Glatiramer Acetate modulates ion channels expression and calcium homeostasis in B cell of patients with relapsing-remitting multiple sclerosis

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To investigate the effects of Glatiramer Acetate (GA) on B cells by an integrated computational and experimental approach. GA is an immunomodulatory drug approved for the treatment of multiple sclerosis (MS). GA effect on B cells is yet to be fully elucidated. We compared transcriptional profiles of B cells from treatment-naïve relapsing remitting MS patients, treated or not with GA for 6 hours in vitro, and of B cells before and after six months of GA administration in vivo. Microarrays were analyzed with two different computational approaches, one for functional analysis of pathways (Gene Set Enrichment Analysis) and one for the identification of new drug targets (Mode-of-action by Network Analysis). GA modulates the expression of genes involved in immune response and apoptosis. A differential expression of genes encoding ion channels, mostly regulating Ca²⁺ homeostasis in endoplasmic reticulum (ER) was also observed. Microfluorimetric analysis confirmed this finding, showing a specific GA effect on ER Ca²⁺ concentration. Our findings unveils a GA regulatory effect on the immune response by influencing B cell phenotype and function. In particular, our results highlight a new functional role for GA in modulating Ca²⁺ homeostasis in these cells.

Traditionally, multiple sclerosis (MS) has been considered to be a CD4⁺ T mediated disorder but recent evidences have challenged this idea by indicating a role also for B lymphocytes in the pathogenesis of MS. Not surprisingly B lymphocytes have become a target of novel pharmacotherapies in MS (i.e. ocrelizumab). However, many MS existing disease-modifying therapies (DMTs), beside their specific mechanism of actions, have the potential to affect B cell behaviour as well. Glatiramer acetate (GA) is a polypeptide-based therapy approved for the treatment of relapsing remitting (RR)-MS. The mode of action of GA is only partially known, and specifically its effect on B cells is not completely understood. Data from literature show that GA treatment of MS patients decreases CD19⁺ B cells and memory B cells and increases peripheral blood naïve B cells leading to a less disease promoting B cell phenotype. On the other hand, GA affects B cells functions, inducing B cell expression of anti-inflammatory cytokines and inhibiting pro-inflammatory cytokines. Elucidating the mechanisms of action of drugs in clinical use for MS is crucial for a concrete improvement of MS pathogenesis knowledge.

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Therefore, we decided to investigate GA effects, at the transcriptional level, on treatment-naive RR-MS patients’ B cells by using an integrated computational and experimental approach. Indeed, it has been shown that transcriptional responses of cells treated with small molecules can be used to elucidate their mechanism of action, in the lead optimization phase of drug discovery project and to reveal similarities among drugs, and quickly transfer indications for drug repositioning. The Connectivity Map (CMAP), the largest peer-reviewed public database of gene expression profiles following treatment of five human cancer cell lines with 1309 different bioactive small molecules has been extensively used by both the academic and industrial communities.

We hypothesized that measuring the transcriptional response on patients’ B cells following drug treatment and comparing this response to those of drugs collected in the CMAP database would help revealing the mechanism of action of GA in these cells. To this end, we applied a bioinformatics tool named Mode of Action by NetwoRk Analysis (MANTRA) (http://mantra.tigem.it) that was recently described in the literature. MANTRA is an automatic and robust approach that assesses similarity in gene expression profiles following drug treatment across thousands of small molecules from the CMAP database to predict similarities in drug effect and Mode of Action (MoA). Briefly, each transcriptional response to a drug treatment is represented as a list of genes ranked according to how much they change with respect to untreated cells. Transcriptional similarity among two drugs (i.e. two ranked lists of genes) is quantified by assessing how much two drugs tend to upregulate and downregulate the same genes. MANTRA represents transcriptional similarities among drugs graphically in the form of a network, where two drugs are connected if they are transcriptionally similar according to a metric based on the Gene Set Enrichment Analysis. The position of a drug within the network provides insights about its MoA by exploiting previous knowledge on neighbouring drugs. In addition, the network can be partitioned into communities consisting of groups of densely interconnected drugs sharing similar mode of action. The CMAP database is thus represented in MANTRA as a network of 1,309 drugs grouped into 106 communities. MANTRA has been shown to be able to predict drug mechanism of action and functional targets from the analysis of gene expression profiles and for drug repositioning.

