T CELL RESPONSES IN NEUROLOGICAL DISORDERS

PhD Thesis

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ABBREVIATIONS

ADCC: antibody-dependent cell-mediated cytotoxicity

APC: antigen presenting cell

CBA: Cytometric Bead Array

CD: cluster of differentiation

cDNA: complementary DNA

CDRs: complementarity-determining regions

CIDP: chronic inflammatory demyelinating polyneuropathy

CIDS: central nervous system injury-induced immune deficiency syndrome

CNS: central nervous system

CRIM: cross-reacting immunologic material

CT: computer tomograph

DN: double negative

DNA: deoxyribonucleic acid

ERT: enzyme replacement therapy

FACS: fluorescence-activated cell sorting

FVIII: factor VIII

FasL: Fas ligand

FITC: fluorescein isothiocyanate

αGalCer: α-galactosylceramide

GAA: acid α-glucosidase

GOS: Glasgow Outcome Scale

ICOS: inducible co-stimulatory molecule

IFN: interferon

Ig: immunoglobulin

IL: interleukin

iNKT: invariant natural killer T

mAb: monoclonal antibodies

MAIT: mucosal-associated invariant T

MHC: major histocompatibility complex

mRNA: messenger RNA

MS: multiple sclerosis

NIHSS: National Institutes of Health Stroke Scale

NK: natural killer

NKT: natural killer T

PB: peripheral blood

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PE: phycoerythrin

PMA: phorbol-myristate-acetate

PNS: peripheral nervous system

RCC: renal cell carcinoma

rhGAA: recombinant human α-glucosidase

RNA: ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SEM: standard error of the mean

SSCP: single-strand conformation polymorphism

TCR: T cell receptor

Th: T helper

TIA: transient ischemic attack

TNF: tumor necrosis factor

Treg: regulatory T cell

1. INTRODUCTION

1.1 The immune system

The major function of the immune system is to recognize colonizing microbes, infected cells and tumors and professionally extinguishes them, while maintains the body's integrity. The immune response provides defense against infectious agents, i.e. bacteria, parasites, fungi and viruses; against non-infectious foreign substances, macromolecules, such as proteins and polysaccharides that are recognized as foreign and also against tumor cells. The immune system involves special organs, a great number of cells, sets and subsets, molecules responsible for immunity – all organized into a complex communication network.[1,2]

The two functional parts of the immune system – the innate and adaptive immunity – mediate the protection against foreign cells and substances. The innate immune system (also called natural or native immunity) involves cells and mechanisms that defend the host against pathogens and tumor cells by a non-specific, early, rapid, first line of protection: 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 many of the activities of the cells of innate immunity. The adaptive immune system provides specific immune responses against microbes, is activated by the innate immunity and has immune memory. This response, that takes days to develop on the first interaction with a pathogen, produces a stronger and faster immune response on subsequent exposures to the same antigen.[3-5]

NK cell is a type of cytotoxic lymphocyte that constitutes a major component of the innate immune system. NK cells play an important role in the rejection of tumors and cells infected by viruses. They are able to lyse target cells through the different mechanisms of cytotoxicity and produce rapidly abundant cytokines, mainly interferon-gamma (IFN-γ). They express the surface marker CD56 in humans and do not express the antigen-specific T cell receptor (TCR) or Pan T marker CD3.[6,7]

Perforin and Fas/FasL pathways are the two major mechanisms of cellular cytotoxicity.[8,9] Small granules in the cytoplasm of cytotoxic cells contain proteins such as perforin and serine proteases known as granzymes. Upon degranulation, the cytotoxic granules cause cell lysis by forming pores in cell membranes of target cells.[10] FasL produced by effector cells induces apoptosis through Fas, its receptor on target cell surfaces. Fas ligand (FasL) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Its binding with its receptor (Fas) induces apoptosis in the target cell through cell-surface ligand-receptor interaction.[11]

T and B lymphocytes are the most important cellular components of the adaptive immune system. The function of these cells is to recognize specific "non-self" antigens, during a process known as antigen presentation. They are liable for the two major features of the adaptive immune response, specificity and memory mediated by antigen-specific receptors.

The TCR is a molecule found on the surface of T cells and is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. The TCR is a heterodimer consisting of two different transmembrane protein chains. In 95% of T cells, this consists of an alpha- (α) and beta- (β) chain, whereas in 5% of T cells this consists of gamma- and delta- (γ/δ) chains, and they are covalently linked to each other by a disulfide bridge. Each α- and β-chain consists of one N-terminal variable (V) domain, one constant (C) domain, a hydrophobic transmembrane region and a short cytoplasmic region. The V domain of both the α- and β-chain of TCR have three hypervariable or complementarity determining regions (CDRs), which are responsible for the versatility of TCRs and antigen recognition. This diversity is a result of the rearrangement of antigen receptor coding genes. The TCR α-chain is generated by the recombination of V and J (joining) gene segments, whereas the β-chain is generated by the recombination of V, D (diversity) and J gene segments (both involve random joining of gene segments to generate the complete TCR chain). The three CDRs in the α- and β-chains form the part of the TCR that specifically recognizes peptide-MHC complexes (**Figure 1**).[1-4]

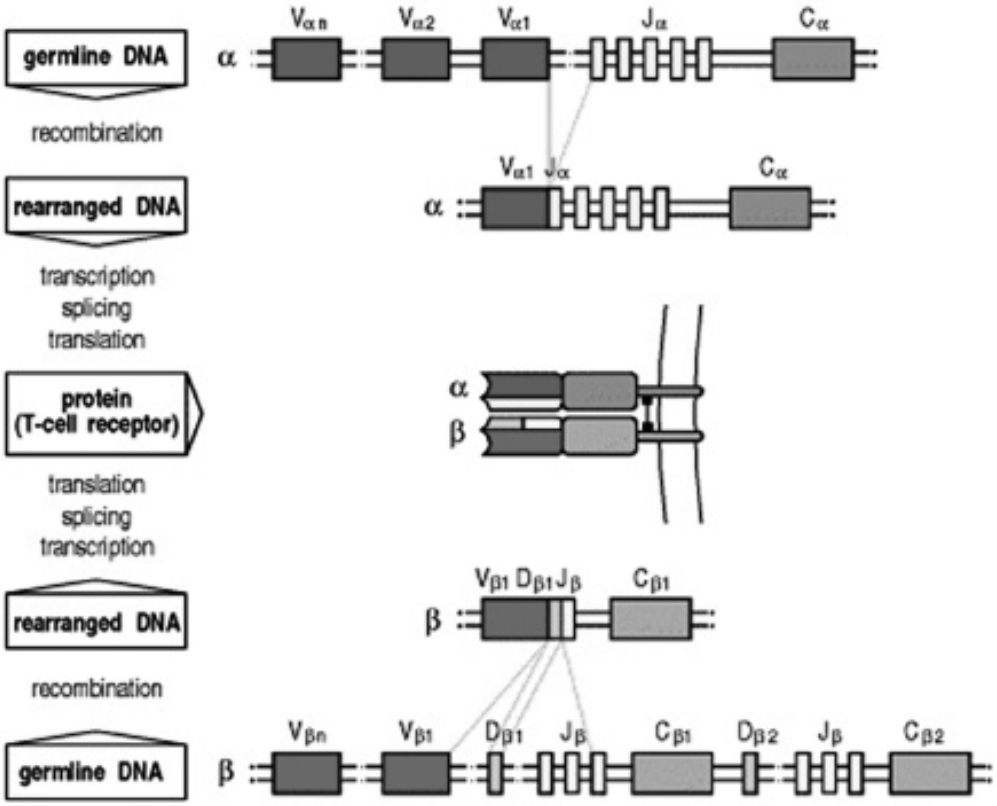


Figure 1. Rearrangement and structure of the T cell receptor[5]

Cytotoxic, $CD8^+$ T cells recognize the specific antigenic peptide produced in the cytosol of cells infected with viruses or intracellular bacteria and in tumor cells, and presented by MHC class I molecules on the surface of target cells: this activates the $CD8^+$ T cell to induce the death of the target cells. With an exception of some cell types, such as non-nucleated cells (including erythrocytes), MHC class I molecules are expressed on almost all cells. $CD4^+$ T cells are commonly divided into two distinct lineages: conventional T helper (Th) and regulatory T (Treg) cells. Th cells control adaptive immunity by activating other effector cells such as $CD8^+$ cytotoxic T cells, B cells and macrophages by production of different cytokines. Treg cells are able to suppress activation of potentially deleterious autoreactive Th cells. $CD4^+$ T cells recognize specific peptide antigens complexed with MHC class II molecules presented by professional antigen-presenting cells (**Figure 2 and 3**).[1,2]

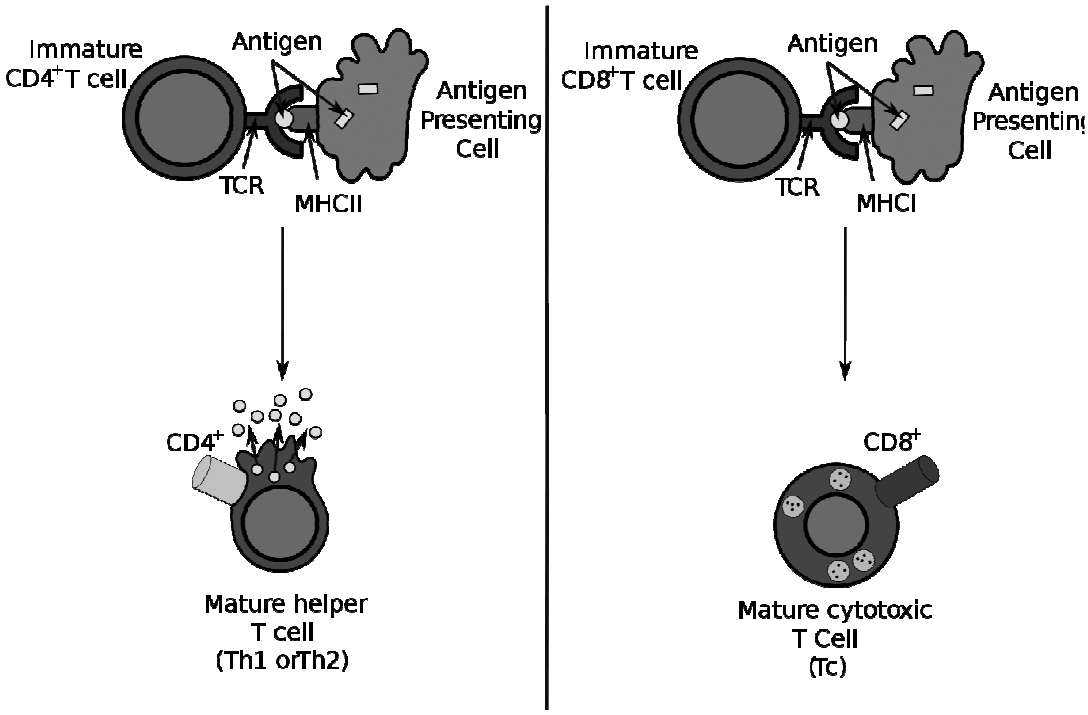


Figure 2. The antigen presentation to $CD4^+$ and $CD8^+$ T cells[5]

Upon activation, all T cells express the late activation marker CD25, the alpha-chain of the high-affinity IL-2 receptor. Among $CD4^+CD25^+$ T cells, only those with high fluorescence intensity of CD25 exert regulatory functions, while expression of low levels of CD25 may indicate T cell activation.[12]

