Distinct disease-modifying therapies are associated with different blood immune cell profiles in people with relapsing-remitting multiple sclerosis

João Canto-Gomes[1,2], Daniela Boleixa[3,4], Catarina Teixeira[3,4], Ana Martins da Silva[3,4], Inés González-Suárez[5,6], João Cerqueira*[1,2,7,8], Margarida Correia-Neves*[1,2,9], Claudia Nobrega*[1,2]

[1]Life and Health Sciences Research Institute, School of Medicine, University of Minho, Braga, Portugal;
[2]ICVS/3B's, PT Government Associate Laboratory, Braga, Guimarães, Portugal;
[3]Porto University Hospital Center, Porto, Portugal;
[4]Multidisciplinary Unit for Biomedical Research (UMIB) - ICBAS, University of Porto, Porto, Portugal;
[5]Álvaro Cunqueiro Hospital, Vigo, Spain;
[6]University Hospital Complex of Vigo, Vigo, Spain;
[7]Hospital of Braga, Braga, Portugal;
[8]Clinical Academic Centre, Hospital of Braga, Braga, Portugal
[9]Division of Infectious Diseases and Center for Molecular Medicine, Department of Medicine Solna, Karolinska Institutet, 17176 Stockholm, Sweden;
*These authors contributed equally

Abstract

Relapsing-remitting multiple sclerosis (RRMS) is a chronic inflammatory neurodegenerative disease of the central nervous system. Several disease-modifying therapies (DMTs) are approved for RRMS, all of which target the immune system, although having different mechanisms of action. DMTs have been shown to reduce disease activity and disability progression in people with RRMS (pwRRMS). However, comprehensive comparisons of their effects on the immune system are scarce. We assessed circulating numbers of B cells, $CD4^+$ and $CD8^+$ T cells, regulatory T cells (Tregs), natural killer (NK) cells and NKT cells, and their subsets, in treatment-naïve pwRRMS or treated with different DMTs. All evaluated DMTs induced diverse effects and no common signature was observed on any of the several cell populations analysed. Cluster analysis was performed and two clusters of pwRRMS were identified. Cluster 1 includes pwRRMS on fingolimod, alemtuzumab and dimethyl fumarate (DMF); and cluster 2 includes pwRRMS on teriflunomide, natalizumab or interferon (IFN)β. The T cell subsets were the ones that contributed the most to cluster discrimination with no clear pattern on the other cell populations. Compared to treatment-naïve pwRRMS, DMTs in cluster 1 lead to lower numbers of $CD4^+$ and $CD8^+$ T cells, and Treg subsets, being fingolimod the therapy causing the strongest effect. For the DMTs in cluster 2, no pattern of changes was seen in the number of any of the immune cell populations analysed. The effect of the DMTs on B cell numbers is extremely diverse, with natalizumab causing a great increase and fingolimod, on the contrary, a pronounced decrease. The effect of DMTs on NKT and NK cell subsets is highly variable. This study emphasises that, despite all being efficient in treating multiple sclerosis, the impact of different DMTs on the numbers of different immune cell populations differs strongly on the cell populations affected, as well as on the magnitude and direction of the effect.

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that affects more than 2.8 million people worldwide (1). Most people with MS (pwMS) are diagnosed with relapsing-remitting MS (RRMS; ~85%), which is characterised by interleaved episodes of disability and recovery (2). There is no cure for MS, but several therapies have been shown to be effective in preventing disease progression in pwRRMS (3-5).

Most therapies available for RRMS dampen the immune response, either by preventing cells from entering the CNS, by lowering specific immune cell activities (including cell polarisation, proliferation, and peptide presentation) or even by inducing immune cell depletion. Several disease-modifying therapies (DMT) are available for MS and the decision supporting the choice of the appropriate DMT for a given person is complex and mostly based on the individual clinical profile (*e.g.,* disease activity, safety, comorbidities, side effects and pregnancy planning). DMTs can be categorised according to their efficacy: modest, moderate, and high. The increasing efficacy of DMTs correlates with increasing side effects and complications, including the risk of lymphopenia, infection, development of secondary autoimmune disorders and development of progressive multifocal leukoencephalopathy (6-9). In severe cases of MS, an intensive therapeutic approach using high-efficacy DMTs is preferred to reduce progression. In pwRRMS with lower disease activity, DMTs with more modest efficacy are chosen initially and escalated to more effective DMTs when needed. DMTs with modest efficacy include glatiramer acetate, interferon (IFN)β and teriflunomide; moderate to high efficacy include fumarate derivatives [e.g., dimethyl fumarate (DMF)], cladribine and sphingosine-1-phosphate (S1P) inhibitors (e.g., fingolimod); and high efficacy include alemtuzumab, mitoxantrone, natalizumab, anti-CD20 monoclonal antibodies, and autologous haematopoietic stem cell transplantation (aHSCT) (10, 11).

Existing studies evaluating the effects of different DMTs on pwRRMS mostly focus on their efficacy in preventing relapses, formation of new lesions and disease progression, providing very scarce data on the effects of different DMTs on immune cell subsets and phenotypes. Although the mechanism of action of some DMTs is partially understood, there are few studies with extensive phenotypic characterisation of immune system cells. Indeed, most studies have focused on the effects of therapies on conventional T and, more recently, on B cells, which are considered the most relevant immune cell subsets involved in the disease (4). However, there is increasing evidence supporting the notion that several other immune system cells with regulatory potential are involved in MS, including regulatory T cells (Tregs), natural killer (NK) cells and NKT cells. Tregs have an essential role in preventing autoimmunity and the percentage of these cells has been found to increase with therapy, including IFNβ (12). NK and NKT cells may have

important implications in MS as they are able to produce both pro- and anti-inflammatory cytokines, depending on the local cytokine *milieu,* and can mediate cellular cytotoxicity against abnormal cells through the engagement of their inhibitory and activating receptors (13-16). Interestingly, treatment with some DMTs, including natalizumab and alemtuzumab, has been shown to be associated with an increased frequency and number of $CD56^{bright}$ NK cells (17). However, data on the phenotype of NK and NKT cell subsets, including the expression of inhibitory (*e.g.*, KIR3DL1, KIR2DL2/3, KLRG1 and NKG2A) and activating (*e.g.*, NKp30, NKp44 and NKp46) receptors, in the context of different MS therapies remains scarce.

