Massive Restrain of Cytotoxic B cells in the Peripheral Blood During Fingolimod and Natalizumab Treatments in Multiple Sclerosis Patients

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Research

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Abstract

Background

Recently, the success of anti-CD20 monoclonal antibody therapy brought a new light over the role of B cells in multiple sclerosis (MS) pathogenesis. Due to the expression pattern of CD20 during B cells ontogeny, this role seems to be extended beyond the antibodies' production and secretion. Therefore, here we investigated whether not only classical cytotoxic CD8\(^+\) T lymphocytes but also non-classical cytotoxic B cells may occur in the peripheral blood from relapsing-remitting MS (RRMS) patients.

Methods

104 RRMS patients during different treatment and 58 healthy donors were studied. CD19, GzmB, Runx3 and CD49d expression was assessed by flow cytometry analyses.

Results

Patients treated with Natalizumab (NTZ) showed an increased percentage of CD8\(^+\)GzmB\(^+\) when compared to other MS therapies, untreated RRMS patients and healthy volunteers. Similarly, and unexpected, massive cytotoxic behavior of B cells CD19\(^+\)GzmB\(^+\) was observed in RRMS patients during Fingolimod (FTY) and NTZ therapies when compared to Glatiramer, Interferon\(\beta\), untreated MS patients and healthy donors.

Conclusions

During different MS treatments, B cells exhibit cytotoxic behavior resembling CD8\(^+\) T lymphocytes. This data suggest a possible involvement of “cytotoxic” B cells during MS pathology. Monitoring cytotoxic subsets might become an available marker for the risk of relapses and even for accessing therapeutic effectiveness in MS patients.

Introduction

Multiple sclerosis (MS) is an immune-mediated and demyelinating disease of the central nervous system (CNS). The inflammatory response is characterized by the perivascular leukocyte infiltration, mainly in the white matter. However, recent magnetic resonance imaging (MRI) studies revealed a much broader CNS damage, including gray matter atrophy and normal-appearing white matter (NAWM) abnormalities [1–3]. Moreover, leptomeningeal contrast enhancement is resent in around 19% of patients during the initial phase of the disease [4]. Noteworthy, brain atrophy is not correlated with the number of relapses, activity lesions, or with inflammatory infiltration in the gray matter [2, 3]. Cortical brain lesions seem to be
regionally associated with meningeal inflammation [3]. Meningeal inflammation leads to local leukocytes aggregates, which are formed mainly by B cells, T cells, macrophages, follicular dendritic cells, and plasmacytoid dendritic cells [4, 5].

Although the pathophysiology of MS is very complex, it is well accepted that the disease is mediated mainly by T lymphocytes [6]. However, recently, it has been shown a successful outcome by targeting B cell in patients with MS using anti-CD20 monoclonal antibodies (rituximab, ocrelizumab, or ofatumumab) [7]. Nevertheless, the CD20 molecule is not expressed on pro-B cells or differentiated plasma cells. Thus, the beneficial effect of anti-CD20 treatment appears to be extended beyond the autoantibody production and release. Besides the central role in the humoral response, B cells can exert antigen presentation, helper, and regulatory functions as well as direct effector activity [8]. Indeed, secretory products of B cells are cytotoxic to oligodendrocytes and neurons in vitro [9, 10].

B cells may differentiate in granzyme B (GzmB)-producing cells upon insufficient T cell help [11, 12]. Recently, we have been demonstrated an enhancement of the cytotoxic activity of B cells during refractory neuromyelitis optica spectrum disorder [13]. Of note, GzmB inhibition induces neuroprotection in MS experimental model, although it did not alter the numbers of CD4+ or CD8+ T lymphocytes infiltrated in the CNS [14].

Thus, in the present study, we evaluated the cytotoxic profile and activity of B cells (CD19+) in RRMS patients and healthy controls.

