In Vitro Treg Immunosuppression Assay of GITR Proteins Could Elucidate the Autoimmune-mediated Mechanism Underlying Type 1 Narcolepsy

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Abstract

Type 1 narcolepsy is a hypersomnia sleep disorder characterized by excessive daytime sleep and shallow NREM nighttime sleep. Deficiency of hypocretin-1 secreting neurons in the lateral hypothalamus is the primary cause of the disorder, and studies demonstrated that these neurons were solely diminished in a brain region of remarkable heterogeneous neuronal population. Specific destruction of targeted neurons could be achieved via antigen presentation to immune cells, a characteristic of cell-mediated response in the adaptive immune system. Since hypocretin-1 neurons are exclusively targeted, numerous studies have speculated an autoimmune-mediated mechanism, but currently lack adequate knowledge and consistent results. In this research proposal, it is hypothesized that enhanced glucocorticoid-induced TNFR-related (GITR) protein expression in T regulatory (Treg) cells results in defective suppression capacity of CD4+CD25+ T helper cells and defective self-tolerance, thereby promoting the destruction of hypocretin-1 secreting-neurons in the lateral hypothalamus. Enhanced GITR expression in Treg cells was shown to inhibit the suppression capacity of an inappropriate immune response, and it is a plausible mechanism through which hypocretin-1 neurons are destroyed. This proposal devises a correlational study to measure cerebrospinal fluid (CSF) hypocretin-1 levels inversely proportional to GITR expression levels in Treg cells to assess the autoimmune nature of the disorder. This study is aimed at investigating how defective Treg cells respond in type 1 narcolepsy patients via CSF hypocretin-1 measurement and in vitro Treg cell suppression assay.

Keywords: Type I narcolepsy, GITR protein expression, Treg immunosuppression assay

Introduction

Imagine you are irresistibly drowsy, and you fall asleep during the day despite making tremendous efforts to stay awake. Imagine you are frequently awakened from nighttime sleep, constantly suffering from poor quality sleep and restlessness. These symptoms are recurring and could profoundly impact loved ones. Type 1 narcolepsy or narcolepsy-cataplexy is characterized by cataplexy (loss of muscle tone), excessive daytime sleepiness associated with dreaming, and fragmented nighttime sleep concomitant with hypocretin-1 deficiency (Alakuijala et al., 2016). Narcolepsy with cataplexy has a prevalence of between 25 and 50 per 100,000 people, and the time onset of the disease predominantly affects adolescent and middle-aged demographics (Longstreth et al., 2007).

The criteria for narcolepsy-cataplexy diagnosis consists of excessive daytime sleepiness (almost every day for at least three months) and a score of two to five sleep-onset rapid eye movement periods (SOREMPs) where each SOREMP is determined by the presence of REM sleep within 15 minutes of sleep onset during a multiple sleep latency test (Dauvilliers et al., 2007). Excessive daytime sleep is characterized by decreased sleep-onset latency (shorter than 8 minutes) to REM sleep (Dauvilliers et al., 2007). Moreover, nocturnal polysomnography revealed that narcolepsy-cataplexy patients had a

significantly higher arousal index during nighttime sleep and higher wake time after sleep onset percentage but shallower stage 2 non-REM (NREM) sleep (Takei et al., 2012). Altogether, these studies concluded that narcolepsy-cataplexy induces frequent awakenings during nighttime sleep and abnormal daytime sleep. Actigraphy studies showed drastically longer sleep latencies and more interrupted sleep bouts in narcoleptics with less than 30 pg/ml of hypocretin-1 levels than those with more than 30 pg/ml of the neuropeptide (Alakuijala et al., 2016). Importantly, the study also suggested that lower hypocretin-1 levels strongly correlated restlessness with compromised sleep efficiency, and these symptoms inversely covaried with hypocretin levels among the patients.

Hypocretin-1, also known as orexin A, is a neuropeptide produced by hypocretin neurons in the lateral hypothalamus crucial for arousal and maintenance of the waking state (Mahoney et al., 2019). Hypocretin peptides are released in vast projections throughout the brain to wake-promoting regions, namely the basal forebrain, tuberomammillary nucleus, periaqueductal grey, dorsal raphe, and locus coeruleus (Mahoney et al., 2019). Moreover, hypocretin is involved in feeding and metabolism; therefore, hypocretin deficiency is also linked to obesity (Hara et al., 2001). Hypocretin neurons additionally co-release

hypocretin-2, dynorphin, glutamate, and neuronal activity-regulated pentraxin (NARP), which synergize with hypocretin-1 effects (Mahoney et al., 2019). Interestingly, studies have implied that narcolepsy is specifically caused by a deficit of hypocretin-secreting neurons rather than the deficit of the hypocretin-1 peptides, since a decrease in the co-release of NARP and dynorphin substrates from hypocretin neurons paralleled the decrease in hypocretin-1 in narcoleptic patients (Crocker et al., 2005).