Here, by performing a bioinformatics analysis of the CMAP dataset together with gene expression profiles of B cells from patients treated with GA, we revealed a possible role of this drug in modulating immune response, apoptosis, and ion channels expression, in particular voltage gated- and store-operated Ca^{2+} channels. To support these findings, we investigated endoplasmic reticulum (ER) Ca^{2+} content in B cells from MS patients treated with GA vs healthy subjects, and vs drugs-naive RR-MS patients or treated with other first line DMTs.

**Results**

**Generation of gene expression profiles.** Ten consecutive treatment-naive patients with RR-MS were enrolled between July 2013 and March 2014. One patient was successively excluded from the study for pregnancy. All patients were females. Mean age at enrolment ± SD was 31.5 ± 2.07. Mean age at MS onset was 29.16 ± 4. Mean EDSS ± SD was 2.5 ± 0.5. Specifically, as shown in Fig. 1A,B cells of each patient were isolated at baseline (treatment free cells, before starting GA) and after six months of 20 mg/mL GA in vivo administration (in vivo treated cells). At baseline, B cells were also cultured and treated or not in vitro with 100 µg/mL GA for six hours (Fig. 1A. Acute (6 hours, in vitro) and chronic (6 months, in vivo) responses to GA treatment in B cells were then analyzed at the transcriptional level by Affymetrix microarrays (GSE110023). RNA quality of three patients was poor therefore microarray data were analyzed only in 6 patients (60%).

**Functional pathway and MANTRA analysis of acute (in vitro) transcriptional response to GA treatment.** Gene expression profiles of B cells following 6 hour GA treatment in vitro were compared to those of untreated cells in order to detect changes in gene expression caused by the drug treatment across the 6 patients. Genes were ordered according to their fold-change in treated versus untreated samples. To assess the molecular pathways modulated in vitro by GA, we performed Gene Set Enrichment Analysis (GSEA). GSEA uses one or more databases of set of genes (i.e. pathways) to identify those gene-sets which are significantly modulated by the treatment. In this study, we selected as pathway databases those of gene expression signatures of immune system cells (C7) and of Gene Ontology (GO) including biological processes, cellular components and molecular functions (C5). Pathway enrichments were evaluated by their normalized enrichment score (NES), nominal P value, and FDR. The most significant GO pathways identified by GSEA included those involved in translation, which were up-regulated, and those involved in ion channels expression, including calcium, which were down-regulated (Table 1). GSEA of immune system signatures highlighted up-regulation of genes specific to naïve T and B cells (Table 1).

We then analysed the ranked list of genes following acute treatment of B cells with GA to identify drugs inducing a significantly similar transcriptional response, and hence which could share a common mechanism of action with GA by means of the MANTRA online tool. MANTRA identified 42 drugs eliciting a significantly similar transcriptional response to the in vitro GA treatment (Fig. 1B and in Supplementary Table S1). The 42 drugs were part of 17 communities sharing similar mode of action. Interestingly, among the 42 drugs we found agents with antiinflammatory effects (estradiol, corticosterone etc.), widely used for the treatment of relapses in MS and drugs such as vigabatrin an antiepileptic able to suppress voltage-sensitive sodium channels, naltrexone, an opioid antagonist proposed against spasticity, pain and fatigue in MS, which has been shown to prevent relapses in MS and to reduce the disease.