Comparative studies on the impact of different DMTs on immune system cells are important to better understand whether the degree of efficacy of DMTs correlates with the magnitude of their impact on immune cell subsets and whether there is a common immune target between the different DMTs. The aim of this study was to compare the effects of different DMTs on blood immune cell subsets. For this purpose, a comprehensive phenotypic characterization of $CD4^+$ and $CD8^+$ T cells, Tregs, NK and NKT cells was performed in treatment-naïve pwRRMS and compared with pwRRMS treated with one of the following DMTs: alemtuzumab, DMF, fingolimod, IFNβ, natalizumab or teriflunomide.

Material and Methods

Participants

This multicentre cross-sectional study was approved by the local Ethical Committees and participants were recruited at the Hospital de Braga (ref. 5888/2016-CESHB; Braga, Portugal), Hospital Geral de Santo António (ref. 098-DEFI/097-CES; Porto, Portugal) and Hospital Alvaro Cunqueiro (ref. 2021/430; Vigo, Spain) between 2018 and 2022. The inclusion criteria were: diagnosis of RRMS according to McDonald 2017 criteria, over 18 years of age and in the same DMT for more than 6 months prior to recruitment. Excluding criteria were: other immune-mediated diseases in addition to MS; history of treatment for cancer; splenectomy or thymectomy; pregnancy; treatment with corticosteroids in the previous 3 months or continuously for more than 6 months. Participants signed a written informed consent and agreed to provide demographic and clinical data, obtained from their medical records, including age, sex, age of MS onset, months living with MS, time since last DMT administration, and disease severity [Expanded Disability Status Scale (EDSS); **Table 1**]. The control group of recently diagnosed treatment-naïve pwRRMS with a diagnosis of RRMS according to McDonald 2017 criteria, aged over 18 old, and not meeting any of the above exclusion criteria has been described previously (18).

Table 1. Clinical and demographic data of the study groups.

	Treatment-naïve	Fingolimod	Alemtuzumab	DMF	Teriflunomide	Natalizumab	IFNβ
n	25	7	8	11	17	24	9
Age (years) Median [range]	32 [25;51]	40 [29;57]	40.5 [27;56]	40 [22;49]	48 [30;57]	40 [26;61]	40 [31;52]
Men. % (*n*)	28 (7)	14.3 (1)	25 (2)	9.1 (1)	29.4 (5)	37.5 (9)	22.2 (2)
Age at RRMS onset (years) Median [range]	32 [23;50]	34 [18;45]	34 [13;39]	30 [17;48]	40.5 [25;54]	30 [14;45]	28 [19;37]
Months since MS diagnosis Median [range]	1 [0;7]	128 [52;172]	103 [20;234]	44 [11;188]	62,5 [5;277]	134.5 [19;257]	86 [16;296]
EDSS Median [range]	1.0 [0.0;4.5]	2.0 [1;3.5]	2.0 [0;3.5]	1.0 [0;4.0]	2.0 [0;4.0]	3.5 [0;6.5]	2 [0;4.0]
Days from last therapy infusion Median [range]	na	na	353 [62;913] [1]	na	na	28 [4;31] [2]	na
anti-HCMV IgG$^+$ % (*n*)	68.0 (17)	71.4 (5)	37.5 (3)	63.6 (7)	76.5 (13)	79.2 (19)	44.4 (4)

[1] Alemtuzumab treatment is complete upon 2 infusions, 1 year apart. All individuals taking this DMT have fulfilled the 2 infusions.

[2] Natalizumab was administered every 28 days in all natalizumab-treated individuals.

DMF, dimethyl fumarate; EDSS, Expanded Disability Status Scale; HCMV, human cytomegalovirus; IFNβ, interferon beta; na, not applicable; RRMS, relapsing-remitting multiple sclerosis.

Sample Processing

Peripheral blood processing, human cytomegalovirus (HCMV) serology and FACS staining were performed as previously reported (18, 19). For FACS staining, per participant, a vial containing 5 million PBMCs was thawed and divided by four distinct panels of antibodies as described in **Supplementary Table 1**.

Statistical Analysis

The sample size and the median number of each cell population studied per each study group are summarized in **Supplementary Table 2**. The contribution of each DMT, controlling for age, sex and HCMV serology, on the number of every immune cell subset, was evaluated through a two-stage multiple linear regression analysis, as previously reported (20). In the first regression model, the number of every immune cell subset (Log_{10}-transformed to overcome the non-normal distribution, determined by the D'Agostino & Pearson test) was included as the dependent variable; age, sex and HCMV serology were included as independent variables. Outputs from these regressions are depicted in **Supplementary Table 3**. The unstandardized residuals from each regression were retrieved to be used in a second regression model; these unstandardized residuals of a regression model represent the variation on the dependent variable (*i.e.,* the number of every immune subset) that is not explained by the independent variables (*i.e.,* age, sex and HCMV serology). In the second regression, the unstandardized residuals were used as the dependent variable and the different DMTs as the independent variables. Therapies were included in the regression as dummy variables having as reference the treatment-naïve pwRRMS.

Statistical outputs of these regressions are shown in **Supplementary Table 4**. The standardized β-values from statistically significant predictors were used to perform a clustering analysis of the DMTs. The standardized β-values reflect the strength of the effect of each treatment on immune cell subset numbers. A hierarchical clustering dendrogram using the Jamovi software v2.3.21 (Sydney, Australia) was performed considering a Euclidean distance measure and a ward.D2 clustering method.

In the case of natalizumab- or alemtuzumab-treated pwRRMS, the impact of the time since the last drug infusion on the immune cell subsets' numbers was evaluated by performing regression models using the unstandardized residuals from the abovementioned first regressions (dependent variable) and time since last drug infusion (independent variable). These regression outputs are represented in **Supplementary Tables 5** and **6**.

