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科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月 ２０ 日現在

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研究課題名（和文）Invesitgation of Eomes+ Th cells: a pathogenic population controlling neuroinflammation during secondary progressive multiple sclerosis

研究課題名（英文）Invesitgation of Eomes+ Th cells: a pathogenic population controlling neuroinflammation during secondary progressive multiple sclerosis

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：多発性硬化症（MS）は免疫細胞が脳内や脊髄内に侵入して活性化し、自己組織を損傷することにより発症し神経障害を起こす。我々はこれまでに、Eomes+ Th cellsという新型リンパ球が慢性的MSに関連することをマウスモデルにおいて見いだした。本研究ではEomes+ Th cellsの増加が二次進行型MS（SPMS）患者の病状悪化と相関することを発見し、Eomes+ Th cellsの測定がSPMSの診断と病気の状態を示すバイオマーカーとして有用であることを明らかにした。さらに、Eomes+ Th cellsを標的としたSPMSの新しい治療法が有効である可能性を示した。

研究成果の学術的意義や社会的意義
This study provides new information about a type of damage-associated T cells in autoimmune disease. This will aid future study of the process involving these cells and increase understanding of cellular immunology in diseased tissues as well as providing new biomarkers and targets for treatment.

研究成果の概要（英文）：The autoimmune disease multiple sclerosis (MS) occurs when activated immune cells enter the brain and spine, causing tissue damage, which leads to peripheral and neurological disabilities. Previously, we found that a new type of T helper cell (Eomes+ Th cells) was associated with a model of chronic MS. In this study, we investigated how Eomes+ Th cells were associated with disease in patients with secondary progressive MS (SPMS), a chronic type of MS.
We found Eomes+ Th cells were increased in the blood of some patients with SPMS and these cells were infiltrating into patient brain tissue. Also, high levels of Eomes+ Th cells were associated with worsening clinical disease. This study highlights Eomes+ Th cell measurement as a biomarker for SPMS diagnosis and disease activity, allowing early treatment with efficacious drugs. As Eomes+ Th cells appear to be a pathogenic cell type associated with damage in SPMS, these cells are a candidate target for developing future SPMS treatments.

研究分野：Autoimmune disease

キーワード：Autoimmune disease　multiple sclerosis　neuroinflammation

１．研究開始当初の背景 ＜ Background ＞

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) and its incidence has been rapidly increasing in Japan and other developed countries. MS most often manifests as a relapsing-remitting form (RRMS), with patients suffering from acute onset of neurological signs followed by recovery to a remission phase that lasts for months to years. RRMS has long been known to be associated with self-reactive T helper (Th) cells as highlighted by large gene association studies and the efficacy of immune cell-targeting treatments (International Multiple Sclerosis Genetics Consortium *et al.* ***Nat Genetics***, 2013; Martin, R., *et. al* ***Eur J Immunol.*** 2016).

For many patients with RRMS, this disease shifts to a progressive course with accompanying chronic neuroinflammation (secondary-progressive MS; SPMS). Transition to SPMS is accompanied by gradual chronic or intermittent deterioration of physical activities required for daily life, such as the ability to walk. Cognitive impairment due to brain atrophy can also manifest as a predominant sign of SPMS. Previously, SPMS was thought to be a purely neurodegenerative disease (Trapp, B. & Nave, K. ***Ann. Rev. Neurosci*** 2008). Recently, evidence from our group and others is gaining acceptance with many expert neurologists that in fact active immune processes instead play key roles in SPMS (Kappos *et al*. ***Lancet***, 2018; Christensen *et al.*, ***Neurology***, 2014). These findings have increased interest in developing new immune-specific drugs for SPMS.

No clear clinical boundary exists between RRMS and SPMS: diagnosis of SPMS is dependent on retrospective medical record analysis by expert neurologists. The absence of reliable biomarkers to indicate SPMS transition and future disease activity, which would enable early diagnosis and treatment without delay, is an urgent unmet clinical need. Treatments that were effective in RRMS lack efficacy in SPMS, suggesting that the development of SPMS involves different and unknown pathogenic mechanisms. Thus, discovery of pathogenic mechanisms driving CNS damage in SPMS would present new and much needed targets to develop modern specific therapy for patients, allowing control of this disease as has already been achieved for RRMS.

２．研究の目的＜ Purpose of research ＞

Currently, mechanisms that drive pathogenicity in SPMS are unknown and the lack of available drugs is an unmet clinical need. Without knowledge of mechanism, no biomarkers indicating transition from RRMS to SPMS exist and this disease change can only be diagnosed retrospectively after permanent disability has occurred. Additionally, there is a lack of treatment targets and the importance of immune responses was unclear.