**Functional pathway and MANTRA analysis of chronic (in vivo) transcriptional response to GA treatment.** Gene expression profiles of patients’ B cells after six months of GA administration (in vivo) were compared to untreated cells of each patient at baseline (before starting GA treatment). We again performed GSEA and found a significant up-regulation of genes encoding ion channels (Table 1), such as genes belonging to the calcium voltage-gated channel subunit (CACC) and to the transient receptor potential (TRP) channel.
(TRPC1-5, TRM2, TRPV2) families. TRPC1, TRPC3 and TRPC4 are molecular component of store-operated Ca\(^{2+}\) entry (SOCE), an ubiquitous Ca\(^{2+}\) entry pathway that is activated in response to depletion of ER-Ca\(^{2+}\) stores\(^{14,15}\). Ca\(^{2+}\) signal, initiated by Ca\(^{2+}\) release from ER is evoked by tyrosine kinase receptors phosphatase C activation\(^{16}\). Interestingly, GSEA shows also the induction of genes involved in protein tyrosine kinase activity, amine-binding function and in nervous system development and function.

A general down-regulation of pathways involved in negative regulation of cell death and cellular biosynthetic process was also identified by GSEA (Table 1). As in the case of acute response, GSEA for pathways related to the immune system identified a significant up-regulation of genes linked to a less mature B cell phenotype. On the contrary, genes specific for lymphocytes vs others cells were down-regulated (naive _bcell_vs_neutrophil_up, bcell_vs_monocyte_up), while in the acute response these were up-regulated.

We then performed MANTRA analysis of the B cells in vivo transcriptional response to GA. MANTRA identified a total of 49 drugs grouped into 18 communities (Fig. 1C and Supplementary Table S1). The communities with the largest number of drugs included community n. 14 (cyclin-dependent kinases 2 (CDK2) and Topoisomerase II inhibitors) with 6 drugs, community n. 89 (hemostatic agents) with 7 drugs, and community 90 (mainly anti-inflammatory, antibacterial and antiretroviral compounds) with 14 drugs.

**Effect of GA on [Ca\(^{2+}\)]\(_i\) homeostasis in B cells.** In order to better investigate the computational results hinting at a role of GA in modulating Ca\(^{2+}\) channels expression, we analyzed the effect of GA treatment on ER Ca\(^{2+}\) homeostasis. We recorded PBMCs form three healthy subjects and from 13 RR-MS patients: three disease-treatment free, four treated with GA, six with INF\(^{3-}\)-1a and 1b (Table 2). Confocal immunofluorescence analysis showed that the anti-CD19 antibodies clearly recognized B cells both in PBMCs isolated from healthy subjects and GA-treated patients (Fig. 2A,a,b). In this latter group, CD19\(^{+}\) cells appeared surrounded by several vesicles when observed in brightfield microscopy. (Fig. 2A,c,d).

Microfluorimetric experiments showed that the ER Ca\(^{2+}\) content was significantly higher in PBMC isolated from GA-treated patients than in those isolated from healthy subjects, disease-treatment free, or INF\(^{3-}\)-1a and 1b treated patients (p = 0.02; Fig. 2B). To assess whether the observed changes in Ca\(^{2+}\) content occurred in B cells, we recorded the ER Ca\(^{2+}\) content in both FITC-CD19\(^{+}\) and FITC-CD19\(^{-}\) cells. Interestingly, microfluorimetric analysis revealed that the ER Ca\(^{2+}\) release elicited by ATP + thapsigargin was significantly higher in FITC-CD19\(^{+}\) cells than in FITC-CD19\(^{-}\) cells from patients treated with GA (p = 0.01), whereas the ER Ca\(^{2+}\) content was similar in FITC-CD19\(^{+}\) and FITC-CD19\(^{-}\) cells obtained from healthy subjects (Fig. 2C,D).

Conversely, when FITC-CD19\(^{+}\) cells from disease-treatment free RRMS patients were exposed in vitro to 100 µg/ml GA for 8/10 hours, the ER Ca\(^{2+}\) content was significantly lower than in untreated cells (p = 0.008; Fig. 2E).
Discussion

Our functional analyses of transcriptional profiles after both acute and chronic GA treatment revealed two main perturbated biological functions: (1) immune response and (2) ion channel activity.

The first GA action on immune response seems to be on B phenotype switch from a more to a less mature one. Indeed the acute response shows a relative decrease in plasma cells or memory B cells with concomitant expansion of B-cell precursors and/or naïve B cells. These data are in line with the literature. Given the short time of GA exposure for acute treatment, we may only appreciate the initiation of the cells phenotypic switch phenomenon.

