Loss of thymic function promotes EAE relapse in anti-CD52-treated mice

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1. Introduction

Alemtuzumab (Campath-1H) is a humanized anti-CD52 monoclonal antibody used to treat relapsing-remitting multiple sclerosis (Hale et al., 1990; Willis and Robertson, 2016). Anti-CD52 therapy causes transient lymphopenia of the T and B cells, which expresses high levels of the CD52 receptor, and to a lesser extent, in natural killer cells and monocytes (Rao et al., 2012). The hematopoietic stem cells and lymphocyte precursor cells lack CD52, thereby providing the potential for lymphocyte repopulation from precursors after anti-CD52 therapy. We recently showed that immune system reset with anti-CD52 promotes lymphocyte repopulation that is initially dominated by double-negative T cells and newly generated T and B cells (Haile et al., 2020). The rapid repopulation of the peripheral lymphoid niche with newly generated T cells in mice with EAE treated with anti-CD52 suggested a possible explanation for the variable efficacy of anti-CD52 in humans (Cohen et al., 2012; Haile et al., 2020; Turner et al., 2015; Ziemssen and Thomas, 2017) compared to the near-complete effectiveness in mice. In humans, repopulation of T cells post anti-CD52 is extremely slow, particularly for CD4 T cells (>2 years to recover), while repopulation in mice occurs within several weeks (Haile et al., 2020; Hill-Cawthorne et al., 2012). This creates a long-term state of lymphopenia in the human setting (Jones et al., 2013) that does not typically occur in the mouse. Lymphopenia is known to promote autoimmune disease (Ellestad and Anderson, 2017; Schulze-Koops, 2004). Whether or not the reduced lymphocyte function in humans and the subsequently delayed repopulation of the T cell repertoire after anti-CD52 treatment contributes to MS relapses is unknown. In this study, we examined the hypothesis that eliminating the rapid repopulation by newly generated T cells in mice would reduce the efficacy of anti-CD52 treatment of EAE and thus model more closely the human condition. The data suggest that the thymus and the ability to generate recent thymic emigrants (RTE) reduces EAE relapse following anti-CD52 treatment.

2. Materials and methods

2.1. Mice

Mice were 6–12-week-old males and females. B6.Cg-Foxp3tm2(EGFP) TcRb/J (B6 Foxp3-GFP) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). B6 Rag2p-GFP mice (Boursalian et al., 2004; Yu et al., 1999) were kindly provided by Pamela Fink (University of Washington, Seattle, WA). In the Rag2p-GFP mice, green fluorescent protein (GFP) expression is restricted to RTE and newly generated B cells (Boursalian et al., 2004; Yu et al., 1999). Animal care was in accordance with the Canadian Council on Animal Care guidelines. The studies were performed under Animal Use Protocol 00000369 approved by the Animal Care and Use Committee Health Sciences of the University of Alberta. Mice were housed under clean conventional housing conditions at the University of Alberta Health Sciences Laboratory Animal Services.

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facilities.

2.2. EAE induction and clinical score

EAE was induced and scored as previously described (Haile et al., 2020) using MOG35-55 peptide in complete Freund’s adjuvant and pertussis toxin. EAE disease was scored as 0 = no signs, 1 = paralyzed tail, 2 = hindlimb weakness, 3 = paralysis of both hindlimbs, 4 = paralysis of both hindlimbs and weakness of one forelimb, 5 = moribund. A score of 0.5 was added to the score where intermediate degrees of disability are observed. Mice were euthanized at a score of ≤4 to prevent suffering. EAE induced mice on the C57BL/6 (B6) background were injected subcutaneously with 10 mg/kg/mouse monoclonal IgG2a anti-mouse CD52 (provided by Genzyme (Turner et al., 2015)) beginning on day 14, when all mice had begun to have symptoms (a clinical score of 0.5 or greater). EAE relapse was defined as an increase in the clinical disease score occurring more than ten days post the termination of anti-CD52 treatment.

2.3. Thymectomy

Mice were anesthetized and restrained on a surgical board. To expose the thymus, a 2 mm incision was made on the base of the manubrium. A suction cannula was used to create negative pressure and to remove both thymic lobes separately. Similarly, sham surgery was performed on another group of mice, or no surgical procedure was performed (euthy whole group). After seven days of recovery, mice were immunized to induce EAE. Thymectomized mice were examined at the end of the experiment to confirm there were no thymic remnants.

2.4. Flow cytometry

The baseline data, depletion, and repopulation of lymphocytes were monitored using flow cytometry. Flow cytometry assessed TCRβ, CD4, CD8α, Foxp3 (antibodies from Thermofisher), and GFP. Data were acquired using an LSR II (Becton Dickson, Sunnyvale, CA) flow cytometer and analyzed with FlowJo™ (Treestar software, OR, USA).

2.5. Statistical analysis

Data are depicted as mean ± standard error (SEM) or as Kaplan–Meier survival curves, and statistical methods employed are indicated in each figure. All statistical analyses were done using Prism (GraphPad Software, La Jolla, CA).