We had identified a new pathogenic Th cell subset in SPMS, Eomes^{+} Th cells, which appear to mediate CNS damage via unusual direct cytotoxic mechanisms (**Raveney *et al.*, *Nat Commun***, 2015). Further, we have recently reported that these cells can be generated by the particular environment in long-term inflamed CNS (Zhang, **Raveney *et al.*, *PNAS***, 2019). We wished to further investigate Eomes^{+} Th cells in detail, using both our unique mouse model for late chronic neuro-inflammation and our access to a large group of MS patients.

３．研究の方法＜ Methods ＞

MS patients and healthy volunteers were recruited and enrolled in the study having obtaining informed consent. During regular appointments in our MS clinics, patients donated a small amount of venous blood and peripheral blood mononuclear cells (PBMC) were prepared from this using density centrifugation. PBMC were evaluated using flow cytometry to define specific human T cell subsets.

T cell subset level in patients with SPMS or RRMS was compared with results from healthy volunteers. Clinical and demographic data were obtained by patients' regular

physicians and used to ensure groups were comparable, avoid any effects caused by current drug treatments and to assess clinical severity of disease. In particular, relapse status was recorded and any clinical worsening (progression), improvement, or disease symptom stability (stationary disease) was measured on follow-up for 1 year after regular blood sampling.

To assess infiltration of T cells into CNS tissues and examine association of local effects with pathogenic T cell subsets, we investigated CNS autopsy samples by flow cytometry.

For functional assessment of Eomes+ Th cells, production of the cytotoxic associated enzyme, granzyme B, by CD4+ PBMC was measured by ELIspot.

４．研究成果＜Results＞

We previously reported that CD4+ Th cells expressing the transcription factor Eomes were associated with chronic neuroinflammation. This unusual population of Th cells, which we termed Eomes+ Th cells, appeared in the CNS of mice in a model of late, chronic MS. These cells were pathogenic, invoking neuroinflammation and peripheral clinical signs on transfer into healthy mice. Eomes was required for full disease in the late, chronic phase. Importantly, this transcription factor is normally only found in CD8+ Cytotoxic T cells and not Th cells – in those cells it controls cytotoxic function and we made the surprising finding that Th cells expressing Eomes could also act in a cytotoxic fashion.

As we found Eomes+ Th cells in murine neuroinflammation only in the late, chronic type of disease, we investigated if similar populations of unusual Th cells could be found in different types of MS patients. We sampled peripheral blood from RRMS and SPMS cases and measured Eomes+ Th cells by intracellular flow cytometry.

Whereas a large proportion of CD8+ Tc cells were Eomes+, only a low number of CD4+ Th cells expressed Eomes (5-10%) in healthy volunteer blood donors (Healthy subjects). Similar levels were seen in RRMS cases – indicating that there was no expansion of Eomes+ Th cells due to RRMS and thus, these cells are unlikely to be linked to pathogenicity in this type of MS. However, in contrast, there was a great expansion of Eomes+ Th cells in the blood of patients with SPMS (Fig. 1). These data highly suggest that Eomes+ Th cells may also be involved in pathogenesis in chronic progressive MS in a similar manner to their role in late, chronic murine MS-like disease model.

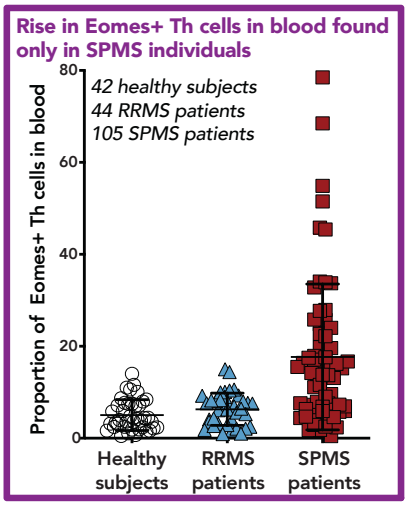
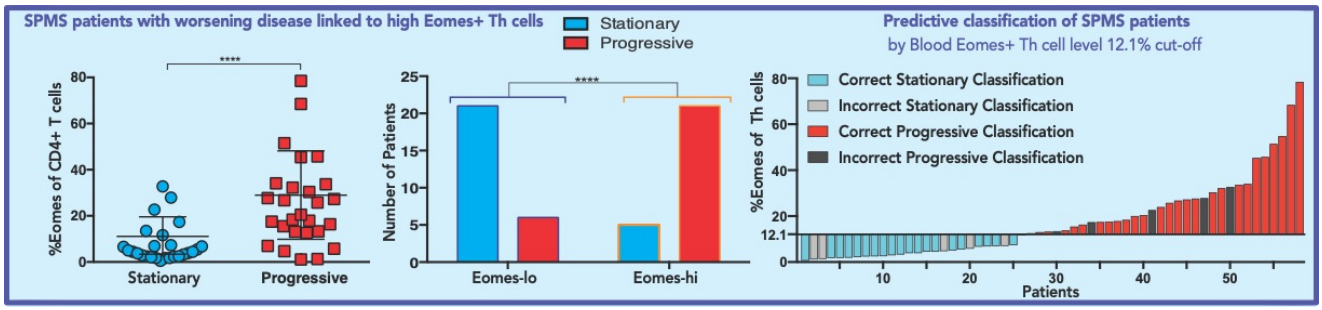