Cytokines are small cell-signaling protein molecules secreted by the cells of innate and adaptive immunity. Th1 cells produce pro-inflammatory (Th1) cytokines, such as IFN-γ, TNF-α, that improve cellular immune responses: maximize the killing efficacy of the macrophages and the proliferation of cytotoxic $CD8^+$ T cells. Anti-inflammatory (Th2) cytokines, such as interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13, produced by Th2 cells trigger humoral immune response: stimulate B-cells into proliferation, induce B-cell antibody class switching and increase their neutralizing antibody production. Recently, another novel T helper subtype, the Th17 cells producing other pro-inflammatory cytokines, IL-23 and IL-17, have been suggested to participate in the induction of several organ-specific autoimmune diseases (**Figure 3**).[13,14]

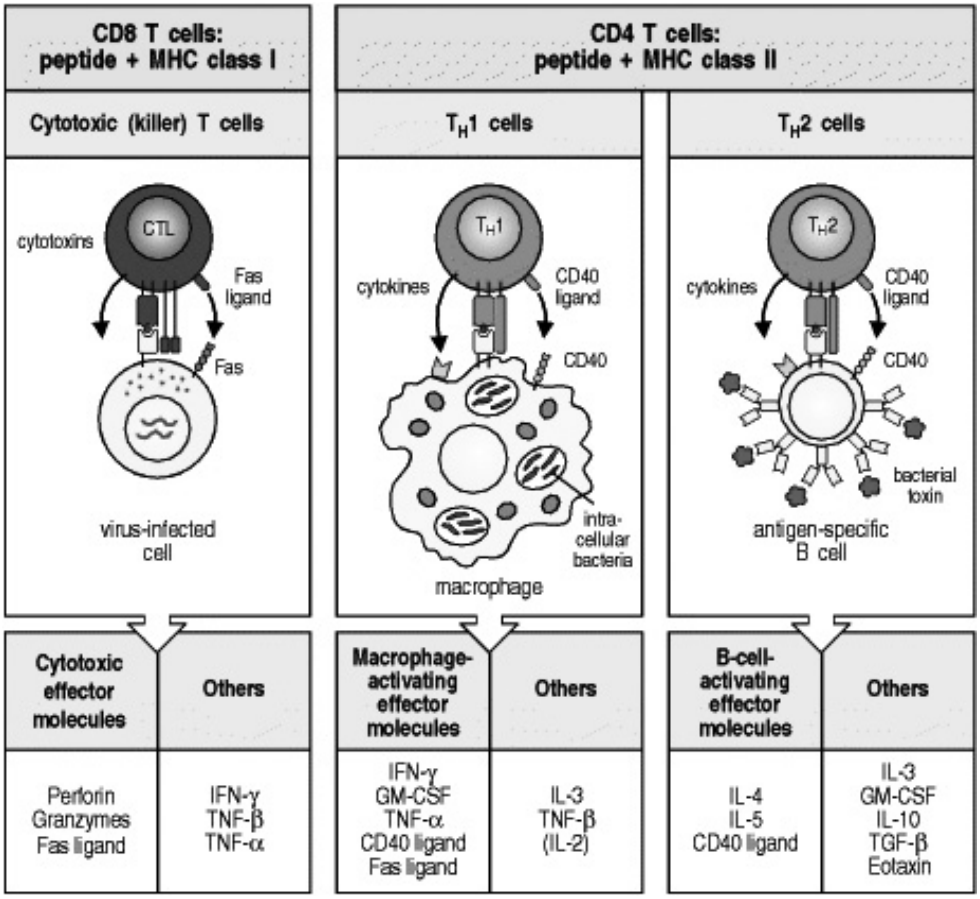


Figure 3. The three main types of effector T cell produce distinct sets of effector molecules[5]

A small subset of T cells, called γδT cells, express a TCR consisting of one γ- and one δ-chain. γδT cells are suggested to bridge the innate and adaptive immune responses. They quickly expand upon pathogen challenge after infection and are able to produce great amounts of IFN-γ, these are characteristic to cells of the innate immune system.[15,16] γδT cells are important immunoregulatory cells, play essential role in the defense against certain pathogens and in tumor immunity.[17] The Vδ2 subset, expressing a δ2TCR variable chain, represents the majority of adult γδT cells mainly with a cytotoxic property.[16,17]

Natural killer T (NKT) cells are a small subset of T cells that co-express αβTCR, but also express molecules specific to NK cells, such as CD56. This population, similarly to the γδT cells, is proposed to serve as a bridge between the innate and the adaptive immune systems. NKT cells have an important role in the regulation of the protection against autoimmunity and tumors. Murine NKT cells are

categorized 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 are characterized by limited repertoire diversity. They express an invariant Vα14-Jα18 variable region of the TCR and are either $CD4^+$ or $CD4^-CD8^-$ (double negative, DN). They recognize lipid antigens rather than peptides, and respond to these when presented by a non-classical MHC class I molecule, CD1d. The majority of NKT cells recognize a glycolipid, α-galactosyl ceramide (α-GalCer).[18,19] The human type I NKT cells expresses an invariant Vα24-JαQ TCR α-chain paired with Vβ11 TCR β-chain, and are either $CD4^-CD8^-$ DN or $CD4^+$. These cells are also called invariant NKT (iNKT) or CD1d-restricted NKT cells.[20-22] Principal involvement of human iNKT cells in tumor immunity and autoimmunity are suggested. Deficiency of human iNKT cells has been described in multiple sclerosis (MS) indicated by a decreased frequency in the peripheral blood and absence of the invariant Vα24-JαQ TCR in central nervous system (CNS) plaques of patients with MS despite the presence of conventional Vα24 TCR. Such deficiencies are not characteristic to the chronic autoimmune demyelinating disease of the peripheral nervous system (PNS), chronic inflammatory demyelinating polyneuropathy (CIDP).[23-27]

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 in various immune processes, including response to infectious agents, tumor rejection and autoimmunity.[28-30]

Like iNKT cells, another DN T cell population, mucosal-associated invariant T (MAIT) cells are also characterized by the expression of an invariant TCR rearrangement (Vα7.2-Jα33).[20,31] In addition to the conserved α-chains, MAIT cells express a restricted Vβ2 and Vβ13 driven by the selecting antigen.[32] MAIT cells derive their name from the findings that the invariant TCRα chain is enriched (in humans and mice) in lymphocyte preparations derived from the gut (the lining lamina propria and Peyer's patches). They recognize possibly glycolipids (similarly to iNKT cells) presented by a non-classical MHC class Ib molecule, MR1.[33] In humans, the invariant TCR of MAIT cells was shown to be expressed in autoimmune

lesions of CNS and PNS, which correlated with the expression of IL-4, suggesting an anti-inflammatory role and regulating autoimmune response.[26,34]

In addition to iNKT 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.[35]

1.2 Pompe disease and enzyme replacement therapy

Late-onset Pompe disease is an inherited metabolic myopathy caused by abnormal lysosomal glycogen storage due to the deficiency of the lysosomal enzyme, acid α-glucosidase (GAA).[36] Synonyms for the disease are glycogen-storage disease type II, glycogenosis type II and acid-maltase deficiency.[36]

Pompe disease is an ultra-orphan disease: only 9 patients with the late-onset form have been diagnosed so far in Hungary among a population of 10 million; in other countries with similar size the number of patients is comparable.[37]

At present, Pompe disease is the only inherited muscle disorder which can be treated by enzyme replacement therapy (ERT) in the form of regular infusions of recombinant human acid α-glucosidase (rhGAA).[36,38] ERT induces an IgG antibody response against rhGAA in most patients and treated animals. In immunodeficient GAA knockout mice, where an antibody response against rhGAA does not develop, the therapeutic effect of ERT is better compared to immunocompetent GAA-deficient mice.[39] This suggests that anti-rhGAA antibodies may interfere with the efficacy of treatment; indeed, anti-rhGAA antibodies probably inhibit rhGAA uptake[39], and induction of immune tolerance in mice improves the treatment potential.[40] In humans, the effect of anti-rhGAA antibodies on the efficacy of ERT is controversial. In patients with Gaucher disease (the most common lysosomal storage disease caused by a hereditary deficiency of the enzyme glucocerebrosidase), reduced efficacy of ERT was attributed to the enzyme neutralizing capacity of the induced antibodies.[41,42] In Pompe disease caused by complete absence of GAA, antibody production against rhGAA is increased due to lack of immune tolerance and the therapeutic response is worse.[43] In contrast, no such antibody-related changes in efficacy were observed in several other human studies[44-46] and antibody titer may

decrease during the course of ERT similar to mucopolysaccharidosis I (another lysosomal storage disorder caused by a hereditary deficiency of the enzyme α-L-iduronidase).[47]

1.3 Stroke and post-stroke infections

The traditional definition of stroke, devised by the World Health Organization is „a focal (or at times global) neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours".

Stroke is currently the second leading cause of death in the Western world, ranking after heart disease and before cancer and causes 10% of deaths.[48] Stroke has a high incidence worldwide – an annual incidence of stroke is estimated in the United States at 700,000, leading to over 150,000 deaths. Of all strokes, approximately 88% are ischemic and 12% are hemorrhagic.[49] The mortality of patients with stroke is also extremely high in Central-Eastern European countries. In Hungary approximately 40,000 patients are admitted to hospital a year with the diagnosis of stroke.[50]

Stroke is associated with high mortality and morbidity, and stroke survivors often remain permanently disabled.[51] While direct neurological deficits cause early deaths, infectious complications prevail in the postacute phase of stroke contributing to the poor outcome.[52-57] Such an increased susceptibility to infections after stroke may suggest early alteration of immune responses. Ischemic injury of the CNS alters the balanced neuroimmune modulation resulting in CIDS, the central nervous system injury-induced immune deficiency syndrome. Due to the immunodepression and reduced pro-inflammatory immune responses, the susceptibility for infection is increased. On the other hand, CIDS may protect against damaging autoimmune responses elicited by exposed CNS antigens.[58-60] Patients with stroke suffer from increased rates of infection (in 18-28% of patients developed a post-stroke infection), especially urinary tract infections and pneumonia.[55,56,61] Impaired T and NK cell responses, particularly a reduced IFN-γ production were described in a mouse model of focal cerebral ischemia, where the animals were susceptible to spontaneous pneumonia. This pulmonary infection was related to a massive apoptosis of

lymphocytes in spleen and thymus.[62,63] Furthermore, stroke led to splenic atrophy characterized by a reduction in organ size and a drastic loss of splenocyte number in animal models.[64] Very few human immunological studies have been performed in stroke patients so far. 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.[65] IL-6 serum levels were increased on admission and continued to rise throughout the observation period (14 days), whereas IL-10 did not differ from control subjects.[65]

1.4 Tumor immunology

Cancer is one of the three leading causes of death in industrialized nations. Cancers are caused by the progressive growth of the progeny of a single transformed cell. Malignant tumors are able to aggressively infiltrate the surrounding healthy tissues and to compose metastasis, a secondary cancerous growth formed by transmission of cancerous cells from a primary growth located elsewhere in the body, usually by way of the blood vessels or lymphatics.

