Alemtuzumab mediates T-regulatory response via macrophagal CD23.

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Abstract

**Background:** Alemtuzumab (ALM) effectively prevents multiple sclerosis (MS) relapses. It causes lymphocytic depletion with subsequent enhancement of T-regulatory cell population. Direct administration of ALM to T-cells causes cytolysis. However, the T-cells may be indirectly affected by myeloid cells, which are resistant to ALM cytotoxicity.

Does ALM modulate monocytes? Does the cross-talk between exposed monocytes and lymphocytes result in anti-inflammatory effects?

**Methods:** CD14$^+$ monocytes of 10 healthy controls and 10 MS (treatment naïve) patients were isolated from peripheral blood mononuclear cells (PBMCs), exposed to ALM and reintroduced to PBMCs depleted from CD14$^+$ cells. After treatment, macrophage profile and T-cells markers were measured.

**Results:** ALM promoted M2 anti-inflammatory phenotype, noted by increased percentage of CD23$^+$, CD83$^+$ and CD163$^+$ cells. CD23$^+$ cells were the most prominently upregulated (7-fold, p=0.0002). Observed effect was larger in MS patients compared to healthy subjects. The exposed macrophages increased the proportion of T-regulatory cells, without affecting T-effector cells. Neutralization of monocytic CD23 reversed the effect on T-regulatory cells.

**Conclusions:** ALM enabled monocytes’ conversion towards anti-inflammatory macrophages, which in turn promoted T-regulatory enhancement, in CD23 dependent manner. These findings suggest an ALM mechanism of action, which may explain some aspects of the MS pathogenesis.

**Keywords:** Alemtuzumab, CD23, multiple sclerosis, monocyte, macrophage, T-regulatory cell
**Introduction**

Alemtuzumab (ALM) is a humanized monoclonal antibody directed against CD52, an antigen primarily present on mature lymphocytes (T and B cells), but also on circulating myeloid cells (monocytes, macrophages, circulating dendritic cells). ALM is an efficient and long lasting treatment for RRMS with yet illusive mechanism of action and considerable side effects [1–4].

ALM administration causes lymphocytic depletion with a gradual reconstitution [5–7]. The remaining lymphocyte population demonstrated a relative increase in proportion of anti-inflammatory T-regulatory (T-reg), which is one of the possible explanations for the long lasting effect of the drug [8]. Nonetheless, it remains unclear by which mechanism ALM alters T-reg differentiation. *In vitro* studies may help to dissect the underlying mechanism. Upon direct administration of ALM cultured T-cells undergo complement dependent cytolysis [9]. Though, there might be indirect mechanisms involved in anti-inflammatory T-cell differentiation.

Interestingly, despite expressing CD52, myeloid cells are less prone to cytolytic effect of ALM: only a modest effect on their total amount was found following ALM exposure [10–12]. Hence, their properties may still be affected by ALM. Imbalance of monocyte activation profile is recognized as a contributing factor to MS relapses [13, 14]. Therefore, modulation of monocytes by ALM may contribute to its mechanism of action.

Myeloid cells are known to sustain a bilateral interaction with lymphocytes in general, and specifically with T-cells. A previous study demonstrated the ability of human mesenchymal stem cells to influence T-cell differentiation through the modulation of monocytes [15].
In the current study, we investigated the modulatory properties of ALM on monocyte activation profile and the influence of ALM treated monocytes on the T-cell differentiation.

The research was performed on the PBMCs derived from untreated RRMS patients and healthy control subjects.

Materials and Methods

Subjects and peripheral blood mononuclear cell isolation

The study followed institutional review board approval of study protocol and informed consents. Diagnosis of RRMS was according to the 2010 and 2017 revised McDonald criteria and MAGNIMS consensus guidelines [16, 17]; patients with RRMS experienced at least two clinical relapses. All subjects were naïve with no prior treatment, including no cortico-steroidal treatment for at least 6 months prior to blood sampling. Subjects with RRMS were 22-38 years old, 80 percent female, with an Expanded Disability Status Scale (EDSS) of 1-3; healthy control subjects were 24-40 years, 80% female.