Narcolepsy involves hypocretin-1 deficiency (Nishino et al., 2000). In human studies, iodine-125 hypocretin-1 radioimmunoassay demonstrated that most narcoleptic patients had a substantial decrease in hypocretin concentrations that was undetected by the limit of the assay (<40 pg/mL) (Nishino et al., 2000). Additionally, studies have shown that this deficiency was exclusively found in hypocretin neurons of the lateral hypothalamus, and no other neuronal population deficits were found in the same brain region (Thannickal et al., 2000). Given the heterogeneous composition of neuronal populations in the lateral hypothalamus (Mickelsen et al., 2017), resident melanin-concentrating hormone (MCH) neurons of the same brain region did not decrease while hypocretin neurons did in narcoleptics (Thannickal et al., 2000). This demonstrated that the sleep disorder resulted from a specific deficit of the hypocretin cell population in the

lateral hypothalamus rather than a general decrease in neuron populations. Consistent with this finding, treating narcoleptic dogs with hypocretin-1 prevented symptomatic sleep durations and cataplexy and restored normal sleep behaviour (John et al., 2000).

Given the specificity of hypocretin neuron deficiency in the lateral hypothalamus, many have hypothesized that autoimmunity may cause narcolepsy (Mahlios et al., 2013). Multiple studies have shown an increased propensity of developing narcolepsy after *Streptococcus pyogenes* and the influenza A virus (2009 H1N1) infection (Mahlios et al., 2013). This immunological aspect of narcolepsy cultivated a significant interest in searching for an autoimmune mechanism, and studies found a strong correlation between the presence of anti-tribbles homolog 2 (TRIB2) autoantibodies in narcoleptic HLA DQB1*06:02 gene carriers and cataplexy symptoms (Kawashima et al., 2010). Mechanisms of autoimmunity against the hypocretin neuron autoantigen, TRIB2, suggested that H1N1 virus epitopes may stimulate a cross-reactivity against TRIB2, producing TRIB2 autoantibodies that target the hypocretin-secreting neurons (Singh et al., 2013). However, no T cell cross-reactivity with influenza antigens was experimentally and consistently reproduced (Latorre et al., 2018).

For context, the immune system consists of delicate coordination between the

innate and adaptive immune responses to prevent pathogenic foreign antigens from infecting cells in the body. Immune cells are selected for self-tolerance meaning that they must tolerate their self-antigens to prevent self-destruction (Silverthorn et al., 2019). An adaptive immune response, unlike an innate immune response, is slower but antigen-specific because it produces memory cells that have acquired the immunity to fight specific pathogens. In an adaptive immune response, cell-mediated responses coordinate with antibody-mediated responses to kill specific pathogens (Silverthorn et al., 2019). Cell-mediated immunity requires contact-dependent signalling between an immune cell and its respective receptors on target cells. It primarily involves lymphocytes, a type of white blood cell, including B lymphocytes (and their derivative plasma cells) and T lymphocytes (cytotoxic T cells, Helper T cells and Regulatory T cells) (Silverthorn et al., 2019). Adaptive immunity requires the presence of a pathogenic antigen to immune cells so they can identify and destroy infected cells. Antigen presentation is facilitated by membrane complexes called major histocompatibility complexes (MHC), which are found on antigen-presenting cells (APC) such as macrophage immune cells. Next, a cell-mediated immune response occurs when an MHC-antigen complex interacts with a T-cell receptor, leading to subsequent activation of CD4+ Helper T cells that help activate other T cells (Silverthorn et al., 2019).

B lymphocytes, in contrast, are activated upon exposure to an antigen matching their receptors. They subsequently differentiate into plasma cells to secrete antigen-specific antibody molecules into the bloodstream. CD8+ cytotoxic T cells destroy cells that display MHC-antigen complexes, whereas Treg cells prevent inappropriate immune responses by suppressing other lymphocyte activities, thus maintaining self-tolerance (Brusko et al., 2008). Treg cells, also known as suppressor T cells, actively suppress effector T cell activity, including CD4+helper T cells, B-cell antibody production, CD8+ cytotoxic T lymphocyte granule release, and APC function (Brusko et al., 2008). Therefore, T lymphocytes play a major role in mediating immune responses that target cells displaying specific proteins.