Renal cell carcinoma (RCC) accounts for approximately 3% of all cancer diagnoses in the USA each year. In the USA in 2010, 58,000 individuals were diagnosed with RCC and approximately 13,000 died. The 82% of malignant neoplasms of RCCs are clear cell renal carcinomas.[66]

Gliomas are the most frequently occurring primary malignancies in the central nervous system, and glioblastoma multiforme is the most common and most aggressive of these tumors. Despite vigorous basic and clinical studies over past decades, the median survival of patients with this disease remains at about one year.[67]

Meningiomas are the most frequently diagnosed primary brain tumor accounting for 33.8% of all primary brain and CNS tumors reported in the United States between 2002 and 2006. Atypical and malignant meningiomas comprise a small fraction of the total (~5%) and have a slight male predominance.[68]

The tumors express antigenic peptides (tumor-associated or tumor-specific antigens) that can become targets of tumor-specific immune responses (especially T

cell mediated processes). In the classic immune surveillance theory it is believed that the cellular immune defense continuously discovers and eliminates newly arisen tumor cells which express such tumor-specific antigens. These peptides of tumor-cell proteins are presented to cytotoxic T cells by MHC class I molecules and they can become the targets of a tumor-specific T cell response, because they are not displayed on the surface of normal cells, at least not at levels sufficient to be recognized by T cells.[69] It has been shown that many tumors express less MHC class I on their surface compared to the normal tissue from which they have arisen, and also that patients with a reduced immune function have an increased incidence of certain forms of cancer. It is necessary for the activation of effectory mechanisms of anti-tumor response that the antigen is first presented to the T cells on antigen presenting cells (APCs). Although macrophages and partly B lymphocytes play the role of APCs, dendritic cells have shown the highest potential for stimulating the anti-tumor immune response. Activated macrophages, beside the antigen-presentation, are able to destroy tumor cells by the production of TNF-α, enzymes and reactive nitrogen intermediers. Beside the cytotoxic T cells, the NK cells also have an important role in the defense against tumor cells. Carbohydrate components of the target cells are recognized by the receptors of NK cells, and this type of connection activates the cytolytic function of NK cells. NK cells also express FcγRs, hereby the tumor cells that has been bound by specific antibodies are also lysed by antibody-dependent cell-mediated cytotoxicity (ADCC). Humoral immune response may be also induced by particular tumor antigens. Tumor cells are destroyed by the complement-mediated cell lysis or by the previously mentioned mechanism of ADCC.[1,2]

To prevent diseases induced by autoimmune attack or to control collateral damage during an immune response, the immune system has developed many mechanisms of negative regulation. In the context of tumor immunity, the strict regulation of immune responses to maintain self-tolerance and prevent autoimmunity can represent a barrier to successful anti-tumor therapy. Recently, several cell types with suppressive function have been described such as the widely studied $CD4^+CD25^+$ T regulatory cell[70], M2 or tumor-associated macrophages[71], myeloid-derived suppressor cells[72], and the CD1d-restricted NKT cell.[73,74] Stimulation of type

I NKT cells protected against tumor growth even when responses were relatively skewed toward Th2 cytokines.[75]

2. AIMS OF THE STUDIES

Here, we investigated the role of T cells in immune responses in different neurological disorders.

2.1 Enzyme replacement therapy induces T cell responses in late-onset Pompe disease

At present, Pompe disease is the only inherited muscle disorder which can be treated by enzyme replacement therapy in the form of regular infusions of recombinant human acid α-glucosidase. ERT induces an IgG antibody response against rhGAA in most patients, which may interfere with the efficacy of treatment, therefore, we examined if ERT also induces rhGAA-specific T cell responses:

1. Are there any differences in lymphocyte frequencies, expression of activation and cytotoxic molecules in peripheral blood of patients with Pompe disease treated with rhGAA?
2. Does the treatment with rhGAA induce specific anti- or pro-inflammatory cytokine production in patients with Pompe disease treated with rhGAA?

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

Acute-onset cerebrovascular diseases are connected to a number of immunological changes. Few human 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 lymphocyte subsets have not been thoroughly examined in the acute phase of ischemic stroke. We may suspect that early changes in innate immune responses 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:

3. Are the frequencies of the innate T cells affected by acute ischemic events?

4. Are the anti- or pro-inflammatory cytokine productions and cytotoxicity of the innate lymphocyte subsets affected in the acute phase of ischemic stroke?

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

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

The selective absence of the invariant Vα24-JαQ TCR of iNKT cells in central nervous system plaques of patients with multiple sclerosis was previously observed, while conventional Vα24 TCRs and invariant TCR of MAIT cells were present. To partly examine whether absence of iNKT 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. Therefore, we addressed:

5. Do iNKT cells infiltrate tumors within the CNS similarly to tumors outside the CNS?
6. Can we detect other invariant T cells in tumors with a special emphasis on MAIT cells, which may possess similar functions to iNKT cells?
7. 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.
8. What is the relation of MAIT and NKT-like cells in tumors?
9. Do MAIT cells express CD56? Do $CD56^+$ MAIT cells participate in anti-tumor immune responses?

3. ENZYME REPLACEMENT THERAPY INDUCES T CELL RESPONSES IN LATE-ONSET POMPE DISEASES

3.1 Materials and methods

3.1.1 Patients and samples. Seven Hungarian patients with late-onset Pompe disease (6 treated, 1 untreated) and 5 healthy controls were examined at least twice at different time points (1-6 months, mean 4.2 months difference). All treated patients exhibited antibodies against rhGAA in the sera, but none had inhibitory antibodies tested at several time points [determined by a non-commercial assay established in the Genzyme Clinical Specialty Laboratory (Genzyme Corporation, Framingham, MA, USA) as part of the protocol and guideline to treat patients with Pompe disease with ERT]. Written informed consent was obtained from all subjects. Peripheral blood was drawn from the cubital vein from each patient and control. The study protocol was approved by the Regional Local Ethics Committee.

3.1.2 Isolation of peripheral blood mononuclear cells (PBMC), $CD4^+$ and $CD8^+$ T cells. Peripheral blood mononuclear cells were isolated from heparinized venous blood on Ficoll-Paque gradient (AP Hungary Kft. Budapest, Hungary) by density gradient centrifugation. $CD4^+$ and $CD8^+$ T cells were separated by MACS Cell Separation Technology (all reagents and instruments from Miltenyi Biotec, Frank Diagnosztika Kft., Budapest, Hungary). PBMCs were first labeled magnetically with CD4 MicroBeads according to the manufacturer's instructions, and $CD4^+$ cells were positively separated on the cell separation column. In the next step, the negatively separated population was labeled magnetically with CD8 MicroBeads, and the $CD8^+$ subpopulation was positively separated. The remaining fraction, which did not bind the CD4 or CD8 MicroBeads composed the antigen presenting cells.

3.1.3 Flow cytometry (FACS) and intracellular cytokine production. After *ex vivo* and *in vitro* activation, $1x10^6$ PBMCs were incubated for 30 minutes at room temperature with different double or triple combinations of the following anti-

human monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD4, anti-CD19; phycoerythrin (PE)-conjugated anti-FasL (CD178), anti-IFN-γ, anti-perforin, anti-CD25 and allophycocyanin-conjugated anti-CD56 and anti-CD8 (all antibodies from BD Pharmingen, Soft Flow Hungary Kft., Pécs, Hungary). In the case of IFN-γ staining, PBMCs were stimulated for 4 hours at 37 °C with phorbol-myristate-acetate (PMA, 25 ng/ml) and ionomycin (1 μg/ml), and Golgi transport was inhibited by brefeldin A (10 μg/ml; 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 phosphate-buffered saline (PBS) and resuspended in 4% paraformaldehyde, stored at 4ºC in the dark to be processed for FACS analysis 24 hours at the latest following fixation. At least 50,000 cells were analyzed using a FACSCalibur 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).

3.1.4 Cell culture. PBMCs and isolated $CD4^+$ and $CD8^+$ T cells in the presence of antigen presenting cells were stimulated with different concentration of rhGAA (1 and 10 μg/ml) in RPMI 1640 medium containing 10% foetal bovine serum, penicillin and streptomycin (all from Invitrogen, Csertex Kft., Budapest, Hungary) for 48 hours *in vitro*. Supernatants were collected and PBMC were restimulated with PMA and ionomycin for examination of cytokine production and intracellular expression, respectively.

3.1.5 Cytometric Bead Array (CBA). The levels of IL-4, IL-6, IL-17A, IFN-γ and tumor necrosis factor (TNF)-α were determined from the culture supernatants with Cytometric Bead Array (CBA) (#560484, BD Biosciences, Soft Flow Hungary Kft., Pécs, Hungary) using different Capture Beads to detect the respective cytokines. 50 μl of each unknown samples were incubated for 3 hours at room temperature with the presence of 50 μl mixed Capture Beads and 50 μl PE

Detection Reagent. After incubation, the samples were washed in Wash Buffer and bead pellet was resuspended in Wash Buffer. Samples were analyzed right after the experiment on a FACSCalibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) calculating the amount of cytokines with CBA Software (BD Biosciences, San Diego, CA, USA).

3.1.6 Statistical analysis. Statistical comparisons were made by using the Student 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.

3.2 Results

3.2.1 *Ex vivo* and *in vitro* lymphocyte frequencies in the peripheral blood (PB) of patients treated with rhGAA. The frequency of lymphocytes was examined *ex vivo* in the PB and also in PBMC cultures stimulated with rhGAA for 48 hours *in vitro*. The *ex vivo* and *in vitro* frequencies of $CD3^+$ T cells, T subsets ($CD4^+$ and $CD8^+$ T cells), $CD19^+$ B cells, $CD3^+CD56^+$ NKT-like T cells and $CD3^-CD56^+$ NK cells in the treated patients were not different from the untreated patient and healthy controls (**Table 1**). The *ex vivo* percentage of T cells expressing the late activation marker CD25 (activated $CD4^+CD25^{low}$ T cells) was significantly elevated in the treated patients (28.7±1.4 vs. 20.4±2.6; $p<0.05$) compared to untreated patient and healthy controls. In contrast, the *ex vivo* frequency of $CD4^+CD25^{high}$ regulatory T (Treg) cells was not different (**Table 1**).

The perforin and Fas/Fas ligand (FasL) pathways are the two major mechanisms of cytotoxicity.[8,9] Intracellular expression of perforin and the surface expression of FasL were analyzed by flow cytometry in $CD3^-CD56^+$ NK cells, $CD3^+CD56^+$ NKT-like and $CD3^+CD8^+$ T cells. There were no significant differences in the expression of perforin and FasL when treated patients and controls were compared (**Table 1**).

	Ex vivo		0 ug/ml[a]		10 ug/ml	
	Controls[b]	Patients	Controls	Patients	Controls	Patients
CD3	53.9±11.7[c]	62.4±4	51±10.1	58.8±4.3	52±10	58.9±3.9
CD4	25.1±8.2	29.8±4	23.9±4.4	32.4±2.8	23.8±5.5	32.3±3.1
CD8	29.6±7.3	28.9±4	24.5±6.2	26.9±3	25.5±6	26.9±3.5
CD19	1.9±0.5	3.3±0.5	4.2±0.8	6.6±0.7	4.3±1	6±0.8
$CD3^-CD56^+$	9.9±2.3	7.6±0.9	8.5±2	6.6±0.9	9±2	6.8±0.9
$CD3^+CD56^+$	3.7±0.7	3.9±0.6	2.2±0.9	2.9±0.4	1.9±0.6	3±0.4
CD25 expression						
by $CD3^+$ cells	12.9±2.8	20.7±1.6	13.3±2.6	14.4±0.8	14±2.3	14.3±1.1
by $CD8^+$ cells	5±1.5	4.6±3.6	8.8±1.7	4.3±1.2	8.7±1.7	5±1.2
$CD25^{low}$expression						
by $CD4^+$ cells	20.4±2.6	28.7±1.4[d]	12.7±1.4	22±1.5	14.7±1.9	21.3±1.9
$CD25^{high}$ expression						
by $CD4^+$ cells	1.3±0.2	1.4±0.2	0.6±0.2	1.6±0.2	0.9±0.2	1.5±0.2
Perf expression						
by $CD3^+CD8^+$ cells	17.2±7.1	21.5±4.8	13.3±7.6	9.1±4.4	7.4±4.1	7.6±1.6
by $CD3^-CD56^+$ cells	33.7±8.2	39.1±5.2	28.2±7.7	32.1±4.9	32.7±10.4	28.5±4.3
by $CD3^+CD56^+$ cells	21±3.3	22.6±4.5	16.5±0.3	18.7±6.7	16.1±0.9	18±6.2
FasL expression						
by $CD3^+CD8^+$ cells	0.8±0.2	0.6±0.1	0.8±0.1	1±0.3	0.6±0.1	0.9±0.2
by $CD3^-CD56^+$ cells	1.1±0.6	3.1±0.8	1.4±0.3	3.5±1.4	2±0.5	2.9±1
by $CD3^+CD56^+$ cells	1.7±0.8	2.6±0.7	1.6±0.3	3.9±1.3	2.7±0.5	2.6±0.7

Table 1. Lymphocyte frequencies, activation of lymphocyte subsets and the expression of perforin and FasL by NK, NKT-like and $CD8^+$ T cells in peripheral blood
[a]concentration of rhGAA used for in vitro stimulation; [b]healthy controls and one untreated patient**;** [c]percentages of positive cells, mean±SEM are indicated; [d]the *ex vivo* percentage of $CD4^+CD25^{low}$ T cells was significantly higher ($p<0.05$) in treated patients compared to control subjects.

