and high mortality of stroke. Therefore, we examined innate T cells and NKT-like cells in the early phase of acute ischemic stroke. We also explored activation of leukocytes and its relation to post-stroke infections:

6. Are innate T cells including $CD3^+CD56^+$ NKT-like cells affected by acute ischemic events?
7. What is the relation of leukocyte activation to post-stroke infections?
8. Does post-stroke leukocyte activation depend on the duration of ischemia and the extent of infarct?
9. Can we use simple bed-side tests to predict outcome and susceptibility for post-stroke infections?

B. Association of multiple sclerosis with polymorphisms of genes involved in shaping immune responses and regulating autoimmune tolerance

CTLA-4 is an important molecule to terminate immune responses and thus to prevent autoimmunity. Data about association of MS with *CTLA4* polymorphisms are conflicting. By using a large number of patients, here we examined:

10. Are polymorphisms of the *CTLA4* gene associated with multiple sclerosis?
11. Do polymorphisms of the *CTLA4* gene influence expression of co-stimulatory molecules important in terminating or shaping immune responses?

A novel functional Th cell subpopulation (Th17) has recently been described as the main autoimmune T cell subset. One of the Th17 differentiation pathways is IL-23-dependent. IL-23 is necessary for the survival and expansion of Th17 cells mediated via the IL-23 receptor (IL-23R) signaling pathway. Association of polymorphisms of *IL23R* gene has recently been suggested in autoimmune inflammatory bowel disease. Therefore, we examined:

12. Are polymorphisms of the *IL23R* gene associated with multiple sclerosis?
13. Is *IL23R* a shared autoimmunity gene?

MS is a complex genetic disease mediated by interaction of several genes and environmental factors. In addition, regulation of gene expression is controlled through the combinatorial action of multiple transcription factors. Therefore, we performed a gene network analysis to examine complex autoimmune processes underlying the pathogenesis of MS:

14. Are there gene expression networks dysregulated in multiple sclerosis?

III. SUMMARY OF PAPERS SUPPORTING THESES

A. THE ROLE OF INNATE T CELLS IN DISEASES AFFECTING THE CENTRAL NERVOUS SYSTEM

Paper 1

Invariant Vα7.2-Jα33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells

(Int Immunol, October 16, 2008, Epub ahead of print)

The anti-tumor response of human iNKT cells is well established. A novel T cell subset, mucosal-associated invariant T (MAIT) cells, possess similar regulatory properties to iNKT cells in autoimmune models and disease, but data about other functions of human MAIT cells are very limited, partly due to the absence of clonotypic antibodies. In addition, our previous data indicated the selective absence of iNKT cells in MS plaques in contrast to autoimmune inflammatory demyelinating lesions of the peripheral nervous system and MAIT cells. To address whether such deficiency of iNKT cells is related to the CNS environment or MS, we examined the presence of four known T cells with invariant αTCRs including that of iNKT cells in tumors within and outside of the CNS. The presence of sequences of Vα24-JαQ (iNKT), Vα7.2-Jα33 (MAIT), Vα4-Jα29 and Vα19-Jα48 TCRs was investigated in 19 biopsy samples of human kidney cancers and brain tumors by using RT-PCR SSCP clonality assay (***Figure 4***).

The MAIT clonotype was identified and co-expressed with iNKT clonotype in half of the tumors. In contrast, invariant Vα4 and Vα19 T cell clonotypes were not present in tumors. Vβ expression of Vα7.2-Jα33 MAIT cells with a restricted Vβ2 and Vβ13 TCR usage was further analyzed in tumor samples containing MAIT cells, as well as their antigen-presenting molecule, MR1 expression and pro- and anti-inflammatory cytokine environment. All kidney tumors with MAIT αTCR also expressed Vβ2 and Vβ13, in contrast to brain tumors, suggesting that MAIT cells in CNS tumors may express other Vβ chains as well. All tumors positive for MAIT invariant TCR express the antigen presenting molecule MR1, indicating that MAIT cells can be locally re-activated. Indeed, a high percentage of infiltrating T cells was $CD8^+$ and expressed HLA-DR suggesting activation. The clonal presence of MAIT cells in tumors correlated with the expression of pro-inflammatory cytokines but no IL-4, IL-5 and

IL-10, suggesting that a pro-inflammatory subset of human MAIT cells may exist. We also examined CD56 expression of MAIT cells both in tumor samples and in the peripheral blood of tumor patients. Although the MAIT αTCR was identified in both peripheral $CD56^+$ and $CD56^-$ subsets, tumor infiltrating lymphocytes were CD56 negative.

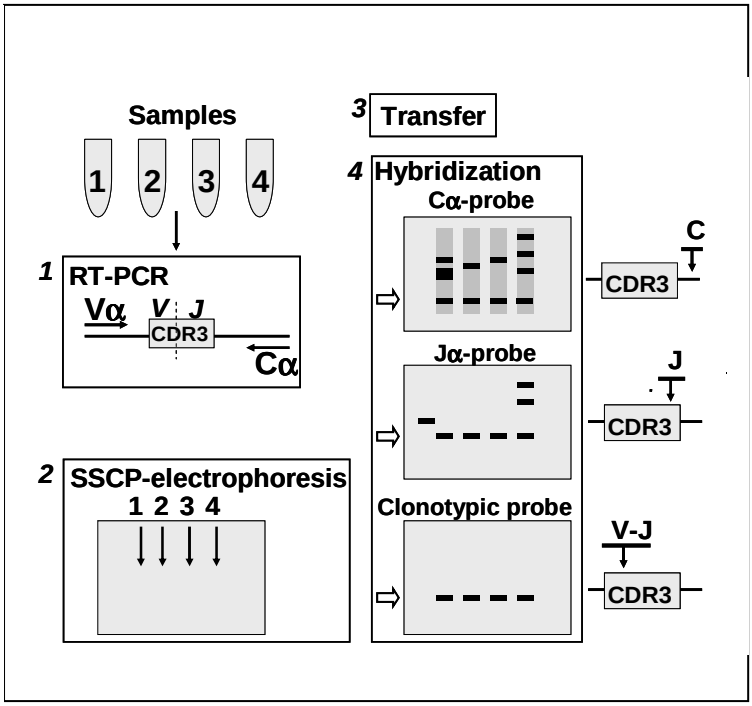

Figure 4.
Identification of invariant Vα4-Jα29, Vα7.2-Jα33, Vα19-Jα48 and Vα24-JαQ T cell receptors. *Following RT-PCR with Vα (4, 7.2, 19 or 24) and Cα primers (**1**), the amplified CDR3α regions were electrophoresed in non-denaturing SSCP gel (**2**). DNA was transferred to a nylon membrane (**3**) and hybridized with biotinylated internal Cα-, Jα-specific (Jα29, Jα33, Jα48, JαQ) or a junctional clonotypic probe complementary to the corresponding invariant CDR3α (**4**). An invariant CDR3α sequence can be identified even by hybridization with Cα or Jα probe by moving into the same position in different samples (**arrow**) among conventional TCR clonotypes.*

Our data imply that a $CD56^-$ subset of MAIT cells may participate in pro-inflammatory tumor immune responses similarly to iNKT cells. In addition, MAIT cells may have a pro-inflammatory T cell subset, similarly to human iNKT and murine MAIT cells. Our data also suggest that the selective absence of iNKT cells in CNS plaques of MS may be specific to the disease and not related to the CNS environment.

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Paper 2

Impaired function of innate T lymphocytes and NK cells in the acute phase of ischemic stroke

(revised version resubmitted to Stroke)

While direct neurological deficits cause early deaths, infectious complications prevail in the postacute phase of stroke contributing to the poor outcome. The increased susceptibility to infections after stroke may suggest early alteration of immune responses, thus immunodepression induced by stroke has been proposed. The few animal and human studies

all addressed the rapid changes in the adaptive arm of the immune system, mainly T cells. We analyzed rapid changes in immunological functions of cells of the innate immunity or lymphocytes bridging the innate and the adaptive arms of the immune system, all capable of shaping subsequent immune responses through rapid production of cytokines and/or cytotoxicity. The analyzed cell subsets were Vδ2 T cells, $CD3^+CD56^+$ natural killer T (NKT)-like cells and $CD3^-CD56^+$ NK cells. Their frequencies, cytokine production, intracellular perforin, surface Fas ligand (FasL) expression, and NK cytotoxicity were measured in 28 patients' peripheral blood obtained within 6 hours and also after 72 hours of ischemic stroke. The paired samples were compared both with each other and with 20 healthy controls.

Percentages of Vδ2, NKT-like and NK cells at 6 and 72 hours after stroke were constant and similar to percentages in healthy subjects. In contrast, pro-inflammatory intracellular IFN-γ expression by Vδ2 T cells, NKT-like cells and NK cells and IFN-γ production by isolated NK cells in culture were low at 6 hours and reached the level of healthy subjects' by 72 hours after stroke. Anti-inflammatory IL-4, IL-5 and IL-10 production of NKT-like and NK cells was not altered. Intracellular perforin expression by Vδ2 T cells, NKT-like cells and NK cells, and NK cytotoxicity were low at 6 hours and reached the level of healthy subjects by 72 hours.

According to our results, pro-inflammatory and cytotoxic but not anti-inflammatory responses of NK, NKT-like and Vδ2 T cells become acutely deficient in ischemic stroke, which may contribute to an increased susceptibility to infections.

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Paper 3

Deficient leukocyte antisedimentation is related to post-stroke infections and outcome

(J Clin Pathol, 2008, in press)

Patients with stroke are more susceptible to infections suggesting possible deficiencies of early immune responses, particularly of leukocytes. Here, we examined whether post-ischemic activation of leukocytes is related to duration of ischemia and extent of infarct. We also addressed, if disregulated leukocyte activation might be related to post-stroke infection and worsen outcome. We used leukocyte antisedimentation rate (LAR) to detect activation of leukocytes and correlated LAR with clinical and laboratory parameters. An additional aim was to test simple bed-side investigations in predicting outcome and susceptibility for post-stroke infections early.

LAR, a simple test to detect activation of leukocytes was serially examined and correlated with blood level of S100β related to extent of infarct, procalcitonin indicating infection and outcome in patients with acute ischemic events. Venous blood samples were taken from 61 healthy volunteers and 49 patients with acute ischemic events: 38 patients with acute ischemic stroke, AIS, and 11 patients with transient ischemic attack, TIA where symptoms disappear in 24 hours and cranial CT scan does not indicate infarct. Sampling was done within 6 hours, at 24 and 72 hours after onset of symptoms. LAR was significantly higher in acute ischemic events within 6 hours after onset of stroke regardless of post-stroke infections. In addition, elevation of LAR was delayed and attenuated in TIA in contrast to AIS and we also observed a positive correlation between LAR and S100β at 72 hours after the onset of ischemic stroke both indicating that the extent of tissue injury correlates with the magnitude of innate immune responses. Importantly, a deficiency in early elevation of LAR was associated with post-stroke infections and a poor outcome measured by Glasgow Outcome Scale in AIS.

We conclude that an early activation of leukocytes indicated by elevation of LAR is characteristic of acute ischemic cerebrovascular events. A delayed and ameliorated leukocyte activation represented by LAR is characteristic to TIA in contrast to definitive stroke. Our data suggest that acute activation of leukocytes, which has been regarded detrimental so far, serves also to prevent post-stroke infections. Our data impy that concept about the post-ischemic role of leukocytes should be changed and dissected: recruitment of leukocytes in the CNS may be damaging but should be separated from the systemic activation, which may prevent post-stroke infections. A disregulated early immune response or deficient leukocyte activation may result in an increased susceptibilty to infections in some patients with stroke.

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B. ASSOCIATION OF MULTIPLE SCLEROSIS WITH POLYMORPHISMS OF GENES INVOLVED IN SHAPING IMMUNE RESPONSES AND REGULATING AUTOIMMUNE TOLERANCE

Paper 4

Multiple sclerosis and the *CTLA4* autoimmunity polymorphism CT60: no association in patients from Germany, Hungary and Poland

(Mult Scler, 2008, 14:153-158)

For the full activation of T cells, besides the antigen-specific stimulation through TCR, a second signal called co-stimulation is necessary. Both APC and T cells will be activated during co-stimulation, a sequence of ligand-receptor interactions on the surface of both cells. Such interactions are required also for the termination of activation, a mechanism important in autoimmune tolerance. This termination is mainly mediated by CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) expressed on the surface of T cells upon activation.

Polymorphisms in the *CTLA4* gene region have been associated with susceptibility to autoimmune diseases. The recently described single nucleotide polymorphism *CT60*, located in the 3' untranslated region (3'UTR) of *CTLA4* is associated with Graves' disease, thyroiditis, autoimmune diabetes and other autoimmune diseases, however, its role in multiple sclerosis (MS) susceptibility has been controversial. Therefore, we conducted a case-control association study in a large number of German, Hungarian and Polish MS patients and regional control individuals for the *CTLA4 CT60* and *+49A/G* polymorphisms. We also performed haplotype analysis. Besides, we examined functional consequences *ie.* genotype differences in the expression of CTLA-4 and ICOS. ICOS is also important in autoimmune tolerance due to its role in Th2-mediated anti-inflammatory responses and susceptibility to experimental autoimmune encephalomyelitis (EAE), the animal model of MS is related to locus containing genes for both CTLA-4 and ICOS. Splice variants of CTLA-4 modify expression of ICOS in such models.

However, we found no significant association of these polymorphisms or respective haplotypes with MS, even when our data were extended with previously published results in a metaanalysis thus evaluating 1228 MS and 1440 controls. No association of *CT60* genotypes with T cell expression of ICOS and CTLA-4 after *in vitro* stimulation was detected. In summary, our data using a large number of cases and controls contradict to a major effect of *CTLA4* in MS susceptibility.

Paper 5

3'UTR C2370A allele of the IL-23 receptor gene is associated with relapsing-remitting multiple sclerosis

(Neurosci Lett, 2008, 431:36-38)

Besides Th1 type cytokines, other pro-inflammatory cytokines, IL-23 and IL-17 have been indicated to play an important role in the establishment of autoimmune diseases, and T cells producing IL-17 have been dubbed Th17 cells. One of the Th17 differentiation pathways is IL-23-dependent and IL-23 is necessary for the survival and expansion of Th17 cells; this effect is mediated through the IL-23 receptor (IL-23R) signaling pathway. The IL-23/IL-17 cytokine axis has been suggested to play an important role in the development of several autoimmune diseases including multiple sclerosis.

We compared the prevalence of C2370A single nucleotide polymorphism (SNP) in the 3' untranslated region (3'UTR) of the IL-23 receptor gene (*IL23R*) between 223 patients with relapsing-remitting multiple sclerosis (RRMS) and 200 healthy controls. The A2370A genotype was significantly over-represented among patients with RRMS (10.8%) and RRMS exhibiting oligoclonal bands in the cerebrospinal fluid (12.9%) when compared to healthy subjects (5.50%). Multiple regression analysis revealed that presence of AA genotype provides a two-fold risk for the development of multiple sclerosis (OR=2.072, 95% CI: 0.988-4.347, $p<0.05$) and presence of oligoclonal bands in the CSF (OR=2.554, $p=0.03$). We did not find significant differences when female patients or HLA-DRB1*1501 positive/negative patients were separately analyzed and compared to controls. Nor did we find association in secondary progressive cases, athough the simple size was small (n=45).

Besides suggesting association with MS, these data indicate that *IL23R* represents a novel shared susceptibility gene as its association with several autoimmune diseases including inflammatory bowel disease (IBD) and psoriasis has recently been verified.

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Paper 6

Aberrant transcriptional regulatory network in T cells of multiple sclerosis

(Neurosci Lett, 2007, 422:30-33)

Although several data indicated altered gene expression profile in MS using microarrays, transcriptional networks, which can regulate a number of genes, have not been

examined in MS. In addition, genetic differences may also contribute to altered expression of genes. The concordance rate of monozygotic twins is approximately 30 %, while it is less than 5 % for dizygotic twins, suggesting the involvement of not a single but multiple susceptibility genes in the pathogenesis of MS. In addition, regulation of these multiple susceptibility gene expressions is controlled again through the combinatorial action of multiple transcriptional factors. Therefore, gene network analysis is necessary to evaluate the complex autoimmune processes underlying the pathogenesis of MS.

Therefore, we studied gene expression profile of purified $CD3^+$ T cells isolated from Hungarian monozygotic MS twins by DNA microarray analysis and performed gene network analysis (***Figure 5***).

Three pairs were concordant, while one pair was discordant for MS. By comparing the three concordant and one discordant pairs, we identified 20 differentially expressed genes (DEG) between the MS patient and the genetically identical healthy subject. Molecular network of 20 DEG analyzed by KeyMolnet, a comprehensive information platform, indicated the close relationship with transcriptional regulation by the Ets transcription factor family and the nuclear factor NF-κB.

This novel bioinformatic approach proposes the logical hypothesis that aberrant regulation of the complex transcriptional regulatory network contributes to development of pathogenic T cells in MS.

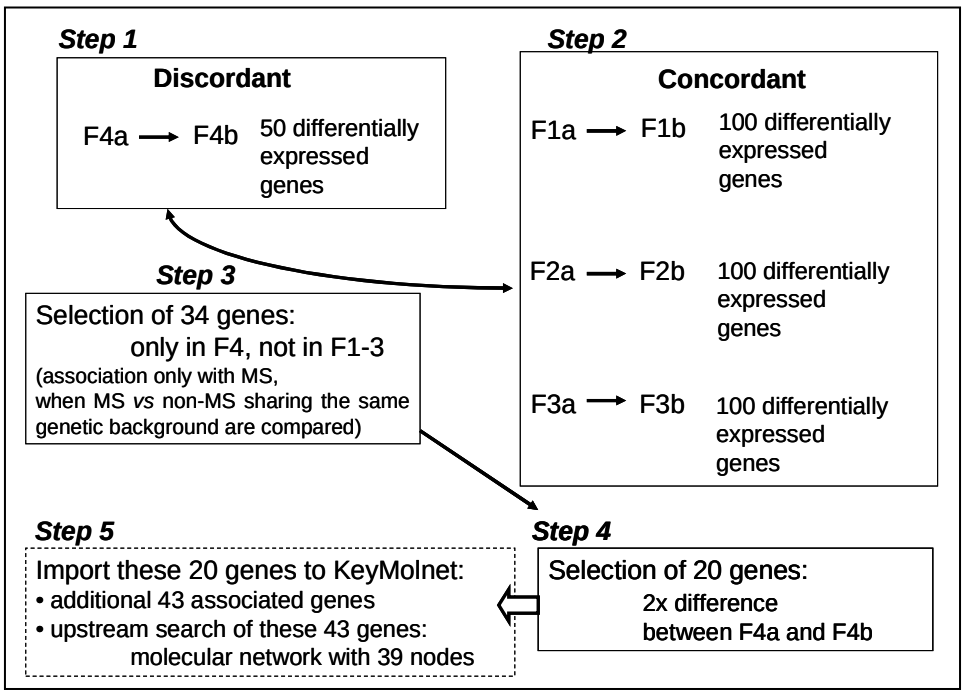

Figure 5. Gene expression network analysis in monozygotic twins with MS

IV. PAPERS

Paper 1

Invariant Vα7.2-Jα33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells

(Int Immunol, October 16, 2008, Epub ahead of print)

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Invariant Vα7.2-Jα33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells

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Keywords: innate, invariant TCR, MAIT, NKT, tumor

Abstract

The anti-tumor response of human invariant NKT (NKT) cells is well established. A novel T cell subset, mucosal-associated invariant T (MAIT) cells, possesses similar regulatory properties to NKT cells in autoimmune models and disease. Here, we examined the clonality of four T cell subsets expressing invariant αTCR, including Vα7.2-Jα33 of MAIT cells, in 19 kidney and brain tumors. The MAIT clonotype was identified and co-expressed with NKT clonotype in half of the tumors. In contrast, two other invariant T cell clonotypes (Vα4 and Vα19) were not present in tumors. Such tumors also expressed Vβ2 and Vβ13, the restricted TCRβ chain of MAIT cells and the antigen-presenting molecule MR1. A high percentage of infiltrating T cells was CD8+ and expressed HLA-DR suggesting activation. Although the MAIT αTCR was identified in both peripheral CD56+ and CD56− subsets, infiltrating lymphocytes were CD56 negative. The clonal presence of MAIT cells in tumors correlated with the expression of pro-inflammatory cytokines but no IL-4, IL-5 and IL-10, suggesting that a pro-inflammatory subset of human MAIT cells may exist. Our data imply that a CD56− subset of MAIT cells may participate in tumor immune responses similarly to NKT cells.