All regression analyses were performed using IBM SPSS Statistics v26 (IBM Corporation, NY, USA); GraphPad Prism v9 (GraphPad Software, CA, USA) was used for the graphical representation of the heatmaps. Differences were considered statistically significant when the p-value <0.050. In the regression models, the magnitude of the effect of each independent variable was inferred based on the standardized β-values and classified as: small, when β-value <0.29; medium, when 0.30< β-value <0.49, or large, when β-value >0.5 (21). During flow cytometry analysis, cell subsets whose parent population was represented with less that 500 events were not considered for the statistical analysis. For this reason, the number of individuals analysed varied between cell subsets of the same group.

Results

Clustering of DMTs effect was based mostly on alterations on T cell subsets numbers

To investigate the changes in blood absolute numbers of $CD4^+$ and $CD8^+$ T cell subsets, Tregs, NK and NKT cells in pwRRMS treated with one out of the six distinct DMTs compared to treatment-naïve pwRRMS, we performed hierarchical clustering analysis and observed the formation of two main clusters. Cluster 1 grouped pwRRMS treated with fingolimod, alemtuzumab and DMF, whereas cluster 2 grouped those on teriflunomide, natalizumab and IFNβ (**Figure 1**). The discriminating effect between cluster 1 and 2 was mostly the impact of DMTs on T cell subsets. DMTs in cluster 1 led to a significant reduction in the number of most T cell subsets. In contrast, the effect of DMTs from cluster 2 on immune cell numbers did not result in a clear pattern.

By analysing the changes in immune cell subsets contributing to the formation of the two clusters, we found that fingolimod- and DMF- treated pwRRMS (belonging to cluster 1) had lower total lymphocyte counts compared to treatment-naïve pwRRMS. In contrast, in cluster 2 individuals treated with

natalizumab presented a slight increase in total lymphocyte counts. The effect of DMTs on the number of B cells was quite heterogeneous across the clusters, with alemtuzumab (cluster 1) and natalizumab (cluster 2) leading to higher numbers of B cells compared to treatment-naive pwRRMS, whereas fingolimod (cluster 1) induced lower numbers of those cells.

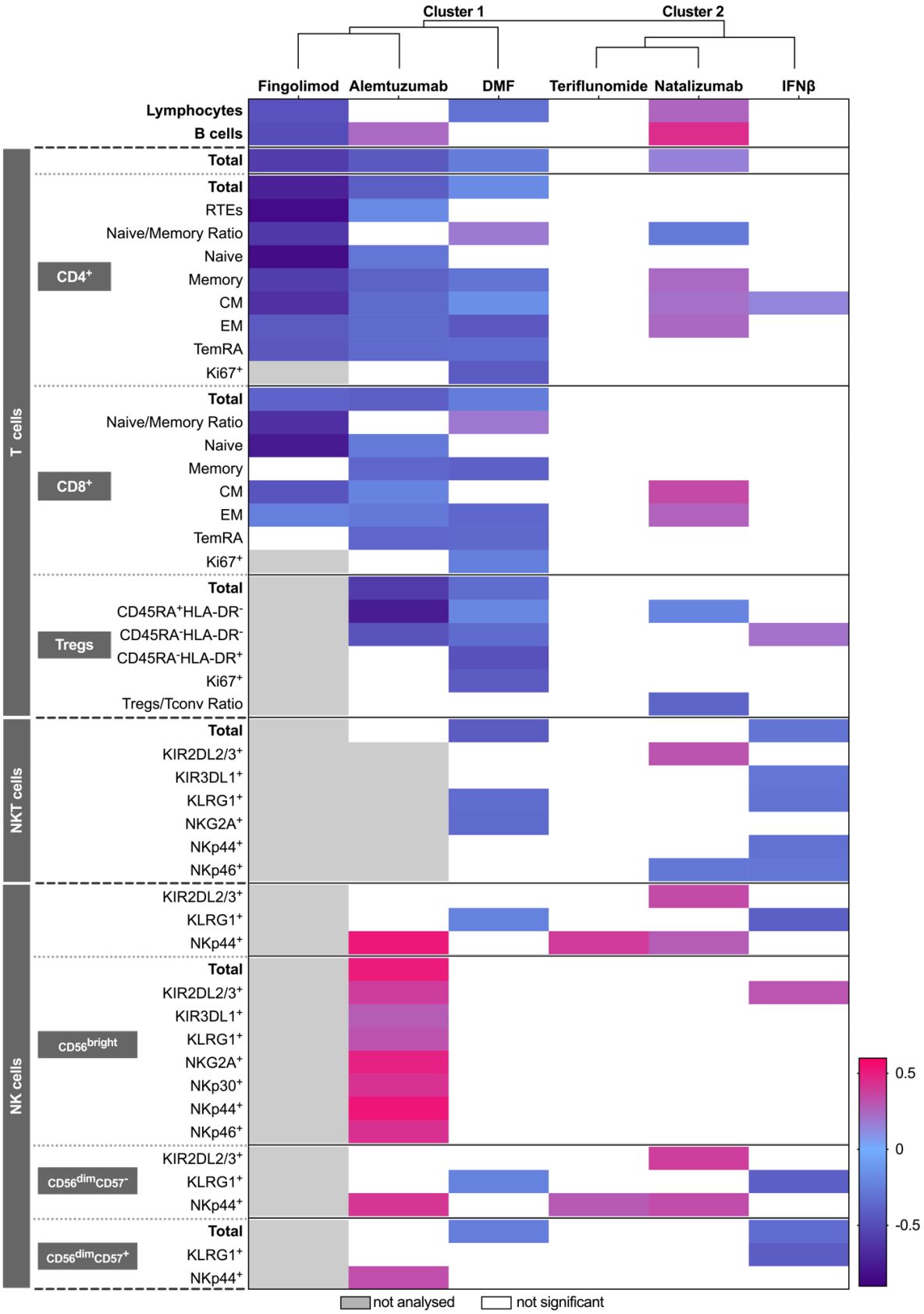

Figure 1. Effect of multiple sclerosis (MS) disease-modifying therapies (DMTs) on the number of immune cell subsets. The effect of DMTs on the number of immune cell populations was assessed using sequential multiple linear regression models. To exclude the effect of age, sex, and human cytomegalovirus (HCMV) serology on immune cell counts, a first multiple linear regression was performed