3.2.2 Cytokine production of rhGAA-specific T cell subsets. Intracellular IFN-γ expression was analyzed by flow cytometry in $CD3^+$, $CD4^+$ and $CD8^+$ T cells.

After *in vitro* stimulation with different concentration of rhGAA, a dose-dependent increase of pro-inflammatory intracellular IFN-γ expression in $CD4^+$ and $CD8^+$ T cells was observed in the treated patients in contrast to the untreated patient and healthy subjects, where no increase or rather, a dose-dependent decrease was found (**Figures 4 and 5**). Such dose-dependent increase in IFN-γ expression was characteristic of all treated subjects (**Figures 4B and 5B**). In addition, expression of IFN-γ by T cell subsets was higher in the treated patients than in the untreated patient and healthy controls after *in vitro* stimulation (**Figures 4 and 5**). Every patient and control subject was examined at least twice at different time points.

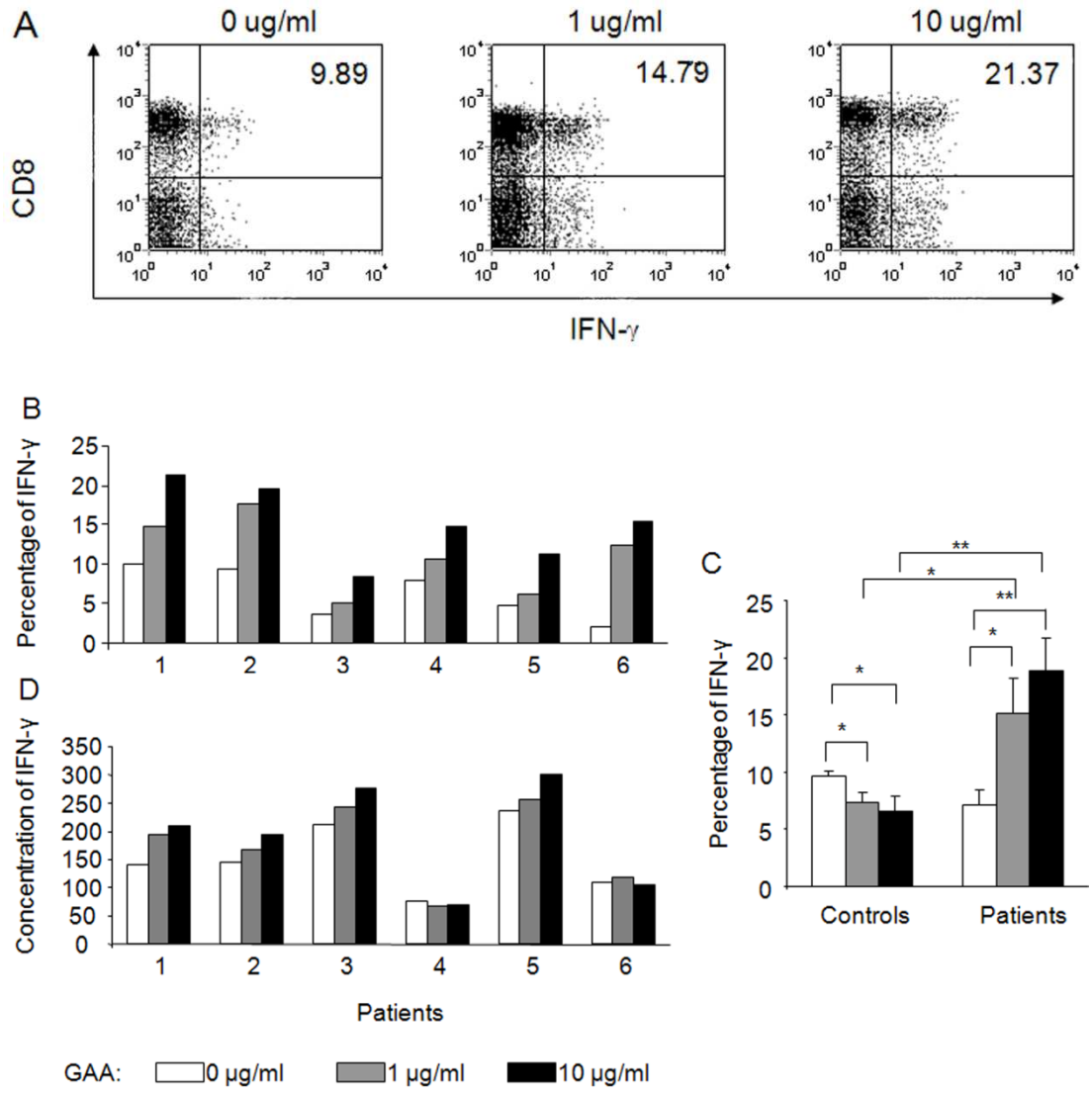


Figure 4. IFN-γ expression by CD8$^+$ cytotoxic T cells in patients seropositive for rhGAA antibodies and controls

A. Intracellular IFN-γ expression by CD8$^+$ T cells in a representative sample from a patient treated by ERT for more than a year. PBMC were stimulated with different concentrations of GAA for 2 days. Percentages indicate IFN-γ$^+$ cells among CD8$^+$ T cells. **B.** Percentage of intracellular IFN-γ expression by CD8$^+$ T cells in individual patients (n=6). PBMC were stimulated with different concentrations of GAA for 2 days. Percentages indicate IFN-γ$^+$ cells among CD8$^+$ T cells. **C.** The mean of percentages of intracellular IFN-γ expression by CD8$^+$ T cells in treated patients (n=6) and in the control group (healthy and one untreated patient with Pompe disease). PBMC were stimulated with different concentrations of GAA for 2 days. The experiments were repeated at least for 2 times in every subject. Mean ± SEM was calculated and is shown. Percentages indicate IFN-γ$^+$ cells among CD8$^+$ T cells. **D.** Concentration of IFN-γ in the supernatants of isolated CD8$^+$ T cell cultures in individual patients (n=6). Cell cultures were stimulated with different concentrations of GAA for 2 days. Concentrations of cytokine are given in ng/ml.

GAA: acid α-glucosidase , SE: standard error of mean; * $p<0.05$; ** $p<0.01$

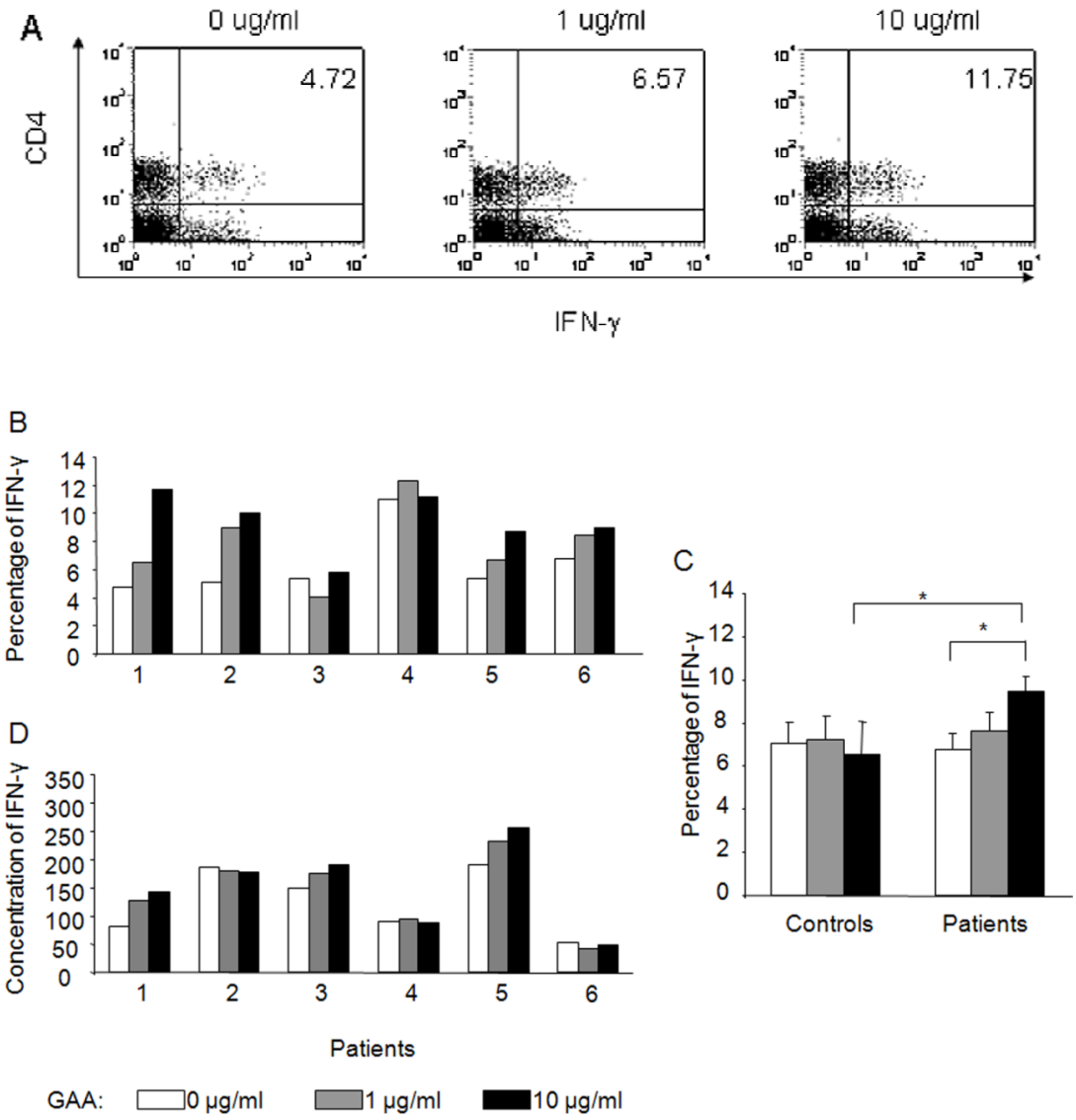


Figure 5. IFN-γ expression by CD4$^+$ helper T cells in patients seropositive for rhGAA antibodies and controls

A. Intracellular IFN-γ expression by CD4$^+$ T cells in a representative sample from a patient treated by ERT for more than a year. PBMC were stimulated with different concentrations of GAA for 2 days. Percentages indicate IFN-γ$^+$ cells among CD4$^+$ T cells. **B.** Percentage of intracellular IFN-γ expression by CD4$^+$ T cells in individual patients (n=6). PBMC were stimulated with different concentrations of GAA for 2 days. Percentages indicate IFN-γ$^+$ cells among CD4$^+$ T cells. **C.** The mean of percentages of intracellular IFN-γ expression by CD4$^+$ T cells in treated patients (n=6) and in the control group (healthy and one untreated patient with Pompe disease). PBMC were stimulated with different concentrations of GAA for 2 days. The experiments were repeated for at least 2 times in every subject. Mean ± SEM was calculated and is shown. Percentages indicate IFN-γ+ cells among CD4$^+$ T cells.**D.** Concentration of IFN-γ in the supernatants of isolated CD4$^+$ T cell cultures in individual patients (n=6). Cell cultures were stimulated with different concentrations of GAA for 2 days. Concentrations of cytokine are given in ng/ml.