3. Results

3.1. Thymectomy promotes EAE relapse in anti-CD52-treated mice

To block the repopulation by RTE post anti-CD52 in the mouse model (Haile et al., 2020), we performed thymectomy on mice of the B6 (B6 Rag2-GFP; and B6 Foxp3-GFP) genetic background (Fig. 1A). These mice would be expected to repopulate their T cells either exclusively from the homeostatic expansion of the remaining residual T cells or homeostatic expansion with a small contribution from RTE exported from cervical thymus tissue that is present in some mice (Smolarchuk et al., 2014; Terszowski et al., 2006). The proportion of RTE (GFP+ T cells) pre- and post-thymectomy was examined to determine if thymectomy was successful. The frequency of RTE was significantly reduced by day 7 post-thymectomy and more fully by day 14 (7 days post-EAE) (Fig. 1B). Consistent with our previous data (Haile et al., 2020), euthymic mice induced with EAE without anti-CD52 treatment maintained an average clinical score of 2 while anti-CD52 treatment given after disease onset reduced the score to 0, and this reversal of disease was maintained long-term (Supp. Fig. 1). We then examined if this lack of relapse in mice given anti-CD52 after disease onset depended on thymic function. Surprisingly, while 11 of 11 mice with intact thymus function (no surgery and sham surgery groups) remained relapse-free, 8 of 12 (66.67%) thymectomized mice had a relapse of EAE from three weeks post-anti-CD52 (Fig. 1C and Supp. Fig. 1). EAE relapse post-anti-CD52 treatment occurred in both male and female mice (Supp. Table 1). Relative to the no surgery and sham surgery groups, thymectomy was associated with less weight gain and eventually a decline in weight in those mice experiencing an EAE relapse (Supp. Fig. 1). In a preliminary analysis of the effect of thymectomy on EAE relapse in mice on the NOD background, relapse was less frequent but again only occurred in the thymectomy group (1 of 5 thymectomized vs 0 of 4 sham surgery; Supp. Fig. 1). Assessment of the lymphocytes in the thymectomized B6 mice showed a long-term reduction in the frequency of CD4 T cells in PBMC together with a low proportion RTE in both CD4 and CD8 T cells relative to the euthymic (no surgery/sham surgery) mice pre- and post-anti-CD52 treatment (Fig. 2). Recovery of T cells, particularly CD4 T cells, was associated with an increased proportion of RTE (Fig. 2). Thymectomy did not reduce the proportion of CD4 T cells expressing Foxp3 at day 7 post-thymectomy (mean and SEM, thymectomy = 11.1 ± 0.85; euthy whole 10.9 ± 0.48).

4. Discussion

Autoreactive T cells, such as the myelin-specific T cells, are part of the normal peripheral T cell repertoire because the thymic negative selection process eliminates most but not all potentially autoreactive T cells (Wekerle et al., 1996). Indeed, priming of the myelin-specific T cells already present in vivo with MOG35-55 peptide is the basis of EAE induction in mice. Anti-CD52 therapy depletes CD52-expressing lymphocytes from the periphery, which results in a transient but long-term lymphopenic state in humans (Hill-Cawthorne et al., 2012; Jones et al., 2013). This strategy of resetting the immune system by purging lymphocytes from the periphery, including the myelin-specific T cells, and allowing repopulation of the peripheral lymphoid organs has been very effective in the treatment of MS but not in all patients (Haile et al., 2020; Ruck et al., 2015). The failure of Alemtuzumab (anti-CD52) to eliminate disease relapse in some MS patients reflects in part the complexity of MS pathology, and the complexity of processes leading to immune tolerance to self (Cohen et al., 2012; Ziemssen and Thomas, 2017).