for each cell subset (**Supplementary Table 3**). A second regression was performed using the residuals of the first regression (the part not explained by age, sex and HCMV serology) as the dependent variable and the DMTs (alemtuzumab, DMF, fingolimod, IFNβ, natalizumab or teriflunomide; using treatment-naïve pwRRMS as reference) as the independent variable. For each cell subset, standardised β-values of the second regression were obtained (**Supplementary Table 4**), reflecting the strength of the effect of each treatment (independent variable) on the number of the cell subset (dependent variable) compared with treatment-naïve pwRRMS. These standardised β-values were used to perform a clustering analysis and generate a heatmap of the effect of the different DMTs on the number of immune cells. On the heatmap, grey cells represent cell subsets that were not analysed; white cells represent predictors that were not significant in the regression models. Only regression models with at least one statistically significant standardised β-values are shown on the heatmap. CM, central memory; EM, effector memory; DMF, dimethyl fumarate; IFNβ, interferon beta, RTE, recent thymic emigrants; Tconv, conventional $CD4^+$ T cells; TemRA, terminally differentiated effector memory T cells re-expressing CD45RA; Treg, regulatory T cells; NK, natural killer cells.

Compared to treatment-naïve, pwRRMS treated with DMTs from cluster 1 showed decreased numbers of most T cell subsets.

A reduction in the absolute number of T cells, and in most of their subsets, was observed for DMTs from cluster 1 compared to treatment-naïve pwRRMS (**Figure 1**). The magnitude of the effect on the naïve and memory T cell subsets was quite heterogeneous across the different DMTs. Fingolimod-treated pwRRMS showed the strongest effect, with reductions in the numbers of naïve and memory subsets among $CD4^+$ and $CD8^+$ T cells. Interestingly, the greatest effect was observed in recent thymic emigrants (RTEs), and in naïve $CD4^+$ and $CD8^+$ T cells, as indicated by the lowest standardised β-values (**Supplementary Table 4**), and by the significant decrease in the ratio of naïve to memory T cells (both $CD4^+$ and $CD8^+$ T cells). Alemtuzumab-treated pwRRMS also showed decreased numbers of naïve and memory T cell subsets in comparison to treatment-naïve pwRRMS. However, due to the similar magnitude of the decrease, no changes in the ratio of naïve to memory T cells were observed in these individuals. Alemtuzumab-treated pwRRMS were the ones presenting the stronger reduction in the numbers of Tregs, particularly of those with a naïve phenotype ($CD45RA^+$). In DMF-treated pwRRMS, the reduction in memory and the lack of effect on naïve T cells, resulted in an increased ratio of naïve to memory $CD4^+$ and $CD8^+$ T cells compared to treatment-naïve pwRRMS. In terms of T cell proliferation, as assessed by Ki67 expression, DMF-treated pwRRMS systematically showed lower numbers of proliferating $CD4^+$ and $CD8^+$ T cells, and Tregs compared to treatment-naïve pwRRMS.

Regarding the effect of DMTs from cluster 2, teriflunomide and IFNβ had no to subtle effects on T cell subsets numbers, respectively. In contrast, natalizumab-treated pwRRMS presented increased numbers of central memory (CM) and effector memory (EM) among $CD4^+$ and $CD8^+$ T cells. Moreover, natalizumab

also induced lower numbers of naïve Tregs ($CD45RA^+HLA\text{-}DR^-$), which may ultimately have contributed to the reduced ratio of Tregs to conventional $CD4^+$ T cells, compared with treatment-naïve pwRRMS.

DMTs mostly led to decreased numbers of NKT cells, with no clear signature on NK cells distinguishing the treated pwRRMS from the two clusters.

Overall, pwRRMS treated with different DMTs were found to present reduced numbers of distinct NKT cell subsets (**Figure 1**). Among DMTs from cluster 1, only DMF-treated pwRRMS were analysed. Aside from the decrease in total NKT cell numbers, a great reduction in the number of cells expressing KLRG1 and NKG2A was also observed. In cluster 2, IFNβ-treated pwRRMS presented lower counts of total NKT cells, and of those expressing KIR3DL1, KLRG1, NKp44 and NKp46 receptors. Teriflunomide had no effect on NKT cell numbers and its subsets, while natalizumab caused an increase in the number of $KIR2DL2/3^+$ NKT cells and a decrease in NKp46 NKT cells.

The effect of different DMTs on NK cell subset counts varied greatly in comparison to treatment-naïve pwRRMS. In cluster 1, alemtuzumab-treated pwRRMS had increased numbers of $NKp44^+$ on all NK cell subsets. Additionally, the numbers of $CD56^{bright}$ NK cell expressing any of the activation and inhibitory receptors analysed was increased in alemtuzumab -treated pwRRMS. In contrast, DMF treatment leads to a reduction in the number of total $CD56^{dim}CD57^+$ NK cells, and in the number of total and $CD56^{dim}CD57^-$ NK cells expressing KLRG1. Regarding the DMTs from cluster 2, teriflunomide treatment resulted in increased numbers of total and $CD56^{dim}CD57^-$ NK cells expressing NKp44. Compared to treatment-naïve individuals, natalizumab-treated pwRRMS had higher numbers of $KIR2DL2/3^+$ and/or $NKp44^+$ cells among total and $CD56^{dim}CD57^-$ NK cells. IFNβ-treated pwRRMS had higher counts of $KIR2DL2/3^+$ $CD56^{bright}$ NK cells, and lower counts of $CD56^{dim}CD57^+$ NK cells and total and of both $CD56^{dim}$ NK cell subsets expressing KLRG1.

Time since the last infusion of alemtuzumab or natalizumab correlates with changes in the number of immune cell subsets.

The therapeutic regimen of alemtuzumab and natalizumab involves intervals between successive drug infusions, as opposed to the other studied DMTs that are administered daily (fingolimod, teriflunomide, DMF) or every other day (IFNβ). As pwRRMS treated with alemtuzumab and natalizumab differ greatly in the time since the last drug administration (**Table 1**), we performed a regression analysis to evaluate the relationship between the time since the last drug infusion and the number of circulating immune cells (**Figure 2**).