Introduction

The heterogeneous T cell repertoire of mainstream lymphocytes is generated by random recombination of V, D and J segments and junctional deletion and insertion of nucleotides. In contrast, innate lymphocytes are characterized by limited repertoire diversity. The best-characterized invariant αβT cell population, NKT cells, was identified in the early 90s in humans (1, 2). NKT cells express the invariant Vα24-JαQ CDR3 and reside mostly in the CD4−CD8− (double negative (DN)) and CD4+ cell subsets (1, 2). The CD1d recognition of DN Vα24 T cells and the restricted Vβ usage defined this subset as the human analog of murine NKT cells (3, 4). Emerging data indicate the functional diversity of human NKT cells and their involvement in tumor immunity and autoimmunity (5–8).

Along with the identification of Vα24 NKT cells, another DN T cell population expressing an invariant Vα7.2-Jα33 TCR has been described (1). Homologous TCR sequences displaying the same CDR3 length could be identified in bovine and murine DN T cell subsets (Vα19.1-Jα26), suggesting an important physiological function (9). Out of the two *TCAV7* genes, most of the conserved Vα7+ clones utilize the *AV7.2* gene segment in humans (10–12). In addition to the conserved CDR3α, mucosal-associated invariant T (MAIT) cells express a restricted Vβ2 and Vβ13 driven by the selecting antigen (9, 10). The preferential location of invariant Vα7.2-Jα33 T cells is the gut lamina propria; hence, the name MAIT cells has been recently suggested (13). Nevertheless, MAIT cells are also present in the peripheral blood at a similar frequency to Vα24 NKT cells (0.1–0.2%) and constitute about up to 15% of DN T cells (10). Vα7.2-Jα33 MAIT cells are selected by a non-classical MHC class Ib molecule, MR1 encoded by chromosome 1, similarly to CD1d. Several lines of evidence suggest that MR1 presents ligands to MAIT cells, possibly glycolipids, similarly to NKT cells (13–16).

Recent data suggest that MAIT cells are similar to NKT cells in a number of aspects: both use a semi-invariant TCR, recognize glycolipids and are activated in a co-receptor-independent manner, selected and restricted by a monomorphic class I-like molecule and characterized by a natural memory phenotype, suggesting a high-avidity interaction with

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self (8, 9, 12, 13, 16). In NKT cell-deficient mice, NK1.1+ hybridomas express the Vα19.1-Jα26+ TCR homologous to human Vα7.2-Jα33 (17). In Vα19.1-Jα26 TCR transgenic mice, the transgenic T cells also express NK1.1, the surface molecule of NKT cells (18).

So far, data about function of human MAIT cells are very limited, partly due to the absence of clonotypic antibodies. In humans, the invariant TCR of MAIT cells was shown to be expressed in autoimmune lesions of the central nervous system (CNS) and peripheral nervous system (PNS), which correlated with the expression of IL-4, suggesting an anti-inflammatory role and regulating autoimmune response similar to mice (12, 18, 19). However, a functional diversity similar to NKT cells has been very recently indicated in mice (20).

In addition to NKT and MAIT cells, two other T cell subsets expressing an invariant α chain have been suggested. These additional T cell populations express a Vα4-Jα29 and Vα19-Jα48 TCR, respectively (11).

Here, we examined expression of the invariant TCRs of the four human innate T cells in tumors using single-strand conformation polymorphism (SSCP) clonotype analysis. This method has been used to detect clonal dominance and expansion of T cell clones in different T cell subsets, including NKT and MAIT in autoimmune lesions of the nervous system (12, 19, 21). The selective absence of the invariant Vα24-JαQ TCR of invariant NKT (NKT) cells in CNS plaques of patients with multiple sclerosis (MS) was previously observed, while conventional Vα24 TCRs and invariant TCR of MAIT cells were present (12, 19). To partly examine whether absence of NKT cells in CNS plaques might be related to the CNS compartment or is specific to MS, here we examined tumors inside and outside the CNS, i.e. malignant brain tumors and kidney cancers. In tumors characterized by clonal presence of MAIT cells, we also examined the expression of CD56, pro- and anti-inflammatory cytokines, restricted TCR-Vβ chains and MR1.

Methods

The study protocol was approved by the Regional Local Ethics Committee. Patients or representatives gave written permission to perform all procedures only performed due to medical purposes supporting diagnostic and therapeutic decisions.

Isolation of messenger RNA and synthesis of cDNA

Nineteen tumor samples were obtained by biopsy or during operation (11 clear cell kidney cancer, 6 glioblastoma and 2 malignant meningioma) and immediately snap frozen or processed. None of the patients was treated with immunosuppressants or irradiation before or at the time of sampling. Messenger RNA (mRNA) was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). The air-dried pellet was re-suspended in 20 μl of RNAse-free water and used for cDNA synthesis by First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden, or Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using oligo-dT as primer.

SSCP analysis

Briefly, mRNA was isolated from tumor tissues, and cDNA of αCDR3 regions was amplified by reverse transcription (RT)–PCR with Vα- and Cα-specific primers. Primers and probes were designed based on the previously published sequences (12, 21). In brief, 1 μl of the diluted cDNA was used for each PCR with one of the TCR-Vα-specific primers and a Cα-specific primer. Sense primers specific for Vα7.2 (GTCGGTCTAAAGGGTACAGT) and Vα19 (GCCACAATAAACATACAGGA) were used in conjunction with the same anti-sense Cα primer (CAGCTGAGAGACTCTAAAT). Sense primer for Vα4 (ACAGAATGGCCTCTCTGG) was used with another anti-sense Cα primer (ATCGGTGAATAGGCAGACAG). To detect Vα24-JαQ-invariant human NKT cells, RT–PCR was performed as described previously with Vα24-specific sense primer (ACACAAAGTCGAACGGAAG) and Cα-specific anti-sense primer (GATTTAGAGTCTCTCAGCTG) (21). cDNA synthesized from mRNA of biopsy samples was amplified for 40 cycles, diluted (1:3) and heat denaturated.

Four microliters of the diluted samples were electrophoresed in non-denaturing 4% polyacrylamide gel. DNA was transfered to Immobilon-S (Millipore Intertech, Bedford, MA, USA) and hybridized with a biotinylated Cα-specific, Jα-specific or α-CDR3-specific clonotypic internal probe. Hybridization with a Cα probe detects all amplified CDR3 sequences (clonotypes) representing distinct T cell clones. In samples with a heterogeneous T cell population, the SSCP pattern is characterized by a smear, while CDR3 sequences of the dominant or expanding clones appear as distinct bands reflecting clonality of the repertoire (12, 19, 21). The probes were as follows: Cα (AAATATCCAGAACCCTGACCCTGCCGTGTACC), Jα29 (CTCTTGTCTTTGGAAAGGGCACAAGACTTTCTGT), Jα33 (TATCAGTTAATCTGGGGCGCTGGGACCAAGCT), Jα48 (ATTAACCTTTGGGACTGGAACAAGACTCACCATC) and Vα24inv, clonotypic (TGTGTGGTGAGCGACAGAGGCTCAACCCTG).

DNA was visualized by subsequent incubations with streptavidin, biotinylated alkaline phosphatase and a chemiluminescent substrate system (Phototope™ Detection Kit, New England Biolabs, Inc., MA, USA).

RT–PCR

cDNAs for human β2-microglobulin, IL-4 and IFN-γ were amplified by RT–PCR as described previously (12, 21). In brief, 1 μl of cDNA was used in 25 μl PCR mixture using PCR Master Mix (Promega, Madison, WI, USA). Similar strategies were used for amplification of other cytokines and MR1 mRNA. The primer sequences and annealing temperatures are indicated in Table 1. cDNA was amplified in GeneAmp 2700 amplifier (Applied Biosystems) using 39 cycles. The clonotypic RT–PCR for the detection of the invariant MAIT TCRα chain was performed as described previously, by using Vα7.2-specific sense and clonotypic invariant Vα7.2-Jα33 anti-sense primers (Table 1) (12).

Flow cytometry and sorting of lymphocyte populations

Thirty milliliter heparinized peripheral blood was obtained from patients with clear cell kidney cancer, brain tumors and from healthy subjects. PBMCs were isolated on Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden) by density gradient centrifugation. Fresh, unfixed tumor tissue blocks (~1 cm^3) obtained by nephrectomy were immediately sampled in the

Table 1. Primer sequences and annealing temperature

	Primer sequences (5′–3′)	Annealing temperature (°C)
TNF-α	F: caatgccctcctggccaat; R: tcggcaaagtcgagatagtc	58
IL-17	F: aatctccaccgcaatgagga; R: acgttcccatcagcgttgat	58
IFN-γ	F: atgtagcggataatggaactc; R: aacttgacattcatgtcttcc	58
IL-12	F: attctcggcaggtggaggt; R: gcagaatgtcagggagaagt	58
IL-4	F: actgcaaatcgacacctatta; R: atggtggctgtagaactgc	58
IL-5	F: gcttctgcatttgagtttgctagct; R: tggccgtcaatgtatttctttattaag	60
IL-10	F: gaaccaagacccagacatc; R: cattcttcacctgctccac	58
MR1	F: tgggagaggtacactcagc; R: agccacattatctacagcca	58
Vβ13A	F: gtatcgacaagacccaggc	62
Vβ13B	F: ggctcatccattattcaaatac	60
Vβ2	F: tcatcaaccatgcaagcctg	60
Cβ	R: gcttctgatggctcaaacac	
Vα7.2-Jα33	F: gtcggtctaaagggtacagt; R: tgatagttgctatctctcac	58
β2-Microglobulin	F: aagatgagtatgcctgccgtg; R: cggcatcttcaaacctccat	58

operation theater from the tumor–kidney border region or from brain tumors, kept on a humid atmosphere at +4°C and transported within an hour time to the flow cytometry laboratory. Tumor tissues were cut into cubic millimeter pieces by a sharp sterile surgical knife, immersed in 2 ml, pH 7.4, PBS and pushed gently through a 100-μm microfilter (Millpore). The cell counts of the filtered tumor cell suspensions were measured by a 'routine' laboratory volumetric hemocytometer (CellCyn3700, Abbott, USA) and adjusted to 5×10^6 cells per ml in PBS. After washing in PBS, 1×10^6 PBMCs and 5×10^6 tumor cells were incubated for 30 min at room temperature with different dual or triple combinations of the following mAbs: FITC-conjugated anti-CD3 and anti-CD4; PE-conjugated anti-CD8, anti-CD4, anti-HLA-DR and allophycoerythrin- or perCP-conjugated anti-CD56 and anti-CD45 (all antibodies from Becton Dickinson, San Diego, CA, USA). At least 50 000 cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium) after single gating on lymphoid cells for all mAb combinations. Tumor cells were gated out from the tumor-infiltrating lymphocyte populations by their large forward/side scatter and $CD45^{dim}$ staining characteristics. The percentages of positive cells were calculated using CellQuest software (Becton Dickinson).

CD56+ cells were positively selected with CD56 MicroBeads on a MACS Cell Separation Column according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Immunohistochemistry

After endogenous peroxidase blocking, slides were incubated with antibodies against CD3 (Dako, prediluted), CD4 (Labvision, 1:40), CD8 (Labvision, 1:50) and CD56 (Novocastra, 1:50). The antibodies were visualized in an automated immunostainer (Ventana Medical System).

Results

Detection of the invariant Vα24-JαQ TCR sequence in biopsy samples of kidney cancer and brain tumors

In order to investigate the presence of human NKT cells in tumor tissues, we applied SSCP clonality assay (21). We could detect Vα24 mRNA in all kidney cancers and six out of eight CNS tumors. Control kidney and brain samples were negative for the Vα24+ TCR (data not shown). The number of infiltrating clonotypes varied between two and nine per sample in kidney cancer, and there was a great variation in the number and dominancy of certain clonotypes. Furthermore, a clonotype in the same position was identified in different samples indicating the presence of a shared, invariant αCDR3 (Fig. 1). Indeed, hybridization with the invariant clonotypic probe revealed a single band. The invariant clonotype was present in 11 out of 19 cancer tissues (7 out of 11 kidney and 4 out of 8 brain) (Fig. 1).

Detection of the invariant Vα7.2-Jα33 TCR sequence in biopsy samples of kidney cancer and brain tumors

The expression of Vα7.2+ clonotypes and the invariant Vα7.2-Jα33 MAIT TCR was examined by previously established SSCP method, similar to the detection of NKT TCR (12, 19). Vα7.2+ clonotypes were detected in 8 out of 11 kidney cancer and 6 out of 8 brain tumors. Similar to the Vα24+ T cell population, CNS samples were characterized by smear with a few, less dominant clonotypes compared with kidney cancers when the membrane was hybridized with a Cα-specific probe. A common clonotype representing the Vα7.2-Jα33-invariant αCDR3 was found in the Vα7.2 repertoire confirmed also by hybridization with a Jα33-specific probe (12). Invariant clonotypes could be detected in 14 out of 19 cancer samples (8 out of 11 kidney and 6 out of 8 brain tumors) (Fig. 2). In both kidney and brain tumors, the MAIT clonotype was more dominant than in the peripheral blood (PB) (Fig. 2, lower panel).

Search for invariant Vα4+ and Vα19+ TCR sequences in tumors

In addition to MAIT and NKT cells, Vα4-Jα29+ and Vα19-Jα48+ T cells have been shown to express non-canonical αCDR3s (11). To investigate their presence among tumor-infiltrating lymphocytes, we established SSCP clonotypic assays.

Vα19+ mRNA was detected in 5 out of 11 kidney cancers (45%) but in none of the brain tumors. Similarly, Vα4+ mRNA could be amplified from six kidney cancers (54%), but was not present in brain tumors. The infiltrating Vα4+ repertoire

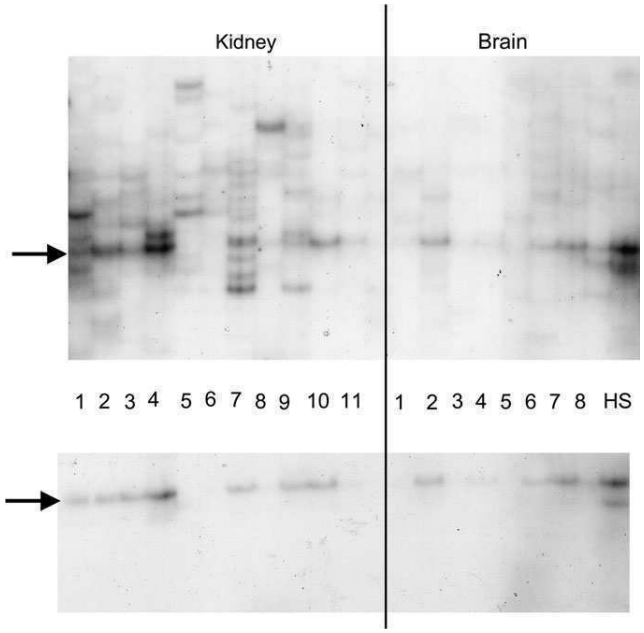

Fig. 1. Clonality of the Vα24+ T cell repertoire and presence of the invariant Vα24-JαQ clonotype representing NKT cells in kidney and brain tumors. Tumor tissues obtained from 11 patients with clear cell kidney cancer (left panel) and eight patients with brain tumor (right panel) were examined by RT–PCR SSCP clonotypic analysis. Amplified Vα24+ CDR3 was hybridized with a Cα probe (upper panel) and an invariant, clonotypic CDR3-specific probe (lower panel). HS indicates a lane for PBMC from a healthy subject. Arrow shows the position of the clonotype representing the invariant Vα24-JαQ CDR3 in several samples indicating the presence of NKT cells.

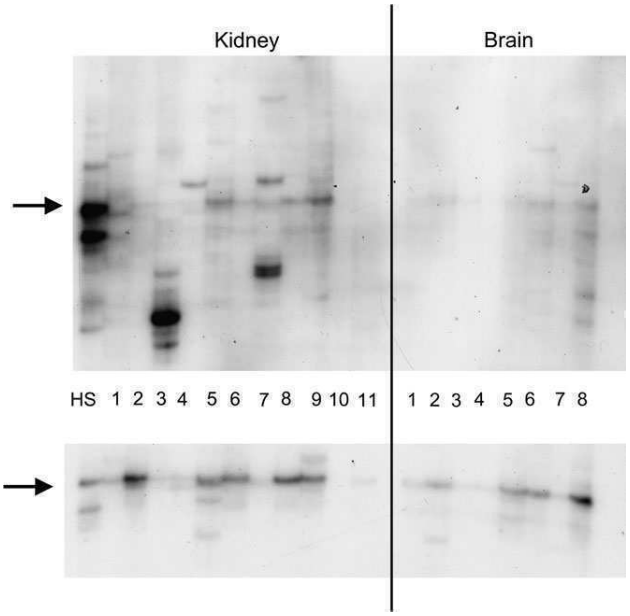

Fig. 2. Clonality of the Vα7.2+ T cell repertoire and presence of the invariant Vα7.2-Jα33 clonotype representing MAIT cells in kidney and brain tumors. Tumor tissues obtained from 11 patients with clear cell kidney cancer (left panel) and eight patients with brain tumor (right panel) were examined by RT–PCR SSCP clonotypic analysis. Amplified Vα7.2+ CDR3 was hybridized with a Cα probe (upper panel) and a Jα33-specific probe (lower panel). HS indicates a lane for PBMC from a healthy subject. Arrow shows the position of the clonotype representing the invariant Vα7.2-Jα33 CDR3 in several samples indicating the presence of MAIT cells.

was very restricted in kidney cancers indicated by the presence of a few, well-demarcated clonotypes. In contrast, the Vα19+ T cells expressed heterogeneous αCDR3 (Fig. 3). We were not able to identify shared Vα4+ or Vα19+ clonotypes hybridizing the samples with Cα- or Jα-specific probes (Fig. 3), suggesting that presence of MAIT and NKT clonotypes in tumors may not be accidental.

Detection of Vβ2 and Vβ13 TCRβ chains expressed by MAIT cells in kidney and brain tumors

Beside the invariant Vα7.2-Jα33 TCRα chain, MAIT cells are characterized by a restricted Vβ2 and Vβ13 TCR usage (9, 10). We examined the expression of these TCRβ chains by RT–PCR. In kidney cancers, all but one sample expressed Vβ2 and Vβ13 mRNA, respectively (Fig. 6). In addition, all the eight samples expressing the MAIT clonotype were positive for both β chains. In contrast, expression of these β chains was more limited in brain tumors: Vβ2 and Vβ13 sequences could be detected in five samples, and half of the brain tumors expressing the invariant MAIT αTCR did not express Vβ2 and Vβ13.

T cell subtypes in tumors expressing the invariant MAIT αTCR

To further characterize tumor samples expressing invariant αTCR and restricted TCRβ chains of MAIT cells, tissues were stained with anti-CD3, anti-CD4, anti-CD8 and anti-CD56

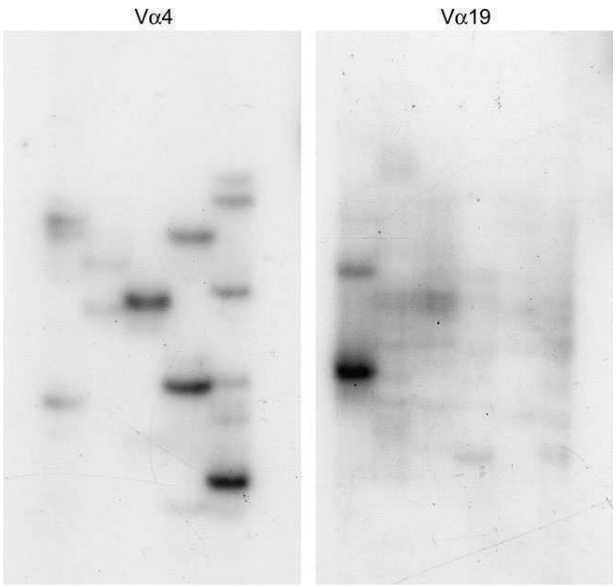

Fig. 3. Clonality of the Vα4+ and Vα19+ T cell repertoire in clear cell kidney cancer. Tumor tissues expressing Vα4+ and Vα19+ TCR were further analyzed by SSCP clonality analysis using Cα-specific probe to detect clonotypes with invariant TCR. No shared clonotypes are present in five kidney cancers positive for Vα4+ TCR (left panel) and seven kidney cancers expressing Vα19+ TCR (right panel)

antibodies. All kidney and brain tissues were positive for CD3+ lymphocytes, as expected. Only a few lymphocytes expressed CD4 in contrast to CD8 co-receptor (Fig. 4). FACS staining of infiltrating lymphocytes confirmed that HLA-DR+CD8low T cells were the major subset in tumors expressing MAIT TCRs (Fig. 4).