**Material And Methods**

**Study participants**

104 RRMS patients (19 Untreated, 15 Glatiramer Acetate [GA], 24 Interferon-β [IFN], 14 Fingolimod [FTY] and 32 Natalizumab [NTZ]), according to the McDonald criteria were recruited in Neurology Clinic at University of Campinas Hospital (UNICAMP). Also, 58 healthy subjects were included in the control groups. Both RRMS patients and healthy subjects included in this work (Table 1) signed a term of consent approved by the University of Campinas Committee for Ethical Research (CAAE: 53022516.3.0000.5404).
### Table 1
Demographic and baseline clinical characteristics of MS patients and controls

| Subjects     | Sample Size | Gender | Time after Age first relapse (years) | Time after last relapse (months) | Treatment duration | OCB | EDSS | CSF (+/-)* |
|--------------|-------------|--------|-------------------------------------|----------------------------------|--------------------|-----|------|------------|
| Healthy      | 58          | 40:18  | 28 (19–50)                          | -                                | -                  | -   | -    | -          |
| RRMS         | 104         | 80:24  | 37 (18–65) 9 (0.5–32)               | 27 (0–166)                       | 3.0                | 2.0 + 1.9 | 60/30 |
| **RRMS patients** |          |        |                                     |                                  |                    |     |      |            |
| Untreated    | 19          | 14:5   | 27 (18–59) 5 (0.5–19)               | 4.5 (0–146)                      | -                  | 1.5 + 2.0 | 12/6* |
| Glatiram er Acetate (GA) | 15          | 13:2   | 42 (23–58) 12.5 (1–32)              | 21 (5–93)                        | 4.0                | 1.5 + 1.4 | 7/5*  |
| Interfero n-β (IFN) | 24          | 20:4   | 41 (28–65) 12.5 (1–22)              | 40 (1–166)                       | 6.5                | 2.0 + 1.6 | 12/8* |
| Fingolim od (FTY) | 14          | 10:4   | 39 (22–65) 11 (4–25)                | 102 (32–132)                     | 3.0                | 2.0 + 1.6 | 8/5*  |
| Natalizu mab (NTZ) | 32          | 23:9   | 35 (23–62) 9 (2–15)                 | 48 (24–120)                      | 2.0                | 2.0 + 2.0 | 21/6* |

All data are represented in median (max – min values)

* Not all patients were tested for Oligoclonal bands (Tested: n = 90; 66% OCB positive in the CSF)

### Blood samples collection and lymphocyte separation

Peripheral blood (PB) (25 ml) samples were collected from RRMS patients and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque® gradient.

### Flow Cytometry Analyses (FACS)

PBMCs were stained with anti-human: CD3 – 5.5 PerCP, CD8 - PE, CD19 - FITC, CD49d - APC (BD Biosciences®). All samples are washed and the pellet resuspended in 200 microliters of buffer Fixation/Permeabilization kit BD Cytofix/Cytoperm™ (BD Biosciences ®) for fixing the antibody molecule
to the membrane and for permeabilizing the cell surface. To investigate the cytotoxic profile held intracellular staining with anti-human GzmB antibody - AlexaFluor® 700 (BD Biosciences®) and anti-human Runx3 - eFluor660 (eBioscience™) in all samples. The acquisition was performed in FACSVerse® flow cytometer (BD Biosciences®), and the analysis used the FlowJo® software.

**B cells isolation and in vitro stimulation**

After PBMCs obtaining, B cells were isolated using the EasySep® Human B Cell Enrichment Kit with EasySep® magnet. After the isolation, $2 \times 10^4$ B cells were stimulated during 16 h in culture, with CPG-ODN (2.5µL/mL) and human recombinant IL-21 (50 ng/mL), as previously described [16]. After the culture period, the supernatants were stored at -80 °C for the investigation of GzmB production using a Cytometric Bead Array (CBA).

**Cytometric Bead Array (CBA)**

According to the manufacturer's protocol, 50 microliters of B cells stimulated supernatants, and solutions for calibration curve construction were incubated with beads containing monoclonal antibodies to GzmB. After incubation for 2 hours, it was added revealing antibody conjugated to the fluorochrome PE. The acquisition was made in flow cytometer FACSCanto (BD Bioscience®), and the analysis used the FCAP Array software (BD Bioscience®).