In chronic response, we were able to confirm that GA targets immune response by: (1) affecting B cells phenotype, (2) affecting B cells function (down-regulation of inflammatory pathways), (3) reducing the number of lymphocytes (down-regulation of lymphocytes vs other blood cells pathways).

| MSigDB collections | Sub-collections | Gene Set name | SIZE | NES | NOM p-val | FDR q-val |
|--------------------|----------------|---------------|------|-----|-----------|-----------|
| Acute response     | MF             | STRUCTURAL_CONSTITUENT_OF_RIBOSOME | 71   | 1.7717452 | 0        | 0.07397725 |
|                    | MF             | VOLTAGE_GATED_POTASSIUM_CHANNEL_ACTIVITY | 32   | -2.7496712 | 0        | 0         |
|                    | MF             | LIGAND_GATED_CHANNEL_ACTIVITY | 36   | -2.384527  | 0        | 0         |
|                    | MF             | VOLTAGE_GATED_CALCIUM_CHANNEL_ACTIVITY | 18   | -2.348026  | 0        | 0         |
|                    | MF             | VOLTAGE_GATED_CATION_CHANNEL_ACTIVITY | 59   | -3.215854  | 0        | 0         |
|                    | MF             | CALCIUM_CHANNEL_ACTIVITY | 32   | -1.4731557 | 0.0      | 0.04459786 |
| C7: immunologic signatures | NAIVE_BCELL_VS_NEUTROPHIL_UP | 175 | 1.6156584 | 0.0      | 0.001     |
| C7: immunologic signatures | NAIVE_BCELL_VS_BM_PLASMA_CELL_UP | 174 | 1.4851125 | 0.0      | 0.00728678 |
| C7: immunologic signatures | NAIVE_MEMORY_CD8_TCELL_UP | 129 | 1.4530996 | 0.001    | 0.01323772 |
| C7: immunologic signatures | NAIVE_TCELL_VS_NEUTROPHIL_UP | 168 | 1.4417284 | 0.0      | 0.01254614 |
| C7: immunologic signatures | BCELL_VS_MONOCYTE_UP | 165 | 1.5381814 | 0.0      | 0.0028463998 |
| Cronic response   | MF             | GATED_CHANNEL_ACTIVITY | 108  | 2.9445336 | 0        | 0.00108302 |
|                    | MF             | CATION_CHANNEL_ACTIVITY | 106  | 2.849798  | 0        | 0.00180962 |
|                    | MF             | SUBSTRATE_SPECIFIC_CHANNEL_ACTIVITY | 135 | 2.832006  | 0        | 0.06168386 |
|                    | MF             | METALION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY | 132 | 2.5542498 | 0        | 0.02961846 |
|                    | MF             | VOLTAGE_GATED_CALCIUM_CHANNEL_ACTIVITY | 18  | 2.4818778 | 0.00180505 | 0.05882143 |
|                    | MF             | VOLTAGE_GATED_POTASSIUM_CHANNEL_ACTIVITY | 32  | 1.8135619 | 0.01344538 | 0         |
|                    | BP             | SODIUM_ION_TRANSPORT | 15   | 2.1463487 | 0.00367647 | 0.00396349 |
|                    | BP             | POTASSIUM_ION_TRANSPORT | 51   | 1.9955596 | 0.00172712 | 0.01392227 |
|                    | MF             | TRANSMEMBRANE_RECEPTOR_PROTEIN_TYROSINE_KINASE_ACTIVITY | 43  | 2.7663453 | 0        | 0.01309908 |
|                    | MF             | AMINE_BINDING | 20   | 2.3429487 | 0        | 0.01807819 |
|                    | BP             | NERVOUS_SYSTEM_DEVELOPMENT | 314  | 2.4207823 | 0        | 0.02556699 |
|                    | BP             | NEUROLOGICAL_SYSTEM_PROCESS | 324  | 2.3578296 | 0        | 0.01907202 |
|                    | BP             | SYNAPTIC_TRANSMISSION | 152  | 2.2413225 | 0        | 0.0320724  |
|                    | BP             | TRANSMISSION_OF_NERVE_IMPULSE | 165  | 2.1886022 | 0.00176991 | 0.03053605 |
|                    | BP             | CELLULAR_BIOSYNTHETIC_PROCESS | 259  | -2.3858764 | 0.0      | 0.02961543 |
|                    | BP             | NEGATIVE_REGULATION_OF_PROGRAMMED_CELL_DEATH | 131 | -2.1043394 | 0.0      | 0.02761854 |
|                    | BP             | CELL_STRUCTURE_DISASSEMBLY_DURING_APOPTOSIS | 16  | -2.103058  | 0.00217391 | 0.02629634 |
|                    | BP             | NEGATIVE_REGULATION_OF_APOPTOSIS | 130  | -2.0976427 | 0.0      | 0.01524475 |
|                    | BP             | POSITIVE_REGULATION_OF_IMMUNE_RESPONSE | 22   | -2.0900369 | 0.002331  | 0.02761854 |
|                    | MF             | INFLAMMATORY_RESPONSE | 107  | -2.0895972 | 0        | 0.02629634 |
| C7: immunologic signatures | CHEMOKINE_ACTIVITY | 37   | -2.2534335 | 0.00228833 | 0.01524475 |
| C7: immunologic signatures | IGM_VS_SWITCHED_MEMORY_BCELL_UP | 175 | 2.538935  | 0        | 0         |
| C7: immunologic signatures | IGM_MEMORY_BCELL_VS_PLASMA_CELL_DN | 176 | 1.8842129 | 0.002     | 0.017     |
| C7: immunologic signatures | NAIVE_VS_SWITCHED_MEMORY_BCELL_UP | 166 | 2.1028352 | 0        | 0.004     |
| C7: immunologic signatures | NAIVE_BCELL_VS_NEUTROPHIL_UP | 175 | 3.4844412 | 0.0      | 0         |
| C7: immunologic signatures | NAIVE_TCELL_VS_NEUTROPHIL_UP | 168 | 3.3898792 | 0.0      | 0         |
| C7: immunologic signatures | NAIVE_TCELL_VS_DC_UP | 173 | 3.1822155 | 0        | 0         |
| C7: immunologic signatures | BCELL_VS_MDC_UP | 162 | 2.8965137 | 0        | 0         |
| C7: immunologic signatures | BCELL_VS_MONOCYTE_UP | 165 | 2.749503  | 0        | 0         |