Increasing time since the alemtuzumab infusion were related with higher numbers of naïve and CM among $CD4^+$ and $CD8^+$ T cells, which in the case of $CD8^+$ T cells resulted in a higher ratio of naïve to memory cells. In addition, higher numbers of proliferating $CD8^+$ T cells and of $KIR3DL1^+$ $CD56^{dim}CD57^-$ NK cells were observed with increasing time since the last alemtuzumab infusion.

The effect of time since the last natalizumab infusion in pwRRMS was observed only on NK cells. A negative association was observed between the time since the last natalizumab infusion and the number of $NKp30^+$ NK cells, and of $CD56^{dim}CD57^+$ NK cells expressing NKp30 and/or NKp46. In contrast, the number of $KIR2DL2/3^+$ $CD56^{bright}$ NK cells increased with time since the last natalizumab infusion.

Overall, while the time since the last alemtuzumab infusion correlated mostly with changes in the number of T cell subsets, the time since the last natalizumab infusion correlated with changes in NK cells.

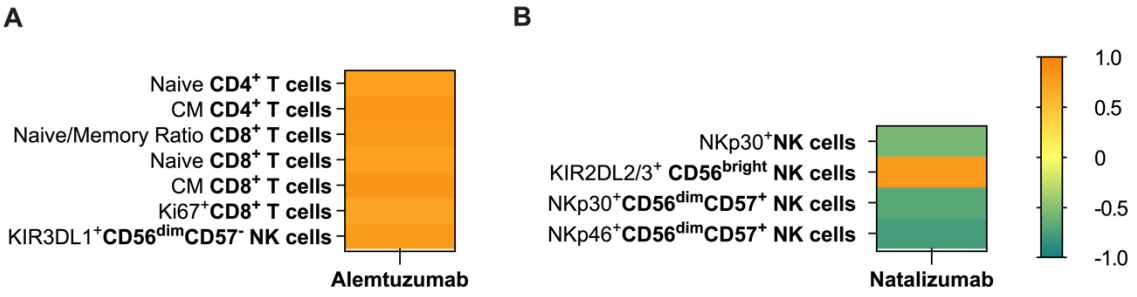

Figure 2. Effect of time since last alemtuzumab or natalizumab infusion on the number of immune cell subsets. The impact of time since the last alemtuzumab (**A**) or natalizumab (**B**) infusion on the number of immune cell populations was assessed using sequential multiple linear regression models. The effect of age, sex, and human cytomegalovirus (HCMV) serology on immune cell counts was assessed using a first multiple linear regression. Using the residuals from the first regression (the part not explained by age, sex and HCMV serology), a second regression was performed for each cell subset with these residuals as the dependent variable, and time since the last drug infusion as the independent variable. From these regressions, standardised β-values were obtained and used to generate a heatmap of the effect of time since the last alemtuzumab (**Supplementary Table 5)** or natalizumab infusion (**Supplementary Table 6)** on the number of immune cell subsets. Only significant linear regression models are shown on the heatmaps. CM, central memory; NK, natural killer cells.

Discussion

We provide a comprehensive comparison of the effect of different DMTs on the number of blood immune cell populations in pwRRMS. Control for age, sex and HCMV serology was essential to generate robust information, as these variables are known to influence immune cell homeostasis (22, 23). The clustering analysis performed in this study allowed two main clusters of pwRRMS treated with different DMTs to be distinguished. Cluster 1 included pwRRMS treated with DMF, alemtuzumab or fingolimod,

whereas cluster 2 included pwRRMS treated with natalizumab, teriflunomide or IFNβ. These two clusters were formed mostly due to the effect of DMTs from cluster 1 on the number of T cell subsets. There was no clear pattern in the effect of DMTs on the number of other immune system cells, either between DMTs from the same cluster or between clusters.

PwRRMS treated with DMTs included in cluster 1 presented a significant reduction of the number of most T cell subsets, in contrast to those on DMTs included in cluster 2. The effect of DMTs from cluster 1 in T cell subsets is consistent with the proposed mechanism of action of these DMTs. Fingolimod acts as a functional antagonist of S1P receptors, which are essential for lymphocyte egress from the lymph nodes, overriding the CCR7 retention signal. By blocking S1P receptors, CCR7-expressing cells (*i.e.,* naive and CM T cells) are retained in the lymph nodes (24). Decreased percentages and numbers of naïve and CM T cells have been described in the blood of fingolimod-treated pwRRMS compared to treatment-naïve pwRRMS (24, 25). In addition, Ghadiri *et al.* also showed that the numbers of RTEs and EM T cells are decreased in fingolimod-treated individuals, which correlates with our observations (25). Though fingolimod acts more strongly on RTEs, naïve and CM T cells, the cumulative sequestration of those cells in lymph nodes might contribute to the reduced number of the cells that succeed in the differentiation process, namely the EM T cells. Regarding DMF, despite its direct antioxidant effects on the nervous system and neuroprotection, it has been described to induce T cell apoptosis, particularly of those with a memory phenotype (EM and TemRA), and reduce their activation and proliferation (26, 27). Consistent with this, we found that DMF-treated pwRRMS showed a reduction in memory, but not in naive T cell subsets, and decreased numbers of proliferating T cells. Alemtuzumab is described to lead to targeted cytotoxicity of all CD52-expressing cells (*i.e.,* T and B cells, and monocytes). Baker *et al.* observed that the numbers of $CD4^+$ and $CD8^+$ T cells, and of Tregs recovered to only ~30% to ~50% of the baseline levels after one year of alemtuzumab infusion (9). Moreover, Haas *et al.* showed a sustained increase in the percentage of $CD45RO^+$ Tregs (28). We now add that the number of naïve Tregs ($CD45RA^+HLA\text{-}DR^-$) is reduced to a greater extent in comparison to memory ($CD45RA^-$) Tregs, in alemtuzumab-treated compared to treatment-naïve pwRRMS. Given the broad effect of alemtuzumab on all T cell subsets, more immune cell subsets were expected to correlate with time since the last alemtuzumab infusion. A possible explanation is that those immune cell subsets whose numbers recover rapidly after drug infusion, may not have been captured in the regression models due to the wide variation of the time elapsed since the last alemtuzumab infusion of our cohort.

