ImmunoTools *FlowISiAM* Award 2025

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Exploring the Role of Glial Fibrillary Acidic Protein (GFAP) in Multiple Sclerosis Pathogenesis: Mechanisms of Macrophage-Mediated Recognition and Triggering Autoreactive Adaptive Immune Responses

Abstract:

Multiple sclerosis (MS) is an autoimmune demyelinating disorder of the central nervous system (CNS) characterized by progressive neuroinflammation and axonal damage. Recent evidence suggests that glial fibrillary acidic protein (GFAP), a key intermediate filament protein expressed predominantly in astrocytes, may play a critical role in MS pathogenesis. While GFAP has long been considered a structural protein, emerging data indicate that it may also function as an autoantigen, potentially triggering inappropriate autoreactive adaptive immune responses. This proposal aims to investigate the mechanisms by which GFAP is recognized and presented by macrophages, leading to the activation of T cells and contributing to the inflammatory milieu in MS. Specifically, we will explore the hypothesis that GFAP, released from astrocytes during neuroinflammation, can be taken up by macrophages, processed, and fragments of GFAP will be presented to T cells on MHC II present on macrophages, thereby initiating a cascade of immune responses that contribute to MS development. This study will enhance our understanding of the role of GFAP in MS and

provide novel insights into macrophage-mediated autoantigen presentation, with the potential to identify new therapeutic targets for the treatment of patients suffering from MS.

Background:

Multiple sclerosis (MS) is a chronic, inflammatory neurodegenerative disease of the CNS in which the immune system erroneously targets myelin, produce autoantibodies, leading to neurodegeneration and progressive disability. The exact trigger for the autoimmune response in MS remains unclear, but it is thought to involve a combination of genetic predisposition and environmental factors, stress, infection and pregnancy. GFAP, a protein primarily expressed by astrocytes, has long been considered a biomarker of astrocyte activation in various CNS diseases, including MS. However, recent findings suggest that GFAP may also serve as an autoantigen in MS, potentially contributing to the activation of both the innate and adaptive immune systems.

Astrocytes, through the release of GFAP during periods of injury or inflammation, may provide a critical link between the CNS and the immune system. Macrophages, as key players in immune surveillance and antigen presentation, may uptake GFAP and present it to T cells, thereby initiating a cascade of immune responses that exacerbate inflammation and tissue damage. Understanding how GFAP is processed by macrophages and presented to T cells could lead to novel insights into MS immunopathogenesis and provide new opportunities for targeted therapies.

Innovation:

This proposal introduces an innovative approach by linking the role of GFAP in MS to macrophage-mediated adaptive immunity. While GFAP has been implicated in neuroinflammation, its role as an autoantigen recognized by the adaptive immune system has not been fully explored. The proposed studies will provide the first direct evidence that GFAP can be processed and presented by macrophages, leading to the activation of autoaggressive T cell responses. This novel concept could redefine our understanding of immune-mediated MS pathogenesis and open the door to new therapeutic strategies targeting macrophage-GFAP interactions or GFAP-specific T cells.

Specific Aims:

1) ***Characterization the expression and release of GFAP in the context of neuroinflammation in MS***.

GFAP is an intracellular protein, which means that, under normal conditions, it is not presented on the surface of cells as part of the antigen-presentation process to T cells. However, in some pathological contexts, such as neuroinflammatory diseases, GFAP may be released or processed, and its fragments could be presented on MHC II molecules by antigen-presenting cells (APCs) like dendritic cells, macrophages, or microglia. There are emerging studies describing GFAP fragments presentation on HLA in human (*Fissolo et al., 2009*) and

elevated levels of GFAP in mouse model (*Linker et al., 2009*). However, there is a scarce literature available about immunodominant fragments of GFAP. Interestingly, GFAP specific $CD8^+$ T cells have been described (*Gklinos et al., 2024; Sasaki et al., 2014*). We hypothesize that GFAP released from astrocytes damaged by ongoing inflammation in patients suffering from multiple sclerosis will be recognized and engulfed by macrophages which in turn present fragments of GFAP on MHCII. To confirm this idea, we will use two approaches. The first will rely on human samples (peripheral blood, liquor) from patients visiting MS center at General University Hospital in Prague, Czech Republic. Thanks to the collaboration with ImmunoTools GmbH we will try to develop antibodies specific against GFAP present on MHCII to test the presence of GFAP complexes in patients suffering from MS in both peripheral blood and liquor.