Venous blood samples taken from 10 healthy donors and 10 untreated RRMS subjects were processed at the neuro-immunology lab at Rambam Health Care Campus. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (STEMCELL, Vancouver, Canada) using a gradient centrifugation technique protocol. For subjects with RRMS, blood samples were taken within 5 years of first symptoms.

Monocytes isolation, exposure to ALM, and examination of myeloid cells

-differentiation
Monocytes (CD14+ cells) were isolated from PBMCs from healthy controls or subjects with RRMS, using magnetic-activated cell sorting (MACS) and CD14 MicroBeads (Miltenyi Biotec, San Diego, CA, USA). Purity of the isolated monocytes (>97%), as well as PBMCs’ depleted-CD14, was confirmed by flow cytometry. Purified monocytes were cultured (0.2 x 10^5 cells) with RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and 1% glutamine (Biological Industries, Beit Ha’Emek, Israel) in U bottom 96 well plate, at 37 °C and 5% CO₂. Cultured monocytes were exposed to either 10 µg ALM (Sanofi Genzyme, Boston, USA) or 10 µg human IgG Kappa isotype control (Cat no. 0151K-14, SouthernBiotech, Birmingham, USA) in addition to 100 ng/ml LPS (Santa Cruz, Texas, USA) for 24 hours. After 24 hours, surface staining was performed according to M1 pro-inflammatory and chemokines profile [FITC-conjugated CD14, PE-conjugated CD80, PE/Dazzle-conjugated CCR7, PerCP/Cy5.5-conjugated CD86, PE/Cy7-conjugated-CCR5, APC/Cy7-conjugated CD16 (BioLegend, San Diego, CA, USA)] or M2 anti-inflammatory profile [APC/Cy7-conjugated CD14, FITC- conjugated CD23, PE/Dazzle-conjugated CD163, PerCP/Cy5.5-conjugated CD83, PE/Cy7-conjugated CD209, APC-conjugated CD206 (BioLegend)]. The data was readout by FACS (BD FACSFortessa, Becton Dickinson, USA) and analyzed using FlowJo software, version 10.1 (Ashland, OR, USA).

*Monocytes exposed to ALM impact on T-cells*
On a U bottom 96 well plate, $0.4 \times 10^4$ monocytes isolated from PBMCs were seeded with complete RPMI 1640 medium at 37 °C and 5% CO$_2$ as described above. Cultured monocytes were exposed to either 10 µg/ml ALM or 10 µg/ml human IgG Kappa isotype control for 24 hours. The concentration was selected based on Havari et al. 2014 [9] and Rao et al. 2012 [10]. An additional $0.16 \times 10^5$ PBMCs depleted from monocytes were seeded and incubated with complete RPMI 1640 medium. After 24 hours, exposed monocytes cells were washed three times with PBS, then PBMCs depleted from CD14$^+$ cells were reunited with the washed monocytes. The cells were incubated for 3 days with addition of activation cocktail containing 0.5 µg/ml anti-human CD3, 0.5 µg/ml anti-human CD28, 50 ng/ml recombinant IL-23, 5 µg/ml anti-human IL-4, and 5 µg/ml anti-human interferon (IFN-γ) (all from BioLegend). On day 3, the cells were stimulated with 10 ng/ml PMA and 1 µg/ml ionomycin (Cayman Chemical, Ann Arbor, MI, USA) and GolgiPlug (BD, San Diego, CA, USA) for 4 hours. Subsequently, surface staining conducted with T-effector (Th1/Th17) pro-inflammatory profile [PerCP/Cy5.5- conjugated CD3, APC/Cy7- conjugated CD4, PE/Cy7- conjugated CD45RA, PE- conjugated CD45R0, FITC- conjugated CD8, PE/Dazzle- conjugated CCR7, Brilliant Violet 421- conjugated IL-17, APC- conjugated IFNγ (all from BioLegend)]. Cells were then fixed and treated with CytofixCytoperm kit (BD, San Diego, CA, USA) and intracellularly stained with Brilliant Violet 421-conjugated IL-17, APC- conjugated IFN-γ (BioLegend). The same was done with T-reg anti-inflammatory profile [PerCP/Cy5.5-conjugated CD3, APC/Cy7-conjugated CD4, PE-conjugated CD25, PE/Dazzle- conjugated DC127, PE/Cy7- conjugated CD39 (BioLegend)]; followed by intracellular staining for FITC-conjugated FOXP3 treated with FOXP3 Fix/Perm Buffer set and IL-17 Brilliant
Interleukins neutralization and examination of the effect on T-cells differentiation