However, infiltrating T lymphocytes did not express CD56 in either tumor, despite of previous data suggesting expression of this molecule by NKT and MAIT cells as well (4, 9) (Fig. 5).

Detection of the invariant MAIT αTCR in CD56 subsets of PBMC obtained from patients with kidney and brain tumors

The absence of CD56+ T cells in tumors expressing MAIT and NKT TCR was unexpected (Fig. 5). Therefore, we examined the expression of the invariant Vα7.2-Jα33 TCRα chain in peripheral CD56+ and CD56− T cell subsets isolated from PB of five patients with kidney cancer, five patients with brain tumors and five healthy subjects. CD56+ and CD56− subsets were isolated by MACS from PB, and the invariant MAIT TCR was amplified by clonotypic RT–PCR. Both CD56+ and CD56− subsets obtained from patients and healthy controls expressed MAIT TCR (Fig. 5).

Cytokine and MR1 expression in kidney and brain tumors infiltrated by MAIT and NKT cells

While the functional heterogeneity of NKT cells is well established, MAIT cells are regarded as a T cell population producing primarily T_h2 cytokines (9, 12, 17, 18). This view has been recently challenged in mice (20). Therefore, we attempted to examine the cytokine environment in tumors expressing MAIT and NKT TCR (Fig. 6). Tumor samples were examined for the presence of MAIT and NKT clonotypes by RT–PCR SSCP and correlated with pro/anti-inflammatory cytokine expression examined by RT–PCR in the same samples (Tables 2 and 3).

The majority of tumors expressed pro-inflammatory (T_h1 and T_h17) cytokines. IL-12 and tumor necrosis factor (TNF)-α mRNA were equally well represented in both kidney and brain tumors. In contrast, only a single brain tumor expressed IL-17 and none was positive for IFN-γ mRNA, although both cytokines were abundantly expressed in kidney cancer (Tables 2 and 3).

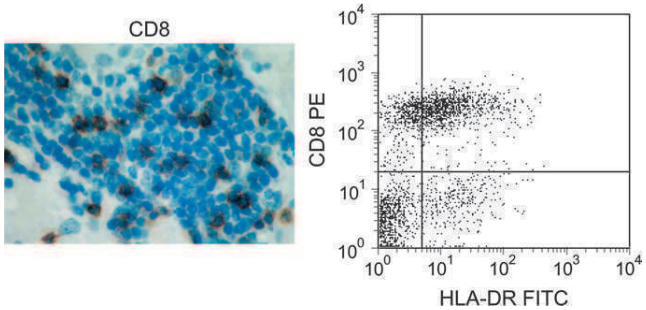

Fig. 4. Phenotype of infiltrating T lymphocytes in tumor tissues expressing the invariant MAIT αTCR. Surface expression of CD8 by immunohistochemistry (×400) and CD8/HLA-DR ontumor-infiltrating lymphocytes analyzed by flow cytometry is shown in a representative sample.

A similar bias was observed in the case of anti-inflammatory cytokines: only IL-10 mRNA was detected in abundance in kidney cancers. Three kidney cancers were also positive for IL-4 and IL-5 mRNA, but these cytokine messages were not detected in brain tumors at all. In addition, three of the kidney cancers and three of the brain tumors expressed only pro-inflammatory cytokines despite the presence of the invariant MAIT TCR (Tables 2 and 3).

To examine whether infiltrating MAIT cells may be locally activated through antigen recognition, expression of MR1 was also checked (Tables 2 and 3 and Fig. 6). All but one kidney cancer and four out of six brain tumors expressing MAIT TCR were positive for MR1 mRNA. In contrast, we identified 4 out of 19 tumors, which expressed MR1 but no MAIT invariant TCR.

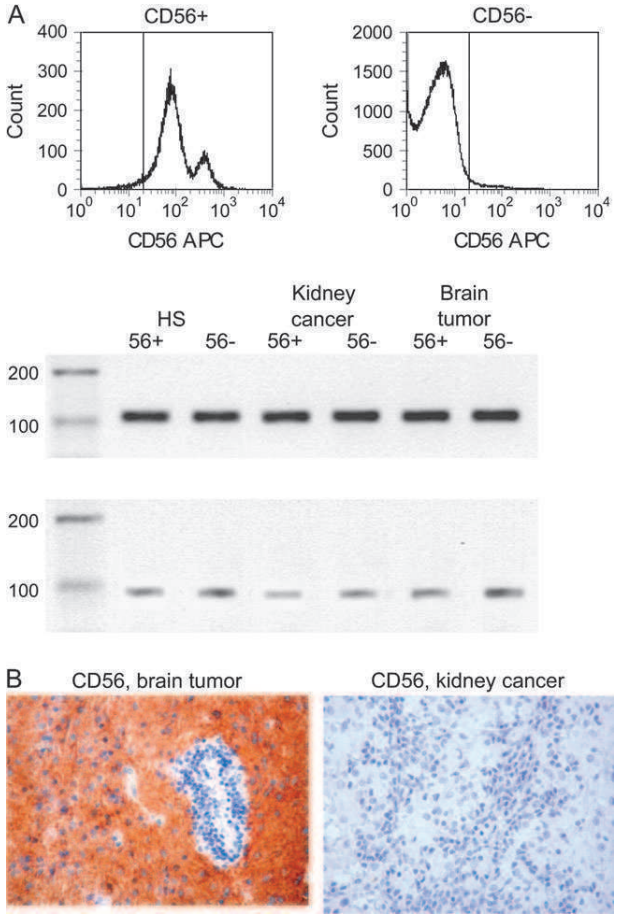

Fig. 5. Expression of CD56 in tumors expressing MAIT αTCR and presence of MAIT αTCR in peripheral CD56+ and CD56− lymphocyte subsets obtained from cancer patients and controls. (A) CD56+ and CD56− lymphocyte subsets were isolated from the peripheral blood of healthy controls, patients with clear cell kidney cancer and patients with brain tumors by MACS. Purity of the sorted population was checked by flow cytometry. Expression of β2-microglobulin (upper panel) and the invariant MAIT αTCR by a clonotypic RT–PCR (lower panel) was examined in the CD56+ and CD56− subsets. (B) Clear cell kidney cancer and brain tumors expressing the MAIT αTCR were examined for the expression of CD56 by immunohistochemistry. The positive staining of CNS served as positive control (brown, ×200).

Discussion

The role of NKT cells in tumor immunity is well established (5, 22, 23). In contrast, other innate T lymphocytes expressing canonical αβTCR have not been examined. A novel innate T cell subset, MAIT cells, is particularly interesting since phenotypic and functional similarities to NKT cells have been already suggested (8, 9, 12, 15–18). Here, we examined the clonality of those T cell repertoires in tumors, which contain innate lymphocytes with invariant TCRα chains (Vα4, Vα7.2, Vα19 and Vα24). The applied method (RT–PCR SSCP) has the advantage of assessing clonality and clonal dominance beside the examination of TCR expression. After the amplified αCDR3 cDNA sequences are separated by SSCP electrophoresis, hybridization with a Cα-specific probe visualizes the particular Vα T cell repertoire. The presence of invariant TCRα chains can be judged in this whole repertoire and further confirmed in a more restricted T cell repertoire by a subsequent hybridization with a Jα- or invariant αCDR3-specific clonotypic probe. After establishing SSCP clonality assay for the identification of NKT cells in autoimmune lesions, our method has been applied in other pathological studies and data were confirmed by different methodologies (24–27). Particularly, we modified the method to detect invariant Vα7.2-Jα33 T cells, later termed MAIT cells, in autoimmune lesions (12, 19).

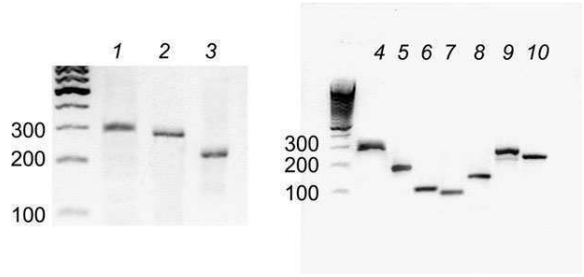

Fig. 6. Expression of Vβ2, Vβ13, MR1 and cytokines in tumor tissues expressing MAIT αTCR. Expression of cytokines, TCR-Vβ chains and MR1 was determined by RT–PCR in a representative sample of clear cell kidney cancer (#1) expressing the invariant MAIT αTCR (see Tables 2 and 3) (lanes indicate: 1 = Vβ13A, 2 = Vβ13B, 3 = Vβ2, 4 = TNF-α, 5 = IFN-γ, 6 = IL-17, 7 = IL-12, 8 = IL-10, 9 = IL-4 and 10 = MR1).

Out of the analyzed four T cell sub-populations, only invariant Vα24-JαQ NKT and Vα7.2-Jα33 MAIT cells could be identified in tumor tissues. Although both Vα4+ and Vα19+ TCR were present in about half of the kidney cancers, no identical clonotypes indicating an invariant αCDR3 were detected in these repertoires. In brain tumors, even the non-canonical Vα4+ and Vα19+ clonotypes were absent. These data suggest that MAIT and NKT cells are the major innate αβTCR lymphocyte subsets to infiltrate human tumors and may indicate that presence of these T cell subsets in tumors is not accidental but rather specific. Indeed, the clonal dominance of MAIT and NKT was superior to PB.

The number of infiltrating Vα7.2 and Vα24 clonotypes varied between two and nine per sample in kidney cancer and there was a great variation in the number and dominancy of certain clonotypes. In contrast, brain tumors had a more restricted number and less dominancy of clonotypes. These data suggested that although Vα7.2+ and Vα24+ T cells were present in both PNS and CNS tumors, kidney cancers were infiltrated with more heterogeneous T cell populations. Alternatively, T cells died in the CNS tumors. We also observed a more restricted Vα7.2+ and Vα24+ T cell repertoire in autoimmune CNS lesions, compared with autoimmune demyelinating lesions of the PNS (12, 21). Accordingly, our data may indicate that within the total Vα24+ and Vα7.2+ repertoire, both MAIT and NKT cells represent relatively more dominant populations in CNS compared with kidney tumors. Moreover, presence of NKT cells in CNS tumors contrasted to autoimmune CNS lesions, where NKT cells were rarely detected despite the presence of conventional Vα24+ T cells (21). Detection of NKT cells in CNS tumors indicates that absence of NKT cells is unique to autoimmune infiltrates and is not related to the special immunoregulation of the CNS.

The presence of MAIT cells in tumors indicated by the expression of invariant MAIT TCR clonotypes is a novel finding, but not unexpected considering the similarities to NKT cells. The anti-tumor response of NKT cells has already initiated human clinical trials to treat cancer (23, 28–30). Presence of NKT cells or the invariant Vα24-JαQ TCR has been shown in

Table 2. Expression of cytokines, MR1 and TCRα chains of T cell populations with invariant CDR3α in clear cell kidney cancers

	1	*2*	*3**	*4*	*5*	*6**	*7*	*8*	*9**	*10*	*11*
TNF-α	+	+	–	+	+	+	+	+	+	+	+
IL-12	+	+	–	+	+	+	+	+	+	+	+
IFN-γ	+	–	+	+	+	+	+	–	–	–	–
IL-17	+	+	–	+	–	+	+	+	–	+	+
IL-4	+	–	–	–	–	–	–	–	–	–	–
IL-5	–	–	–	+	–	–	+	–	–	–	–
IL-10	+	+	–	+	+	–	+	+	–	–	–
MR1	+	+	+	+	+	+	+	+	–	+	+
NKT	+	+	+	+	–	–	+	–	+	+	–
MAIT	+	+	+	+	+	+	–	+	+	–	–

Numbers in italics indicate individual tumor samples. The number of samples corresponds to those in Figures 1 and 2. MAIT and NKT indicate the presence of the invariant Vα7.2-Jα33 and Vα24-JαQ TCR, respectively. The expression of invariant Vα7.2-Jα33 and Vα24-JαQ TCR was examined by RT–PCR SSCP clonotypic analysis, and the expression of cytokines and MR1 was examined by RT–PCR (see Methods). Asterisks indicate those samples where no IL-4, IL-5 and IL-10 mRNA could be detected despite the presence of the invariant MAIT TCRα chain.

Table 3. Expression of cytokines, MR1 and TCRα chains of T cell populations with invariant CDR3α in brain tumors

	*1**	*2**	*3*	*4*	*5*	*6*	*7*	*8**
TNF-α	+	+	+	+	+	+	+	−
IL-12	+	+	+	+	+	+	+	+
IFN-γ	−	−	−	−	−	−	+	+
IL-17	−	−	+	−	−	−	−	−
IL-4	−	−	−	−	−	−	−	−
IL-5	−	−	−	−	−	−	−	−
IL-10	−	−	−	−	+	+	+	−
MR1	−	+	+	+	−	+	+	+
NKT	−	+	−	−	−	+	+	+
MAIT	+	+	−	−	+	+	+	+

Numbers in italics indicate individual tumor samples. The number of samples corresponds to those in Figures 1 and 2. MAIT and NKT indicate the presence of the invariant Vα7.2-Jα33 and Vα24-JαQ TCR, respectively. The expression of invariant Vα7.2-Jα33 and Vα24-JαQ TCR was examined by RT–PCR SSCP clonotypic analysis, and the expression of cytokines and MR1 was examined by RT–PCR (see Methods). Asterisks indicate those samples where no IL-4, IL-5 and IL-10 mRNA could be detected despite the presence of the invariant MAIT TCRα chain.

human tissue samples (21, 31–33), including tumors *in situ* (34–36). However, the expression of the Vα7.2-Jα33 rearrangement was only examined and found in autoimmune and cutaneous sarcoid lesions, suggesting that MAIT cells can infiltrate tissues as well (12, 31). Although the invariant MAIT TCR has not been examined in tumors so far, the restricted Vβ2 and especially Vβ13 TCRs expressed by MAIT cells have been already shown in a number of tumors (37–41). In addition, several studies indicated an *in vitro* cytolytic activity of infiltrating Vβ13+ T cells, particularly with a CD8 phenotype, both characteristic of MAIT cells (39–41). Indeed, kidney and brain tumors expressing the invariant MAIT TCR were infiltrated by CD8+ T cells in our study. These CD8+ T cells also expressed HLA-DR, indicating an activated state. Of note, the invariant MAIT and NKT TCR were co-expressed in about half of the tumor samples regardless of compartmentalization, suggesting that MAIT and NKT cells infiltrate cancers together.

We also examined the presence of CD56 molecule on infiltrating T cells, which can be connected to cytotoxicity and can be expressed by both NKT and MAIT cells (4, 9). However, T cells in kidney cancer and brain tumors were negative for CD56. Therefore, we examined whether peripheral MAIT cells obtained from patients with these cancers alter expression of CD56 compared with healthy controls. Since no antibody is available to detect MAIT cells, we sorted CD56+ and CD56− subsets from the peripheral blood and applied a clonotypic PCR to identify the presence of MAIT cells in these subsets. The invariant Vα7.2-Jα33 TCR message could be amplified in both CD56+ and CD56− subsets, similar to healthy subjects. Thus, although part of peripheral MAIT cells express CD56 in cancer patients, the tumor-infiltrating MAIT cells may comprise a CD56− subset.

MAIT cells represent a novel T cell population with similar phenotypic and functional properties to NKT cells (8, 9, 12, 15–18). Their regulatory role has been already addressed in autoimmunity and may be related to anti-inflammatory cytokines produced or induced by MAIT cells. In mice, they can protect against autoimmune inflammation of the CNS by an increased IL-10 production through interactions with B cells (18). In humans, the invariant MAIT TCR was detected in autoimmune lesions in connection with expression of IL-4 and IL-10 mRNA (12). However, recent data indicated a heterogeneous cytokine production by murine MAIT cells, similar to NKT cells (20, 42). Considering the presence of MAIT cells in tumors, the suggested similarities to human NKT cells with anti-tumor activity due to production of pro-inflammatory cytokines and the functional heterogeneity of murine MAIT cells, we correlated pro- and anti-inflammatory cytokine expression in tumors with the presence of MAIT clonotypes. Pro-inflammatory cytokines were widely expressed in both kidney cancers and brain tumors, as expected. The only abundantly expressed anti-inflammatory cytokine was IL-10, which may both suppress and stimulate anti-tumor immune responses (43). In addition, only pro-inflammatory cytokines were detected in six tumors expressing the invariant MAIT TCR. The correlation of pro-inflammatory cytokines with MAIT clonotypes may indicate that human MAIT cells may have a pro-inflammatory subset, similar to human NKT and murine MAIT cells (20, 42). Besides expression of cytokines, tumors also expressed MR1, the antigen-presenting molecule of MAIT cells. In the majority of tumors, MR1 was co-expressed with the invariant MAIT TCR, indicating that MAIT cells may have the possibility to be locally activated by ligands presented by MR1 and possibly contribute to the cytokine environment and cytotoxicity (15, 16).

Our data also emphasize the immunological differences of tumors located outside and within the CNS. First, there was a difference in the number and dominancy of both Vα24 and Vα7.2 clonotypes between tumors located in different compartments. In addition, while Vβ2 and Vβ13 expression was obvious in all tumors presenting the invariant MAIT clonotypes in kidney cancer, some of the brain tumors did not disclose Vβ2 and Vβ13 TCR, indicating that a subset of MAIT cells may express other β chains in CNS tumors. In kidney cancer, pro-inflammatory cytokines IFN-γ and IL-17 were abundantly present. In contrast, none of the brain tumors expressed IFN-γ and only one tumor was positive for IL-17 mRNA. This bias was characteristic only of T_h1/T_h17 cytokines since TNF-α and IL-12, cytokines important in anti-tumor responses, were equally well represented in kidney and brain tumors. The number of malignant gliomas and meningiomas was not enough to examine differences regarding MAIT, NKT cells and cytokines.

In summary, our data indicate that a novel NKT-like T cell population, MAIT cells infiltrate tumors similar to NKT cells, while other invariant T cell subsets may not be present. The co-expression of MR1 and MAIT TCR in tumors suggests that MAIT cells may be locally activated. The co-expression of pro-inflammatory cytokines and the invariant MAIT TCR in the absence of T_h2 cytokine messages in tumors may suggest functional heterogeneity of human MAIT cells. Our data also imply that MAIT cells in tumors may belong to a CD56− subset and express CD8 and HLA-DR. Considering the importance of NKT cells in anti-tumor responses represented even by human drug trials, the functional similarities between MAIT and NKT cells and the co-expression of the two invariant TCRs in tumors, our data indicate that beside NKT cells, MAIT cells may be also considered in anti-cancer treatment strategies.