Fig. 1 Eomes+ Th cell level amongst blood Th cells in MS patients and healthy controls

SPMS patients are known to form a heterogenous group, in terms of disease course, progression of clinical symptoms, presence of relapses/recovery, prognosis and which treatment are effective. We predicted that this heterogeneity could result from heterogenous immune mechanisms that drive pathology in different individuals. Indeed, as we observed that there was a great range of Eomes+ Th cell level in SPMS cases, some subjects had very high levels, whilst others had low levels similar to healthy subjects or RRMS cases. To investigate if SPMS cases could be split into those with high Eomes+ Th cells, which may be linked to Eomes+ Th cell-associated pathogenesis, and those SPMS cases with normal Eomes+ Th cell level, we turned to mathematical modelling. Using a gaussian mixture model, we generated a statistically significant cut-off value that split SPMS subjects into a predicted 2 groups both with a normal distribution of Eomes+ Th cell level. We termed these two groups Eomes-hi SPMS and Eomes-lo SPMS patients.

Our suggestion was that if Eomes+ Th cells were pathogenic in some SPMS, then there would be a relationship between Eomes+ Th cell level and disease severity. Therefore, we tested if Eomes-hi and Eomes-lo SPMS groups had different magnitudes of disease burden. Some SPMS cases maintain relapse activity, even after transitioning from RRMS to SPMS. We compared Eomes+ Th cell level for SPMS currently undergoing active disease at a time of relapse versus stabilized SPMS cases. We found no link to Eomes level indicating that active relapses were not associated with any potential pathogenic action of Eomes+ Th cells. The other type of disease worsening in SPMS is the chronic progressive symptoms where disease burden is increasing year on year. Therefore, we measured disease severity at the time of blood sampling and at 1 year follow up after measure blood cells using the EDSS score (Expanded Disability Status Scale – a clinician-assessed severity measure). Patients could then be divided into progressive disease – EDSS was increased 1 year after sampling, or stationary – no clinical worsening over the follow-up year.

Fig. 2 Eomes-hi SPMS cases are linked to worsening progressive clinical symptoms



SPMS patients in a progressive phase (i.e. cases where symptoms would be worse 1 year after sampling) had significantly higher levels of Eomes+ Th cells (Fig. 2 left). Over 80% of Eomes-hi SPMS patients would go on to have worse disease in the next year, whereas 80% of Eomes-lo SPMS had stationary disease (Fig. 2 Center). Eomes+ Th cell level was found to be an effective biomarker; ROC analysis indicated a similar Eomes level cut-off to the predicted level from the modelling and gave an accuracy of 83% in predicting future disease progression (Fig. 2. Right), with a low false discovery rate (FDR = 0.188).

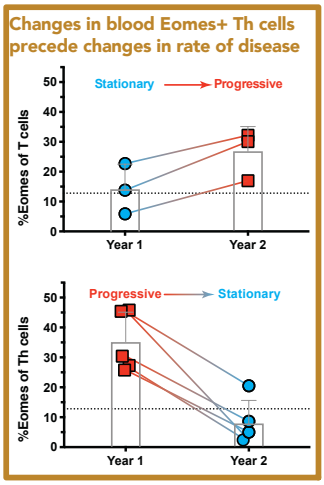
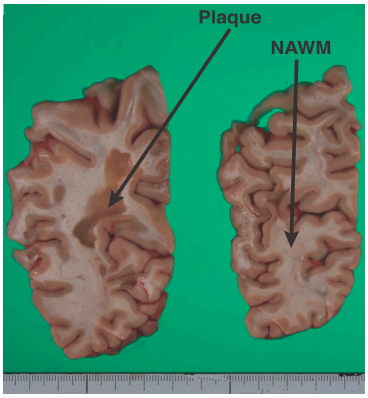


Fig. 3 Eomes+ Th cells level predicts future disease worsening in SPMS

Using further follow up of a smaller number of SPMS patients (7 shown in Fig. 3), we were able to show that Eomes+ Th cell level was not fixed and was able to dynamically predict clinical outcome in these cases. Briefly, Eomes level was assessed at the start of year 1 and again at the start of year 2. Clinical assessments were carried out at these times and at the end of year 2. Thus, the progression/stationary status of these patients could retrospectively be calculated for both year 1 and year 2 and the preceding Eomes+ Th cell measurement be checked for predictive value. As before, Eomes-hi status was predictive of progression in year 1 (Fig. 3 lower). But despite this worsening, a reduced level of Eomes after 1 year was predictive of a change to stationary status in year 2 (Fig. 3 lower). In contrast, when an increase in Eomes+ Th cell level was seen over year 1, where patients were in a stationary disease phase, this was indicative of future worsening in year 2 (Fig. 3 upper).