Monocytes were isolated and cultured with ALM for 24 hours as described above. After 24 hours, ALM were washed and the PBMCs depleted from monocytes reunited with the monocytes. To examine the effect of neutralizing antibodies on T-reg profile cells, we added 10 µg anti-human IL-10, 10 µg anti-human Granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 µg anti-human IL4, 10 µg anti-human TNFα (BioLegend) or 0.5 µg anti CD23 antibody (Suothernbiotech, birmingham, AL, USA) for 3 days (in addition to the activation cocktail as mentioned above). On day 3, the cells were stimulated for 4 hours with PMA, ionomycin, and GolgiPlug followed by surface staining for T-reg profile (as described above). We did not examine the effect of neutralizing antibodies on Th1/Th17 profile cells because we did not see any significant effect of ALM on those cells.

Statistical analysis

Statistical analysis and graphs were performed using Prism version 5 software (GraphPad, San Diego CA, USA). All conditions were compared using a paired t-test, and differences between subjects in both the healthy and RRMS groups were compared using an unpaired t-test. We compared reversal conditions using repeated measure One-Way ANOVA. A cutoff of $p \leq 0.05$ was considered statistically significant.

Results
**ALM promotes differentiation of monocytes towards M2 anti-inflammatory macrophages**

M1 macrophage pro-inflammatory markers were not significantly changed upon 24h ALM-treatment in healthy subjects and RRMS patients alike. In contrast, M2 anti-inflammatory markers significantly increased following ALM treatment in CD23\(^+\) (7.43-fold, p=0.0002), CD23\(^+\)CD83\(^+\) (7.48-fold, p=0.0006), CD23\(^+\)CD163\(^+\) (6.24-fold, p=0.005), CD23\(^+\)CD206\(^+\) (7.82-fold, p=0.019), CD83 (1.56-fold, p=0.0017), and CD163\(^+\) (1.27-fold, p=0.0177) (Fig. 1).

Additionally, we observed significant reduction in CCR5\(^+\) (0.91-fold, p=0.0081), CCR7\(^+\) (0.83-fold, p=0.0003), and CCR5\(^+\)CCR7\(^+\) (0.8-fold, p=0.0001) (Fig. 2).

**ALM-treated monocytes promote T-reg differentiation**

We examined the influence of monocytes pre-treated with ALM on T-cells differentiation. No significant changes were found in the T helper population expressing IL-17 (Th17) or IFN \(\gamma\) (Th1), neither in double producer cells nor in the general population or as part of naive (CD45RA\(^+\)) or memory cells (CD45RO\(^+\)). CD8\(^+\) population showed similar results, regarding general, naïve, and memory sub-populations. In contrast, T-reg relative percentage of the general CD4\(^+\) cells was significantly increased, with a 1.2-fold increase (p=0.0056) in CD4\(^+\)CD39\(^+\) and 1.25-fold (p=0.021) increase in CD4\(^+\)CD25\(^+\)CD127\(^{lo}\)FOXP3\(^+\) (Fig. 3).

**The influence of ALM on M2 differentiation is greater in RRMS patients**
We examined the differences between healthy and RRMS subjects following ALM-exposed CD14 cells on M1/M2 markers and T-effector cells' differentiation or T regulatory cell response. We observed a significant increase in M2 anti-inflammatory CD23^+CD163^+ cells (2-fold, p=0.046) (Fig. 4) in subjects with RRMS compared to healthy control subjects. Regarding CD23 alone, only tendential increase (p=0.083, Fig. 4) was observed.

No statistical differences were found between the group of patients with RRMS and the healthy control group regarding the effect of exposed monocytes on T-effector cells' differentiation or T regulatory cell response.