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Abbreviations

CNS	central nervous system
DN	double negative
MAIT	mucosal-associated invariant T
mRNA	messenger RNA
MS	multiple sclerosis
NKT	invariant NKT
PB	peripheral blood
PNS	peripheral nervous system
RT	reverse transcription
SSCP	single-strand conformation polymorphism

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Paper 2

Impaired function of innate T lymphocytes and NK cells in the acute phase of ischemic stroke

(revised version resubmitted to Stroke)

IMPAIRED FUNCTION OF INNATE T LYMPHOCYTES AND NK CELLS IN THE ACUTE PHASE OF ISCHEMIC STROKE

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ABSTRACT

Background and Purpose: Functional alterations of innate lymphocytes, which can amount rapid immune responses and shape subsequent T cell reactions, were examined in the acute phase of ischemic stroke. **Methods:** Frequencies, intracellular perforin and IFN-γ expression of Vδ2 T cells, $CD3^+CD56^+$ natural killer T (NKT)-like and $CD56^+$ natural killer (NK) cells were examined sequentially in the peripheral blood of 20 healthy controls and 28 patients within 6 hours of the onset of acute ischemic stroke and after 72 hours by flow cytometry. Pro- and anti-inflammatory cytokine production of isolated NKT-like and NK cells following in vitro activation was measured by cytometric bead array. NK cytotoxicity was examined in the peripheral blood mononuclear cells. **Results:** Percentage of Vδ2, NKT-like and NK cells at 6 and 72 hours after stroke were constant and similar to percentages in healthy subjects. In contrast, pro-inflammatory intracellular IFN-γ expression by Vδ2 T cells, NKT-like cells and NK cells and IFN-γ production by isolated NK cells in culture was low at 6 hours and reached the level of healthy subjects by 72 hours after stroke. Anti-inflammatory IL-4, IL-5 and IL-10 production of NKT-like and NK cells was not altered. Intracellular perforin expression by Vδ2 T cells, NKT-like cells and NK cells, and NK cytotoxicity was low at 6 hours and reached the level of healthy subjects by 72 hours. **Conclusions:** Pro-inflammatory and cytotoxic responses of NK, NKT-like and Vδ2 T cells become acutely deficient in ischemic stroke, which may contribute to an increased susceptibility to infections.

INTRODUCTION

Stroke is associated with high mortality and morbidity, and stroke survivors often remain permanently disabled. While direct neurological deficits cause early deaths, infectious complications prevail in the postacute phase of stroke contributing to the poor outcome.[1-6] Such an increased susceptibility to infections after stroke may suggest early alteration of immune responses.[7-9] Recently, acute deficiency of T cells has been indicated in experimental stroke models.[10,11] Very recently, dramatic loss of T cells in the peripheral blood of patients with acute ischemic stroke, within 12 hours from onset of symptoms, has been indicated.[12] These few studies all addressed the rapid changes in the adaptive arm of the immune system, mainly T cells. Cells of the other part of the immune system, innate immunity can initiate immediate non-antigen specific reactions in contrast to T cells. In addition, particular lymphocyte subsets including specific innate T cell populations bridge the adaptive and innate immune responses and are able to amount a rapid immune response either through inflammatory cytokine production or cytotoxicity. These mechanisms are important in defense against pathogens and also shaping subsequent adaptive T cell responses. Such innate lymphocyte subsets have not been thoroughly examined in the acute phase of ischemic stroke.

γδT cells express a T cell receptor (TCR) consisting of a γ-chain combined with a δ-chain, composing a distinct T cell population from the well-known αβT cells. γδT cells have been proposed to bridge the innate and adaptive immune responses.[13,14] They are able to produce great amounts of interferon-γ (IFN-γ) in a short period of time, which is characteristic to cells of the innate immune system.[13,14] γδT cells are important regulators of immune responses, play a necessary role in protective immune responses against pathogens and tumors through provision of an early source of IFN-γ.[15] The Vδ2 subset, expressing a δ2 TCR variable chain, represents the majority of adult γδT cells mainly with a cytotoxic property.[14,16]

Natural killer T (NKT)-like $CD3^{+}CD56^{+}$ cells are unique T cells co-expressing a T-cell receptor complex (CD3) and NK receptors (CD56).[17] The role of NKT-like cells have been suggested and investigated in various immune responses, including response to infectious agents,[18] tumor rejection,[19,20] and autoimmunity.[21,22] This subset also includes invariant NKT (iNKT) cells being able to produce large quantities of pro-inflammatory IFN-γ and anti-inflammatory cytokines such as interleukin (IL)-4 and IL-10,[17,23] and capable of activating NK cells as well.[24]

Natural killer (NK) cells are crucial components of the innate immune system, which are able to rapidly produce abundant cytokines, mainly IFN-γ, and lyse target cells without prior sensitization.[25,26] NK cells play a key role in resistance to infections and destruction of

tumor cells.[25,26] They express CD56 on their surface, which is widely used as an NK cell marker to identify them, and lack the expression of CD3.[25,26] Assistance by NK cells in the development of adaptive immune responses has recently been shown as well.[27]

Perforin and Fas/Fas ligand (FasL) pathways are the two major mechanisms of cytotoxicity.[28,29] Exocytosis of cytotoxic granules containing pre-formed perforin and serine esterase molecules leads to cell lysis by forming pores in target cell membranes.[28-30] Perforin is a marker of cytotoxic cells.[30,31] FasL produced by effector cells induces apoptosis through Fas, its receptor on target cell surfaces. The Fas/FasL-mediated apoptotic pathway thus depends on cell-surface ligand-receptor interaction.[28,29,32]

Here, we analyzed functional alterations in cytokine production and cytotoxicity of innate lymphocyte subsets capable of shaping adaptive T cell responses in the acute phase of ischemic stroke.

MATERIALS AND METHODS

The study protocol was approved by the Regional Local Ethics Committee.

Patients and samples. Altogether 28 Hungarian patients with acute ischemic stroke (16 males, 12 females, mean age: 62 years ± 2.08 years) were prospectively studied and 20 healthy controls were included in this study. All patients were admitted to the Department of Neurology, University of Pecs, Hungary. Written informed consent was obtained from all patients or their authorized representative. Exclusion criteria were infectious diseases, fever <4 weeks before stroke, elevated white blood cells (WBC), erythrocyte sedimentation rate (ESR), high-sensitivity C-reactive protein (hsCRP, cut-off value <10 mg/L), procalcitonin (PCT, cut-off value <0.05 ng/mL) on admission, positive chest X-ray, hemorrhagic stroke defined by an acute cranial CT scan, transient ischemic attack (TIA, based on the non-persistency of clinical signs and cranial CT scan after 24 hours) and decline to participate in the study. Patients admitted to hospital more than 6 hours after symptom onset were excluded too. Twenty-two patients had large territorial and 6 patients had lacunar infarct. In 8 cases, stroke was caused by embolism either cardiogenic (n=5) or arterial (n=3). All but one patients had infarct in the anterior circulation. Control CT scan showed hemorrhagic transformation in a single case only. Peripheral blood was drawn from the cubital vein two times from each patient within 6 hours from symptom onset and after 72 hours. Clinical data were collected on admission and at 72 hours. Outcome measures were obtained at discharge from hospital (**Table 1**). The National Institutes of Health Stroke Scale (NIHSS) was assessed as a quantitative measure of stroke-related neurological deficit on admission and at 72 hours.

Table 1

Demographic and clinical data of subjects		
	Patients, acute ischemic stroke n=28	**Healthy subjects** n=20
Age (year, mean, range)	62 (40-83)	45.1 (26-64)
Sex : Male	16	7
Female	12	13
NIHSS 6h (median, range)	10 (4-19)	N/A
NIHSS 72h (median, range)	6 (1-22)	N/A
GOS at discharge (median, range)	4 (1-5)	N/A
Length of stay (day, mean, range)	9.04 (4-27)	N/A

Cell isolation and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood on Ficoll-Paque gradient (AP Hungary Kft. Budapest, Hungary) by density gradient centrifugation. After washing in phosphate-buffered saline (PBS), $1x10^6$ PBMCs were incubated for 30 minutes at room temperature with different dual or triple combinations of the following anti-human monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-Vδ2 T cell receptor, phycoerythrin (PE)-conjugated anti-FasL (CD178), anti-IFN-γ, anti-perforin, and allophycocyanin (APC)-conjugated anti-CD56 (all antibodies from BD Pharmingen, Soft Flow Hungary Kft., Pécs, Hungary). In the case of IFN-γ staining, PBMCs underwent a 4-hour cytokine stimulation with ionomycin, brefeldin A and phorbol-myristate-acetate (PMA) (all from Sigma-Aldrich Kft., Budapest, Hungary) prior to the immunostaining. In the case of perforin and IFN-γ stainings PBMCs were permeabilized with FACS Permeabilizing Solution 2 (BD Biosciences, Soft Flow Hungary Kft., Pécs, Hungary) before adding the respective antibody. After incubation, the samples were washed in PBS and resuspended in 4% paraformaldehyde, stored at 4ºC in dark to be processed for FACS analysis 24 hours at the latest following fixation. At least 50,000 cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Erembodegen, Belgium) after single gating on lymphoid cells for all mAb combinations. The percentages of positive cells were calculated using CellQuest software (Becton Dickinson, San Diego, CA, USA).

NK and NKT-like $CD3^+CD56^+$ T cell separation and Cytometric Bead Array (CBA). Natural killer and $CD3^+CD56^+$ T cells were separated by MACS Cell Separation Technology (all reagents and instruments from Miltenyi Biotec, Frank Diagnosztika Kft., Budapest, Hungary). PBMCs were first magnetically labelled with CD56 MicroBeads according to the manufacturer's instructions and $CD56^+$ cells were positively selected on the cell separation column. In the next step, the magnetic beads bound to the cell surface were enzymatically released from the $CD56^+$ cells, which were then magnetically labelled with CD3 MicroBeads and the $CD3^+$ subpopulation positively selected to compose the $CD3^+CD56^+$ T cell population. The remaining fraction of the $CD56^+$ cells, which did not bind the CD3 beads composed the $CD3^-CD56^+$ NK cell population. Both cell populations were stimulated with ionomycin and PMA (Sigma-Aldrich, Sigma-Aldrich Kft., Budapest, Hungary) in RPMI 1640 Medium containing 10% foetal bovine serum, penicillin and streptomycin (all from Invitrogen, Csertex Kft., Budapest, Hungary) overnight for cytokine production. The levels of IL-2, IL-4, IL-5, IL-10, IFN-γ and tumor necrosis factor (TNF)-α were determined from the culture supernatants with Cytometric Bead Array (CBA) (#550749, BD Biosciences, Soft Flow Hungary Kft., Pécs, Hungary) using different capture beads according to the manufacturer's instructions to detect the respective cytokines. Samples were analyzed right after the experiment on a FACS calibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) calculating the amount of cytokines with CBA Software (BD Biosciences, San Diego, CA, USA).

Cytotoxicity. The cytotoxic activity of NK cells was determined with a non-radioactive, colorimetric cytotoxicity assay (#G1780, Promega, Bio-Science Kft., Budapest, Hungary). PBMCs containing NK cells were co-cultured with K562 cells, conventional target cells of NK cells (NK-sensitive erythroleukaemia cell line)[33,34] for 4 hours at 37ºC in 40:1, 20: 1 and 10:1 effector-to-target ratios in a U-bottom 96-well tissue culture plate, according to the manufacturer's instructions. Absorbance of the final color product was recorded by a standard 96-well plate reader (Thermo Labsystems, Franklin, MA, USA) at 492 nm. Cytotoxicity was expressed as the percentage of lysed target cells in each effector-to-target ratio.

Statistical analysis. Statistical comparisons were made by using one- and two-tailed Student's t-tests. The results were expressed as the mean value±standard error of the mean (SEM). Differences were considered significant when the value of P was equal to or less than 0.05.

RESULTS

Innate peripheral lymphocyte frequencies in the acute phase of ischemic stroke

In order to investigate the acute innate cellular immune responses in ischemic stroke, peripheral blood (PB) samples were acquired within 6 hours and after 72 hours of symptom onset and compared both with each other and with healthy controls. We particularly focused on innate T lymphocytes like γδT cells, $CD3^+CD56^+$ NKT-like cells and $CD3^-CD56^+$ NK cells, which are capable of rapidly producing cytokines and performing cytotoxicity. The frequency of $V\delta2^+$, NKT-like T cells and NK cells in the PB was not different, when samples obtained within 6 hours after onset of stroke were compared to samples obtained 72 hours later. In addition, samples taken from patients did not differ when compared to healthy controls (**Table 2**).

Table 2

Innate lymphocyte frequencies in the peripheral blood

Percentage of PBL	**Healthy subjects**	**Patients, acute ischemic stroke**	
		within 6 hours	***after 72 hours***
Vδ2 T %	2.02 ± 0.54	1.37 ± 0.24	1.66 ± 0.25
CD3+CD56+ NKT-like %	4.41 ± 1.56	6.22 ± 1.37	5.75 ± 1.37
CD3-CD56+ NK %	17.34 ± 2.23	16.34 ± 2.19	16.63 ± 2.34

mean±SE

Cytokine production of innate lymphocyte subsets in the acute phase of ischemic stroke

Intracellular IFN-γ production. Next, intracellular IFN-γ expression was analyzed by flow cytometry in $V\delta2^+$ T cells, $CD3^+CD56^+$ NKT-like, and NK cells (**Figure 1**). The expression of IFN-γ was significantly higher in all subsets after 72 hours compared to samples obtained within 6 hours from the beginning of ischemic stroke. In order to define whether the baseline IFN-γ at 6 hours was deficient or normal, data were obtained from healthy individuals as well. IFN-γ expression in healthy subjects corresponded to percentages of IFN-γ producing cells at 72 hours of patients in all subsets, and differed significantly from percentages at 6 hours in $CD3^+CD56^+$ NKT-like and $V\delta2^+$ T cells.

Pro- and anti-inflammatory cytokine production. Pro- and anti-inflammatory cytokine production of isolated $CD3^+CD56^+$ NKT-like and NK cells were also analyzed. NK and NKT-like subsets were separated by magnetic beads, stimulated for 24 hours by PMA/ionomycin and IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10 production was measured in the supernatants. In case of NK cells, significantly higher IFN-γ levels were detected at 72 hours compared to 6 hours after the beginning of stroke in accordance with data obtained by the flow cytometric analysis (37,6 ng/ml vs. 21,8 ng/ml $p<0,05$). The levels of the other investigated cytokines were not different (*data not shown*).

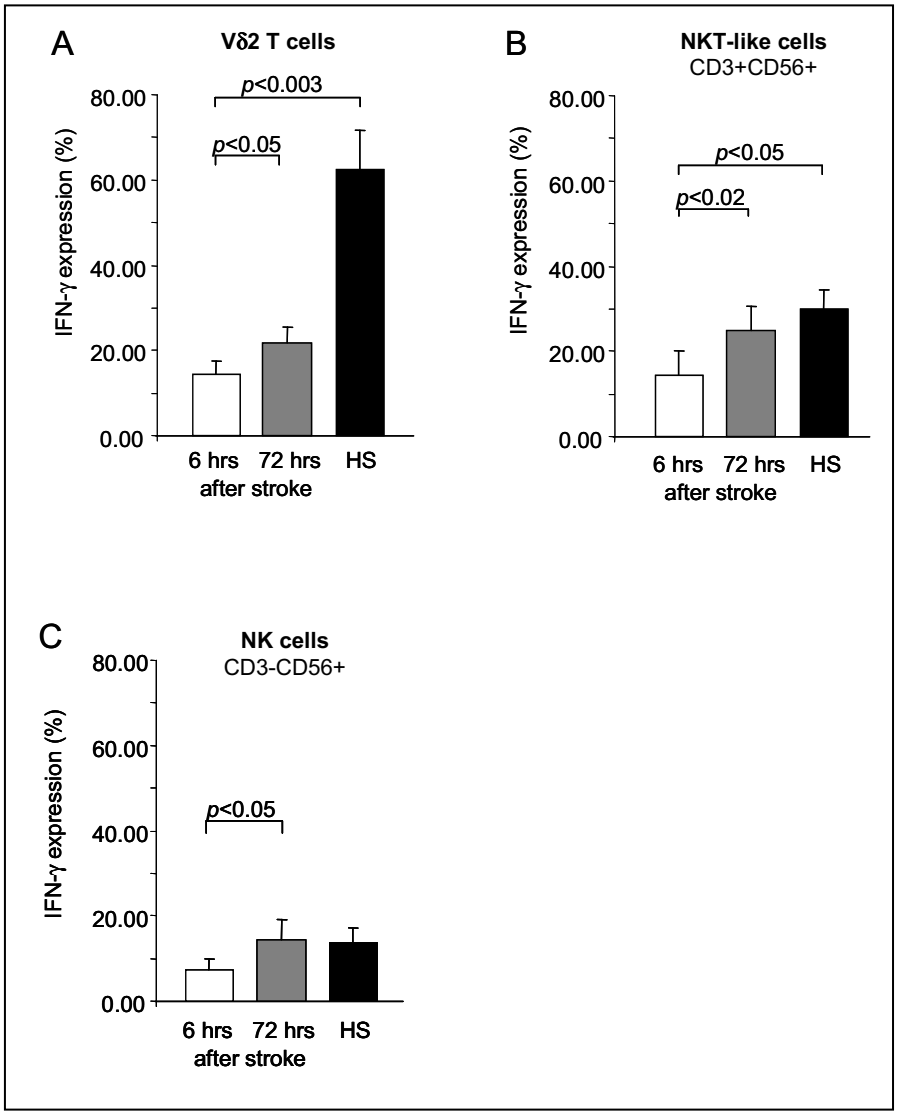

Figure 1

Intracellular IFN-γ expression by innate lymphocytes in acute ischemic stroke.

Intracellular IFN-γ expression by Vδ2 T cells (A), $CD3^+CD56^+$ NKT-like (B) and NK ($CD3^-CD56^+$) (C) and in the peripheral blood of acute ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and those of healthy subjects is indicated.

Cytotoxicity of innate lymphocytess in the acute phase of ischemic stroke

Perforin expression. Intracellular expression of perforin was analyzed by flow cytometry in $V\delta2^+$ T cells, $CD3^+CD56^+$ NKT-like and NK cells (**Figure 2**). Significantly increased percentages of perforin-expressing $V\delta2^+$ T cells, NKT-like and NK cells were revealed in samples obtained at 72 hours compared to 6 hours after onset of stroke. Perforin expression in healthy subjects corresponded to percentages of perforin producing cells at 72 hours of patients in all subsets, and differed significantly from percentages at 6 hours in $CD3^+CD56^+$ NKT-like cells.

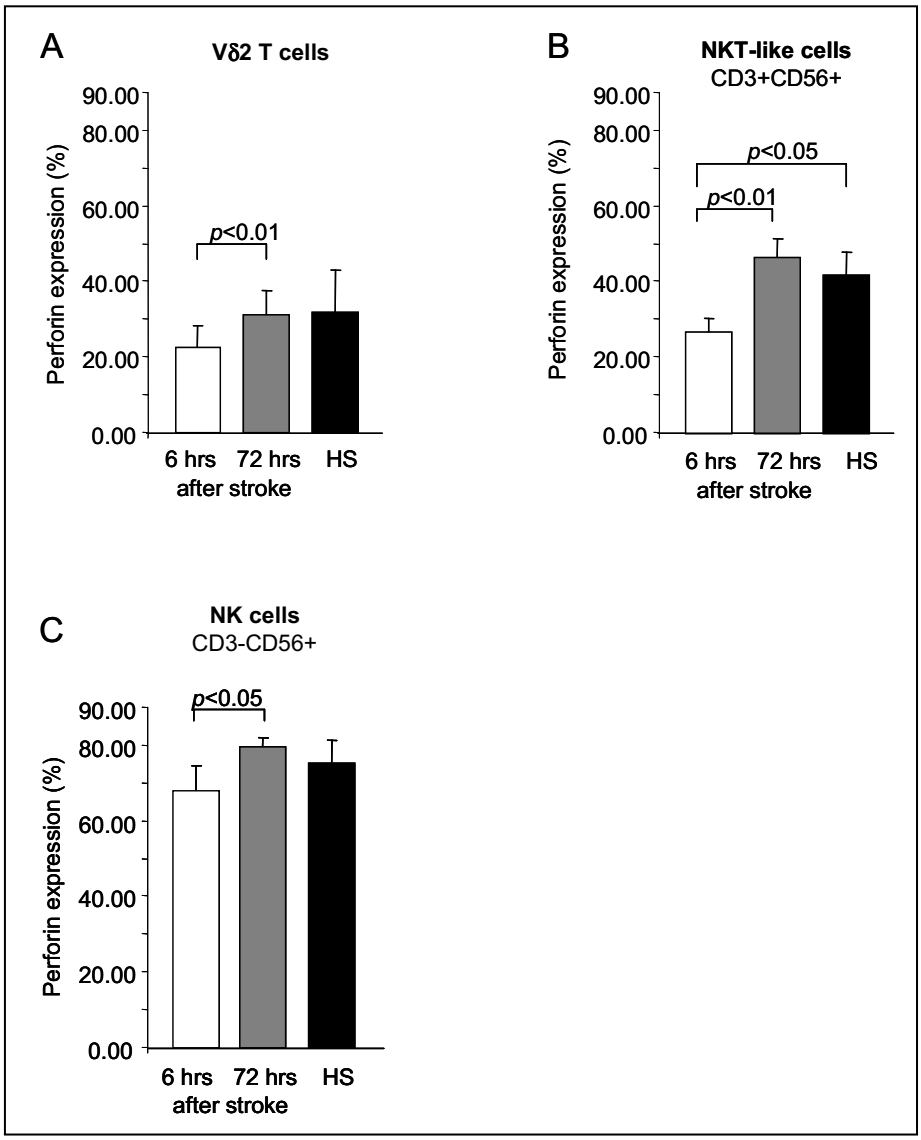

Figure 2

Intracellular perforin expression by innate lymphocytes in acute ischemic stroke.