Table 1. Significant gene sets down-regulated and up-regulated obtained by GSEA performed after in vitro glatiramer acetate treatment (acute response) and after in vivo six months glatiramer acetate administration to patients (chronic response).
The apoptosis induction, revealed by GSEA analysis, may be consequence of GA modulation on immune system rather than a direct effect.

Ion channels genes are down-regulated in acute treatment (in vitro) but up-regulated in chronic GA treatment (in vivo).

A possible explanation of this opposite response to GA may be a compensatory effect where GA treatment acutely down-regulates ion channels genes in B cells, which in turn causes a chronic upregulation of these genes to maintain homeostasis.

### Table 2. Demographic and clinical characteristics of patients and controls enrolled for Ca²⁺ homeostasis study.

|                         | Controls | GA RR-MS patients | INF-β1b RR-MS patients | INF-β1a RR-MS patients | Drug naïve RR-MS patients |
|-------------------------|----------|-------------------|------------------------|------------------------|--------------------------|
| Total                   | 3        | 4                 | 3                      | 3                      | 3                        |
| Age (years; mean ± SD)  | 30 ± 7.0 | 33.25 ± 5.4       | 34.5 ± 1.1             | 35.5 ± 3.5             | 31.5 ± 3.5               |
| Age at onset (years; mean ± SD) | 30.5 ± 7.4 | 26.5 ± 3.5      | 28 ± 6.5               |                        |                          |
| DD (years; mean ± SD)   | 8.75 ± 5.8 | 11.5 ± 3.7       | 9.5 ± 4.0              |                        |                          |
| EDSS (mean ± SD)        | 2.375 ± 1.0 | 2.25 ± 0.3       | 3.225 ± 0.2            | 2.5 ± 0                |                          |