GAA: acid α-glucosidase , SE: standard error of mean; * $p<0.05$; ** $p<0.01$

		IL4	IL6	TNFα	IFNγ	IL17A
				$CD4^+$		
0 ug/ml[a]	Controls[b]	6.3±0[c]	4,343.5±401.2	9,088±2,792.2	76.9±60.2	16±5.6
	Patients	7.3±0.6	5,112±1,360	3,126.8±2,535.1	129.2±61.9	13.2±4.9
1 ug/ml	Controls	6±0.5	4,148.3±325.1	5,687.9±1,767.9	79.9±57	14.1±4.3
	Patients	7.5±1.2	4,978.6±1,178.1	5,987.3±1,134.5	135.7±67.9	13.5±2.9
10 ug/ml	Controls	7.2±0.7	3,769.5±19.3	5,850.4±163.3	84.5±72.2	14±2.7
	Patients	7.7±1.5	4,860.4±1,063.8	6,401.2±7.8	165.4±54.3	12.7±1.8
				$CD8^+$		
0 ug/ml	Controls	5.9±0.1	4,070.6±358.1	8,518.4±1,956.1	105.7±90.8	12.7±1.4
	Patients	7.4±1	7,552.1±937	3,749.6±2,300	172.1±77.5	16±4.9
1 ug/ml	Controls	7.4±0.9	4,110.7±1,012.2	6,056.9±878.2	101.3±77.9	13.2±1.7
	Patients	7.2±1.7	6,320.4±1,278.9	5,712.5±578.3	188.3±101.2	16.4±5.3
10 ug/ml	Controls	7.1±0.6	4,168.4±406.7	5,806.4±590.2	92.4±80	11.9±1.8
	Patients	8.5±1.3	6,419.9±387.1	5,904.2±356.8	203±98.9	17.3±1.1

Table 2. The Th1, Th2 and Th17 cytokine production of isolated T cell ($CD4^+$ and $CD8^+$) cultures

[a]concentration of rhGAA used for in vitro stimulation; [b]healthy controls and one untreated patient; [c]concentration of cytokines are given in ng/ml, mean±SEM are indicated

Pro- and anti-inflammatory cytokine production (IFN-γ, TNF-α, IL-4, IL-6 and IL-17A) was also examined in the supernatants of isolated $CD4^+$ and $CD8^+$ T cell cultures stimulated with different concentrations of rhGAA in the presence of antigen presenting cells (**Table 2**). Increased production of IFN-γ by $CD4^+$ T cells was observed in 3 out of 6 cases; while increased production of IFN-γ by $CD8^+$ T cells was observed in 4 out of 6 cases, including the very same patients (**Figures 4D and 5D**). Increased production of TNF-α by $CD4^+$ and $CD8^+$ T cells was also observed in the same three cases. Two of the patients (#3 and #5), who had the highest antibody titers in the sera (data not shown), also had the highest concentration of IFN-γ in the supernatants of both $CD4^+$ and $CD8^+$ T cell cultures after stimulation with rhGAA (**Figures 4D and 5D**). Both of them require respiratory support but have had the longest disease course as well. One of them (#3) responds to ERT well; the treatment response in the other patient (#5), who has had a prolonged increase in antibody titers, is less obvious. We also examined the IFN-

γ/IL-4 and TNF-α/IL-4 ratios in individual patients (**Table 3**). The highest IFN-γ/IL-4 ratios were found in the supernatants of $CD8^+$ T cell cultures established from patients #3 and #5. Patient #5 had also the highest ratio regarding $CD4^+$ T cultures. In addition, we observed a dose-dependent increase in such ratios suggesting that a dose-dependent production of IFN-γ was more pronounced than the production of IL-4.

	Patients					
	#1	*#2*	*#3*	*#4*	*#5*	*#6*
	$CD4^+$					
IFN-γ/IL-4[a]						
0 μg/ml	21.3	32.0	27.6	21.2	43.7	11.1
1 μg/ml	33.3	42.5	33.7	20.8	44.0	11.9
10 μg/ml	36.1	39.1	31.0	19.1	57.6	10.7
TNF-α/IL-4						
0 μg/ml	321.3	585.2	574.9	818.6	637.5	354.0
1 μg/ml	665.0	775.7	762.6	720.2	652.8	633.5
10 μg/ml	709.6	490.1	676.6	724.5	875.0	740.3
	$CD8^+$					
IFN-γ/IL-4						
0 μg/ml	49.8	49.6	65.9	25.0	61.5	23.4
1 μg/ml	67.7	39.7	103.7	21.1	104.5	20.1
10 μg/ml	60.6	55.5	93.2	30.2	87.8	18.4
TNF-α/IL-4						
0 μg/ml	453.2	596.7	547.9	861.2	610.4	277.8
1 μg/ml	649.3	467.7	696	753.1	704.6	244.4
10 μg/ml	561.7	598.3	669.2	747.3	421.2	228.3

Table 3. Ratio of pro-inflammatory and anti-inflammatory cytokines in T cell cultures stimulated with rhGAA

[a]ratio were calculated by dividing mean of pro- and anti-inflammatory cytokine concentrations measured in the supernatants of T cells stimulated with rhGAA at different time points

3.3 Discussion

Here we show that enzyme replacement therapy by rhGAA infusions generates T cell responses in all patients. They exhibit antibodies against the recombinant enzyme, indicating that T cell tolerance against GAA is incomplete, similar to B cell tolerance. Such rhGAA-specific T cell responses existed at least for six months.

The *ex vivo* percentage of activated $CD4^+CD25^{low}$ T cells was higher in treated patients compared to controls including one untreated patient. Such activation of T cells may be related to chronic stimulation with rhGAA infusions. The chronic antigen-specific activation is also supported by the observed decrease in percentage of activated $CD25^{low}$ T cells in culture after *in vitro* stimulation with rhGAA. This may be caused by activation-induced cell death of pre-activated T cells re-activated by their specific antigen.[76,77]

We also performed functional experiments using different methods. First, antigen-specific activation of T cells was examined by measuring individual $CD4^+$ and $CD8^+$ T cells producing intracellular IFN-γ in response to rhGAA. A dose-dependent increase in the number of T cells expressing IFN-γ was observed in patients treated with ERT compared to controls. In fact, every single patient's PBMC responded to *in vitro* stimulation with rhGAA. Next, $CD4^+$ and $CD8^+$ T cells were isolated and stimulated with rhGAA, and Th1, Th2 and Th17 cytokine secretion was measured in the supernatants. Although a dose-dependent increase in pro-inflammatory IFN-γ and TNF-α levels was also observed in the supernatants of cultured $CD4^+$ and $CD8^+$ T cell subsets, this increase was not significant when patients and controls were compared, in contrast to the increased expression of intracellular IFN-γ. However, when individual patients were analyzed, we found an antigen-specific increase of IFN-γ and TNF-α in half of the patients. This may suggest heterogeneous T cell responses similar to heterogeneous antibody responses in patients. Stronger cytokine responses could be detected by both CBA in supernatants and by intracellular FACS, while weaker responses could be measured only intracellularly in individual cells. Altogether, these data may suggest that although rhGAA-specific pro-inflammatory cytokine production (IFN-γ and TNF-α)

is characteristic of all treated patients, some of them have a very strong cytokine response. The heterogeneous antibody response against rhGAA may be related to the presence of natural GAA indicated by residual enzyme activity; in the absence of the enzyme B cell tolerance is incomplete.[40,43,78] All of our patients with late-onset Pompe disease had residual enzyme activity, therefore expression levels of the enzyme could hardly be responsible for the observed heterogeneity of T cell responses. However, our patients also had a heterogenous antibody response, and two of them had consistently high levels of anti-rhGAA antibodies (data not shown).

Expression of cytotoxic molecules (FasL and perforin) by NK, NKT-like and $CD8^+$ T cells was not increased *ex vivo*. A cytotoxic response against the native GAA and specifically against muscles is unlikely considering the intracellular nature of the enzyme.

The relevance of antibody responses to ERT in lysosomal storage disorders has often been downplayed, although cross-reacting immunologic material (CRIM)-negative patients without residual enzyme activity often formed antibodies and responded poorly to ERT.[78] IgG antibodies generated during ERT in patients with Fabry disease exert an inhibitory effect on alpha-galactosidase A enzyme activity fibroblast cells and tissues from Fabry mice.[79] Mucopolysaccharidosis type I patients, who formed high titer antibodies also responded poorly to ERT.[80] Regular infusion of other proteins impacts efficacy of almost all biological therapies in other diseases.[81,82] So far, T cell responses in these lysosomal storage diseases treated by ERT have not been examined to our knowledge. We can presume that similar responses might be elicited by regular infusion of recombinant alpha-galactosidase in Fabry disease, acid β-glucosidase in Gaucher disease and alpha-L-idurinodase in mucopolysacharidosis I. Since some of these diseases are more prevalent than Pompe disease, the impact of T cell responses may have a broad relevance.

The antibodies against rhGAA react with the native protein and are generated presumably against conformational epitopes. In contrast, linear epitopes, small peptide sequences are important in eliciting T cell responses. In our experiments, we used the native rhGAA to stimulate T cells and the immunodominant epitopes of GAA have not been investigated. It would be interesting to determine if T cell responses are focused or rather target a number of different epitopes.

Whether T cell responses are related to the magnitude of B cell responses is a crucial question. Cytokine production by rhGAA-activated T cells may influence the nature of the immune responses and antibody production. Indeed, inhibitory anti-factor VIII (FVIII) antibodies are generated in hemophilia A patients treated with recombinant human FVIII. In such patients a polarized anti-inflammatory/regulatory T cell phenotype was observed in contrast to a mixed pattern with a bias towards an inflammatory cytokine profile in patients without antibodies.[83,84] Due to the ultra-orphan nature of Pompe disease, the number of patients in our study was limited, although all patients in Hungary were examined. This complicated the analysis of clinical and antibody correlations. Nevertheless, two patients with the highest antibody titers had the highest IFN-γ production in both $CD8^+$ and $CD4^+$ T cell cultures; when IFN-γ/IL-4 ratios were calculated, these two patients had the highest dose-dependent ratio as well. We may speculate that, despite an antigen-specific increase in IL-4 production, pro-inflammatory cytokine production may be more pronounced. Such increased IFN-γ production was also found in all hemophilia A patients treated with FVIII compared to blood donors.[83,84] We may also expect that GAA-specific T cell responses might be more prominent in patients with infantile-onset Pompe disease without any residual enzyme activity due to incomplete T cell tolerance, similar to deficient B cell tolerance.[78] Since the prevalence of infantile Pompe disease is even lower than the prevalence of late-onset disease, due to the absence of such patients in Hungary we could not address that assumption. In order to explore such correlations, i.e. clinical and serological, international multicenter trials are needed.

In summary, we show here that ERT used to treat an ultra-orphan lysosomal storage disease generate long-term specific $CD4^+$ and $CD8^+$ T cell responses, mainly production of pro-inflammatory cytokines besides the well-known antibody response. Although the number of examined patients was relatively low, the consistent results in all single individuals suggest that, despite being heterogeneous in magnitude, T cell responses are general characteristics of ERT.

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

4.1 Materials and methods

4.1.1 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. The study protocol was approved by the Regional Local Ethics Committee. Exclusion criteria were infectious diseases, fever <4 weeks before stroke, elevated white blood cells, erythrocyte sedimentation rate, high-sensitivity C-reactive protein (cut-off value <10 mg/L), procalcitonin (cut-off value <0.05 ng/mL) on admission, positive chest X-ray, hemorrhagic stroke defined by an acute cranial computer tomograph (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 patient 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 4**). 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.