Activation in situ and homeostatic expansion of myelin-specific T cells is partly controlled in healthy individuals by competition with other T cells for survival resources, such as peptide:MHC and homeostatic cytokines (Ernst et al., 1999; Viret et al., 1999; Vivien et al., 2001). This T cell competition for survival resources is reduced during anti-CD52-mediated lymphopenia due to T cell lymphopenia. Given that anti-CD52-mediated T cell depletion is not complete in peripheral lymphoid tissues (Haile et al., 2020), it is conceivable that myelin-specific T cells that escaped depletion can outcompete other T cells during lymphopenia to promote disease relapse. Surprisingly, there were no substantial EAE relapses reported post anti-CD52 treatment in the B6 and SJL mouse strains, unlike the variable efficacy in human MS (Cohen et al., 2012; Haile et al., 2020; Turner et al., 2015; Ziemssen and Thomas, 2017), which we attribute to the rapid repopulation of T cells in mice. This rapid repopulation of CD4 T cells post anti-CD52 treatment in mice increases intra- and inter-clonal competition among T cells for resources required for their survival (Singh et al., 2012). Indeed, we previously showed that the thymus is an important source of repopulating T cells in mice post anti-CD52-mediated lymphopenia (Haile et al., 2020). However, in severely lymphopenic mice, such as the anti-CD52 treated thymectomized used in our study, competition for survival resources is reduced, enabling the clonal expansion and survival advantage in the myelin-specific CD4 T cells causing disease (Askin et al., 2020; Cosburn et al., 2013; Pryce et al., 2005). CD4 T cell lymphopenia in Alemtuzumab-treated patients can persist through year 4, and the MS relapse rate increased through year 3 (Gilmore et al., 2020). In addition, epitope spreading of the repopulating myelin-specific CD4 T cells would be expected to repopulate their T cells either exclusively from the homeostatic expansion of the remaining residual T cells or homeostatic expansion with a small contribution from RTE exported from cervical thymus tissue that is present in some mice (Smolarchuk et al., 2014; Terszowski et al., 2006). The proportion of RTE (GFP+ T cells) pre- and post-thymectomy was examined to determine if thymectomy was successful. The frequency of RTE was significantly reduced by day 7 post-thymectomy and more fully by day 14 (7 days post-EAE) (Fig. 1B). Consistent with our previous data (Haile et al., 2020), euthymic mice induced with EAE without anti-CD52 treatment maintained an average clinical score of 2 while anti-CD52 treatment given after disease onset reduced the score to 0, and this reversal of disease was maintained long-term (Supp. Fig. 1). We then examined if this lack of relapse in mice given anti-CD52 after disease onset depended on thymic function. Surprisingly, while 11 of 11 mice with intact thymus function (no surgery and sham surgery groups) remained relapse-free, 8 of 12 (66.67%) thymectomized mice had a relapse of EAE from three weeks post-anti-CD52 (Fig. 1C and Supp. Fig. 1). EAE relapse post-anti-CD52 treatment occurred in both male and female mice (Supp. Table 1). Relative to the no surgery and sham surgery groups, thymectomy was associated with less weight gain and eventually a decline in weight in those mice experiencing an EAE relapse (Supp. Fig. 1). In a preliminary analysis of the effect of thymectomy on EAE relapse in mice on the NOD background, relapse was less frequent but again only occurred in the thymectomy group (1 of 5 thymectomized vs 0 of 4 sham surgery; Supp. Fig. 1). Assessment of the lymphocytes in the thymectomized B6 mice showed a long-term reduction in the frequency of CD4 T cells in PBMC together with a low proportion RTE in both CD4 and CD8 T cells relative to the euthymic (no surgery/sham surgery) mice pre- and post-anti-CD52 treatment (Fig. 2). Recovery of T cells, particularly CD4 T cells, was associated with an increased proportion of RTE (Fig. 2). Thymectomy did not reduce the proportion of CD4 T cells expressing Foxp3 at day 7 post-thymectomy (mean and SEM, thymectomy = 11.1 ± 0.85; euthy whole 10.9 ± 0.48).
cells may have contributed to the EAE relapses in our study (Lehmann et al., 1992; Tuohy et al., 1998). Spontaneous EAE relapse post anti-CD52 treatment has been observed in the ABH mouse strain when treatment was given late, after onset of severe disease (von Kutzleben et al., 2017). This relapse might be due to the late initiation of treatment. However, it may instead reflect strain differences in T cell repopulation kinetics. No CD4 T cell recovery was seen in the ABH strain within the first four weeks post anti-CD52 treatment (von Kutzleben et al., 2017), while we observed substantial recovery of CD4 T cells in B6 mice within the first 2–3 weeks post treatment [Fig 2 and (Haile et al., 2020)]. Thus, prolonged T cell lymphopenia in the ABH strain may have predisposed it to relapses.

A role for the maintenance of thymus function in disease prevention is increasingly appreciated (Alawam et al., 2022; Velardi et al., 2021). Spontaneous remission of EAE in rodents is severely impaired in the absence of a functional thymus (Ben-Nun et al., 1980; Chen et al., 2009). Also, reduced thymic function post Alemtuzumab has been reported to drive T cell repopulation by homeostatic expansion of the few residual T cells that escaped depletion, which results in an oligoclonal T cell repertoire, with decreased repertoire diversity (Jones et al., 2013). Although several studies showed an increase in the frequency of regulatory T cells post Alemtuzumab (Gilmore et al., 2020; Haile et al., 2020; von Kutzleben et al., 2017), RTEs are the main precursor population of de novo generated regulatory T cells during lymphopenia (Ellestad et al., 2014; Paiva et al., 2013).

In this study, we provide compelling new evidence indicating that the thymus contributes to the maintenance of EAE remission. The results suggest that the thymus contributes to preventing EAE relapse post anti-CD52 treatment through generation and export of T cells and the consequent amelioration of T cell lymphopenia. Continuous thymic output appears critical in ameliorating EAE relapse, as T cell repopulation by the homeostatic expansion of the few cells that escaped depletion is not sufficient for complete regulation of myelin-specific T cells. These findings suggest that monitoring the thymic function of MS patients treated with anti-CD52 may help identify those patients who will require further interventions to prevent relapses.

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CRediT authorship contribution statement

Adeolu O. Adegoke: contributed to the conception, design, data generation, data analysis, and writing of the manuscript. Jiaxin Lin: performed thymectomy, and contributed to design, data generation, and editing of the manuscript. Colin C. Anderson: contributed to conception, design, data analysis, and writing of the manuscript.
Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

- The authors declare that the research was funded by Sanofi Genzyme and that there are no other commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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