### Figure 2. Effect of glatiramer acetate on [Ca²⁺]i homeostasis.

(A) Confocal immunofluorescence images depicting FITC-CD19⁺ B cells in PBMCs isolated from naïve (a) or glatiramer acetate (GA) (b) patients. Panel c shows the brightfield image of panel b; d, higher magnification of the frame in c showing a single FITC-CD19⁺ cell. Arrows point to FITC-CD19⁺ cells, arrowheads point to FITC-CD19⁻ cells. (B) Quantification of ex vivo microfluorimetric experiments showing the effect of ATP + thapsigargin on [Ca²⁺]i in PBMCs from healthy subjects (Ctl) (N = 80 cells), from patients treated with GA (N = 40 cells), interferon (INF) β-1b (N = 40 cells), INFβ-1a (N = 40 cells), and from drugs naïve patients (N = 50 cells). ∗p < 0.05 vs all. (C) Representative traces of the effects of ATP + thapsigargin on [Ca²⁺]i in FITC-CD19⁺ and FITC-CD19⁻ cells of PBMCs isolated from GA treated patients. (D) Quantification of the effects of ATP + thapsigargin on [Ca²⁺]i in FITC-CD19⁺ and FITC-CD19⁻ cells from GA treated patients and healthy subjects. ∗p < 0.05 vs all. (E) Quantification of the effects of ATP + thapsigargin on [Ca²⁺]i in FITC-CD19⁺ cells obtained from drugs naïve RR-MS patients treated or not in vitro with GA. ∗p < 0.05 vs drug naïve RR-MS. Data are expressed as mean ± S.E.M of three independent experiments for each condition. Statistical analysis was performed using the one-way ANOVA or t-test followed by Newman Keul’s test. p < 0.05 was considered statistically significant.
Ion channels genes, whose expression changes following GA treatment, belong to the repertoire of ion-conducting proteins like potassium and chloride, Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) and TRP channels. These last channels participate to ER Ca\(^{2+}\) refilling\(^{27,28}\), which represents the major Ca\(^{2+}\) influx mechanism in B lymphocytes controlling antigen-mediated lymphocyte activation, cytokine/chemokine production, exocytosis, enzyme control, proliferation and apoptosis\(^{19-21}\). In line with the bioinformatic findings, intracellular Ca\(^{2+}\) concentration was reduced in vitro by GA but increased ex vivo.

A role of GA on intracellular Ca\(^{2+}\) has been already reported in platelets where, similar to our findings, intracellular Ca\(^{2+}\) was reduced after 100 µg of GA in vitro\(^{22}\). However, if ion channels expression represents a trigger or a consequence of Ca\(^{2+}\) homeostasis modifications is still to be elucidated.

After acute treatment, according to MANTRA, Brefeldin-A (BFA) and Ambroxol had the most similar gene expression profile to GA. BFA is an inhibitor of intracellular protein transport that leads to blockade the forward transport between the ER and the Golgi apparatus\(^{23}\). When anterograde transport is suppressed, retrograde transport from the Golgi to the ER becomes apparent. This BFA-induced retrograde transport depends on Ca\(^{2+}\) sequestered into intracellular stores\(^{24}\). Therefore, it is possible that, due to GA modulation on Ca\(^{2+}\) homeostasis, and through second messengers and transcription factors, the mode of action of the two drugs could be similar at transcriptional level.

Ambroxol is a mucolytic agent acting by inhibiting Na\(^+\) channel and by reducing nitric oxide levels\(^{25}\). Ambroxol has also antiinflammatory properties including inhibition of oxidative stress, increased of local defense and reduction of pro-inflammatory cytokines\(^{26}\). By blocking voltage gated Na\(^+\) channels and Ca\(^{2+}\) channels in animal models Ambroxol promotes axon regeneration in the injured adult mouse central nervous system\(^{27}\).