Intracellular perforin expression by Vδ2 T cells (A), $CD3^+CD56^+$ NKT-like (B) and NK ($CD3^-CD56^+$) (C) in the peripheral blood of acute ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and those of healthy subjects is indicated.

FasL expression. We were also interested in the role of the other major cytotoxic mechanism, the Fas/FasL-mediated apoptotic pathway.[28,29,32] Surface expresion of FasL was examined on $CD3^+CD56^+$ NKT-like cells and NK cells. FasL expression was significantly decreased by $CD3^+CD56^+$ NKT-like and NK cells in samples obtained after 72 hours of symptom onset (**Figure 3**). FasL expression in healthy subjects corresponded to percentages of FasL expressing cells at 72 hours of patients in both subsets, and differed significantly from percentages at 6 hours in NK cells.

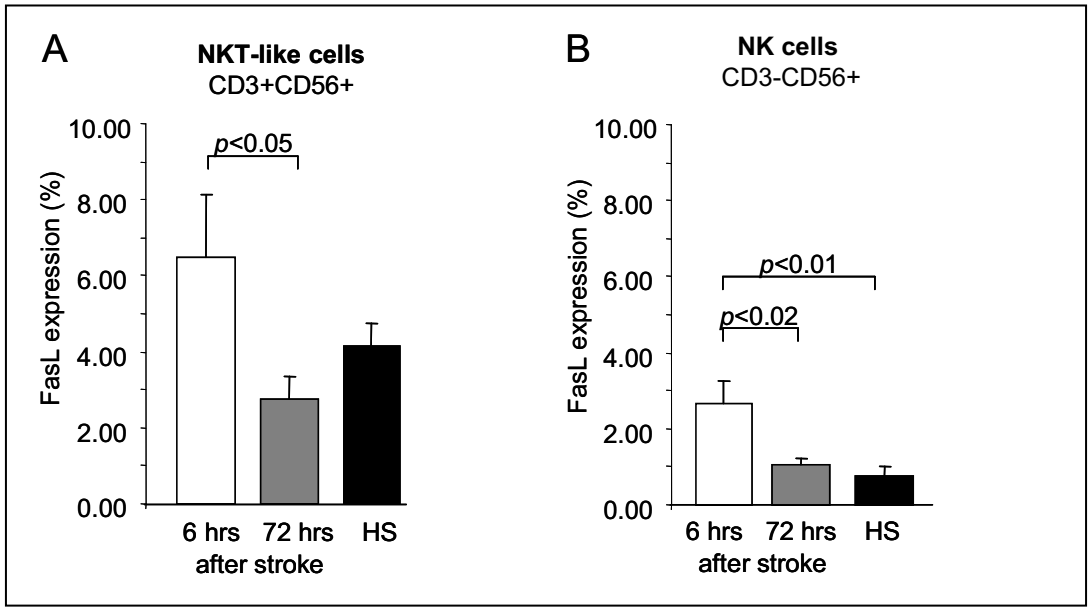

Figure 3. **FasL expression by innate lymphocytes in acute ischemic stroke.** *Surface FasL expression by $CD3^+CD56^+$ NKT-like (A) and NK ($CD3^-CD56^+$) (B) in the peripheral blood of acute ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and those of healthy subjects is indicated. HS: healthy subjects, mean±SE is shown.*

NK cytotoxicity. Since we observed significant alterations in expression of mediators of major cytotoxic pathways, we analyzed functional changes of NK cytotoxicity in acute ischemic stroke. Significantly increased percentages of lysed target cells in samples acquired after 72 hours of stroke compared to the 6-hour samples were found in each experimental effector-to-target cell ratio (40:1, 20:1 and 10:1) (**Figure 4**).

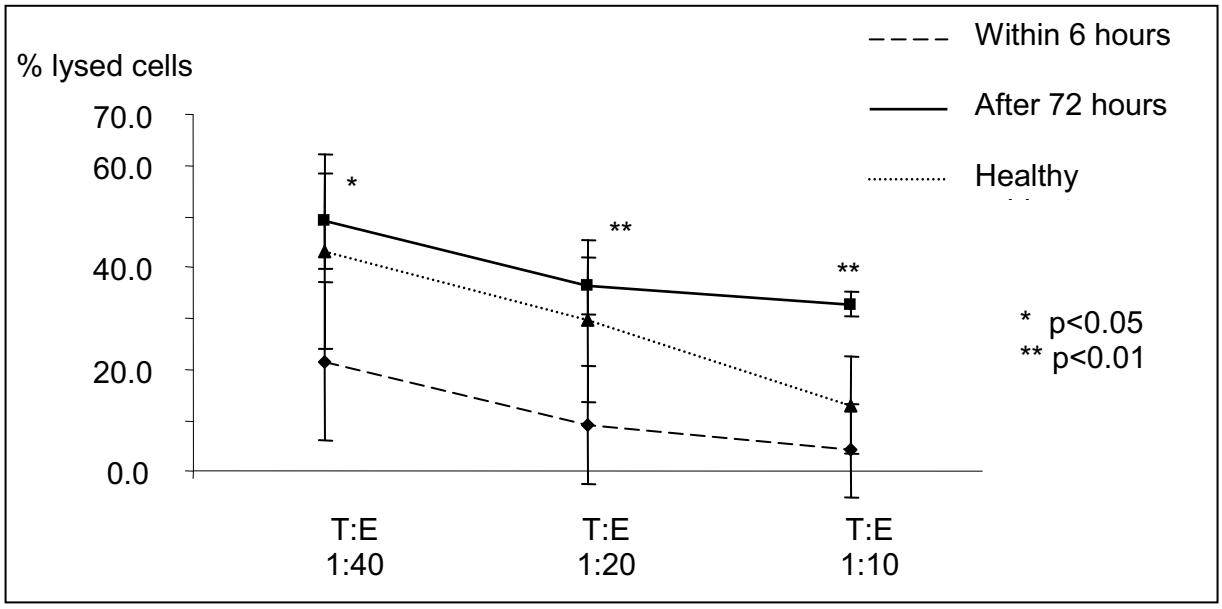

Figure 4. **NK cell cytotoxicity in acute ischemic stroke.** *Cytotoxic activity of NK cells as a percentage of lysed cells is indicated in ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and that of healthy subjects, at different target and effector cell ratios. T=target cell, E=effector cell, mean±SE is shown.*

DISCUSSION

Though the nature of immunological changes due to acute ischemic stroke is not fully described and understood, they seem to occur rapidly, within hours after the cerebrovascular attack. Therefore, in the present study, we analyzed rapid changes in immunological functions of innate lymphocytes, which are capable of rapidly producing cytokines influencing subsequent adaptive immune responses, and performing cytotoxicity. We particularly focused on γδT cells, $CD3^+CD56^+$ NKT-like cells and $CD3^-CD56^+$ NK cells.

We found that counts of innate T lymphocytes and NK cells were consistent in the acute phase of stroke, and did not differ from control subjects. Similarly, a recent report has not found any alterations in NK cell number, only adaptive T lymphocyte subsets showed a decreased percentage in stroke.[12] In contrast to T cells participating in adaptive immune responses, number and percentages of innate T lymphocytes were found to be unaltered here. However, extensive functional changes of these cell types were revealed. Cell counts and percentages do not always reflect faithfully the underlying immunological changes, and functional changes may occur without differences of cell counts and percentages even in cell subsets with regulatory functions.[35]

Rapid cytokine production is one of the major mechanisms to influence and regulate subsequent immune responses. Therefore, we analyzed pro- and anti-inflammatory cytokine expression and production in two different ways: expression of intracellular IFN-γ production and anti- and pro-inflammatory cytokine levels in supernatants of isolated innate lymphocyte cultures. Intracellular production of IFN-γ was reduced in all lymphocyte subsets, and NK cell supernatants showed decreased level of IFN-γ in the hyperacute phase of stroke. Such decreased IFN-γ expression and levels normalized by 72 hours. IFN-γ is a major Th1, pro-inflammatory cytokine with widespread functions in immune cell regulation, which plays a key role in the response to infectious agents.[36,37] The low IFN-γ production after stroke contributes to acute immunodeficiency in ischemic stroke. Similarly impaired T and NK cell responses, particularly a reduced IFN-γ production were described in a mouse model of focal cerebral ischemia.[10] Of note, production of anti-inflammatory cytokines (IL-4, IL-5, IL-10) was not altered in our patients, and expression of inducible co-stimulatory molecule (ICOS) connected to Th2 cytokine responses was also normal (*data not shown*). In a previous study, serum levels of IL-10 was normal as well, supporting our data obtained by stimulation of isolated innate lymphocytes.[12]

Beside cytokine production, we also examined a major effector pathway, alteration of cyotoxicity in the early phase of stroke. The defective cytotoxicity of NK cells within 6 hours from onset indicates a rapid loss of immune functions, and provides further evidence for the existence of stroke-induced immunodepression in humans. Such early deficiency of cytotoxicity correlated well with the decreased production of IFN-γ by NK cells. Since two major pathways are responsible for cytotoxicity,[28,29] we analyzed expression of these molecules on $V\delta2^+$ T, NKT-like and NK cells. Interestingly, expression of such mediators showed anti-parallel alterations: decrease of intracellular perforin expression after acute ischemic stroke correlates with decreased IFN-γ production in all three innate lymphocyte subsets and functional cytotoxicity in NK cells, but apparently not with increase of FasL expression in NK and NKT-like cells. However, perforin and FasL represent two different and independent mechanisms of cytotoxicity, and the processes of NK cell-mediated necrotic and apoptotic cytotoxic pathways mediated by perforin and FasL are differentially regulated by cytokines.[38] Thus expression of perforin and FasL may change inversely and the overall cytotoxic effect is a consequence of the two pathways together: early decrease in NK cytotoxicity and decreased IFN-γ production and perforin expression by all three subsets was characteristic of acute ischemic stroke.

Our data indicate functional impairment of innate lymphocytes in the hyperacute phase of stroke. Considering that innate lymphocytes provide acute defense against infections, particularly by production of IFN-γ and cytotoxicity, such early deficiency or its disregulated normalization may substantially influence susceptibility to infections similar to animal models of cerebral ischemia.[10,39]

Decreased number of T lymphocytes related to adaptive immune responses has recently been shown in the hyperacute phase of stroke but functional changes have not been addressed.[12] Here, we show that besides a decreased frequency of adaptive T cells, function of innate T lymphocytes and NK cells is also deficient in the early phase of stroke, similarly to animal models.[10] Such functional impairment of innate lymphocytes may complicate the reported decreased number of adaptive T lymphocytes in the early phase of stroke and contribute to susceptibility to post-stroke infections.[12]

Altogether, our study offers further evidence to support the development of stroke-induced early immunodepression in humans, suggesting the deficient function of innate lymphocytes. These cells may serve as potential new targets in treatments to avoid stroke-induced immunodepression and its possible consequences, infectious diseases. Controversial results of studies where prophylactic antibiotics were administered in the acute and postacute phase of stroke[40,41] highlight the importance of further investigations on the exact, detailed pathomechanism of the phenomenon and the search for new therapeutic targets.

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Paper 3

Deficient leukocyte antisedimentation is related to post-stroke infections and outcome

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Original article

Deficient leucocyte antisedimentation is related to post-stroke infections and outcome

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ABSTRACT

Background: Patients with stroke are more susceptible to infections, suggesting possible deficiencies of early immune responses, particularly of leucocytes.

Aims: To serially examine leucocyte antisedimentation rate (LAR), a simple test to detect activation of leucocytes, and correlate it with S100β, procalcitonin and outcome in patients with acute ischaemic events.

Methods: Venous blood samples were taken from 61 healthy volunteers and 49 patients with acute ischaemic events (acute ischaemic stroke (AIS), n = 38; transient ischaemic attack (TIA), n = 11) within 6 hours, at 24 and 72 hours after onset of symptoms.

Results: LAR was significantly higher in acute ischaemic events compared to controls within 6 hours after onset of stroke regardless of post-stroke infections. In addition, the increase of LAR was delayed and attenuated in TIA in contrast to AIS. A deficiency in early increase of LAR was associated with post-stroke infections and a poor outcome, measured by the Glasgow Outcome Scale in AIS. There was a positive correlation between LAR and S100β at 72 hours after the onset of ischaemic stroke. Increased levels of S100β at 24 and 72 hours after stroke were associated with poor outcome.

Conclusions: An early activation of leucocytes indicated by an increase of LAR is characteristic of acute ischaemic cerebrovascular events. A delayed and ameliorated leucocyte activation represented by LAR is characteristic of TIA in contrast to stroke. Deficient early activation predisposes to post-stroke infections related to poor outcome. In addition, the extent of tissue injury correlates with the magnitude of innate immune responses.

Cerebrovascular diseases of acute onset, including ischaemic stroke, are associated with diverse immune responses. The post-ischaemic brain tissue per se promotes various inflammatory pathways.[1 2] In addition, infectious complications have been frequently reported within the first few days after stroke, resulting in an increased mortality rate and worse clinical outcome.[3–6] The high incidence of infections in stroke patients is likely to be a result of an impaired immune function similar to other life-threatening conditions.[7 8]

Leucocytes accumulate in the region of cerebral ischaemia in the early stage of stroke, within hours.[9 10] The neurological outcome was shown to be worse and the infarct larger in patients with severe polymorphonuclear leucocyte accumulation.[11] A significant correlation between stroke volume or stroke severity and an acute increase of white blood cells (WBC) in the peripheral blood have been reported.[12–14] Increased aggregation of peripheral leucocytes in the absence of WBC increase was also reported in major stroke.[13] Increased in vitro adhesive properties, activation of leucocytes indicated by increased plasma oxidation of adrenaline to adrenochrome, and higher plasma levels of cytokines and proteases have been shown in ischaemic stroke and transient ischaemic attack (TIA).[15–18] While these studies addressed the detrimental effect of leucocyte activation, it may also play a major role in defence against pathogens, and participate in combating infections. A deficient activation of leucocytes may thus contribute to an increased susceptibility to post-stroke infections.

We have recently established a simple test to examine activation of leucocytes by measuring upward floating in a tube during one hour of gravity sedimentation. The leucocyte antisedimentation rate (LAR) indicates the percentage of leucocytes crossing the middle line of the blood column upwards during 1 hour of sedimentation.[19] It has been shown that a raised LAR is positively correlated with enhanced leucocyte adherence proportional to activation, with an increased cell volume and higher vacuole content of polymorphonuclear leucocytes.[20] LAR correlated significantly with serum procalcitonin (PCT) and C-reactive protein (CRP) concentration in critically ill patients with severe sepsis.[21] The simple LAR test was a good positive predictor of bacteraemia in critically ill patients presenting their first febrile episode without preceding antibiotic treatment.[22 23]

Here, we serially examined LAR, S100β and PCT in patients within hours after onset of acute ischaemic stroke and TIA to characterise the innate immune response and its relation to post-stroke infections. S100β is an astroglial protein, which is increased in the peripheral blood in patients with stroke; its peak concentration around day 3 after stroke correlates with the volume of brain lesions and clinical outcome.[24–29] Increased PCT levels suggest an infectious origin of fever complicating an underlying non-infectious febrile disease.[30] While the prognostic value of serum PCT concentrations is not clear in stroke, its predictive value for post-stroke infections has been recently implicated.[31]

MATERIALS AND METHODS

The study protocol was approved by the regional local ethics committee.

Patients and samples

A total of 49 patients suffering from acute ischaemic cerebrovascular event and 61 matched healthy controls were selected for this study. All patients were admitted to the Department of Neurology, University of Pecs, Hungary. Exclusion criteria were infectious diseases, fever

<4 weeks before stroke, a raised WBC count, erythrocyte sedimentation rate (ESR), high-sensitivity CRP (hsCRP, cut-off value <10 mg/l) and PCT on admission (cut-off value <0.05 ng/ml), positive chest *x* ray, haemorrhagic stroke defined by an acute cranial CT scan, and declining to participate in the study. Clinical data were collected on admission and at 72 hours; outcome measures were obtained at discharge from hospital (table 1).

Glasgow Outcome Scale (GOS) at discharge from hospital or death were used as measures of clinical outcome. Venous blood samples were taken serially for measurement of LAR, S100β and PCT within 6 hours after onset of first symptoms (T0), and at 24 (T24) and 72 hours (T72). After 24 hours, patients were enrolled into a definitive acute ischaemic stroke (AIS) or transient ischaemic attack (TIA) group based on the persistency of clinical signs and cranial CT scan. Levels of PCT were immediately analysed. Blood samples taken for the analysis of S100β were centrifuged at 3000 *g* for 10 minutes. Supernatants were frozen and stored at −80°C until analysis. PCT was measured by automated fluorescence immunoassay (BRAHMS Kryptor, B.R.A.H.M.S., Berlin, Germany). Serum levels of S100β were examined by automated electro-chemiluminescent immunoassay (Liaison Sangtec 100 system, DiaSorin, Bromma, Sweden).

Diagnosis of AIS, TIA and detection of infectious complications

Patients with acute neurological deficits were evaluated by a certified neurologist within 6 hours of onset of symptoms. Cranial CT scan was performed acutely to exclude haemorrhagic stroke. TIA and definitive stroke were defined according to international guidelines.[32] An evidence-based guideline was followed to detect infectious complications (in short, physical and laboratory measures including WBC, ESR, hsCRP, PCT, fever, abnormal urine, chest *x* ray or positive cultures).[33]

Leucocyte antisedimentation rate

LAR was determined as described previously.[19 20] In short, Westergren blood sedimentation rate technique was modified for measuring leucocyte motion during gravity sedimentation of the whole blood. After one hour sedimentation, the leucocyte counts of upper and lower half sections were measured with an automatic cell counter (Coulter Counter CBC5, Coulter Electronics, Luton, UK). The formula $LAR = 100 \times (upper - lower)/(upper + lower)$ was then used to calculate the percentage of leucocytes that crossed the middle line of the sedimentation blood column upwards during one hour of sedimentation (normal range <10%).

Statistical analysis

Statistical calculations were performed using SPSS 11.0. Student t test, χ^2 test, Spearman correlation and Mann–Whitney U test were used. Data were presented as mean, 95% CI, median and interquartile range (IQR). A p-value <0.05 was regarded as significant.

RESULTS

Clinical data

Based on clinical and neuroimaging data, 38 patients were categorised into the AIS group and 11 patients into the TIA group. Eleven of 38 AIS were caused by embolism, either cardiogenic (n = 8) or arterial (n = 3). Thirty patients had large territorial and eight had multiple lacunar infarcts. All but one patient had an infarct in the anterior circulation. Control CT showed haemorrhagic transformation in a single case only. Post-stroke infectious complications occurred only in the AIS group, with a 10/38 (32%) rate on the 4–5th day as an average (8 cases with lower airway infection and 2 cases with urinary tract infection) (table 1). All interventions were done beyond 48 hours, except in one patient (bladder catheter in 7, nasogastric tube in 10, artificial ventilation in 5 patients).