**Statistical Analyses**

The statistical significance of the results was determined using a nonparametric analysis of variance (Kruskal–Wallis test) and a Mann–Whitney test (U-test). Dunn's multiple comparison test was used as post hoc of Kruskal–Wallis. ROUT (Q = 1%)’ test was used to determine the presence of outlier values. $p < 0.05$ values were considered statistically significant.

**Results**

**GzmB expression in CD8$^+$ T and CD19$^+$ B lymphocytes by untreated RRMS patients**

Flow cytometry analysis of PBMCs (Fig. 1a-b) showed that CD8$^+$GzmB$^+$ T lymphocytes from untreated RRMS patients are not different in terms of percentage when compared to healthy donors ($p = 0.0567; CI 95\%: -0.4–20.9$) (Fig. 1c). On the other hand, after we excluded the outlier values (open circles), the RRMS group presented a significantly higher percentage of CD19$^+$GzmB$^+$ cells in comparison with the healthy individual ($p = 0.0198; CI 95\%: -0.007–2.28$) (Fig. 1d). In order to evaluate the cytotoxic activity of those cells, we sorted out CD19$^+$ cells from RRMS patients and healthy controls and analyzed the releasing of GzmB after ODN-CPG and IL-21 stimulation. Purified CD19$^+$ cells from RRMS patients presented a significantly higher release of GzmB in comparison with CD19$^+$ B cells from healthy individuals ($p = 0.0346$), even though some of those patients were under treatment (Fig. 1e).
GzmB expression in CD8\(^+\) T and CD19\(^+\) B lymphocytes by treated RRMS patients

Next, we explored the effect of treatments (GA, IFN, FTY, and NTZ) over those cytotoxic lymphocytes (Fig. 2a and 2d). Flow cytometry analysis did not reveal differences in the percentage of total circulating CD8\(^+\) or B lymphocytes between all groups (Fig. 2b and 2e). However, the expression of GzmB was significantly higher in CD8\(^+\) T lymphocytes from patients treated with NTZ concerning the other treatments (NTZ vs GA: \(p < 0.01\), NTZ vs IFN: \(p < 0.001\), NTZ vs FTY: \(p < 0.01\)), untreated MS patients (\(p < 0.001\)), or healthy donors (\(p < 0.001\)) (Fig. 2a and 2c). In parallel, the expression of GzmB was significantly higher in B cells from patients treated with NTZ concerning the first line immunomodulatory therapies (NTZ vs GA: \(p < 0.001\), NTZ vs IFN: \(p < 0.001\)), untreated patients (\(p < 0.001\)), or healthy donors (\(p < 0.001\)) (Fig. 2f). Surprisingly, the expression of GzmB was also significantly higher in B cells from patients treated with FTY in relation to GA (\(p < 0.05\)), untreated patients (\(p < 0.05\)), or healthy donors (\(p < 0.001\)) (Fig. 2f).

Expression of Runx3 and CD49d in cytotoxic B lymphocytes

Classically, the expression of Runx3 is a pivotal mechanism of the cytotoxic program in CD8\(^+\) T lymphocytes [15]. It has been shown that the ectopic expression of Runx3 is directly related to the cytotoxic activity of non-classic cytotoxic cells [16]. Consistently, there is a strong positive correlation (\(R^2 = 0.8430; p < 0.0001\)) between the expression of Runx3 and GzmB, which is independent of the treatment (Fig. 3a and 3b). Strikingly, considering only Runx3\(^+\) B lymphocytes, NTZ-treated RRMS patients present a significantly higher expression of GzmB in comparison with other treatments (Fig. 3c). Therefore, it seems that NTZ treatment tackles specifically the cytotoxic B cells. In order to evaluate this hypothesis, we analyzed the expression of GzmB in CD19\(^+\)CD49d\(^-\) or CD19\(^+\)CD49d\(^+\) lymphocytes (Fig. 3d). As we hypothesize, the expression of GzmB is significantly higher in CD49d-expressing B lymphocytes (Fig. 3e and 3f).