	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

Table 4. Demographic and clinical data of subject

4.1.2 Cell isolation and flow cytometry. PBMCs were isolated from heparinized venous blood on Ficoll-Paque gradient (AP Hungary Kft. Budapest, Hungary) by density gradient centrifugation. After washing in 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: FITC-conjugated anti-CD3, anti-Vδ2 T cell receptor; PE-conjugated anti-FasL (CD178), anti-IFN-γ, anti-perforin and allophycocyanin-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 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 FACSCalibur 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).

4.1.3 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 MicroBeads 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 TNF-α were determined from the culture supernatants with CBA (#550749, BD Biosciences, Soft Flow Hungary Kft., Pécs, Hungary) using different Capture Beads to detect the respective cytokines. 50 μl of each unknown samples were incubated for 3 hours at room temperature with the presence of 50 μl mixed Capture Beads and 50 μl PE Detection Reagent. After incubation, the samples were washed in Wash Buffer and bead pellet was resuspended in Wash Buffer. Samples were analyzed right after the experiment on a FACSCalibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) calculating the amount of cytokines with CBA Software (BD Biosciences, San Diego, CA, USA).

4.1.4 Cytotoxicity. The cytotoxic activity of NK cells was determined with a nonradioactive, 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)[85,86]

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.

4.1.5 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.

4.2 Results

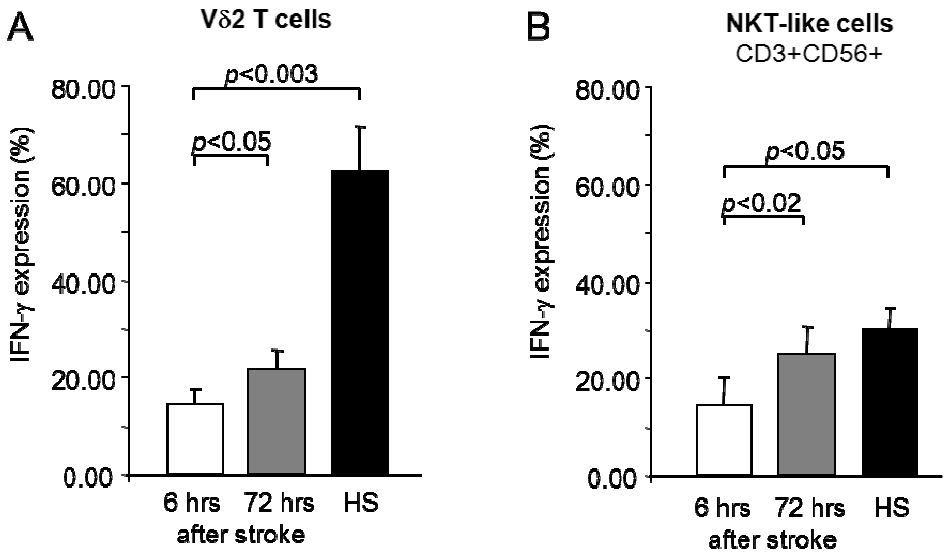
4.2.1 Innate peripheral lymphocyte frequencies in the acute phase of ischemic stroke. In order to investigate the acute innate cellular immune responses in ischemic stroke, peripheral blood samples were acquired within 6 hours and after 72 hours of symptom onset and compared both with each other and 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 5**).

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

Table 5. Innate lymphocyte frequencies in the peripheral blood
Mean ± SEM are indicated.

4.2.2 Cytokine production of innate lymphocyte subsets in the acute phase of ischemic stroke. Next, intracellular IFN-γ expression was analyzed by flow cytometry in $V\delta2^+$ T cells, $CD3^+CD56^+$ NKT-like, and $CD3^-CD56^+$ NK cells (**Figure 6**). 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 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*).



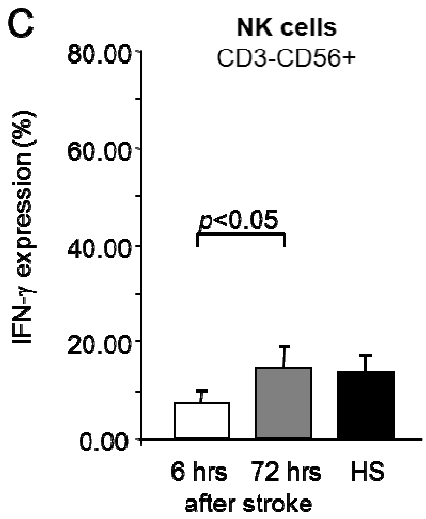


Figure 6. Intracellular IFN-γ expression by innate lymphocytes in acute ischemic stroke
A. Intracellular IFN-γ expression by Vδ2$^+$ T cells, **B.** CD3$^+$CD56$^+$ NKT-like and **C.** CD3$^-$ CD56$^+$ NK in the peripheral blood of acute ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and those of healthy subjects is indicated.
HS: healthy subjects; mean ± SEM is shown.

4.2.3 Cytotoxicity of innate lymphocytes in the acute phase of ischemic stroke. Intracellular expression of perforin was analyzed by flow cytometry in Vδ2$^+$ T cells, CD3$^+$CD56$^+$ NKT-like and NK cells (**Figure 7**). Significantly increased percentages of perforin-expressing Vδ2$^+$ 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.

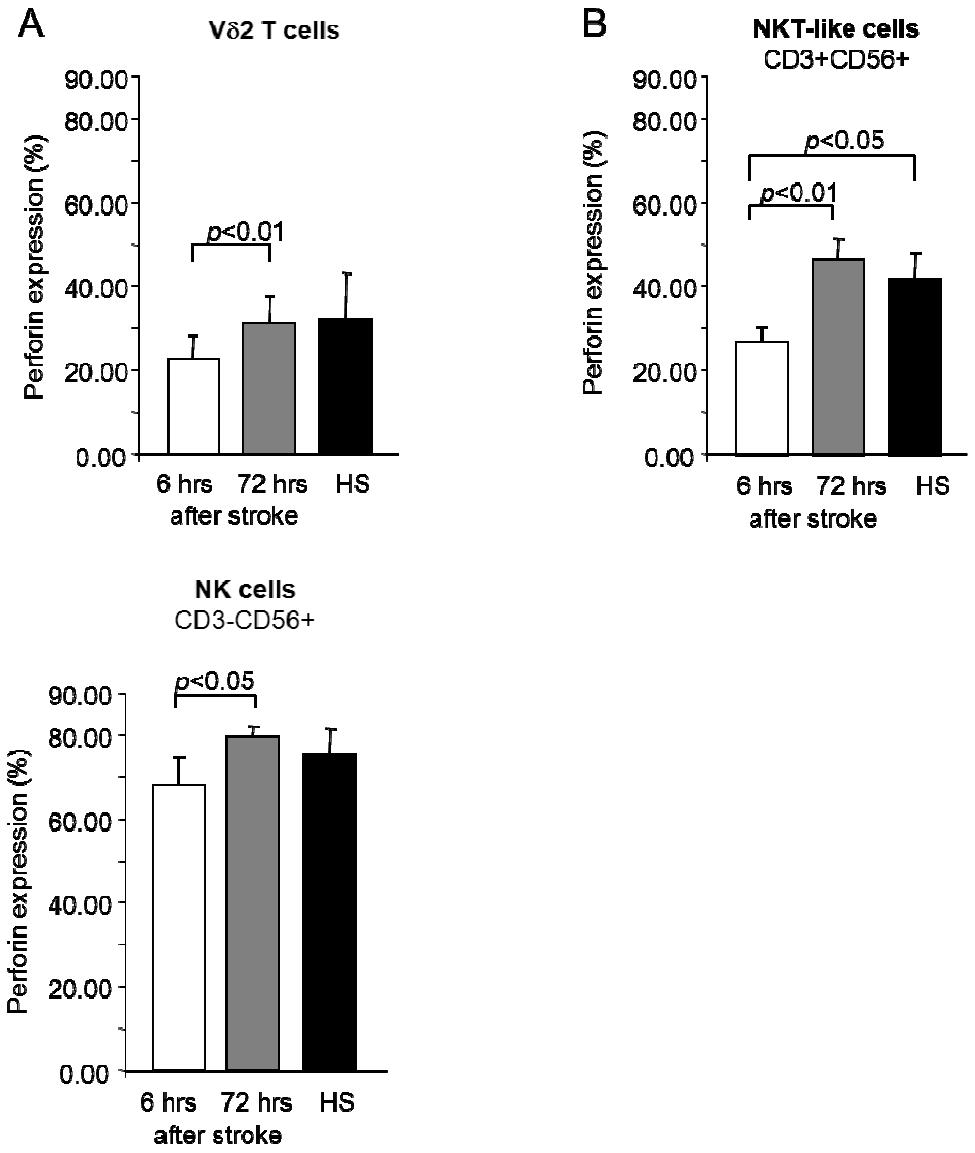


Figure 7. Intracellular perforin expression by innate lymphocytes in acute ischemic stroke

A. Intracellular perforin expression by $V\delta2^+$ T cells, **B.** $CD3^+CD56^+$ NKT-like and **C.** $CD3^-CD56^+$ NK 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 ± SEM is shown.

We were also interested in the role of the other major cytotoxic mechanism, the Fas/FasL-mediated apoptotic pathway.[8,9,11] Surface expression 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 8**). 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.

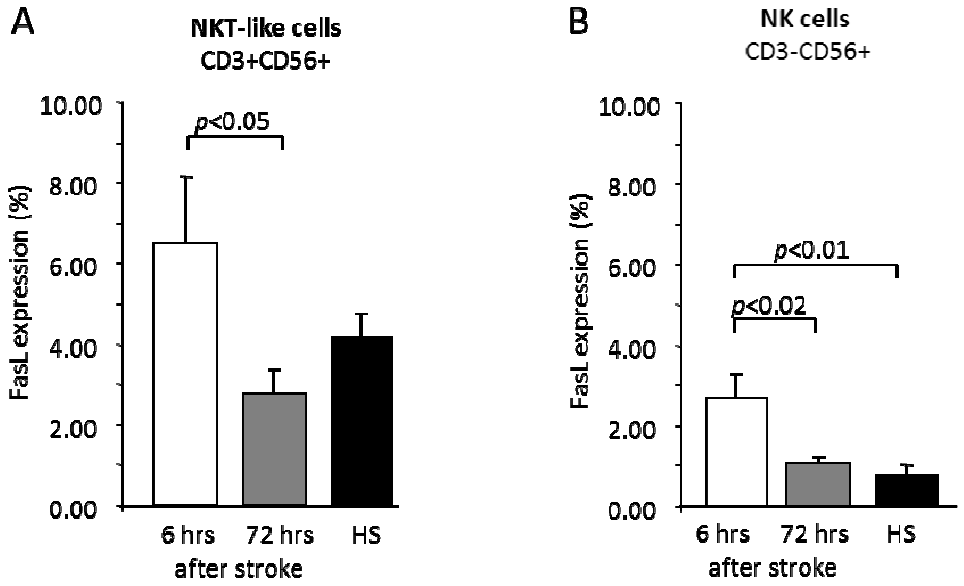


Figure 8. FasL expression by innate lymphocytes in acute ischemic stroke
A. Surface FasL expression by $CD3^{+}CD56^{+}$ NKT-like and **B.** $CD3^{-}CD56^{+}$ NK in the peripheral blood of acute ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and those of healthy subjects is indicated. HS: healthy subjects, mean ± SEM is shown.

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 9**).