There was no clear pattern on T cell subsets on the effect of DMTs belonging to cluster 2, with teriflunomide and IFNβ having little to no effect. Teriflunomide inhibits the dihydroorotate dehydrogenase

and consequently the *de novo* pyrimidine synthesis, thus limiting cellular proliferation, particularly of activated T cells (29, 30). Despite this, no effect of teriflunomide was observed on the number of proliferating $CD4^+$ or $CD8^+$ T cells. The IFNβ is described to induce T cell polarisation towards a Th2 phenotype, to the detriment of Th1 or Th17 (31, 32). We now added that IFNβ does not lead to major alterations in T cell subset numbers. Regarding natalizumab, by binding the α-4 integrin of VLA-4 on lymphocytes, it inhibits cell migration to the CNS and leads to their accumulation in the peripheral blood (33-35). We showed an increase mainly in the numbers of memory T cell subsets in natalizumab-treated pwRRMS, compared to treatment-naïve. Picker *et al.* suggested that the accumulation of memory T cells in the periphery, but not of naïve T cells, may be related to the higher expression of the α-4 integrin on memory subsets (36). The greater increase in peripheral CM $CD8^+$ T cells in comparison to other $CD8^+$ or $CD4^+$ memory T cell subsets showed by us may correlate with the fact that CM T cells predominate in the CSF and that $CD8^+$ T cells are the most abundant T cells in MS lesions (37).

We found that pwRRMS treated with fingolimod had reduced numbers of B cells. Fingolimod prevents B cells migration from lymphoid organs, leading to their reduction in the peripheral blood. In contrast, alemtuzumab and natalizumab treatment increased the numbers of B cells in the peripheral blood. While in natalizumab-treated pwRRMS the higher number of B cells in the peripheral blood may be due to blockage of cell migration into the tissues, in alemtuzumab-treated pwRRMS the higher number of B cells is intriguing and seems to contradict the depleting effect of the therapy. However, Baker *et al.* observed that although alemtuzumab treatment leads to an immediate and dramatic reduction in B cell counts, it is followed by a rapid hyper-repopulation to values exceeding baseline levels one year after drug infusion (9). In line with this report, here the alemtuzumab-treated group of pwRRMS had a median time since the last drug infusion of nearly 12 months. The rapid repopulation of B cells together with the lower Treg counts in alemtuzumab-treated pwRRMS may be a relevant finding considering that this drug predisposes to B cell-mediated secondary autoimmune diseases (7).

In treated pwRRMS, no apparent pattern of DMT's effect on NK and NKT cell subset numbers could be seen. The numbers of distinct NKT cell subsets were reduced in response to DMF and IFNβ. Others have previously found fewer NKT cells in DMF-treated pwRRMS, but the impact of DMF on the underlying NKT cell subsets was not examined (27, 38). In IFNβ- or DMF-treated pwRRMS, fewer $KLRG1^+$ NKT cells were observed. $KLRG1^+$ NKT cells have been found to be long-lived effector cells that can react to antigens (namely, α-Galactosylceramide), according to data from mice (39). Further research is necessary to determine whether these NKT cells have a role in the pathogenesis of MS in humans, and whether their decline is connected to the effectiveness of DMF and IFNβ in the treatment of pwRRMS.

Regarding the DMTs effect on NK cells, we found that alemtuzumab, teriflunomide and natalizumab resulted in increased numbers of NKp44^{+} NK cells, suggesting that these cells may play a role in the mechanism of action or efficacy of these DMTs. Our study supports previous findings showing increased CD56^{bright} NK cells in alemtuzumab-treated pwRRMS (40, 41). The expression of VLA-4 by NK cells provides a plausible explanation for the increased numbers of NK cell subsets in circulation during natalizumab treatment (42). Notably, the time since the last natalizumab infusion negatively correlated with NK cell subset counts. Nevertheless, it remains unknown whether this decline is associated with the recovery of migratory potential and with the migration of these cells to tissues.

DMTs described as less effective (*e.g.,* teriflunomide and IFNβ) are the ones with the least effect on the number of T cell subsets. Nevertheless, conclusions from these observations should be drawn with caution as these DMTs are used in pwRRMS with slower and less severe disease progression, as opposed to DMTs of higher efficacy (*e.g.,* alemtuzumab, natalizumab) (10, 11). It is not known whether the effect of high *vs.* modest efficacy DMTs is an exclusive consequence of the DMT itself and/or a consequence of intrinsic changes in immune cell alterations that predispose to distinct disease severity and consequently the need for high/modest efficacy DMTs.

Overall, we observed moderate to strong alterations in the number of blood immune cells in pwRRMS in all DMTs evaluated, including in those groups with small sample sizes, namely pwRRMS treated with fingolimod or alemtuzumab (n=7 and n=8, respectively). Although the differences observed in the number of T cell subsets correlate with the expected and described mechanism of action of different DMTs, this study highlights that these therapies differ in the magnitude of their impact on immune cells, in particular the T cells. In addition, this study emphasizes the effect of different DMTs on NK and NKT cell subsets and reinforces that these cells may be potential players in MS pathophysiology and/or DMT efficacy.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

Author Contributions

JC-G, MC-N, JJC, and CN contributed to conception and design of the study. JJC, AMdS, DB, CT and IG-S included patients and provided clinical data. JC-G and CN organized the database; performed the experimental tasks and statistical analysis; generated the figures and tables; and wrote the first draft. All authors contributed to the article, including to manuscript revision, and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any conflict of interest.

Funding

This work has been funded by National funds, through the Foundation for Science and Technology (FCT; 2022.05294.PTDC, UIDB/50026/2020 and UIDP/50026/2020) and by the project NORTE-01-0145-FEDER-000039, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) and by a research grant from the Academic Clinical Center of the Hospital of Braga J. C-G. has been supported by FCT PhD grants (PD/BD/137433/2018 and COVID/BD/152629/2022).

Acknowledgments:

The authors would like to thank the people with multiple sclerosis that agreed to participate in this study.