Discussion

Here, we demonstrated that B cells from patients with RRMS present cytotoxic behavior. Moreover, NTZ-treatment seems to restrain these cells in the peripheral blood effectively. These results may present a novel pathophysiological mechanism for the disease as well as for its treatment.

Until now, a vast collection of studies has consistently demonstrated the enhancement of cytotoxic activity during MS. Despite a possible role of GzmB in regulatory pathways [17], CD8 \(+\) T cells are a classical cytotoxic subset that can induce direct axonal and/or neuron injury through the MHC class I-mediated manner and GzmB releasing [18]. Relapses during RRMS are associated with increased CD8\(^+\) T lymphocytes in CSF [19]. Noteworthy, cytotoxic behavior seems not to be restricted to these classical CD8\(^+\) T lymphocytes. Recently, Larochelle and colleagues described the migratory capacity of
CD8\(^{+}\)GzmB\(^{+}\) and CD4\(^{+}\)GzmB\(^{+}\) T lymphocytes into the CNS-parenchyma from fatal MS relapse after NTZ discontinuation [20]. Moreover, our group also has previously identified the cytotoxic activity of B cells in neuroinflammatory diseases [13].

Our results demonstrated a slight but significant increase percentage of GzmB-producing B cells in untreated patients with RRMS in comparison with healthy individuals. Those cytotoxic B cells are capable of producing GzmB as well as to release the protease after IL-21 and CpG stimulation. Of note, untreated RRMS patients are significantly younger and with shorter time of disease diagnosis than treated RRMS patients. Many authors have proposed that B cells would take a later role in MS pathophysiology, especially in the formation of ectopically leukocytes aggregates [21]. Therefore, it is plausible that during the early stage of the disease, the cytotoxic activity of B cells is less prominent. Nevertheless, it is difficult to test this possibility since the patients are treated at a very early stage upon diagnosis.

Regarding treatment, our data showed that FTY-treated RRMS patients present a significantly higher percentage of CD19\(^{+}\)GzmB\(^{+}\) in comparison with healthy, untreated or GA-treated RRMS patients. The effect of FTY over the B cell population seems to be a lesser extent than T cells. On the one hand, the treatment with FTY inhibits the pro-inflammatory B cell function favoring the regulatory phenotype [22, 23]. On the other hand, FTY-treatment does affect the T-independent response [24]. In this context, human cytotoxic B cells differentiate upon incomplete T-cell help [11, 25]. Thus, it is possible that FTY-treatment does not act over those cells, which would explain the increased proportion of granzyme B-expressing cells in the peripheral blood.

Besides, NTZ-treated RRMS patients present a consistent and significant restraints of CD8\(^{+}\) T lymphocytes and cytotoxic B cells in the peripheral blood. The restrain of activated T cells is the rationale of the NTZ treatment [26]. In fact, during fatal rebound following NTZ withdrawal CNS-infiltrating lymphocytes, mainly CD8\(^{+}\) T cells, express high amounts of GzmB. Curiously, monocytes/macrophages, and B cells were enriched in the CNS parenchyma compared to the CSF [20]. Here, we demonstrated that GzmB-expressing B cells are preferentially CD49d positive, which would explain the consistent restrain of cytotoxic B cells by NTZ treatment.

In contrast, NTZ promotes activation and pro-inflammatory enhancement of B cells in vitro [22]. Moreover, those B cells with an inflammatory profile increase in the PB of NTZ-treated RRMS patients progressively [22]. Nevertheless, the restrain of those pro-inflammatory B cells in the PB seem to be crucial for the effectiveness of the treatment.