The compounds most transcriptionally similar to chronic GA treatment belonged to the Topoisomerase II and CDK2 inhibitors drug class, agents widely used for human cancer treatment that prevent unregulated cancer cell proliferation leading to apoptosis. Considering these prosapoptotic drugs, MANTRA results are in line with GSEA analysis in vivo, that showed a downregulation of pathways involved in negative regulation of apoptosis (Table 1). Among these compounds, MANTRA identified Mitoxantrone (Fig. 1C), currently used for the treatment of secondary progressive or worsening RR-MS\(^{28}\). This is noteworthy, since it proves that our protocol was able to identify a previously proven drug against MS.

In conclusion, our combined computational and experimental study hints at a possible role of GA in B cells as a modulator of Ca\(^{2+}\) homeostasis. We suggest that GA interferes with B cell activity by tuning ion channels expressions and Ca\(^{2+}\) influx, crucial for the regulation of B cell differentiation, cytokine production and apoptosis.

**Methods**

**Subjects.** Patients were recruited at MS center of the Department of Neurosciences, Reproductive and Odontostomatological Sciences of Federico II University of Naples. Patients’ inclusion criteria were: (1) new diagnosis of RR-MS according to Mc Donald revised criteria\(^{29}\); (2) presence of oligoclonal bands in the cerebrospinal fluid; (3) DMTs naïve; (4) age between 30 and 35 years; (4) patients should have not been treated with corticosteroids for at least one month at enrolment. Written informed consent was obtained from each patient. All methods were performed in accordance with the relevant guidelines and regulations and all experimental protocols were approved by the local ethical review committee of the University of Naples Federico II. Patients underwent GA treatment 20 mg daily s.c. as for clinical practice, and followed up for six months.

In a second phase, to ascertain computational results, we enrolled new healthy controls and patients treated with GA, with INF\(^{-}\)-1a and 1b, and treatment naïve.

**B cells isolation and treatment.** Patients’ peripheral blood samples were collected at baseline and after six months of GA in vivo administration.

Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Paque centrifugation (GE Healthcare); B cells were purified by autoMACS Pro Separator (Miltenyi biotec) using B cell Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells, were analyzed using a FACS CANTO II (BD Biosciences, CA, USA) to confirm the purity of B cells (>98%). Cells at baseline were divided in two group. One underwent RNA extraction like B cells successively isolated from the same MS patient after six months of GA. The second group was seeded (1 × 10\(^6\) cells/ml) with RPMI-1640 medium supplemented with 100 UI/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and 5% autologous serum. Cultured cells were treated in vitro or not with 100 µg/ml GA for six hours in a humidified atmosphere containing 5% CO\(_2\) at 37 C\(_\circ\). All experiments were carried out in duplicate.

**Microarray experiments.** Microarray hybridization experiments on total RNA extracted with TRIzol reagent (Ambion), were performed at Coriell Institute for Medical Research, Camden, NJ. The Affymetrix Gene-Chip Human Genome HG-U133A_2 hybridization platform (Affymetrix, Santa Clara, CA) was performed as described previously\(^{30}\). Prior to hybridization, RNA was reverted to cDNA using the NuGen Ovation RNA-Seq System V2 (NuGen Technologies, San Carlos, CA).

**Bioinformatics analysis.** **Microarray analysis: GSEA and MANTRA.** Microarray data were pre-processed using the Bioconductor package Affy and normalized with the RMA method\(^{31}\). Differentially expressed genes (DEGs) between conditions (GA-treated versus untreated B cells in vitro and in vivo) were identified using a Bayesian t-test\(^{32}\). For each P value, the Benjamini–Hochberg procedure was used to calculate the false discovery rate (FDR) to correct for multiple testing.