References

1. The Multiple Sclerosis International Federation. Atlas of MS 2020. https://www.msif.org/wp-content/uploads/2020/10/Atlas-3rd-Edition-Epidemiology-report-EN-updated-30-9-20.pdf (Assessed 23 January 2023).
2. M. Filippi, A. Bar-Or, F. Piehl, P. Preziosa, A. Solari, S. Vukusic, *et al*. Multiple sclerosis. *Nat Rev Dis Primers*, 4(1), 43 (2018) doi:10.1038/s41572-018-0041-4
3. I. Callegari, T. Derfuss and E. Galli. Update on treatment in multiple sclerosis. *Presse Med*, 50(2), 104068 (2021) doi:10.1016/j.lpm.2021.104068
4. S. L. Hauser and B. A. C. Cree. Treatment of Multiple Sclerosis: A Review. *Am J Med*, 133(12), 1380-90.e2 (2020) doi:10.1016/j.amjmed.2020.05.049
5. M. P. McGinley, C. H. Goldschmidt and A. D. Rae-Grant. Diagnosis and Treatment of Multiple Sclerosis: A Review. *JAMA*, 325(8), 765-79 (2021) doi:10.1001/jama.2020.26858
6. G. Bloomgren, S. Richman, C. Hotermans, M. Subramanyam, S. Goelz, A. Natarajan, *et al*. Risk of Natalizumab-Associated Progressive Multifocal Leukoencephalopathy. *N Engl J Med*, 366(20), 1870-1880 (2012) doi:10.1056/nejmoa1107829
7. O. Tuohy, L. Costelloe, G. Hill-Cawthorne, I. Bjornson, K. Harding, N. Robertson, *et al*. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. *J Neurol Neurosurg Psychiatry*, 86(2), 208-15 (2015) doi:10.1136/jnnp-2014-307721
8. T. Menge, O. Stüve, B. C. Kieseier and H. P. Hartung. Alemtuzumab: the advantages and challenges of a novel therapy in MS. *Neurology*, 83(1), 87-97 (2014) doi:10.1212/WNL.0000000000000540
9. D. Baker, S. S. Herrod, C. Alvarez-Gonzalez, G. Giovannoni and K. Schmierer. Interpreting Lymphocyte Reconstitution Data From the Pivotal Phase 3 Trials of Alemtuzumab. *JAMA Neurol*, 74(8), 961 (2017) doi:10.1001/jamaneurol.2017.0676
10. A. Simpson, E. M. Mowry and S. D. Newsome. Early Aggressive Treatment Approaches for Multiple Sclerosis. *Curr Treat Options Neurol*, 23(7), 19 (2021) doi:10.1007/s11940-021-00677-1
11. B. Casanova, C. Quintanilla-Bordás and F. Gascón. Escalation vs. Early Intense Therapy in Multiple Sclerosis. *J Pers Med*, 12(1) (2022) doi:10.3390/jpm12010119
12. M. Ebrahimimonfared, A. Ganji, S. Zahedi, P. Nourbakhsh, K. Ghasami and G. Mosayebi. Characterization of Regulatory T-Cells in Multiple Sclerosis Patients Treated with Interferon Beta-1a. *CNS Neurol Disord Drug Targets*, 17(2), 113-8 (2018) doi:10.2174/1871527317666180327122435

13. L. Airas, M. Saraste, S. Rinta, I. Elovaara, Y. H. Huang, H. Wiendl, *et al*. Immunoregulatory factors in multiple sclerosis patients during and after pregnancy: relevance of natural killer cells. *Clin Exp Immunol*, 151(2), 235-43 (2008) doi:10.1111/j.1365-2249.2007.03555.x
14. J. O'Keeffe, C. M. Gately, T. Counihan, M. Hennessy, T. Leahy, A. P. Moran, *et al*. T-cells expressing natural killer (NK) receptors are altered in multiple sclerosis and responses to alpha-galactosylceramide are impaired. *J Neurol Sci*, 275(1-2), 22-8 (2008) doi:10.1016/j.jns.2008.07.007
15. S. M. Poznanski and A. A. Ashkar. What Defines NK Cell Functional Fate: Phenotype or Metabolism? *Front Immunol*, 10, 1414 (2019) doi:10.3389/fimmu.2019.01414
16. K. Takahashi, T. Aranami, M. Endoh, S. Miyake and T. Yamamura. The regulatory role of natural killer cells in multiple sclerosis. *Brain*, 127(Pt 9), 1917-27 (2004) doi:10.1093/brain/awh219
17. A. Laroni and A. Uccelli. CD56bright Natural Killer Cells: A Possible Biomarker of Different Treatments in Multiple Sclerosis. *J Clin Med*, 9(5), 1450 (2020) doi:10.3390/jcm9051450
18. J. Canto-Gomes, C. S. Silva, R. Rb-Silva, D. Boleixa, A. M. da Silva, R. Cheynier, *et al*. Low Memory T Cells Blood Counts and High Naïve Regulatory T Cells Percentage at Relapsing Remitting Multiple Sclerosis Diagnosis. *Front Immunol*, 13, 901165 (2022) doi:10.3389/fimmu.2022.901165
19. J. Canto-Gomes, S. Da Silva-Ferreira, C. S. Silva, D. Boleixa, A. Martins da Silva, I. González-Suárez, *et al*. People with Primary Progressive Multiple Sclerosis Have a Lower Number of Central Memory T Cells and HLA-DR. *Cells*, 12(3) (2023) doi:10.3390/cells12030439
20. P. Marques, P. Moreira, R. Magalhães, P. Costa, N. Santos, J. Zihl, *et al*. The functional connectome of cognitive reserve. *Hum Brain Mapp*, 37(9), 3310-22 (2016) doi:10.1002/hbm.23242
21. J. Cohen. Statistical power analysis for the behavioral sciences. Academic press, (2013) doi:10.4324/9780203771587
22. P. Brodin, V. Jojic, T. Gao, S. Bhattacharya, C. J. Angel, D. Furman, *et al*. Variation in the human immune system is largely driven by non-heritable influences. *Cell*, 160(1-2), 37-47 (2015) doi:10.1016/j.cell.2014.12.020
23. P. Brodin and M. M. Davis. Human immune system variation. *Nat Rev Immunol*, 17(1), 21-9 (2017) doi:10.1038/nri.2016.125
24. J. Chun and H.-P. Hartung. Mechanism of Action of Oral Fingolimod (FTY720) in Multiple Sclerosis. *Clin Neuropharmacol*, 33(2), 91-101 (2010) doi:10.1097/wnf.0b013e3181cbf825
25. M. Ghadiri, A. Rezk, R. Li, A. Evans, P. S. Giacomini, M. H. Barnett, *et al*. Pre-treatment T-cell subsets associate with fingolimod treatment responsiveness in multiple sclerosis. *Sci Rep*, 10(1), 356 (2020) doi:10.1038/s41598-019-57114-2
26. D. Mehta, C. Miller, D. L. Arnold, E. Bame, A. Bar-Or, R. Gold, *et al*. Effect of dimethyl fumarate on lymphocytes in RRMS: Implications for clinical practice. *Neurology*, 92(15), e1724-38 (2019) doi:10.1212/WNL.0000000000007262
27. Q. Wu, Q. Wang, G. Mao, C. A. Dowling, S. K. Lundy and Y. Mao-Draayer. Dimethyl Fumarate Selectively Reduces Memory T Cells and Shifts the Balance between Th1/Th17 and Th2 in Multiple Sclerosis Patients. *J Immunol*, 198(8), 3069-80 (2017) doi:10.4049/jimmunol.1601532
28. J. Haas, C. Würthwein, M. Korporal-Kuhnke, A. Viehoever, S. Jarius, T. Ruck, *et al*. Alemtuzumab in Multiple Sclerosis: Short- and Long-Term Effects of Immunodepletion on the Peripheral Treg Compartment. *Front Immunol*, 10, 1204 (2019) doi:10.3389/fimmu.2019.01204
29. M. Zeyda, M. Poglitsch, R. Geyeregger, J. S. Smolen, G. J. Zlabinger, W. H. Hörl, *et al*. Disruption of the interaction of T cells with antigen-presenting cells by the active leflunomide metabolite teriflunomide: Involvement of impaired integrin activation and immunologic synapse formation. *Arthritis Rheum*, 52(9), 2730-2739 (2005) doi:10.1002/art.21255