To better clarify the role of GFAP in triggering MS, an experimental autoimmune encephalomyelitis (EAE) mouse model will be employed as well. We plan to quantify GFAP levels in the CNS during active disease phases and to determine complexes of GFAP on macrophages in mice with induced EAE. Immunohistochemistry and western blotting will be employed to evaluate the localization of GFAP in astrocytes, with a special focus on identifying GFAP release in the extracellular space and detection of GFAP complexes on macrophages during inflammation.

2) ***Investigation of the uptake of GFAP by macrophages and its presentation to T cells***.
To elucidate how macrophages interact with released GFAP and process GFAP for antigen presentation to T cells we will use macrophages directly isolated from MS patients (or EAE mice) or derived from monocytes (obtained from human peripheral blood) or murine bone marrow precursors (cocultured with M-CSF). Macrophages will be exposed to GFAP protein, and phagocytosis of GFAP will be assessed using flow cytometry and confocal microscopy. The GFP labelled GFAP will be detected in macrophages by flow cytometry as well. T cell activation assays will be performed to assess the ability of GFAP-loaded macrophages to stimulate GFAP-specific T cell responses. Specifically, GFAP-loaded macrophages will be cocultured with naïve $CD4^+$ T cells in ratio 1:10 for seven days to as described previously *(Súkeníková et al., 2017; Hrdý et al., 2020; 2022*). The proportion of particular $CD4^+$ T cell subsets will be detected using flow cytometry with special focus on Th1 and Th17 playing a key role in MS triggering.

3) ***Assessment of the role of GFAP in modulating T cell responses in patients suffering from MS.***
To investigate the impact of GFAP presentation on T cell activation and the progression of neuroinflammation in MS, we will track GFAP-specific T cell responses in both MS patient samples and the EAE model using flow cytometry to identify $CD4^+$ and $CD8^+$ T cells reactive to GFAP-derived peptides. We will examine cytokine production, proliferation, and differentiation into effector T cell subsets (Th1, Th17, etc.) in response to GFAP.

Furthermore, the effect of blocking macrophage-GFAP interactions (via antibodies developed by ImmunoTools) on the severity of EAE will be evaluated to determine the therapeutic potential of targeting GFAP recognition pathways. For detailed analyses, possibly additional grant project will be required.

This project will be solved in collaboration between First Faculty of Medicine, Charles University and General University Hospital in Prague (assoc. prof. Jiri Hrdy) and Multiple Sclerosis Center at General University Hospital in Prague (Petra Nytrova, MD, PhD).

Potential Impact:

The successful completion of this project could have significant implications for MS treatment strategies. By understanding how GFAP is processed and presented by macrophages, we could identify novel biomarkers for early MS diagnosis or progression. Moreover, targeting GFAP-specific T cell responses or macrophage-GFAP interactions may provide new avenues for immunomodulatory therapies aimed at halting disease progression or preventing relapse in MS patients.

Cooperation Partner

Associate professor Dr. Jiri Hrdy and assistant professor Dr. Petra Nytrova will work together with ImmunoTools to adjust the experimental and instrumental set-up to conduct *FlowISiAM* analysis at the Institute of Immunology and Microbiology (Prague). ImmunoTools and its partner SME, INVIGATE, will share specific know-how for computer-aided scoring from *FlowISiAM* raw data for optimal test results. ImmunoTools' partner SME, INVIGATE, will assist in developing peptide-specific monoclonal antibodies designed to detect peptides derived from GFAP in inflammatory macrophages by *FlowISiAM*. The partners hope to collect preliminary data that could be used to prepare a joint research grant application.

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IN CASE OF ACCEPTANCE

ImmunoTools *FlowISiAM* AWARD for
Jiri Hrdy and **Petra Nytrova** includes

antibodies for *FlowISiAM*, know how transfer and protocol, support regarding selection of specific antibodies against specific biomarkers from INVIGATE, expert assistance in evaluating the results obtained, and integration into the ImmunoTools *FlowISiAM* network.