Hypocretin deficiency in narcoleptics is caused by T lymphocytes targeting hypocretin cells. Latorre et al. (2018) demonstrated that CD4+ and CD8+ T lymphocytes can target the hypocretin autoantigen tribbles homologue 2 (TRIB2), which contributes to the specific destruction of hypocretin neurons in the lateral hypothalamus. Therefore, it is highly plausible that hypocretin cell loss is mediated by autoimmunity against self-antigens.

A study found increased system-wide T cell levels in narcoleptics compared to control groups, suggesting an overactive T

cell activation (Lecendreux et al., 2017). Specifically, there was a significant increase in memory CD45RA-CD62L⁺ CD4⁺ T lymphocytes with an increased CD25 and CD69 expression. Increased CD69 and decreased CD28 phenotype in CD8⁺ cells were found, whereas an increase in Treg cell count with an increased GITR expression surfaced (Lecendreux et al., 2017). The research team hypothesized that the expression of these Treg cell phenotypes caused defective immune cell suppression and that T cells expressing GITR were resistant to programmed cell death. Since an overactive GITR expression in Tregs caused defective suppression of effector cells (Shimizu et al., 2002), examining specific Treg expression profiles in relationship with hypocretin-1 measurements enables us to test Lecendreux et al.'s (2017) hypothesis. GITR is predominantly expressed on CD25⁺CD4⁺ regulatory T cells, Treg cells that effectively suppress activation of CD4⁺ and CD8⁺ T cells (Shimizu et al., 2002). Treg cells additionally suppress effector T cells, B-cell antibody production, and antigen-presenting cell (APC) function (Brusko et al., 2008). Because Treg suppresses immune cells, it is implicated in the maintenance of self-tolerance to endogenous antigens (Wing & Sakaguchi, 2010). Lecendreux et al. (2017) believed that the identified Treg phenotypes in narcoleptics were dysfunctional. However, the mechanism through which these specific Treg cells in narcoleptics interact with hypocretin neuron antigens remains

elusive, and no Treg cell suppression assay was performed to test the hypothesis of a defective Treg cell function.

Research Question & Hypothesis

The research objective is to understand how defective Treg immune cells can lead to an autoimmune attack on hypocretin neurons in type 1 narcolepsy patients. According to Lecendreux et al.'s (2017) hypothesis, defective Tregs could induce type 1 narcolepsy by lack of CD8⁺ T cell suppression, leading to hypocretin cell death in the lateral hypothalamus. If Treg cells expressing GITR were defective in suppressing immune cells, then an increased Treg cell concentration should correlate with decreased hypocretin-1 levels.

Alternatively, the null hypothesis predicts that there could be an adaptation in antigen-presentation to T cell receptors (TCR). The T cell receptor alpha locus encodes the alpha-chain of the TCR $\alpha\beta$ -heterodimer, a protein expressed by T lymphocytes, and this protein interacts with both CD8 cytotoxic T cells and CD4 helper T cells (Hallmayer et al., 2009). Studies have demonstrated a robust correlation of narcolepsy with human leukocyte antigens (HLA) and demonstrated an association between narcolepsy and polymorphisms in the T-cell receptor alpha locus, which encodes the major receptors for HLA-peptide presentation (Hallmayer et al., 2009).

Thus, if there is no correlation between defective Treg cells and narcolepsy, then it is plausible that there is a dysfunction downstream of the mechanisms involved in antigen presentation.

Methods & Predicted Results

Animals

10 healthy mice (5 M: 5 F) and 10 type 1 narcoleptic mice (5 M: 5 F) are selected for measuring T cell levels and phenotypes. Healthy subjects with no history of H1N1 infection are selected since infections may be a confounding variable in testing T cells. Narcolepsy-cataplexy subjects are body mass index (BMI) and age matched with the healthy group, because hypocretin-deficient patients tend to be obese, and it is preferable to limit confounding variables in the experiment.