Runx3 is a master regulator of the cytotoxic program in CD8\(^{+}\) T lymphocytes and natural killer cells [27, 28]. The ectopic expression of Runx3 has been extensively associated with non-classical cytotoxic cells [13, 16, 29]. Our data demonstrated a correlation between the expression of GzmB and Runx3 by B cell, which was independent of the treatment. Mechanistically, the ectopic expression of Runx3 by RRMS patient’s B cells might explain the enhancement of cytotoxic activity. Of note, the expression of Runx3 is required for the proliferation of immortalized B cell during the Epstein-Barr virus (EBV) infection, which
has been extensively associated with MS [30]. Noteworthy, the ectopic expression of Runx3 by dendritic cells regulates the activity of the CD49d gene promoter [31].

Collectively, our findings support that beyond antigen presentation to T cells, releasing of lytic factors, secretion of anti and pro-inflammatory cytokines, cytotoxic behavior might emerge in the context of antibody-independent functions developed by B cells during MS. In this context, anti-CD20 monoclonal antibodies have been reported as a viable option for escalation, aiming to prevent relapses after NTZ therapy in MS patients [32]. Anti-CD20 monoclonal antibodies main deplete naïve and memory B cells, preserving antibody-secreting B cells, which is confirmed by the sustained levels of immunoglobulins in the CSF from MS treated patients [33]. The future investigation might reveal whether memory B cells responsible for antigen presentation, secretion of pro-inflammatory cytokines, and even, GzmB-expressing subsets are targeted by anti-CD20 depleting therapies during MS.

Limitations

We are aware that the size of the cohort and the transversal nature of our study did not allow us to understand the clinical relevance of our finds better. Moreover, although we were able to establish a strong correlation between Runx3 and GzmB expression by B cells, there are not enough tools to imply causality to the phenomenon. In vitro generation of cytotoxic B cells will be necessary to clarify the role of Runx3 expression in the ectopic cytotoxic activity of these cells.

Conclusions

Further comprehension of the cytotoxic activity in MS physiopathology might provide an accessible and powerful tool for monitoring progression and therapy effectiveness during disease. Moreover, the cytotoxic mechanism might be a valuable target to develop new therapeutic strategies.

Abbreviations

**BBB**: blood-brain barrier; **CBA**: cytometric bead array; **CSF**: cerebrospinal fluid; **CNS**: central nervous system; **EBV**: Epstein-Barr virus; **EDSS**: expanded disability status scale; **ELFs**: ectopic lymphoid follicles; **FACS**: flow cytometry analyses; **FTY**: Fingolimod; **GA**: Glatiramer Acetate; **GzmB**: granzyme-B; **HD**: healthy donors; **IFN**: Interferon-β; **MRI**: magnetic resonance image; **MS**: Multiple Sclerosis; **NTZ**: Natalizumab; **OCBs**: oligoclonal bands; **PB**: peripheral blood; **PBMC**: peripheral blood mononuclear cells; **RRMS**: relapsing-remitting MS

Declarations

Competing interests
L.M.B.S. received a research grant from BIOGEN and a consultation honorarium from BIOGEN and ROCHE.

Consent for publication

Not applicable.

Availability of data and materials

All data are stored in the university databank and will be provided under reasonable request.

Ethics approval and consent to participate

This study was approved by the University of Campinas Committee for Ethical Research (CAAE: 53022516.3.0000.5404), according to the Term of Consent.

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Author Contributions

V.O.B., R.P.S.Q. and A.SM. performed most of the experiments. C.S., A.D., R.P.D.C. and C.O.B. diagnosed, treated, selected MS patients, as well as, recruited all healthy donors. R.P.S.Q. collected the blood samples from all volunteers. V.O.B., A.S.M. and M.D.A. designed and performed flow cytometry. A.L.F.L. and I.S. performed cell sorting experiments. A.S.F. and L.M.B.S. designed the experimental work. A.S.F. coordinated the study. V.O.B. and A.S.F. wrote the manuscript with inputs from co-authors.