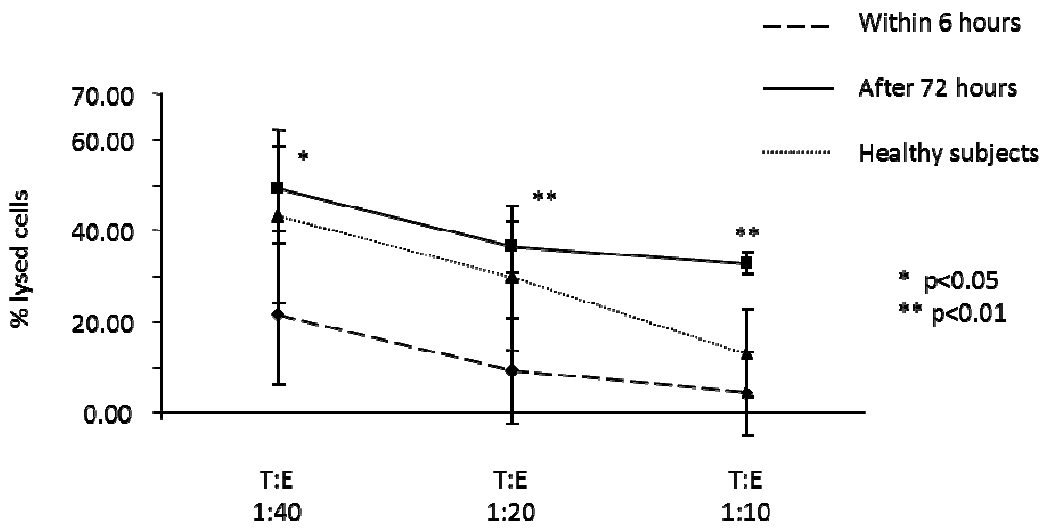


Figure 9. NK cell cytotoxicity in acute ischemic stroke
Cytotoxic activity of NK cells as a percentage of lysed cells is indicated in ischemic stroke patients within 6 hours and after 72 hours from the onset of stroke symptoms, and that of healthy subjects, at different target and effector cell ratios. T=target cell, E=effector cell, mean ± SEM is shown.

4.3 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.[65] 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.[87]

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.[88,89] 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.[62] 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 were normal as well, supporting our data obtained by stimulation of isolated innate lymphocytes.[65]

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[8,9], 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.[90] 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.[62,63]

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.[65] 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.[62] 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.[65]

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 post acute phase of stroke[91,92] highlight the importance of further investigations on the exact, detailed pathomechanism of the phenomenon and the search for new therapeutic targets.

5. INVARIANT Vα7.2-Jα33 TCR IS EXPRESSED IN HUMAN KIDNEY AND BRAIN TUMORS INDICATING INFILTRATION BY MUCOSAL-ASSOCIATED INVARIANT T (MAIT) CELLS

5.1 Materials and 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.

5.1.1 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 resuspended in 20 μl of RNAse-free water and used for complementary DNA (cDNA) synthesis by First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden, or Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using oligo-dT as primer.

5.1.2 SSCP (single-strand conformation polymorphism) 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.[27,34] 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 Vα1 (GCCACAATAAACATACAGGA) were used in conjunction with the same antisense 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 antisense primer (GATTTAGAGTCTCTCAGCTG).[27] 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.[26,27,34] Probes were as follows: Cα (AAATATCCAGAACCCTGACCCTGCCGTGTACC), Jα29 (CTCTTGTCTTTGGAAAGGGCACAAGACTTTCTGT), Jα33 (TATCAGTTAA-TCTGGGGCGCTGGGACCAAGCT), Jα48 (ATTAACCTTTGGGACTGGAACA-AGACTCACCATC) and Vα24 inv, clonotypic (TGTGTGGTGAGCGACAGAGG-CTCAACCCTG).

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).

5.1.3 RT–PCR. cDNAs for human β2-microglobulin, IL-4 and IFN-γ were amplified by RT–PCR as described previously.[27,34] 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 6**. 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 6**).[34]

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

Table 6. Primer sequences and annealing temperature

5.1.4 Flow cytometry and sorting of lymphocyte populations. 30 ml 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 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 $5x10^6$ cells per ml in PBS. After washing in PBS, $1x10^6$ PBMCs and $5x10^6$ tumor cells were incubated for 30 min at room temperature with different dual or triple combinations of the following mAbs: FITC-conjugated anti-CD3, anti-CD4 and anti-HLA-DR; PE-conjugated anti-CD8 and anti-CD4, and allophycocyanin- 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 FACSCalibur 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).

5.1.5 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).

5.2 Results

5.2.1 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.[27] 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 $V\alpha24^+$ 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 (**Figure 10**). 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) (**Figure 10**).

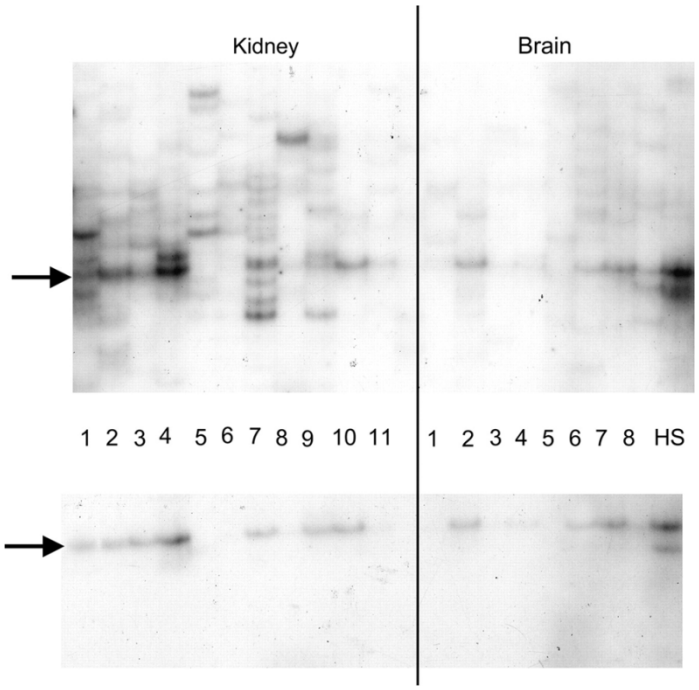


Figure 10. **Clonality of the Vα24$^+$ T cell repertoire and presence of the invariant Vα24-JαQ clonotype representing NKT cells in kidney and brain tumors.**
Tumor tissues obtained from 11 patients with clear cell kidney cancer (left panel) and eight patients with brain tumor (right panel) were examined 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.

5.2.2 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.[26,34] 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.[34] Invariant clonotypes could be detected in 14 out of 19 cancer samples (8 out of 11 kidney and 6 out of 8 brain tumors) (**Figure 11**). In both kidney and brain tumors, the MAIT clonotype was more dominant than in the peripheral blood (**Figure 11**, lower panel).

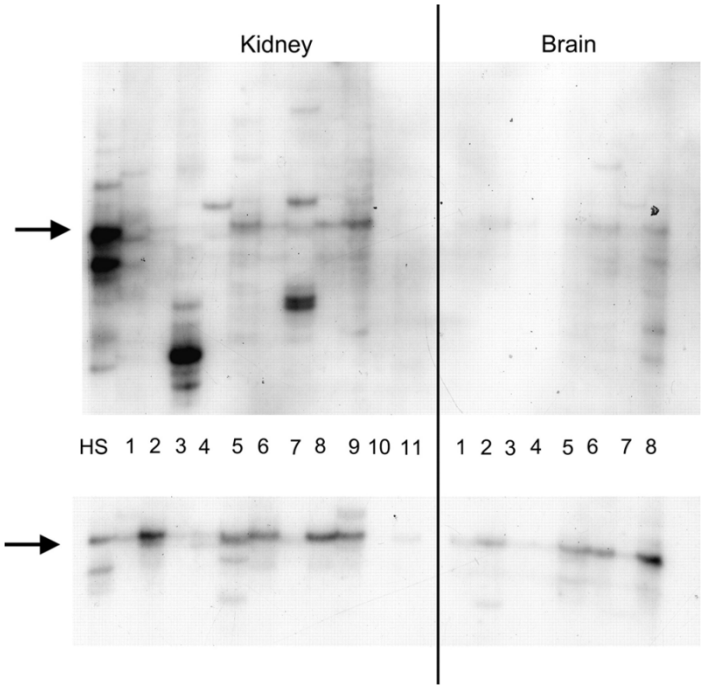


Figure 11. **Clonality of the Vα7.2$^+$ T cell repertoire and presence of the invariant Vα7.2-Jα33 clonotype representing MAIT cells in kidney and brain tumors.**
Tumor tissues obtained from 11 patients with clear cell kidney cancer (left panel) and eight patients with brain tumor (right panel) were 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.

5.2.3 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.[35] 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 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 (**Figure 12**). We were not able to identify shared Vα4$^+$ or Vα19$^+$ clonotypes hybridizing the samples with Cα- or Jα-specific probes (**Figure 12**), suggesting that presence of MAIT and NKT clonotypes in tumors may not be accidental.

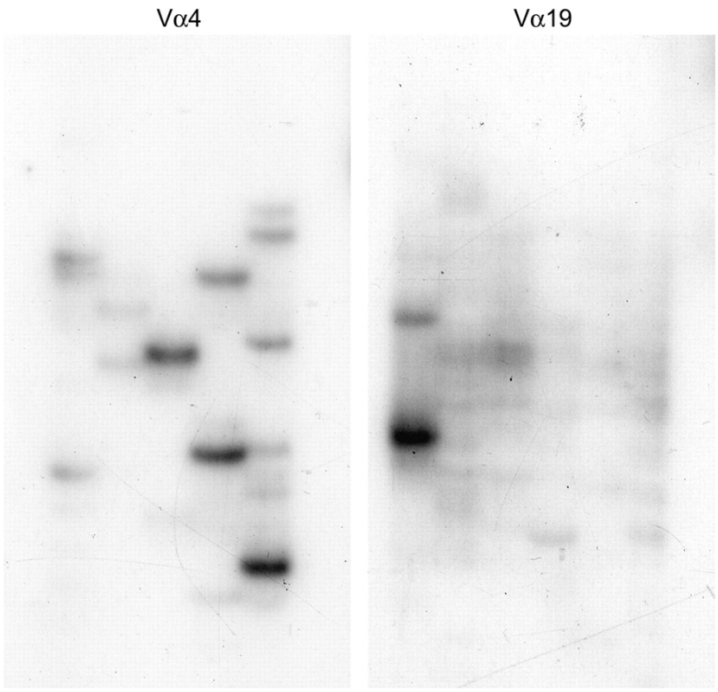


Figure 12. Clonality of the Vα4$^+$ and Vα19$^+$ T cell repertoire in clear cell kidney cancer. Tumor tissues expressing Vα4$^+$ and Vα19$^+$ TCR were further analyzed by SSCP clonality analysis using Cα-specific probe to detect clonotypes with invariant TCR. No shared clonotypes are present in five kidney cancers positive for Vα4$^+$ TCR (left panel) and seven kidney cancers expressing Vα19$^+$ TCR (right panel).

5.2.4 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.[32,93] We examined the expression of these TCRβ chains by RT–PCR. In kidney cancers, all but one sample expressed Vβ2 and Vβ13 mRNA, respectively (**Figure 13**). 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.

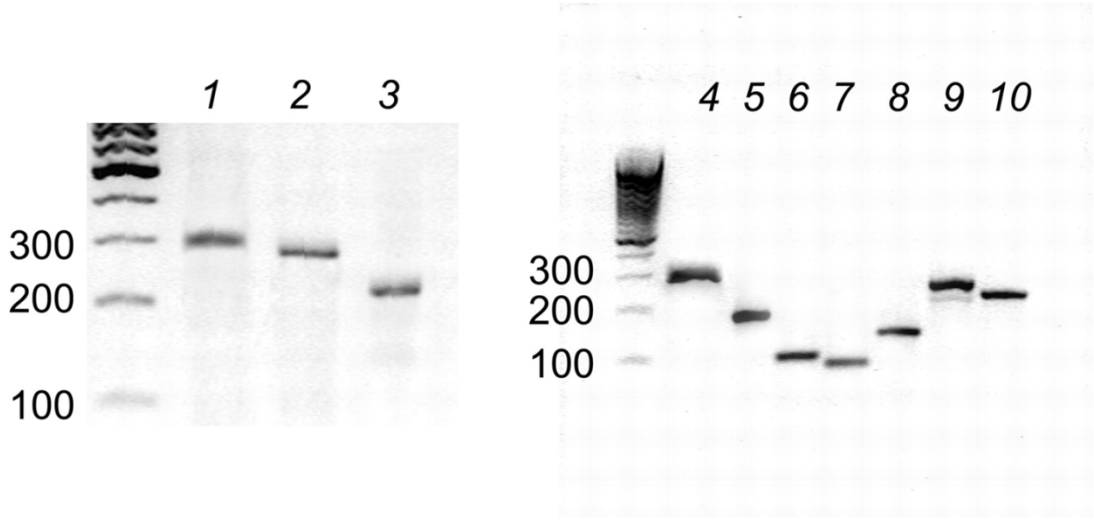


Figure 13. Expression of Vβ2, Vβ13, MR1 and cytokines in tumor tissues expressing MAIT αTCR.
Expression of cytokines, TCR-Vβ chains and MR1 was determined by RT–PCR in a representative sample of clear cell kidney cancer (#1) expressing the invariant MAIT αTCR (see **Tables 7 and 8**) (lanes indicate: 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).