CSF hypocretin-1 sample and multiple reaction monitoring (MRM) liquid chromatography mass spectrometry (LC/MS)

Next, we measure cerebrospinal fluid (CSF) hypocretin-1 levels to provide baseline measurements of hypocretin-1 concentrations in a healthy and a narcoleptic group. This enables experimenters to compare hypocretin-1 levels to a control group and a reference point (Kanbayashi et al., 2002). To measure hypocretin levels, a sample of CSF is removed from anesthetized subjects

during a lumbar puncture. The CSF hypocretin-1 samples are then centrifuged, purified, and quantified. Iodine-125 hypocretin-1 radioimmunoassay was used as a standard for measuring hypocretin-1 levels, but it is limited by its precision as cross-reactivity of metabolized components often occurs; thus, quantifying in mass spectrometry in MRM mode produces superior assay accuracy (Hirtz et al., 2016). Standard curves of hypocretin-1 concentrations of the two experimental groups (healthy and NT1) are then constructed from the spectrometry data (BMI, age and sex matched). Healthy subjects should be in the range of 291 +/- 66 pg/ml of hypocretin-1 (Kanbayashi et al., 2002) while narcoleptic patients should expect data points ranging between 0 – 40 pg/ml of hypocretin-1 (Nishino et al., 2000).

In vitro Treg cell suppression assay

Finally, a Treg cell suppression assay is essential in identifying whether Treg cells are functioning normally by labelling CD4+CD25+ T cells (McMurchy & Levings, 2012). The flow cytometry-based method of labelling CD4+CD25+ T cells with 5,6-carboxyfluorescein succinimidyl ester (CFSE) could identify Treg and helper T cells independently (McMurchy & Levings, 2012). Crucially, it enables experimenters to see if GITR Treg cells have a direct effect on T helper cell activity and concentration levels and if these experimental outcomes collectively

contribute to CSF-hypocretin-1 deficiency. Since Treg cells divide throughout the assay, each producing two daughter cells, using a flow-cytometry analysis software (i.e. FlowJo TreeStar) allows for an accurate estimation of the substrates by considering the Treg proliferation index, the average number of divisions in the starting population, and the percentage of cells in the starting population that have divided at least once to prevent underestimation of its suppression capability (McMurchy & Levings, 2012). Controls are implemented to prevent underestimation of Treg count by measuring T-cell proliferation with and without the cytokine interleukin 12, since cells can die throughout the assay which could be misinterpreted as normal suppression of immune cells (McMurchy & Levings, 2012) (Moon et al., 2015). If GITR Treg cells are defective, then GITR Treg and T helper cell counts should negatively correlate with the CSF hypocretin-1 levels.

Discussion

Identifying markers for Treg cell assay is a daunting task requiring expertise. In human models, Treg cells express several surface markers, and although GITR is a plausible candidate for Treg cell marking, it was only represented in a small CD4⁺CD25⁺ population. While CD4⁺CD25⁺ T cells increase GITR expression right after T cell activation, this GITR upregulation only lasts a few days (Ronchetti et al., 2015). Thus, it is not a permanent Treg marker. Mice studies have

suggested that increased GITR expression in CD25⁺CD4⁺ Treg cells are unable to suppress T cells regardless of the enhanced GITR expression (Shimizu et al., 2002), but this problem could be circumvented if we could correlate increased T helper cells with increasing GITR Treg and decreased CSF hypocretin-1 levels. Because Treg suppression activity is assayed in vitro and Treg cells die throughout the assay, experimental results may not reflect physiological conditions such as higher Treg proliferation index in vivo (McMurchy & Levings, 2012).

Significance of Research

In the last decade, research on narcolepsy focused on genetic associations and autoimmune-mediated mechanisms that may lead to hypocretin-1 neuron deficiency in the lateral hypothalamus. However, no studies were conducted on how defective Treg cells respond in narcoleptic patients. Treg cells physiologically suppress effector T cells, B-cell antibody production, and antigen-presenting cell (APC) function, thereby preventing excessive autoimmune response (Brusko et al., 2008). Lecendreux et al. (2017) identified specific Treg cell phenotypes that increased in type 1 narcolepsy. To extend his research, a suppression assay of Treg cells expressing GITR will determine if Treg cells were dysfunctional in suppressing cytotoxic CD8 cells against TRIB2 hypocretin antigen. Correlating increased GITR expression in Treg cells with CSF hypocretin-1

deficiency will test the hypothesis of an autoimmune-mediated attack on hypocretin neurons by lack of Treg suppression capacity. If the Treg cells in narcoleptics were indeed defective, then this study will provide a ground for the immunopathology. On the contrary, if we fail to identify a deficit in Treg cells' capacity to suppress T effector cells, then it implies that T effector cells tolerated Treg cells' suppression and further T cell receptor studies can test that. Regardless of the experimental outcome, this study still provides insight into the mechanisms of autoimmunity in type 1 narcolepsy, helping neuroscience close the gap between sleep disorders and immunology. The immune system is a sophisticated and fascinating field of study. Its implications for sleep disorders may contribute to a plethora of clinical treatment options for narcolepsy, viral infections, and autoimmune diseases.

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