Serial measurement of LAR in acute ischaemic stroke and TIA

LAR indicating activation of leucocytes was measured on admission within 6 hours, and at 24 and 72 hours after onset of stroke. LAR on admission was significantly higher in patients with acute ischaemic events, ie AIS and TIA, compared to healthy controls (AIS, median 0.342, IQR 0.229–0.426; TIA, median 0.14, IQR 0.069–0.182; vs control, median 0.060, IQR 0.024–0.088; $p<0.001$, $p = 0.007$, respectively) (fig 1A). In addition, LAR was significantly higher at T0 and T72 in patients with definitive stroke compared to patients with TIA (T0, AIS, median 0.342, IQR 0.229–0.426, vs TIA, 0.14, 0.069–0.182, $p = 0.008$; T72, AIS, median 0.463, IQR 0.264–0.552, vs TIA, median 0.255, IQR 0.147–0.388, $p = 0.012$) (fig 1B). To exclude the possibility that post-stroke infections in AIS were responsible for the early difference between the two subgroups, TIA was also compared to AIS without post-stroke infections (n = 28). Difference in median LAR at T0 remained significantly higher in the AIS group without post-stroke infections (median 0.338, IQR 0.240–0.406 vs 0.14, 0.069–0.182, $p = 0.01$) (fig 2A). Of note, no differences in WBC and serum levels of PCT on admission were observed when AIS and controls, or AIS and TIA were compared to each other; ESR also did not indicate infections (table 1).

Table 1 Demographic and clinical data of subjects

	Acute cerebrovascular event (n = 49)			
	AIS (n = 38)	TIA (n = 11)	Control (n = 61)	p Value
GOS at discharge*	3.1 (2.6–3.5)	4.3 (3.6–4.9)	NA	0.006
Length of stay (day)*	8.4 (6.7–10.2)	5.9 (4.0–7.8)	NA	NS
Infection rate†	10/38	0/11	NA	<0.001
Onset of infection (day)*	4.3 (3.47–5.13)	NA	NA	NA
PCT on admission (ng/ml)*	0.09 (0.06–0.11)	0.06 (0.02–0.09)	<0.05	NS
WBC count on admission (g/l)*	8.2 (7.3–9.1)	7.4 (6.6–8.1)	7.8 (7.3–8.4)	NS

*Median and interquartile range, comparisons by Mann-Whitney U test.
†χ^2 test.
AIS, acute ischaemic stroke; TIA, transient ischaemic attack; GOS, Glasgow Outcome Scale; WBC, white blood cell; PCT, procalcitonin; NA, not applicable; NS, not significant.

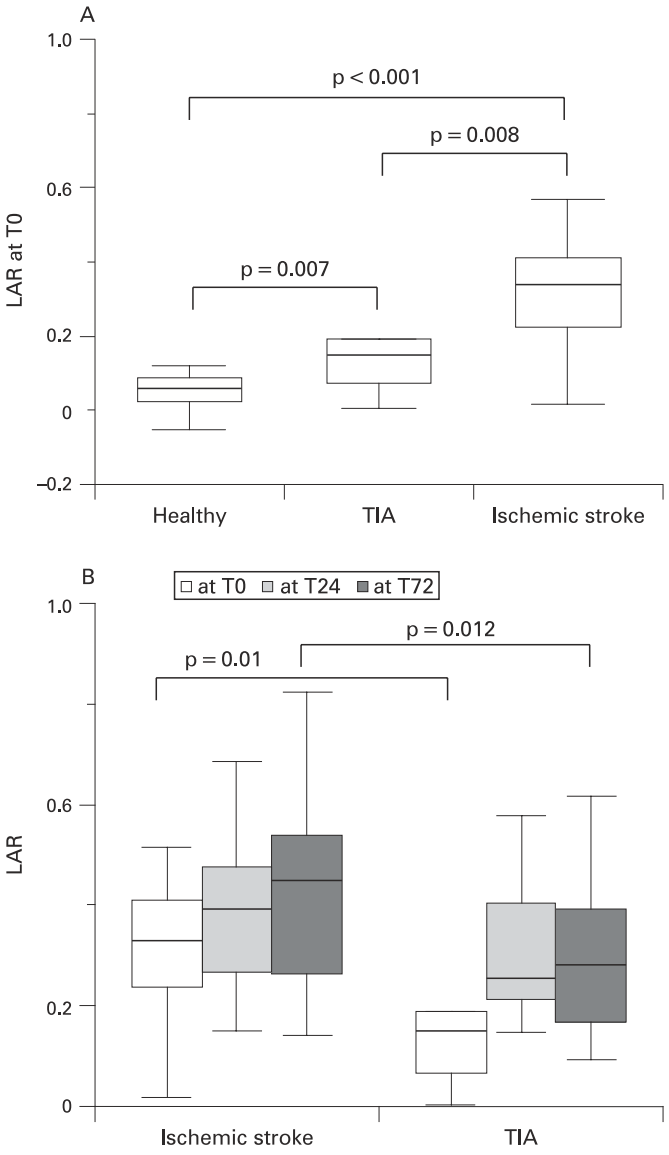

Figure 1 Leucocyte antisedimentation rate (LAR) in healthy controls, patients with transient ischaemic attack (TIA) and ischaemic stroke. (A) LAR measured at 6 hours after onset of symptoms in patients with definitive ischaemic stroke, TIA and healthy controls. LAR (median, interquartile range, minimum–maximum) was measured on admission, within 6 hours after onset of clinical symptoms in 38 patients with definitive ischaemic stroke, 11 patients with TIA and 61 healthy volunteers. Definitive stroke and TIA were determined retrospectively after 24 hours based on clinical and neuroimaging findings (Mann–Whitney U test was used). (B) Serial examination of LAR in patients with TIA and ischaemic stroke. LAR (median, interquartile range, minimum–maximum) was measured on admission (T0), at 24 hours (T24) and 72 hours (T72) after onset of symptoms in 38 patients with acute ischaemic stroke without infectious complications and in 11 patients with TIA (Mann–Whitney U test was used).

Serial measurement of LAR in patients with post-stroke infection and poor outcome

When LAR was serially analysed (T0, T24 and T72) in 38 patients with AIS, a continuous increase of LAR was observed (fig 1B). However, when patients with post-stroke infections were separately examined, a significant decrease of LAR was observed at 24 hours compared to AIS without infectious complications ($p = 0.028$) (fig 2A). Similar although non-significant reduction was also observed at 72 hours. These data may suggest that a deficient increase of LAR could be related to an increased risk of post-stroke infection due to a dysregulated activation of leucocytes. Therefore, we examined whether LAR, indicating activation of leucocytes, is related to the outcome of stroke measured by GOS, and compared AIS patients with poor and favourable outcome. AIS patients with favourable outcome had significantly higher LAR_{24} (median 0.436, IQR 0.273–0.531 vs median 0.262, IQR 0.236–0.312, $p = 0.02$) (fig 2B); the mean value of GOS in patients with post-stroke infections was

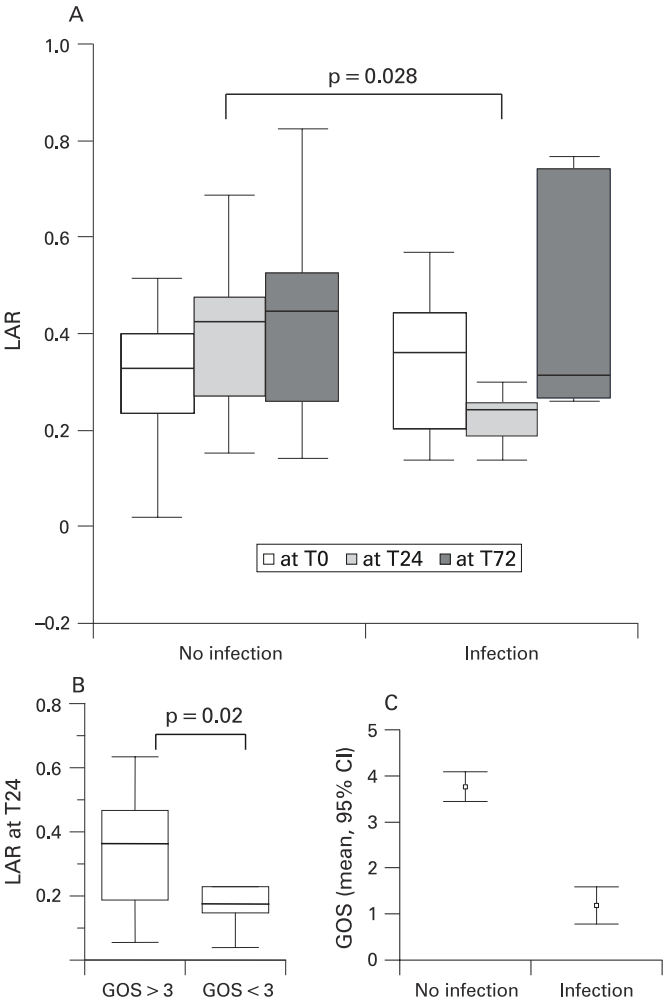

Figure 2 Leucocyte antisedimentation rate (LAR) in patients with ischaemic stroke related to post-stroke infections and clinical outcome. (A) Serial examination of LAR comparing patients with definitive stroke with and without post-stroke infections. LAR (median, interquartile range, minimum–maximum) was measured on admission (T0), at 24 hours (T24) and 72 hours (T72) after onset of symptoms in 28 stroke patients without post-stroke infection and 10 patients with infectious complications (Mann–Whitney U test was used). (B) Comparison of LAR measured at T24 in patients with good and poor outcome after definitive ischaemic stroke. LAR (median, interquartile range, minimum–maximum) was examined in 38 patients measured at 24 hours after acute ischaemic stroke in good (GOS>3) versus poor (GOS ⩽ 3) outcome defined by Glasgow Outcome Scale at hospital discharge (Mann–Whitney U test was used). (C) Clinical outcome of patients with acute ischaemic stroke. Glasgow Outcome Scale (GOS, mean, 95% CI) obtained at discharge from hospital or death in 28 patients without infection compared to 10 patients with infection after acute ischaemic stroke (Student t test, $p<0.001$).

significantly lower ($p<0.001$), indicating a poor outcome (fig 2C).

Relation of LAR to S100β

An important marker of stroke outcome is S100β, which is also related to the extent of infarct.[24–29] Median S100β was significantly higher among patients with definitive stroke at 24 hours (median 0.218, IQR 0.09–0.75, $p = 0.001$) and 72 hours (median 0.228, IQR 0.075–1.035, $p = 0.002$) compared to S100β on admission (median 0.10, IQR 0.06–0.22), but was not increased in patients with TIA. In addition, serum S100β protein at T24 and T72 was significantly higher in AIS patients with poor outcome ($GOS \leqslant 3$) compared to good outcome ($GOS > 3$) (T24, median 0.393, IQR 0.13–1.69 vs median 0.121, IQR 0.08–0.24, $p = 0.01$; T72, median 1.35, IQR 0.20–2.01 vs median 0.136, IQR 0.06–0.27, $p = 0.014$, respectively) (fig 3A). When LAR and serum levels of S100β were compared in patients with definitive stroke, a weak, but positive correlation was found on the 3rd post-stroke day (regression analysis with a Spearman rank correlation coefficient of $r = 0.457$, $p<0.05$) (fig 3B). These data may indicate that increased levels of S100β correlate with high LAR at T72, suggesting that bigger extent of infarct may be related to increased activation of leucocytes.

DISCUSSION

LAR was previously reported in the critical care setting as a novel method for detecting activation of circulating leucocytes that can be the earliest sign of systemic inflammatory response syndrome.[22] Here, we found that an early activation of leucocytes occurs during acute cerebrovascular events, since patients with both definitive stroke and TIA had significantly higher LAR compared to healthy controls as early as 6 hours after the onset of symptoms. In addition, the increase of LAR was proportional to the extension of brain damage: first, LAR was significantly higher in individuals with definitive stroke compared to TIA within 6 hours after onset of symptoms; in addition, measured on the third post-stroke day, LAR correlated with serum levels of S100β, a marker of infarction size.[24–29] Indeed, a possible association between leucocyte activation within the brain and stroke volume has been shown previously.[11] In the peripheral blood, association between leucocyte aggregation and infarct size has also been shown, similar to our data.[13] Despite the functional abnormalities, we did not find an increase of WBCs. A similar alteration of leucocyte functions without any change in number has been reported, but increased leucocytes has also been found in the acute phase of stroke.[12–16]

In the TIA group, we detected a significant increase of LAR compared to healthy controls, but this was delayed and attenuated compared to AIS. We considered the possibility that infectious complications in the AIS group might be responsible for the difference between TIA and AIS. However, such early activation of leucocytes was unlikely to be related to infections 4 days later in AIS. Indeed, when patients with post-stroke infections were excluded from the AIS group, the early difference regarding LAR still remained significant between AIS and TIA. Thus, an early activation of leucocytes represented by an increase of LAR within 6 hours after onset of stroke was ameliorated in TIA compared to AIS regardless of post-stroke infections.

Previous reports emphasised the detrimental effect of leucocyte activation in stroke, supported by the association between infarct size and leucocyte infiltration in the ischaemic tissue.[10 11] Accordingly, the magnitude of the increase of LAR also correlated with serum concentration of S100β. However, activation of leucocytes in the systemic circulation may also reflect the mobilisation of innate immune responses following tissue damage, which may serve to combat infections by mounting a pro-inflammatory response. Ideally, a delicate balance between detrimental and beneficial effects of early innate immune responses should be required to reduce tissue damage but also fight infections in the acute phase of stroke.[34] In our cohort, a progressive increase of LAR was detected within the first 72 post-stroke hours if patients with post-stroke infections were excluded from the analysis. In contrast, a deficient increase of LAR at 24 hours was found in those patients, who became febrile on the 4th–5th days with apparent clinical and laboratory signs of either respiratory or urinary tract infections. A similar, but non-significant defect in the increase of LAR was also seen at 72 hours. This may refer to a dysregulated activation of leucocytes providing insufficient barrier to bacterial infections.

Although PCT was measured serially in order to detect early infections in our cohorts, we also analysed its relation to definitive stroke, TIA, LAR and S100β, since PCT has been proposed as an indicator of systemic inflammatory response in non-infectious situations.[35 36] We did not find any difference in PCT levels measured at 6, 24 and 72 hours after stroke in any subgroup analyses, except that a slight increase of PCT at 72 hours indicated subsequent post-stroke infections. Similar data regarding PCT have been reported by others.[31]

In summary, although the number of recruited patients is relatively small, our pilot prospective study suggests several important messages: (1) activation of leucocytes represented by increase in LAR happens within hours after ischaemic stroke; (2) although LAR was increased in TIA compared to healthy subjects, it was delayed and ameliorated compared to definitive ischaemic stroke; (3) decreased activation of leucocytes reflected by a deficient increase of LAR may predispose to post-stroke infections and predict worse outcome; and (4) a positive correlation exists between LAR and S100β on the third post-stroke day, indicating a relationship between extent of infarct and innate immune responses.

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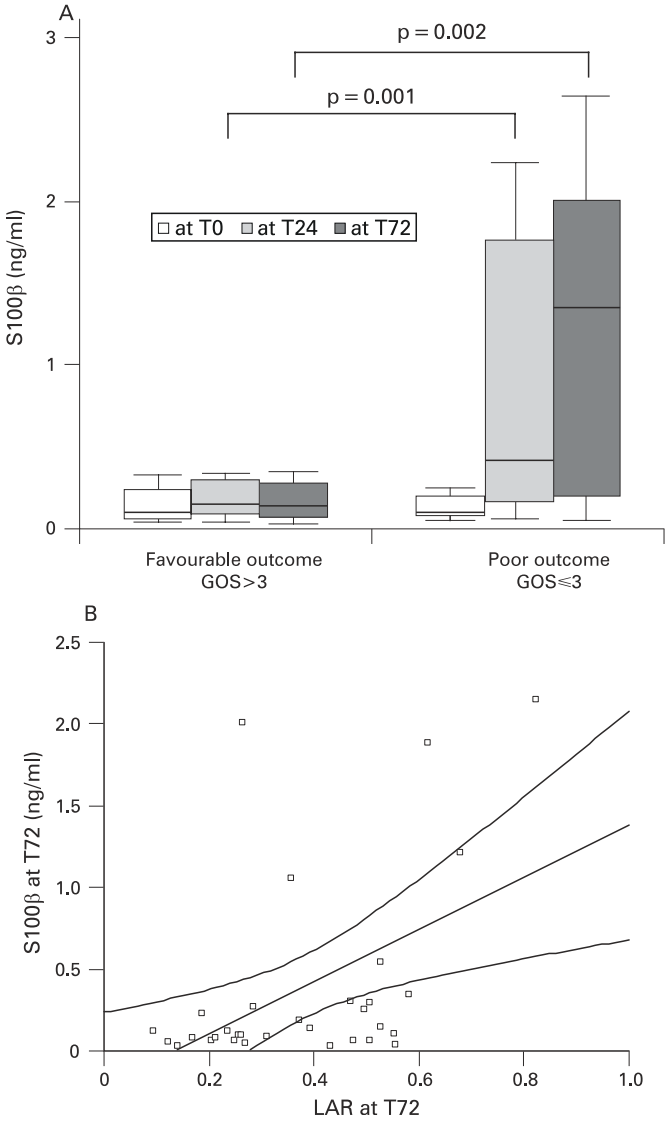

Figure 3 Correlations of S100β with outcome measures and leucocyte antisedimentation rate (LAR). (A) Comparison of serum S100β in patients with poor and good outcome after definitive ischaemic stroke. Plasma S100β protein concentrations (median, interquartile range, minimum–maximum) were examined in 38 patients measured on admission (T0), at 24 hours (T24) and 72 hours (T72) after acute ischaemic stroke in good (GOS>3) versus poor (GOS ⩽ 3) outcome defined by Glasgow Outcome Scale at hospital discharge (Mann–Whitney U test was used). (B) Association between LAR and S100β measured at 72 hours after onset of symptoms in patients with definitive stroke. Scatterplot of serum S100β level versus LAR measured at T72 was created in 38 patients after acute ischaemic stroke. A regression analysis with a Spearman rank correlation coefficient of r = 0.457 ($p<0.05$) is shown.

Take-home messages

- Within hours of ischaemic stroke systemic activation of leucocytes occurs.
- The magnitude and sequence of activation depends on whether stroke is transient or definitive.
- Activation of leucocytes positively correlates with the size of infarct.
- Deficient activation predisposes to post-stroke infection and poor outcome.

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Paper 4

Multiple sclerosis and the *CTLA4* autoimmunity polymorphism CT60: no association in patients from Germany, Hungary and Poland

(Mult Scler, 2008, 14:153-158)

RESEARCH PAPER *Multiple Sclerosis* 2008; **14**: 153–158

Multiple sclerosis and the *CTLA4* autoimmunity polymorphism CT60: no association in patients from Germany, Hungary and Poland

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Polymorphisms in the *CTLA4* gene region have been associated with susceptibility to autoimmune diseases. The recently described single nucleotide polymorphism *CT60*, located in the 3′ untranslated region of *CTLA4* is associated with Graves' disease, thyroiditis, autoimmune diabetes and other autoimmune diseases. A case-control association study was conducted in German, Hungarian and Polish multiple sclerosis (MS) patients and regional control individuals for the *CTLA4 CT60* and *+49A/G* polymorphisms. No significant association of these polymorphisms or respective haplotypes with MS was found. No association of *CT60* genotypes with T cell expression of ICOS and CTLA-4 after *in vitro* stimulation was detected. *Multiple Sclerosis* 2008; **14**: 153–158. http://msj.sagepub.com

Key words: association; CTLA-4; genetics; multiple sclerosis; polymorphism

Introduction

Multiple sclerosis (MS) is a complex genetic disease in which susceptibility and resistance alleles determine the individual threshold for developing overt autoimmune disease (AID) [1]. These polymorphisms may be common variants and overlap between species and different AID. Prime candidates for shared autoimmunity genes are the MHC, *PTPN22* and *CTLA4* [2]. The *CTLA4* region has been mapped as a susceptibility region in mice (*Idd5.1*) and men (*IDDM12*) for various AID. *Idd5.1* influences both experimental autoimmune encephalomyelitis (EAE) and diabetes in mice in an opposite way and is associated with differential expression of inducible costimulator (ICOS) and a murine splice variant of cytoxic T-lymphocyte-associated antigen-4 (CTLA-4) (ligand-independent [li]CTLA-4) [3–5]. In humans, a polymorphism in the 3′UTR of *CTLA4* (*CT60*) has been shown to be associated with Graves' disease, autoimmune thyroiditis, diabetes and genotype-dependent regulation of a CTLA-4 splice variant (soluble [s]CTLA-4) [6]. Since then, association of the *CT60*G* allele with various AID has been reported (as examples [7–13]).