**CD23 contribute to CD39+ T-reg differentiation**

Since CD23^+ cells showed the most drastic increase upon ALM treatment, we hypothesize that this receptor plays a role in T-reg differentiation. Upon neutralization of CD23^+ on ALM stimulated macrophages, their ability to promote CD39+ T-regs significantly dropped in a dose dependent manner (Fig. 5). This result suggests the contribution of CD23 to T-reg differentiation. Finally, in order to investigate the contribution of the cytokines to T-reg differentiation, we neutralized different cytokines, including TNFα, TGFβ, GMCSF, IL-10, IL-4, in ALM treated monocytes. No changes in T-reg markers' expression were found when neutralizing IL-4, TNFα or TGFβ, IL-10, or GMCSF (Fig. 5).

**Discussion**

The current study demonstrated new aspects of RRMS pathogenesis through exploring the ALM mechanism of action.
For the first time we showed that ALM has a modulatory effect on monocytes by converting them into M2 anti-inflammatory macrophages, expressing CD83, CD163 and especially CD23. On the contrary, M1 pro-inflammatory lineage was not affected by ALM. Furthermore, ALM had a greater impact on CD23 in subjects with RRMS in comparison with healthy subjects. Myeloid cells are known to be involved in the mechanism of RRMS [18], and were shown to be influenced by certain disease-modifying therapies [19]. Our finding further supports the importance of monocytes in RRMS progression and proposes them as a potential therapeutic target. Large proportion of ALM-related side effects is associated with lymphocytes depletion [1]. Modulating monocytes without depleting lymphocytes may exert therapeutic potential with less adverse effects.

The relative frequencies of T-regulatory lymphocytes are increased in RRMS patients after ALM administration [6–8], which is believed to contribute to ALM’s therapeutic effect [1]. However, direct administration of anti-CD52 to T-cells causes complement-dependent cytolysis [9]. Since myeloid cells and lymphocytes have a complex and elaborate internal interaction [20], we hypothesize that ALM effect on T-cells may be mediated by monocytes. Indeed, ALM-activated CD14+ mediated differentiation of T-cells in the anti-inflammatory direction, indicated by increase in T-reg markers CD4+CD39+ and CD4+CD25HI/CD127LO/FOXP3, without influencing T-effector cells (Th1/Th17). As the T-cell population gets largely depleted upon ALM treatment, M2 macrophages may be used as a template for the anti-inflammatory T-cells subpopulation recovery. M2 macrophages were previously shown to promote T-reg differentiation [21]. Our study further supports this direction.
Our findings point out that ALM treatment works mostly through regulatory mechanisms. Considering high efficacy of ALM [3], this underlines the importance of weakened regulatory properties in the pathogenesis of MS.

As mentioned above, CD23^+ macrophages were most highly elevated upon ALM exposure. Moreover, CD23 tended to be more upregulated in RRMS patients-derived cells compared to healthy subjects. Neutralizing CD23 diminished the ability of ALM treated monocytes to promote CD4^+CD39^+ T-reg differentiation in a dose-dependent manner, suggesting CD23 as a potential mediator. CD23 is a low affinity IgE with a multitasking profile [22]. It is expressed by M2 anti-inflammatory macrophages and may therefore be involved in anti-inflammatory response [23, 24]. Elevated levels of CD23^+ macrophages were observed in patients with active RRMS [25], potentially as a marker for tissue repair [26]. At the same time, soluble CD23 upregulation is associated with a number of autoimmune diseases [22]. Interestingly, one of the common adverse effects, which limits ALM use, is secondary autoimmunity [1, 5]. In case CD23 is also upregulated in vivo upon Alemtuzumab treatment, it may contribute to this side effect.

**Conclusions**

Our study indicates the involvement of monocytes and subsequent T-reg modulation in the mechanism of ALM treatment and perhaps in the pathogenesis of RRMS. We introduce CD23 as a mediator of the anti-inflammatory effect of ALM. On the other hand, CD23 may also be responsible for ALM associated adverse effects. Our findings open a new avenue to explore in the downstream pathways, in order to understand how CD23 affects the T-reg population.
In-vivo and additional clinical experiments are needed in order to validate our findings regarding CD23 expression and its effect on T-cells properties; it should also be studied whether those changes are persistent over time.