5.2.5 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 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 (**Figure 14**). FACS staining of infiltrating lymphocytes confirmed that HLA-DR^+CD8^{low} T cells were the major subset in tumors expressing MAIT TCRs (**Figure 14**). 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 (**Figure 15**).[22,32]

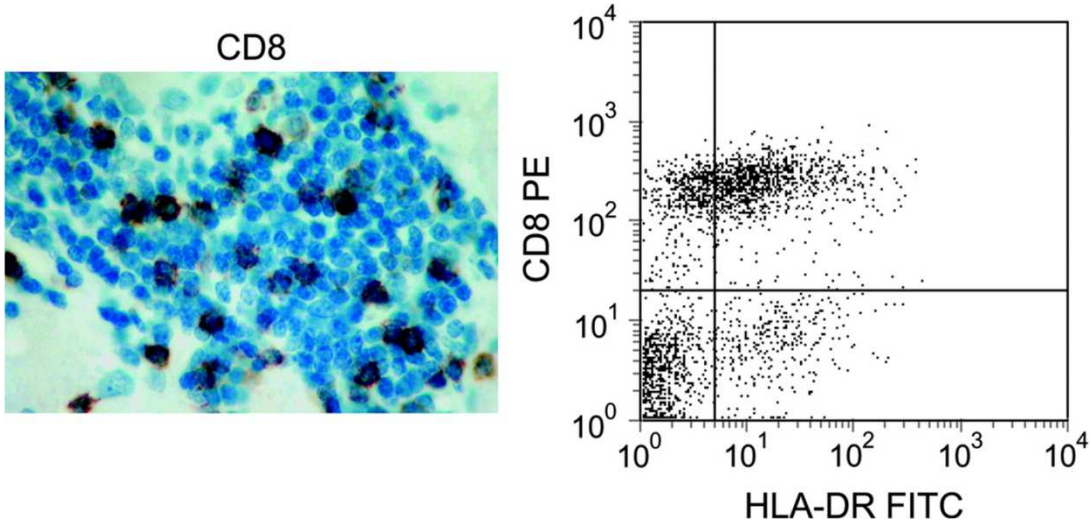


Figure 14. Phenotype of infiltrating T lymphocytes in tumor tissues expressing the invariant MAIT αTCR.
Surface expression of CD8 by immunohistochemistry (x400) and CD8/HLA-DR on tumor-infiltrating lymphocytes analyzed by flow cytometry is shown in a representative sample.

5.2.6 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 (**Figure 15**). 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 (**Figure 15**).

5.2.7 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 Th2 cytokines.[32,34,94,95] This view has been recently challenged in mice.[96] Therefore, we attempted to examine the cytokine environment in tumors expressing MAIT and NKT TCR (**Figure 13**). 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 7 and 8**).

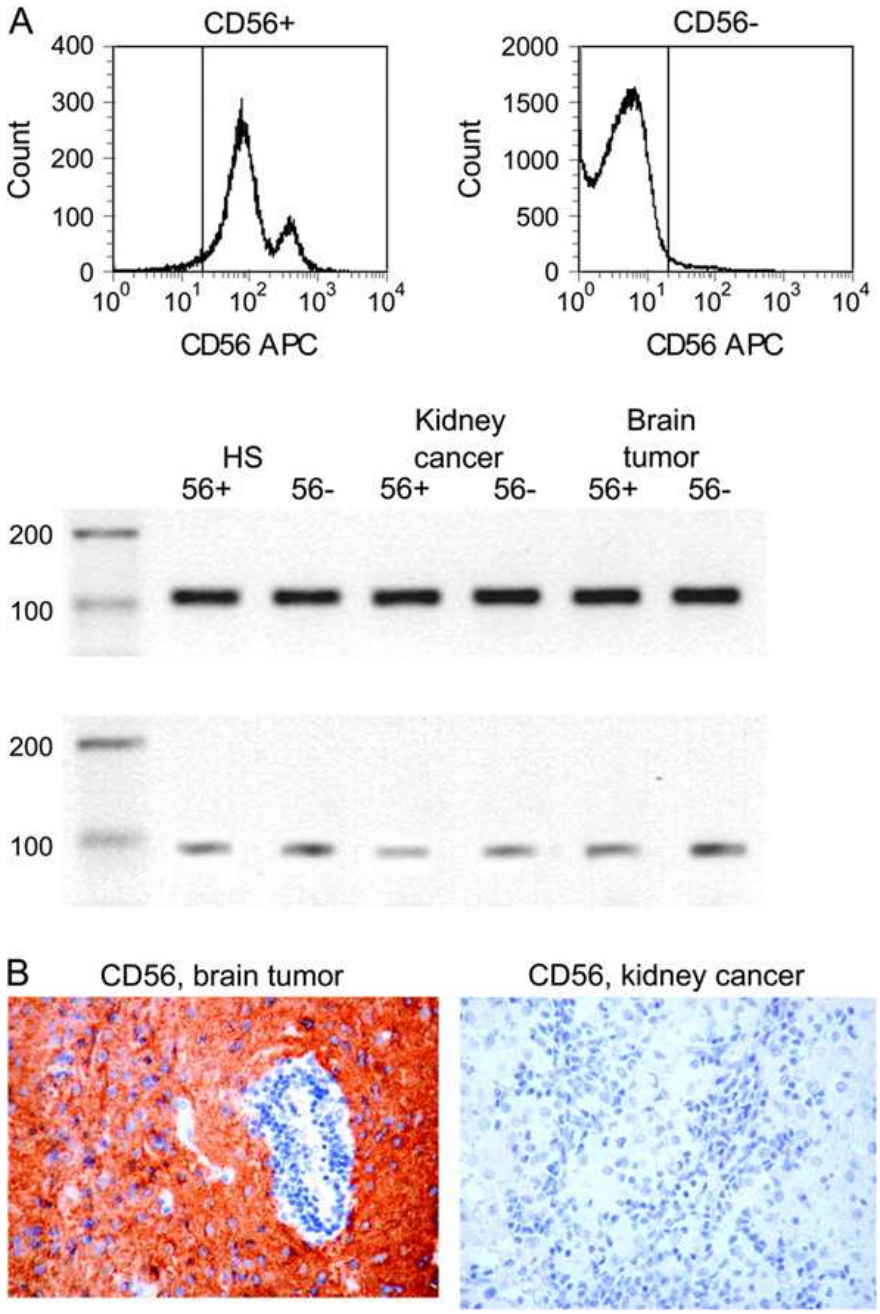


Figure 15. Expression of CD56 in tumors expressing MAIT αTCR and presence of MAIT αTCR in peripheral CD56$^+$ and CD56$^-$ lymphocyte subsets obtained from cancer patients and controls.

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, x200).

The majority of tumors expressed pro-inflammatory (Th1 and Th17) cytokines. IL-12 and 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 7 and 8**).

	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	+	+	+	+	+	+	-	+	+	-	-

Table 7. Expression of cytokines, MR1 and TCRα chains of T cell populations with invariant CDR3α in clear cell kidney cancers.
Numbers indicate individual tumor samples. The number of samples corresponds to those in **Figures 10 and 11**. 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.

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 7 and 8**).

	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	+	+	-	-	+	+	+	+

Table 8. Expression of cytokines, MR1 and TCRα chains of T cell populations with invariant CDR3α in brain tumors.
Numbers indicate individual tumor samples. The number of samples corresponds to those in **Figures 10 and 11**. 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.

To examine whether infiltrating MAIT cells may be locally activated through antigen recognition, expression of MR1 was also checked (**Tables 7 and 8 and Figure 13**). 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.

5.3 Discussion

The role of NKT cells in tumor immunity is well established.[23,97,98] 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.[25,32,94,95,99,100] 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.[101-104] Particularly, we modified the method to detect invariant Vα7.2-Jα33 T cells, later termed MAIT cells, in autoimmune lesions.[26,34]

Out of the analyzed four T cell subpopulations, only invariant Vα24-JαQ NKT and Vα7.2-Jα33 MAIT cells could be identified in tumor tissues. Although both $V\alpha4^+$ and $V\alpha19^+$ 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 $V\alpha4^+$ and $V\alpha19^+$ 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 $V\alpha7.2^+$ and $V\alpha24^+$ 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 $V\alpha7.2^+$ and $V\alpha24^+$ T cell repertoire in autoimmune CNS lesions, compared with autoimmune demyelinating lesions of the PNS.[27,34] 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.[26] 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.[98,105-107] Presence of NKT cells or the invariant Vα24-JαQ TCR has been shown in human tissue samples[27,108-110], including tumors in situ.[111-113] 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.[34,108] 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.[114-118] 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.[116-118] 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.[22,32] 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. 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.[25,32,34,94,95,99,100] 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.[95] In humans, the invariant MAIT TCR was detected in autoimmune lesions in connection with expression of IL-4 and IL-10 mRNA.[34] However, recent data indicated a heterogeneous cytokine production by murine MAIT cells, similar to NKT cells.[96,119] 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.[120] 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.[96,119] 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.[99,100]

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 Th1/Th17 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 Th2 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.

6. SUMMARY OF THESES

1. The *ex vivo* and *in vitro* frequencies of lymphocyte subsets in the treated patients with Pompe disease were not different from the untreated patient with Pompe disease and healthy controls. The *ex vivo* percentage of activated $CD4^+CD25^{low}$ T cells was significantly elevated in the treated patients with Pompe disease. Expression of cytotoxic FasL and perforin molecules by NK, NKT-like and $CD8^+$ T cells were not increased *ex vivo*.
2. rhGAA stimulation *in vitro* generated a dose-dependent increase in intracellular IFN-γ expression in $CD4^+$ and $CD8^+$ T cells in the treated patients with Pompe disease. Isolated $CD4^+$ and $CD8^+$ T cells produced increased amounts of IFN-γ and TNF-α in half of the treated patients after *in vitro* stimulation with rhGAA, while IL-4, IL-6, and IL-17A levels were not elevated. We show that enzyme replacement therapy induces pro-inflammatory T cell responses besides an antibody response in Pompe disease.
3. 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.
4. 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 IFN-γ 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.
5. MAIT and iNKT cells are the only known invariant T cells infiltrating brain and kidney tumors.
6. Since iNKT cells are present in CNS tumors, their absence in MS plaques is disease-specific and not related to the CNS environment.
7. MAIT cells may have a pro-inflammatory subset, which infiltrates tumors.

8. 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.
9. MAIT and iNKT cells in tumors do not express CD56 although both $CD56^{+}$ and $CD56^{-}$ subsets are present in the peripheral blood even in patients with cancer.

7. BIBLIOGRAPHY

Cumulative impact factor:	***15.85***
Cumulative impact factor of articles related to Theses:	***9.25***
Cumulative impact factor of articles not related to Theses:	***6.60***

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7.2 Articles not related to Theses

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