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Figures
Increased CD19+GzmB+ B cells in untreated RRMS patients. (A) Gate strategy for total CD8+ T and CD19+ B cells. (B) Representative samples of CD8+GzmB+ and CD19+GzmB+ lymphocytes found in untreated RRMS (uRRMS) patients and healthy donors. (C) Proportion (%) of CD8+GzmB+ T lymphocytes in uRRMS patients (red) and healthy donors (blue). (D) Proportion (%) of B cells CD19+GzmB+ in uRRMS
patients (red) and healthy donors (blue). (E) Concentration (pg/mL) of GzmB-derived from B cells CD19+ in RRMS patients (red) and healthy donors (blue) (RRMS: n=13 [RRMS subgroups: Untreated: n=2; Glatiramer Acetate (GA): n=4; Interferon-β (IFN): n=4; Natalizumab (NTZ): n=3], Healthy donors: n=7). *p<0.05, **p<0.01, ***p<0.001.
Figure 2

A

|   | GA  | IFN | FTY | NTZ |
|---|-----|-----|-----|-----|
| GzmB | 20 | 20 | 21 | 88 |
| CD8 |    |    |    |    |

B

|   | Healthy | Untreated | GA | IFN | FTY | NTZ |
|---|---------|-----------|----|-----|-----|-----|
| CD8+ (%) | 50 | 40 | 30 | 20 | 10 | 0 |

C

|   | Healthy | Untreated | GA | IFN | FTY | NTZ |
|---|---------|-----------|----|-----|-----|-----|
| CD8+ GzmB (%) | 80 | 70 | 60 | 50 | 40 | 30 |

D

|   | GA  | IFN | FTY | NTZ |
|---|-----|-----|-----|-----|
| GzmB | 2.4 | 4.7 | 30  | 93  |
| CD19 |    |    |     |     |

E

|   | 25 | 20 | 15 | 10 |
|---|----|----|----|----|
| (%) |    |    |    |    |

F

|   | 100 | 80 | 60 | 40 |
|---|-----|----|----|----|
| (%) |    |    |    |    |
**Figure 2**

Increased CD19+GzmB+ B cells during Fingolimod and Natalizumab treatments in RRMS patients. (A) Representative samples of CD8+GzmB+ lymphocytes found in treated RRMS patients and healthy donors. (B) Proportion (%) of total CD8+ T lymphocytes in RRMS patients (red) and healthy donors (blue). (B) Proportion (%) of CD8+GzmB+ T lymphocytes in treated RRMS patients [Glatiramer Acetate (GA), Interferon-β (IFN), Fingolimod (FTY) and Natalizumab (NTZ)], uRRMS (red) and healthy donors (blue). (D) Representative samples of CD19+ lymphocytes found in treated RRMS patients and healthy donors. (E) Proportion (%) of total CD19+ B cells in RRMS patients (red) and healthy donors (blue). (F) Proportion (%) of CD19+GzmB+ B cells in treated RRMS patients [Glatiramer Acetate (GA), Interferon-β (IFN), Fingolimod (FTY) and Natalizumab (NTZ)], uRRMS (red) and healthy donors (blue). *p<0.05, **p<0.01, ***p<0.001.
Figure 3

Increased CD19+Runx3+ B cells in RRMS patients. Expression of Runx3 in CD19+ B cells was identified by FACS. (A) Gate strategies for Runx3 in CD19+ B cells and GzmB-derived CD19+Runx3+ (red) or CD19+Runx3- (blue) B cells are shown. (B) Correlation between the expression of Runx3 and GzmB in CD19+ B cells in treated RRMS patients. (C) Proportion (%) of GzmB-derived CD19+Runx3+ B cells in RRMS patients treated or not (red) and healthy donors (blue). (D) Representative dot plot for GzmB-derived CD19+CD49d+ B cells in RRMS patients (red) and healthy donors (blue). (E) Histogram for GzmB-derived CD19+CD49d+ B cells in RRMS patients (red) and healthy donors (blue). (F) Mean fluorescence intensity (MFI) for GzmB-derived CD19+CD49d+ (red) and CD19+CD49d- (blue) B cells in RRMS patients.