**List of abbreviations:** ALM - Alemtuzumab, CD - cluster of differentiation, FACS - fluorescence-activated cell sorting, GM-CSF - Granulocyte-macrophage colony-stimulating factor, IFN - interferon, IG - immunoglobulin, IL - interleukin, MS - multiple sclerosis, PBMCs - peripheral blood mononuclear cells, PMA - phorbol 12-myristate 13-acetate, RRMS - relapsing-remitting multiple sclerosis, T-eff - T-effector cells, Th - T-helper cells, T-reg - T-regulatory cells, TNF - tumor necrosis factor.
Declarations

*Ethics approval and consent to participate*

The study was approved by the ethical committee in accordance with the World Medical Association Declaration of Helsinki, 275-2015.

*Consent for publication*

not applicable

*Availability of data and materials*

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

*Competing interests*

The authors declare that they have no competing interests

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*Authors’ contributions*

EGC, MAH, IL, AV, AS, AR contributed to conception and design of the study. LR contributed to acquisition and analysis of data. AR, LR, DS, NB, CB contributed to drafting and revising of the manuscript.
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Figure 1. Alemtuzumab mediated M2 anti-inflammatory differentiation of the monocytes.

Effect of 10 μg ALM on isolated CD14 cells for 24 hours with 100 ng/ml LPS stimulation compared to isotype control (IgG). Significant increase in the following anti-inflammatory markers was observed, % of CD14+ shown: (A) CD23 (7.43-fold, p=0.0002), (B) CD163 (1.27-fold, p=0.0177) (C) CD83 (1.56-fold, p=0.0017) (D) CD23+CD206+ (7.82-fold, p=0.019) (E) CD23+CD163+ (6.24-fold, p=0.005) (F) CD23+CD83+ (7.48-fold, p=0.0006) (n=10). All conditions were compared using paired t-test. A cutoff of $p \leq 0.05$ was considered statistically significant.
Figure 2. Alemtuzumab reduced chemokine levels on monocytes (CD14).

Isolated CD14+ cells were treated with 10 μg ALM on for 24 hours with 100 ng/ml LPS stimulation. After 24 hours, surface staining was performed according to the M1 pro-inflammatory and chemokines profile. We observed significant reductions in chemokine markers on CD14+ cells: (A) CCR7 (0.83-fold, p=0.0003), (B) CCR5 (0.91-fold, p=0.0081), (C) CCR5*CCR7+ (0.8-fold, p=0.0001) (n=10). All conditions were compared using paired t-test. A cutoff of p ≤ 0.05 was considered statistically significant.
Figure 3. Monocytes treated with ALM promoted CD39+ T-reg differentiation.

Isolated monocytes were exposed to 10 µg ALM for 24 h, subsequently reintroduced to PBMCs and incubated for 3 days. After 3 days we examined the influence of exposed monocytes on T-cells. We observed a significant increase in T-reg percentage of CD4⁺: (A) CD39⁺ (1.2-fold, p=0.0056), (B) CD25^{HI}CD127^{LO}FOXP3⁺ (1.25-fold, p=0.021) (n=10). All conditions were compared using paired t-test. A cutoff of $p \leq 0.05$ was considered statistically significant.
Figure 4. ALM increase in CD23\(^+\)CD163\(^+\) was higher in RRMS subject-derived cells. ALM-exposed CD14\(^+\) cells from RRMS subjects had 2-fold elevation in CD23\(^+\)CD163\(^+\) double-positive cells. (p=0.046). Tendential increase in CD23 was observed in RRMS derived cells treated with ALM (p=0.083). All conditions were compared using an unpaired t-test. A cutoff of \(p \leq 0.05\) was considered statistically significant.
Figure 5. CD23 neutralization abolished the effect of ALM treated monocytes on CD39+ T-reg. Monocytes were isolated and cultured with 10 μg ALM for 24 h. After 24 h monocytes were washed and the PBMCs depleted from monocytes were reunited with the monocytes and incubated with 0.5 or 1 μg/ml anti-CD23 for 3 days (in addition to the activation cocktail as mentioned above). 0.5 μg/ml anti-CD23 reduced CD4+CD39+ by 22% and 1 μg/ml anti-CD23 by 48% (p<0.05). One way Anova with Bonferroni correction was used. A cutoff of \( p \leq 0.05 \) was considered statistically significant.