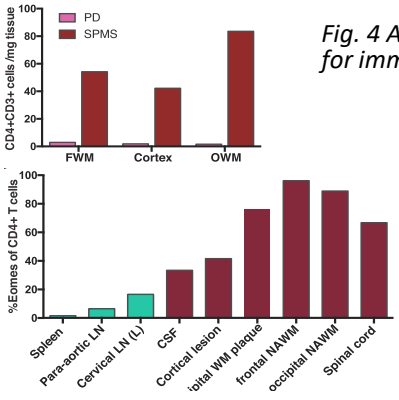
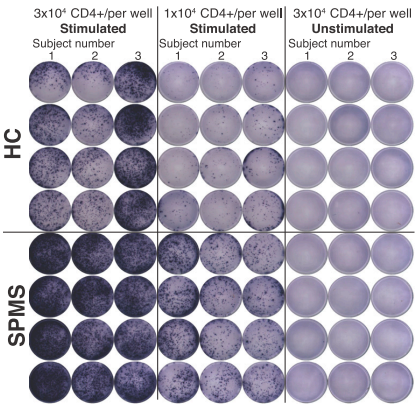


Fig. 4 Autopsy CNS samples were examined for immune cell infiltrate by flow cytometry.

As we have now linked Eomes+ Th cell level to disease worsening in SPMS, we wished to further investigate if these cells are genuinely linked also to SPMS pathogenesis. To truly appreciate the phenotype and mechanisms of Eomes+ Th cells in SPMS, it is critical to examine pathogenic cells in the target site, rather than only peripheral immune cells. To this end, we adopted our mouse CNS workflow to develop a new approach to examine human brain-infiltrating immune cells. This technique offers many advantages over traditional histological examination of tissue sections. We compared Th cell infiltrated into different autopsy tissue samples from different CNS regions from a SPMS subject with a control autopsy case with Parkinson's disease (PD). We observed a significant Th cell infiltrate in SPMS but not PD (Fig. 4 Upper right). Of these CNS-infiltrating Th cells, a high proportion of these expressed Eomes (Fig. 4 Lower Right). These data are highly suggestive of direct pathogenic roles for Eomes+ Th cells in SPMS as they are found to be the majority of cells in the tissue undergoing damage



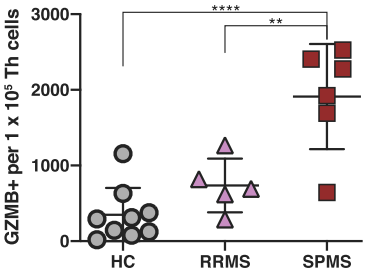


Fig. 5 Granzyme B production by Th cells (ELIspot assay)

As our exciting new data have confirmed Eomes^{+} Th cells *in situ* for the first time, we wished to investigate the potential pathogenic mechanism in the target organ. Data from our mouse model suggested that Eomes+ Th cells may have an unusual function as cytotoxic cells – which is very surprising for Th type T cells. Thus, we investigated if Th cells in SPMS were able to make the cytotoxic enzyme granzyme B.

As expected, Th cells from healthy subjects (HC) or RRMS secrete only low levels of granzyme B (Fig. 5). Importantly, Th cells from SPMS patients elaborated high granzyme B level following stimulation (Fig. 5).

< Summary >

Our study adds important new information to the understanding of SPMS. We propose Eomes+ Th cell level could act as a biomarker to dynamically predict patients at risk of worsening. Furthermore, our study demonstrated that Eomes+ Th cells are implicated in direct damage of CNS tissues as we found these cells infiltrate into the CNS tissue and increase level of Th cells with cytotoxic properties, making cytolytic enzyme granzyme B, in SPMS. Not only does our study now add further weight of evidence for a role of active immune responses in driving SPMS progression, but also it gives new indication of a pathogenic mechanism in this disease.

Finally, our experiments revealing a potential pathogenic mechanism for these cells give hope to a new target for future therapies that could allow a direct treatment of patient with high level of pathogenic Eomes+ Th cells.

５．主な発表論文等

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２．論文標題 Involvement of cytotoxic Eomes-expressing CD4+T cells in secondary progressive multiple sclerosis	５．発行年 2021年
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掲載論文のDOI（デジタルオブジェクト識別子） 10.1073/pnas.2021818118	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

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オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関