The Microarray data have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEOseries accession number GSE110023\(^{33}\).
Gene set enrichment analysis (GSEA) was then performed using the freely available software GSEA v2.0 from the Broad Institute. To run the GSEA algorithm, RMA-normalized microarray data were used. The GSEA algorithm collapsed the probe sets into gene symbols (13,300 genes) and ranked the genes on the basis of the fold change after GA treatment (in vitro or in vivo). An enrichment score was calculated for each gene-set by applying the Kolmogorov–Smirnov statistics as described in the GSEA algorithm. Gene-sets were collected from the Molecular signatures database (MsigDB) available as part of the GSEA software by restricting the output to the two MSigDB collections C5 (e.g. GO gene sets) and C7 (e.g. immunologic signatures). The significant gene sets were obtained by using p value < 0.01 and FDR < 0.1 as threshold. Ranking of the genes set was done using GSEA v2.0.

Microarrays before and after acute (six hours in vitro) and chronic (six months in vivo) treatment with GA were also analyzed with the Mode-of-action by Network Analysis (MANTRA) online tool [http://mantra.tigem.it]. MANTRAs output is a network where each drug is a node and drugs are connected to each other if they elicit a similar transcriptional response. The ‘distance’ between the connected drugs is a measure of the similarity of their gene expression profiles. The transcriptional distance threshold of ≤0.8 from GA treatment was established to obtain the significant compounds.

**[Ca^{2+}]_i Measurement.** PBMCs of three healthy subjects and of four patients treated with GA, three with INF-β1a, three with INF-β1b, and three drug naïve patients, were isolated by Ficoll-Paque density gradients centrifugation.

[Ca^{2+}]_i was measured by single-cell Fura-2 acetoxymethyl-ester (AM) videomaging, as previously described. PBMCs placed on glass coverslips were first preincubated with anti-CD19 conjugated to fluorescein isothiocyanate (FITC-CD19) for 3 hours to identify B cells. Then, cells were loaded with 10 µmol/L Fura-2 AM for 30 minutes at 37°C in normal Krebs solution containing 5.5 mM KCl, 160 mM NaCl, 1.2 mM MgCl_2, 1.5 mM CaCl_2, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). Coverslips were placed into a perfusion chamber (Medical System, Co, Greenvalle, NY) mounted on an inverted Zeiss Axiovert 200 microscope (Carl Zeiss) and samples were alternatively illuminated at 340 nm, 380 nm and 490 nm wavelengths. ER Ca^{2+} content was evaluated as cytosolic Ca^{2+} increase elicited by ATP (100 µM) plus thapsigargin (1 µM) in a Ca^{2+}-free solution. In ex vivo experiments, [Ca^{2+}]_i measurements were conducted 3 hours after plating PBMCs. In in vitro experiments, PBMCs were first exposed to 100 µg/ml GA for 8–10 hours, and then analyzed.

**Confocal microscopy.** Confocal immunofluorescence procedures in PBMCs were performed as described. Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30 minutes. After blocking with 3% BSA, cells were incubated with FITC-CD19 antibodies (1:100; Beckman Coulter) overnight. Nuclei were counterstained with Hoechst (1 µg/ml, Sigma, Milan, Italy). Images were observed using a Zeiss LSM510 META/ laser scanning confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Single images were taken with an optical thickness of 0.7 µm and a resolution of 1024 × 1024.

**Data Availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

C.C. conceived the project and designed experiments, follows the MS patients, isolated B cells, wrote the manuscript. A.C. isolated B cells and performed the *in vitro* experiments and RNA extractions, wrote the manuscript. R.L. follows the MS patients, reviewed the manuscript. D.C. performed microarrays and data analysis. C.A. all statistical analysis. N.F. performed MANTRA analysis and prepared Figure 1. R.D.C. performed experiments of calcium measurements and prepared Figure 2. G.M. discussed the results and reviewed the manuscript. E.B. performed immunofluorescence experiments and prepared Figure 2. A.S. planned and performed microfluorimetric analysis experiments of calcium measurements. D.d.B. conceived the project and designed same experiments, discussed the results. V.B.M. discussed the results, reviewed the manuscript and follows the patients.

Additional Information

Competing Interests: Dr. V.B.M. and R.L. received personal fees for public speaking or consultancy from Merck, Novartis, Biogen, Genzyme, Teva and Almirall. The remaining authors declare no competing interests.

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