Polymorphisms in the *CD28/CTLA4/ICOS* gene cluster have been subject of intense investigation for association with MS. Lorentzen *et al*. found no association with MS across the CD28/CTLA-4/ICOS gene region [14]. In another study, two markers in the CTLA-4 gene region among 17 tested within 2q33 showed some weak association in Finnish MS families [15]. Recently, association of ICOS haplotypes with IL10-secretion and relapsing–remitting MS has

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been reported [16]. Among other polymorphisms of *CTLA4*, the *−318 C/T*, *+49A/G* and $(AT)_n$ repeat and their respective haplotypes have been intensely investigated showing inconsistent results so far (for combined analyses [17–19] and detailed overview [20]).

No association of MS with *CT60* has been reported so far in Australian, Norwegian and British patients [14,19,21]. However, in MS patients from Flanders, association with the *+49A/G*G-CT60*G* haplotype has been reported [20] and recently an association of the *CT60*G* allele with MS was detected in multicase MS families with a history of other autoimmune diseases [22].

In the present study a possible association of *CT60* with MS in patients from Germany, Hungary and Poland was investigated. Although the *CT60*G/G* genotype was consistently overrepresented in MS patients, no significant association was found. No genotype-dependent regulation of ICOS and CTLA-4 expression was detected.

Patients and methods

Patients

German MS patients were recruited at the Neurology Department, University of Tübingen. German controls were recruited from age and sex-matched participants of the Dortmund Health Study, a health survey of the general population in the west and from participants of the MEMO-Study (Memory and Morbidity in Augsburg Elderly) conducted in the south of Germany [23] as well as individuals from the Tübingen region. Polish MS patients were recruited at the Department of Neurology, Medical University of Lodz. Hungarian MS patients were recruited from the Department of Neurology, University of Pecs and Jahn Ferenc Teaching Hospital, Budapest. All patients were confirmed MS patients according to the Poser or McDonald criteria [24,25]. The study was approved by the local Ethics committees and patients gave informed consent prior to inclusion.

Genotyping

The *CT60* SNP was genotyped by the pyrosequencing method [26] on a PSQ™ 96MA System (Biotage AB, Uppsala, Sweden) and the *+49A/G* SNP was genotyped using a melting-curve-based analysis on a Light cycler® 480 system (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. DR2 typing was performed by PCR using DRB1*15/16-specific primers [http://www.ihwg.org/protocols/sbt/sbtprot.htm, chapter 10-A]. All nucleotide sequences are available upon request from the corresponding author.

ICOS and CTLA-4 expression analysis

Whole blood was subjected to density gradient separation as described previously [27]. Peripheral blood mononuclear (PBMC) were incubated at 5×10^6 cells per well in 24-well plates in 2 ml RPMI 1640 cell medium supplemented with 10% FCS, L-glutamine and penicillin/streptomycin, containing 5 μg/ml PHA for up to 48 h. Cells were stained with the following antibodies: anti-CD4-FITC, anti-ICOS-PE, anti CTLA-4-PE and the respective isotype controls (all from BD Pharmingen). Prior to CTLA-4 staining, cells were fixed and permeabilized (Cytofix/Cytoperm, BD Pharmingen, Franklin Lakes, NJ, USA). Dead cells were excluded after staining with 7-amino-actinomycin D (7-AAD). Flow cytometry was performed on a FACSsort (BD Biosciences, Mountain View, CA, USA) and data were analysed using FlowJo software (TreeStar, San Carlos CA, USA). Delta Mean fluorescence intensity (deltaMFI) was calculated as MFI (antibody) – MFI (respective isotype control).

Statistical analysis

Statistical analysis was performed using the JMP5 software package (SAS Institute, Cary, NC, USA). For haplotype analysis the Haploview software was used [28]. For meta-analysis the RevMan software (Version 4.2 for Windows. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003) was employed. χ^2 test was used to test for Hardy-Weinberg equilibrium.

Results

Association of the CT60 and +49A/G polymorphism with MS

In all populations, genotype counts did not deviate significantly from those expected under Hardy-Weinberg equilibrium for both SNPs.

No differences were found between genotypes and allele frequencies when patients and control individuals were compared within our three populations (Table 1). Since numbers of patients and control persons as well as allele frequencies differed substantially among populations, a population-stratified meta-analysis rather than pooling the samples for a combined analysis was performed. This meta-analysis showed no association of MS with *CT60* or +49A/G allele frequencies but an overrepresentation of the *CT60*G/G* genotype among MS patients from all three populations. However, this finding did not reach statistical significance (meta-analysis in 621 MS patients and

Table 1 Genotype and allele frequencies among German, Hungarian and Polish MS patients and control persons for the *+49A/G* and *CT60* SNP

+49AG		n	A/A	A/G	G/G	A	G
Germany	MS	200	0.38	0.45	0.18	0.60	0.40
	controls	469	0.38	0.46	0.15	0.62	0.38
Hungary	MS	193	0.33	0.50	0.17	0.58	0.42
	controls	91	0.35	0.53	0.12	0.62	0.38
Poland	MS	180	0.33	0.53	0.14	0.60	0.40
	controls	171	0.29	0.53	0.19	0.55	0.45
CT60		**n**	**G/G**	**A/G**	**A/A**	**G**	**A**
Germany	MS	227	0.31	0.52	0.17	0.57	0.43
	controls	473	0.31	0.49	0.20	0.55	0.45
Hungary	MS	206	0.37	0.47	0.16	0.61	0.39
	controls	141	0.30	0.57	0.12	0.59	0.41
Poland	MS	188	0.40	0.45	0.15	0.63	0.37
	controls	190	0.37	0.55	0.08	0.65	0.35

804 controls, odds ratio (OR) for the *CT60*G/G* genotype 1.13 [95% CI 0.90–1.42]).

Genotype frequencies were then included among MS patients and controls extracted or calculated from two previously published case-control association studies [14,19] into a meta-analysis. Combining these published data with the present data allowing the analysis of 1228 MS patients and 1440 controls, the OR was 1.12 [95% CI 0.95–1.31] for the association of the *CT60*G/G* genotype with MS (Figure 1). Previously published family-based linkage studies were not included into this analysis. Stratification of our genotype data for DR2 revealed an overrepresentation of the CT60*G/G genotype in both DR2+ patients and control individuals from Hungary and Poland (data not shown).

For haplotype association analysis only patients with known genotypes at both polymorphisms were included. No association with *+49A/G-CT60* haplotypes was detected, even after stratification of patients for DR2 (Table 2 and data not shown).

Information about the disease course was available in 528 patients (relapsing–remitting 67.0%, secondary-progressive 23.5%, primary-progressive 9.5%) and about disease progression (onset, disease duration, EDSS scores) in 435 German and Hungarian MS patients. No association of genotypes with age of onset, progression and disease course was found in these patients.

CT60 genotype-dependent expression of co-stimulatory molecules

CT60 genotype-dependent expression of sCTLA4 has been reported previously [6] and the mouse orthologous *Idd5.1* region is associated with differential surface expression of ICOS [4]. ICOS and

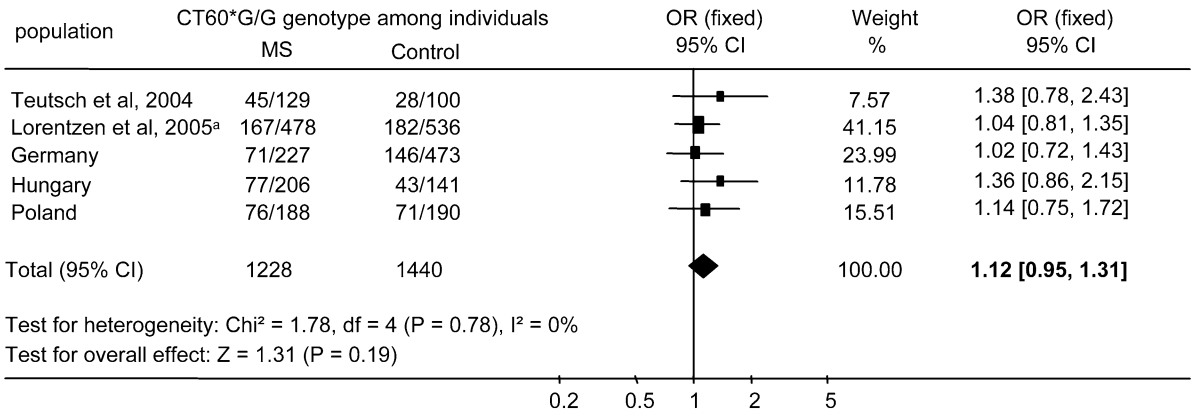

Figure 1 Meta-analysis for the *CT60*G/G* genotype of two previously published case-control association studies in Australian and Norwegian MS patients and data from German, Hungarian and Polish patients. Data from previously published family-based linkage studies are not incorporated. [a]genotype numbers were calculated by multiplying published genotype frequencies and numbers of MS patients and controls, respectively.

Table 2 *+49A/G-CT60* haplotype frequencies among German, Hungarian and Polish MS patients and control persons

	n		Frequency	
	MS/controls	Haplotype	MS	Controls
Germay	200/416	AA	0.417	0.408
		GG	0.389	0.340
		AG	0.186	0.214
		GA	0.008	0.038
Hungary	170/90	GG	0.384	0.383
		AA	0.361	0.366
		AG	0.227	0.245
		GA	0.028	0.006
Poland	178/171	GG	0.398	0.443
		AA	0.372	0.344
		AG	0.226	0.206
		GA	0.004	0.007

Except for the *GA* haplotype in German MS patients and controls ($\chi^2 = 8.7$; $P = 0.0031$) no significant differences were detected.

CTLA-4 expression in CD4+ T cells of healthy individuals with known *CT60* genotypes by flow cytometry was analysed. No significant differences were found in expression levels of these molecules *ex vivo* or after 24 and 48 h of *in vitro* stimulation (Figure 2).

Discussion

CTLA4 is a prime candidate for a shared autoimmune gene [2], and a recently described polymorphism in the 3′UTR of *CTLA4*, *CT60*, has been shown to be associated with different autoimmune diseases [6–13]. These observations are consistent with findings in the respective mouse models showing the association of the orthologous *Idd5.1* locus with autoimmune diabetes and EAE [4,29]. No consistent association of *CTLA4* with MS has been described so far and recent publications including genotyping data of *CT60* have been negative [14,19,21] except for one study demonstrating the *+49A/G*G-CT60*G* haplotype being associated with MS [20] and another demonstrating an association of *CT60*G* with MS in multicase MS families with a history of other autoimmune diseases [22]. In the present study, including MS patients from three different European populations, a significant effect of this polymorphism on susceptibility to MS or association with different disease courses, disease progression and onset of disease could not be demonstrated. A slight overrepresentation of the *CT60*G/G* genotype among MS patients was found, including all three populations and two previously published association studies, totaling 1228 MS patients and 1440 controls, into a meta-analysis.

In German, but not Hungarian or Polish MS patients, the frequency of the *+49A/G*G-CT60*G* haplotype was slightly higher compared with control persons, while a reverse pattern was found for the *+49A/G*A-CT60*G* haplotype. This correlates in part with the observation by Suppiah *et al.* [20]; however, our findings were not statistically significant.

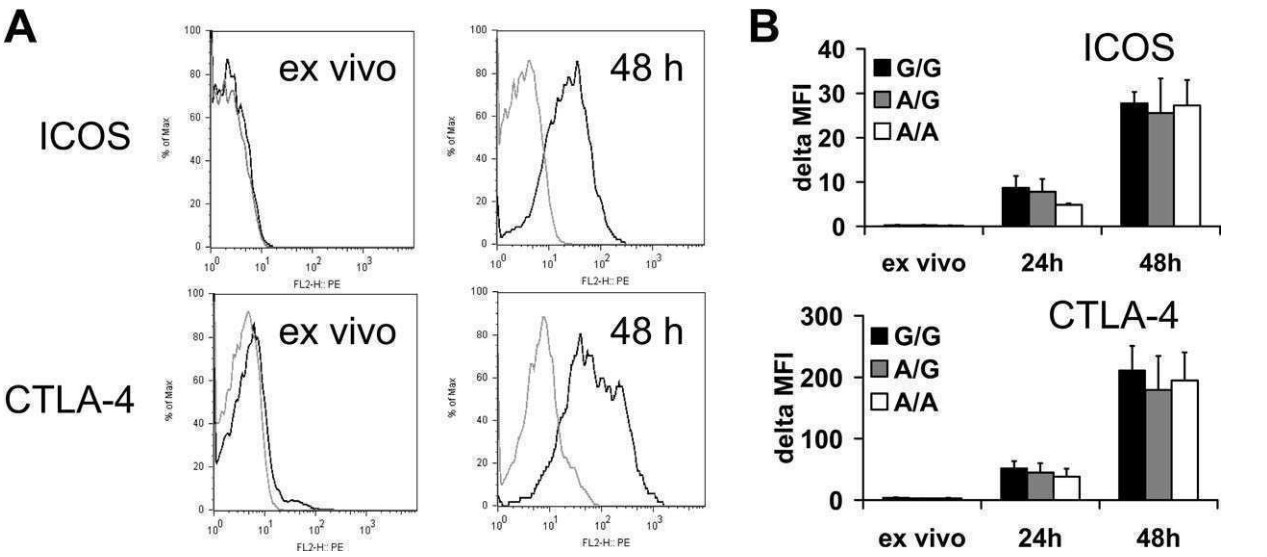

Figure 2 Expression of ICOS and CTLA-4 on $CD4^+$ T cells. PBMC were isolated and stimulated as described in the Methods section. **(A)** ICOS and CTLA-4 expression *ex vivo* and after 48-h stimulation with PHA. Shown are histograms of cells gated on $CD4^+7\text{-}AAD^-$ cells; grey line: isotype control, black line: anti-ICOS or anti-CTLA-4, respectively. **(B)** ICOS and CTLA-4 expression on CD4+ cells of individuals with different *CT60* genotypes, *ex vivo* and after 24 h or 48 h of stimulation with PHA *in vitro*. Data are mean delta MFI, SEM indicated (total $n = 4$ for each the G/G and A/G genotype $n = 3$ for the A/A genotype). No significant expression differences between individuals with different genotypes were found by ANOVA.

This might be due to differences in the respective study-populations. Furthermore, a family-based study might be more powerful than the present case-control approach to detect a weakly MS-associated haplotype [30].

Also, selection of a MS-subphenotype, defined by the presence of a family history of other autoimmune diseases and MS, seems to increase the power to detect an underlying common autoimmune-polymorphism, as demonstrated by Barcellos *et al.* [22]. The present study did not stratify the study population for such a subphenotype.

On the other hand, our negative findings regarding the *+49A/G* polymorphism match well with a meta-analysis on previously published studies examining this polymorphism in MS patients, showing no association in 2453 MS patients and 1851 control persons [19]. *CT60* genotype-dependent variation of mRNA levels of the sCTLA-4 splice variant has been shown previously [6]. However, these findings have been challenged later [31–33], showing no genotype-dependent differences in mRNA and protein levels of sCTLA. It has been shown that the orthologous mouse locus *Idd5.1* regulates ICOS expression on T cells in C57BL/10 congenic NOD mice [4]. Expression levels of a mouse-specific CTLA-4 splice variant (li-CTLA-4) are also dependent on genetic variation in this region [5]. Genotype-dependent expression differences were not found for ICOS and CTLA-4 in healthy individuals with known CT60 genotype.

In summary, it was not possible to demonstrate an association of this common autoimmune polymorphism with multiple sclerosis. However, this does not rule out a possible weak association, especially in a subpopulation of patients with an underlying autoimmune-prone genetic background.

Acknowledgements

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Paper 5

3'UTR C2370A allele of the IL-23 receptor gene is associated with relapsing-remitting multiple sclerosis

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3′UTR C2370A allele of the IL-23 receptor gene is associated with relapsing-remitting multiple sclerosis

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Abstract

The interleukin (IL) -23/IL-17 cytokine axis has been suggested to play an important role in the development of several autoimmune diseases including multiple sclerosis. Here, we compared the prevalence of C2370A single nucleotide polymorphism (SNP) in the 3′ untranslated region (3′UTR) of the IL-23 receptor (IL23R) between 223 patients with relapsing-remitting multiple sclerosis (RRMS) and 200 healthy controls. The A2370A genotype was significantly over-represented among patients with RRMS (10.8%) and RRMS exhibiting oligoclonal bands in the cerebrospinal fluid (12.9%) when compared to healthy subjects (5.50%). Multiple regression analysis revealed that presence of AA genotype provides a two-fold risk for the development of multiple sclerosis (OR = 2.072, 95% CI: 0.988–4.347, $p < 0.05$). These data indicate that *IL23R* represents a novel shared susceptibility gene as its association with inflammatory bowel disease (IBD) has recently been verified.

Keywords: Multiple sclerosis; IL-23; IL23R; IL-17; Shared autoimmunity gene

Cytokines play an important role in the initiation and maintenance of autoimmune diseases. Previous data emphasized the importance of IL-12 driving differentiation of naïve T cells into IFN-γ producing Th1 cells essential for cell-mediated immunity [4]. Since targeting molecules of the IFN-γ pathway in mice resulted in more severe autoimmune diseases, additional inflammatory cytokines, particularly IL-23 and IL-17 have recently been suggested to play an important role in the establishment of autoimmune diseases [7,11]. One of the Th17 differentiation pathways is IL-23-dependent and IL-23 is necessary for the survival and expansion of Th17 cells; this effect is mediated through the IL23R signaling pathway [6]. A genome-wide association study has recently identified several polymorphisms in the IL23R gene associated with inflammatory bowel disease (IBD). The C2370A allele in the 3′UTR showed one of the most significant associations with IBD [2]. Here, we examined the association of this SNP with multiple sclerosis.

Patients: MS patients were recruited at the Department of Neurology, University of Pecs and at Jahn Ferenc Teaching Hospital, Budapest, Hungary. All patients were confirmed MS patients according to McDonald's criteria [10]. Patients gave informed consent prior to inclusion, approved by the local ethics committee of University of Pecs. Altogether, 223 patients with relapsing-remitting MS were involved in the study (61 males, 162 females, mean age 41.1 ± 0.71 years). Forty-five patients have progressed into a secondary progressive phase following a relapsing-remitting course. Cerebrospinal fluid (CSF) was examined in 97 cases and 87.6% was positive for oligoclonal bands (OCB) determined by isoelectric focusing. The control population consisted of 200 clinically healthy subjects (110 males, 90 females, mean age 38.7 ± 0.81 years). None of the patients including those with predominantly optic and spinal cord symptoms (OS-MS) had Devic's disease according to the recently proposed criteria [14].

Genotyping: Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method. For the amplification of the target sequence the following primers were designed and used: 5′-ATCGTGAATGAGGAGTTGCC-3′ as

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the sense and 5′-TGTGCCTGTATGTGTGACCA-3′ as the antisense primer. Digestion by MnlI restriction endonuclease was used to determine C2370A (CA), C2370C (CC), and A2370A (AA) genotypes. Genotyping for HLA-DRB1*1501 was done in 216 cases (Olerup HLA SSP, GenoVision, Qiagen).

Statistics: Statistical analysis was carried out using Excel and SPSS 11.5 for Windows. We performed chi-square tests and regression analyses to uncover the associations between the SNP and MS. Odds ratios were calculated with 95% confidence intervals (95% CIs). All p-values refer to a two-sided test and $p < 0.05$ was considered significant. The examined parameters were the following: sex, disease subtype (relapsing-remitting and secondary progressive), presence of oligoclonal bands and carrying the HLA-DRB1*1501 susceptibility allele.