30. A. Bar-Or, A. Pachner, F. Menguy-Vacheron, J. Kaplan and H. Wiendl. Teriflunomide and Its Mechanism of Action in Multiple Sclerosis. *Drugs*, 74(6), 659-74 (2014) doi:10.1007/s40265-014-0212-x
31. D. Jakimovski, C. Kolb, M. Ramanathan, R. Zivadinov and B. Weinstock-Guttman. Interferon β for Multiple Sclerosis. *Cold Spring Harb Perspect Med*, 8(11), a032003 (2018) doi:10.1101/cshperspect.a032003
32. B. C. Kieseier. The Mechanism of Action of Interferon-β in Relapsing Multiple Sclerosis. *CNS Drugs*, 25(6), 491-502 (2011) doi:10.2165/11591110-000000000-00000
33. R. Rudick, C. Polman, D. Clifford, D. Miller and L. Steinman. Natalizumab: bench to bedside and beyond. *JAMA Neurol*, 70(2), 172 (2013) doi:10.1001/jamaneurol.2013.598
34. R. A. Rudick and A. Sandrock. Natalizumab: alpha 4-integrin antagonist selective adhesion molecule inhibitors for MS. *Expert Rev Neurother*, 4(4), 571-80 (2004) doi:10.1586/14737175.4.4.571
35. C. H. Polman, P. W. O'Connor, E. Havrdova, M. Hutchinson, L. Kappos, D. H. Miller, *et al*. A Randomized, Placebo-Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis. *N Engl J Med*, 354(9), 899-910 (2006) doi:10.1056/nejmoa044397
36. L. J. Picker, L. W. Terstappen, L. S. Rott, P. R. Streeter, H. Stein and E. C. Butcher. Differential expression of homing-associated adhesion molecules by T cell subsets in man. *J Immunol*, 145(10), 3247-55 (1990)
37. G. P. van Nierop, M. M. van Luijn, S. S. Michels, M. J. Melief, M. Janssen, A. W. Langerak, *et al*. Phenotypic and functional characterization of T cells in white matter lesions of multiple sclerosis patients. *Acta Neuropathol*, 134(3), 383-401 (2017) doi:10.1007/s00401-017-1744-4
38. S. Sainz de la Maza, S. Medina, N. Villarrubia, L. Costa-Frossard, E. Monreal, A. Tejeda-Velarde, *et al*. Factors associated with dimethyl fumarate-induced lymphopenia. *J Neurol Sci*, 398, 4-8 (2019) doi:10.1016/j.jns.2019.01.007
39. K. Shimizu, Y. Sato, J. Shinga, T. Watanabe, T. Endo, M. Asakura, *et al*. KLRG+ invariant natural killer T cells are long-lived effectors. *Proc Natl Acad Sci U S A*, 111(34), 12474-9 (2014) doi:10.1073/pnas.1406240111
40. W. Gilmore, B. T. Lund, P. Li, A. M. Levy, E. E. Kelland, O. Akbari, *et al*. Repopulation of T, B, and NK cells following alemtuzumab treatment in relapsing-remitting multiple sclerosis. *J Neuroinflammation*, 17(1), 189 (2020) doi:10.1186/s12974-020-01847-9
41. S. Palmeri, M. Ponzano, F. Ivaldi, A. Signori, C. Lapucci, V. Casella, *et al*. Impact of Natural Killer (NK) Cells on Immune Reconstitution, and Their Potential as a Biomarker of Disease Activity, in Alemtuzumab-Treated Patients with Relapsing Remitting Multiple Sclerosis: An Observational Study. *CNS Drugs*, 36(1), 83-96 (2022) doi:10.1007/s40263-021-00875-0
42. I. Gandoglia, F. Ivaldi, P. Carrega, E. Armentani, G. Ferlazzo, G. Mancardi, *et al*. In vitro VLA-4 blockade results in an impaired NK cell-mediated immune surveillance against melanoma. *Immunol Lett*, 181, 109-15 (2017) doi:10.1016/j.imlet.2016.11.015