Genotyping data were in Hardy–Weinberg Equilibrium for C2370A SNP tested for Hungarian individuals. The AA genotype was significantly over-represented in the 223 MS patients compared to 200 healthy controls (10.8% vs. 5.50%, $p < 0.05$) (Table 1). Multiple regression analysis revealed that presence of AA genotype provides a two-fold risk for the development of multiple sclerosis (OR = 2.072, 95% CI: 0.988–4.347, $p < 0.05$). The AA genotype was also significantly over-represented among patients exhibiting OCB in the CSF ($n = 85$) compared to HS (12.9% vs. 5.50%, $p < 0.05$). Multiple regression analysis indicated a two-fold risk for the presence of oligoclonal bands in the CSF (OR = 2.554, $p = 0.03$). No such differences were observed when HLA-DR15-positive ($n = 84$) (10.7% vs. 5.50%, $p = 0.117$) or negative ($n = 132$) (11.4% vs. 5.50%, $p = 0.052$) subgroups were separated and compared to healthy subjects, respectively. In addition, the A allele frequency was not significantly different between MS patients and control individuals (31.2% vs. 29.8%). Nor did we find any significant differences when female patients ($n = 162$) were separately analyzed. Although the sample size was small ($n = 45$), no association was found with secondary progressive disease course.

Our data indicate that a genetic association exists between relapsing-remitting MS and the C2370A polymorphism of the IL23R gene. The susceptible genotype provides a two-fold risk for developing multiple sclerosis. This polymorphism was also associated with patients exhibiting oligoclonal bands in the CSF. The susceptibility was independent from HLA-DRB1*1501 positivity indicating that cytokine responses rather than presentation of certain autoantigens might be affected.

The IL-23/IL-17 axis has already been suggested to play an important role in MS. The number of IL-17 mRNA expressing mononuclear cells is increased in MS, especially in relapse and within the CSF [9]. Accordingly, elevated IL-17 transcripts have been reported in MS plaques [8]. Recent data also indicated an elevated secretion of IL-23 by dendritic cells (DC) and an increased IL-17 production by T cells from MS patients, which could be modulated to result in IL-10 production by DC [13].

IL-17 levels in the CSF had significant correlation with higher CSF/serum albumin ratio suggesting the role of IL-17 in the destruction of the blood–brain barrier [5]. Since IL-23 is necessary for the survival and expansion of Th17 cells mediated through the IL23R signaling pathway [6], we examined whether blood–brain barrier damage might be associated with *IL23R* polymorphism. The AA genotype was not associated with high CSF/serum albumin ratio in RR-MS, although it was significantly over-represented in our patients with OCB in the CSF. In addition, in patients with opticospinal MS (OS-MS), IL-17 levels were significantly increased in the CSF [5]. However, none of our 20 RR-MS patients with predominant opticospinal symptoms incompatible with the diagnostic criteria of Devic's disease exhibited an AA genotype, while CC and CA genotypes were equally represented [14].

Since association of the A2370A genotype with inflammatory bowel disease has recently been reported, our data also suggest that *IL23R* may represent a novel common autoimmunity gene shared by several distinct disorders, particularly MS and IBD [2]. Indeed, the A2370A genotype has been very recently shown to be associated with an increased risk for rheumatoid arthritis and psoriasis [1,3,12]. However, no association was found with scleroderma and SLE, suggesting that this particular polymorphism may be associated with diseases mediated by cellular rather than humoral immune responses [3,12].

Table 1
Prevalence of the 3′UTR C2370A single nucleotide polymorphism of the IL-23 receptor gene in MS patients and healthy controls

3′UTR C2370A SNP genotypes	Multiple sclerosis ($n = 223$)	Controls ($n = 200$)
CC	108 (48.4%)	92 (46.0%)
CA	91 (40.8%)	97 (48.5%)
AA	24 (10.8%)*	11 (5.50%)*
A allele frequency	31.2%	29.8%

* $p < 0.05$.

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Paper 6

Aberrant transcriptional regulatory network in T cells of multiple sclerosis

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Aberrant transcriptional regulatory network in T cells of multiple sclerosis

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Abstract

To identify the molecular network of the genes deregulated in multiple sclerosis (MS), we studied gene expression profile of purified $CD3^+$ T cells isolated from Hungarian monozygotic MS twins by DNA microarray analysis. By comparing three concordant and one discordant pairs, we identified 20 differentially expressed genes (DEG) between the MS patient and the genetically identical healthy subject. Molecular network of 20 DEG analyzed by KeyMolnet, a comprehensive information platform, indicated the close relationship with transcriptional regulation by the Ets transcription factor family and the nuclear factor NF-κB. This novel bioinformatic approach proposes the logical hypothesis that aberrant regulation of the complex transcriptional regulatory network contributes to development of pathogenic T cells in MS.

Keywords: KeyMolnet; Microarray; Monozygotic twin; Multiple sclerosis; T cells; Transcriptional regulation

Multiple sclerosis (MS) is an inflammatory demyelinating disease mediated by an autoimmune process that is triggered by a complex interplay of both genetic and environmental factors. MS twin studies showed that the concordance rate for monozygotic (MZ) twins is approximately 30%, while it is less than 5% for dizygotic (DZ) twins, suggesting the possible involvement of not a single but multiple susceptibility genes in development of MS [13]. However, most of the candidate genes reported previously have not well been validated. Because regulation of gene expression is controlled through the combinatorial action of multiple transcription factors that activate or repress transcription via binding to *cis*-regulatory elements of target genes, the gene network analysis is more important to clarify the complex autoimmune process underlying the pathogenesis of MS.

DNA microarray technology is an innovative approach that allows us to systematically monitor the expression of thousands of genes in disease-affected tissues and cells. The comprehensive gene expression profiling has given new insights into molecular mechanisms promoting the autoimmune process in MS [19]. By using this technology, we recently showed that interferon-beta (IFNβ) treatment elevates the expression of a set of IFN-responsive genes in highly purified peripheral blood $CD3^+$ T cells of relapsing-remitting MS patients [6]. IFNβ immediately induces a burst of gene expression of proinflammatory chemokines with potential relevance to IFNβ-related early adverse effects in MS [17]. T-cell gene expression profiling classifies a heterogeneous population of Japanese MS patients into four distinct subgroups that differ in the disease activity and therapeutic response to IFNβ [16]. The majority of differentially expressed genes in T cells between untreated MS patients and healthy subjects were categorized into apoptosis signaling regulators [15]. However, we could not exclude the possibility that the heterogeneous genetic backgrounds of the study population might affect gene expression data. In the present study, to identify the molecular network of the genes deregulated in MS, we performed microarray analysis of peripheral blood T cells isolated from the genetically homogeneous population of Hungarian MZ MS/MS and MS/healthy subject twins

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followed by gene network analysis using a novel bioinformatic tool. We focused highly purified $CD3^+$ T cells because autoreactive pathogenic and regulatory cells might be enriched in this fraction.

Hungarian MZ MS twins were followed up for 2–15 years by certified neurologists (ZI and CR) at the MS Clinic of Pecs and Budapest. After written informed consent was taken, $CD3^+$ T cells were isolated form heparinized peripheral blood of three pairs of concordant MZ MS twins (Families #l to #3) and a pair of discordant MZ MS/healthy subject twin (Family #4), as described previously [6,16,15]. All subjects are women with the mean age of 33 ± 5 years and the mean disease duration of 6 ± 5 years. MS was diagnosed following the established criteria [8]. The patients were clinically active, showed variable lesion distributions on MRI, and exhibited a typical relapsing-remitting clinical course, except for one patient in Family #3 with clinically isolated syndrome (CIS). The patients showed the mean Expanded Disability Status Scale (EDSS) score of 1.3 ± 0.8. The clinical profile of the patients is shown in Supplementary Table 1 online. To minimize the influence of confounding factors on gene expression, none of the patients have received corticosteroids, interferons, glatiramer acetate, mitoxantrone or other immunosuppresive medications at least for 8 weeks before blood sampling. The present study was approved by the ethics committee of all institutes involved.

We utilized a custom microarray containing duplicate cDNA spots of 1258 well annotated genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, signal transducers, cell cycle regulators and housekeeping genes (Hitachi Life Science, Saitama, Japan) [6,16,15]. Five micrograms of total RNA isolated from $CD3^+$ T cells by using RNeasy Mini Kit (Qiagen, Valencia, CA) was *in vitro* amplified, and the antisense RNA (aRNA) was labeled with a fluorescent dye Cy5, while universal reference aRNA was labeled with Cy3. The arrays were hybridized at 62 °C for 10 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals.

Because microarray analysis generally produces a large amount of gene expression data at one time, it is often difficult to identify the meaningful relationship between gene expression profile and biological implications from available data. To overcome this difficulty, we conducted a novel bioinformatic approach to extract the molecular network that is the most relevant to the microarray data. The molecular network of the genes identified by microarray was analyzed by using the software named KeyMolnet originally developed by the Institute of Medical Molecular Design Inc., Tokyo, Japan (IMMD) [14]. KeyMolnet constitutes a comprehensive content database, composed of information on relationships among human genes, molecules, diseases, pathways and drugs, which have been carefully curated from selected review articles, literature, and public databases by expert biologists of IMMD. The contents are quarterly updated, composed of approximately 12,000 molecules in the version of April 1, 2007, and categorized into either the core contents collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of the PubMed database.

When DNA microarray data, i.e. the lists of either GenBank accession number or probe ID, were imported into KeyMolnet, it automatically provided corresponding molecules as a node on networks [7,14]. Among four different modes of search, the common upstream search method enables us to extract the most relevant molecular network composed of the genes coordinately regulated by putative common upstream transcription factors. The generated network was compared side by side with total 346 established canonical pathways of human cells pre-installed in KeyMolnet. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations, composed of approximately 90,000 different sets pre-installed in KeyMolnet (the version of 1 April 2007), and X = the sigma variable that defines incidental agreement [3].

$$\text{score} = -\log_2 \left(\sum_{x=O}^{\text{Min}(C,V)} f(x) \right)$$

$$f(x) = \frac{{}_C C_x \cdot {}_{T-C} C_{V-x}}{{}_T C_V}$$

By microarray analysis, we identified top 50 and 100 differentially expressed genes (DEG) in T cells between each MS twin pair numbered #1 to #4 described above. Then, we selected a panel of 34 genes that were listed in 50 DEG of the discordant twin (MS/healthy subject) of Family #4 but were not included in 100 DEG of the concordant (MS/MS) twin pairs of Families #l to #3. Among the 34 genes, we further extracted a set of 20 DEG by a cut-off point greater or equal to two-fold difference between the Family #4 pair. Then, to identify the molecular network with the most close relationship with 20 DEG, the gene list was imported into KeyMolnet (the version of 1 January 2007). This automatically extracted 43 genes directly linked to 20 DEG. The common upstream search of 43 genes illustrated a complex molecular network composed of 39 nodes (Fig. 1). By statistical analysis described above, the generated network showed the most significant relationship with gene regulation by the Ets transcription factor family presenting with the score of 10.2. This was followed by gene regulation by the nuclear factor NF-κB in the second rank (the score 9.0), the Myc/Mad family in the third rank (7.0), the IFN-regulatory factor (IRF) family in the fourth rank (6.0), and the estrogen receptor (ER) family in the fifth rank (4.7).

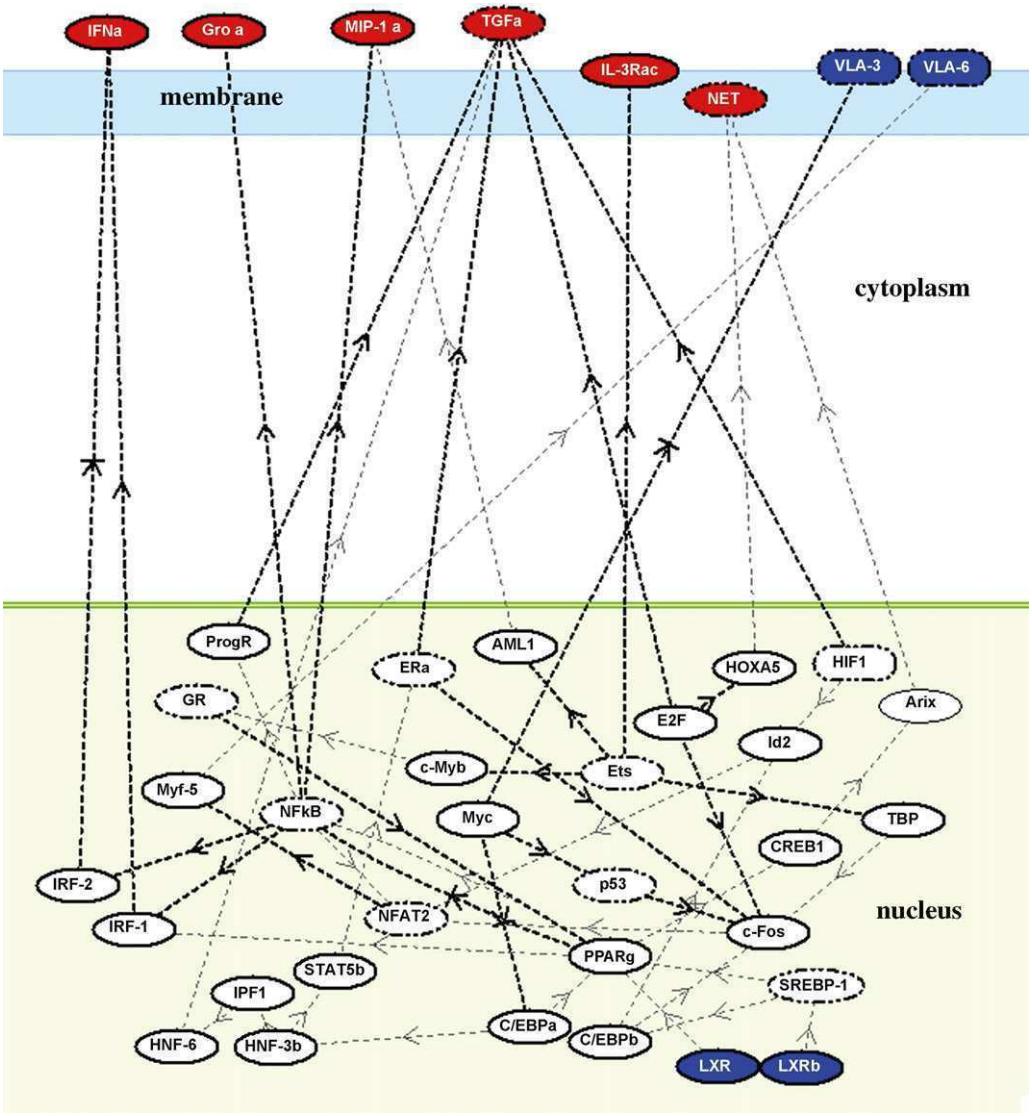

Fig. 1. The common upstream search of 20 differentially expressed genes in T cells of the discordant twin. The microarray data of 20 DEG in T cells differentilally expressed between the discordant MZ twin pair (Family #4) were imported into KeyMolnet that extracted 43 genes directly linked to 20 DEG. The common upstream search of 43 genes generated a molecular network composed of 39 nodes arranged according to the subcellular distribution. Red nodes represent upregulated genes, whereas blue nodes represent downregulated genes in MS. White nodes exhibit the genes automatically settled by KeyMolnet to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet.

The Ets family transcription factor, by interacting with various co-regulatory factors, controls the expression of a wide range of target genes essential for cell proliferation, differentiation, transformation, and apoptosis [18]. Ets-1, the founder member of the Ets family, is pivotal for survival and differentiation of T cells [10]. The Ets family transcription factors regulate the gene expression of autoimmune regulator (AIRE) that directs the expression of self-antigens in thymus potentially related to the autoimmune process of MS [9]. The NF-κB family, consisting of NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel, acts as a central regulator of innate and adaptive immune responses, cell proliferation, and apoptosis [1]. More than 150 target genes for NF-κB are currently identified [12]. A significant subset of NF-κB target genes, including proinflammatory cytokines TNFα and IL-1β, activate the expression of NF-κB, providing a positive regulatory loop that amplifies and perpetuates inflammatory responses [1]. Importantly, RelA, c-Rel, and p50 are overexpressed in macrophages in active demyelinating lesions of MS [4]. Furthermore, RelA expression is enhanced on oligodendrocytes that survive in the lesion edge [2]. Recently, we found that the expression of the orphan nuclear receptor NR4A2, a direct target gene of NF-κB, is elevated at the highest level in $CD3^+$ T cells of untreated MS patients [16,15]. Furthermore, targeted disruption of the NFKB1 gene confers resistance to development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [5]. *In vivo* administration of selective inhibitors of NF-κB activation protects mice from EAE [11]. All of these observations suggest the principal involvement of aberrant regulation of NF-κB in the pathogenesis of MS.

We have recently identified 286 genes differentially expressed in purified $CD3^+$ T cells between 72 untreated Japanese MS patients and 22 age- and sex-matched healthy subjects of heterogeneous genetic backgrounds [16]. When the list of 286 DEG was imported into KeyMolnet (the version of 1 April 2007), it extracted 456 genes directly linked to 286 DEG. The common upstream search of 456 genes illustrated a complex molecular network composed of 335 nodes (Supplementary Fig. 1 online). The generated network showed the most significant relationship with gene regulation by NF-κB (the score 13.0), IRF (12.3), LRH-1 (12.0), BLIMP-1 (11.4), and Ets (7.4) transcription factor families. Thus, these observations suggested again the central role of aberrant transcriptional regulatory network that involves NF-κB, Ets, IRF and other transcription factors in development of pathogenic T cells in the genetically heterogeneous population of Japanese MS patients.

Although the sample size in the present study is fairly small because of limited availability of the samples of MZ MS twins, the novel bioinformatic approach proposes the logical hypothesis that aberrant regulation of the complex transcriptional regulatory network might contribute to development of pathogenic T cells in MS. This hypothesis warrants evaluation by using a large cohort of the genetically identical MS and healthy subject twins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2007.05.056.

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V. SUMMARY OF THESES

1. MAIT and NKT cells are the only known invariant T cells infiltrating brain and kidney tumors.
2. Since NKT cells are present in CNS tumors, their absence in MS plaques is disease-specific and not related to the CNS environment.
3. MAIT cells may have a pro-inflammatory subset, which infiltrates tumors.
4. Brain and kidney tumors differ in infiltrating T cell and MAIT cell subsets: brain tumor infiltrating MAIT cells may express additional TCRβ to Vβ2 and Vβ13.
5. MAIT and NKT cells in tumors do not express CD56 although both $CD56^+$ and $CD56^-$ subsets are present in the peripheral blood even in patients with cancer.
6. The percentages of particular innate lymphocytes, Vδ2, NKT-like and NK cells do not change in the acute phase of ischemic stroke in contrast to the reported decrease of adaptive T cells.
7. In contrast to unaltered frequency, an acute functional deficiency of innate lymphocytes occurs in the acute phase of ischemic stroke, within 6 hours: pro-inflammatory cytokine production, expression of perforin and NK cytotoxicity are decreased, while there is no change in production of Th2 cytokines and Th2-related ICOS expression. We may hypothesize that such early deficiency or its disregulated normalization may substantially influence susceptibility to infections similarly to animal models of cerebral ischemia.
8. Activation of leukocytes represented by elevation in LAR happens within hours after onset of ischemic stroke.
9. Although LAR was elevated in TIA compared to healthy subjects, it was delayed and ameliorated compared to definitive ischemic stroke.
10. Decreased activation of leukocytes reflected by a deficient elevation of LAR may predispose to post-stroke infections and predict worse outcome.
11. Concept about uniformly harmful post-ischemic role of leukocytes should be changed and dissected: recruitment of leukocytes to the ischemic brain may be damaging by amplifying brain injury. However, systemic activation of leukocytes plays an important role in preventing post-stroke infections.
12. A positive correlation exists between LAR and S100β on the 3^{rd} post-stroke day indicating a relationship between extent of infarct and innate immune responses.

13. There is no significant effect – if at all – of the *CT60*G* allele on susceptibility to MS compared to other autoimmune diseases. No genotype-dependent expression differences in CTLA-4 and ICOS by T cells were found. Taken together, our data, together with previously published studies, suggest lack of association of this common autoimmune gene with multiple sclerosis.

14. A genetic association exists between RRMS and the C2370A polymorphism (AA) of the *IL23R* gene: the susceptible genotype provides a two-fold risk for developing MS.

15. *IL23R* may represent a novel shared autoimmunity gene.

16. Aberrant regulation of the complex transcriptional regulatory network contributes to the development of pathogenic T cells in MS.

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