

RESEARCH ARTICLE

Extracellular Mg concentration and Ca blockers modulate the initial steps of the response of Th2 lymphocytes in co-culture with macrophages and dendritic cells

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ABSTRACT. Magnesium is highly involved in the metabolic network such that even subtle disturbances in its homeostasis affect many cellular functions, including calcium homeostasis, signal transduction, energy metabolism, membrane stability and cell proliferation. Recently, magnesium level has been proposed to modulate the priming and activity of immune cells. We studied the behavior of antigen-presenting cells (APCs) and T lymphocytes after altering the magnesium/calcium balance. We used two different populations of primary APCs, i.e. bone marrow-derived dendritic cells and bone marrow-derived macrophages, while D10.G4.1 cells served as a model of responding Th2 cells. Our principal findings are the following: (i) the extracellular magnesium concentration had no significant impact on endocytosis by bone marrow-derived APCs, (ii) high concentrations of extracellular magnesium, with or without calcium antagonists, significantly decreased IL-4 and IL-10 secretion by Th2 cells in a co-culture system of APCs and Th2 lymphocytes, (iii) proliferation of Th2 cells in co-culture systems was significantly inhibited by calcium antagonists independently from extracellular magnesium concentrations. Our results suggest that alterations of magnesium and calcium homeostasis impact on some crucial steps of the immune response.

Key words: magnesium, calcium, dendritic cells, macrophages, T lymphocytes

Recognition of a pathogen is a fundamental function of the immune system. Important players in this process are the so-called antigen-presenting cells (APCs) which engulf, process, and then present antigens to T lymphocytes. APCs also produce the signals necessary for the activation of lymphocytes into effector cells that actively carry out immune defenses. In addition, they prime self-reactive T cells [1]. APCs are a heterogeneous population that includes Langerhans cells, macrophages, dendritic cells (DCs) and B lymphocytes. In particular, DCs are powerful APCs, which express both MHC I and MHC II, thus being excellent in presenting antigens to naive CD8 T and CD4 T lymphocytes. On the other hand, macrophages, which express MHC II, phagocytose antigens, process them intracellularly, and then present them on MHC-II to T lymphocytes.

The activities related to antigen presentation in these cells are governed by calcium (Ca) signals resulting from Ca influx across the plasma membrane and its release from intracellular stores, mainly the endoplasmic reticulum and mitochondria. Ca is known to be a multifunctional messenger regulating many cell functions that are

potentially relevant to antigen processing, including antigen adherence and entry, receptor-mediated endocytosis, actin depolymerization, phagosome-lysosome membrane fusion, vesicle formation in the trans-Golgi network, and exocytosis. It is noteworthy that the addition of Ca ionophores to immature DCs induces the acquisition of morphological and functional properties of activated, mature DCs [2]. Accordingly, intracellular Ca chelators prevent DC maturation [2]. In macrophages, the capacity to adhere to a substrate and antigen processing are dependent on the availability of extra- and intracellular Ca, and on plasma membrane Ca channels [3]. The role of Ca signaling pathways is also well established in lymphocyte activation [4].

Magnesium (Mg) also modulates the immune response. Apart from regulating hundreds of different enzymes, Mg is crucial in controlling transporters, ion channels and second messengers [5]. Mg, in addition, modulates intracellular, free Ca concentrations [6]. Leukocytes are sensitive to extracellular Mg concentrations [7]. *In vitro*, the activation of polymorphonuclear cells (PMNs) is markedly dependent on extracellular Ca and Mg concentra-

tions [8-10]. High extracellular Mg has also been shown to affect the antigen-presenting capacity of human epidermal Langerhans cells *in vitro* and *in vivo* [11]. In lymphocytes, high extracellular Mg impacts on the production of cytokines [12]. Recently, a rapid, transient, Mg influx induced by antigen-receptor stimulation has been shown in normal T lymphocytes [13]. Interestingly, mutations in the Mg transporter MagT1 abrogated this influx and decreased the Ca influx, thus impairing the T cell response to antigen presentation [13].

Both Ca and Mg therefore, play a part in intracellular signaling in immune cells. Until now however, these two metals have been studied individually in different cell types and very little is known about the relevance of their balance in modulating cell function, in particular in APCs and lymphocytes.

With the aim of elucidating some events involved in the process of antigen presentation by APCs, such as endocytosis by APCs, and growth and cytokine production in the effector Th2 cells, we cultured macrophages, dendritic cells and T lymphocytes in the presence of low or high concentrations of extracellular Mg to alter the extracellular Mg/Ca ratio. To investigate whether and how extracellular and intracellular Ca contribute to the aforementioned events, we also exposed the cells to verapamil and TMB-8.

Verapamil, a widely used L-type Ca channel-blocker, targets L-type voltage-dependent Ca channels (also known as dihydropyridine-sensitive DHP-receptor) in their open state by interfering with the binding of Ca ions to the extracellular opening of the channel [14, 15]. Functional L-type Ca channels have been detected in a variety of immune cells, including dendritic and T cells. It is the interplay between L-type Ca channels and store-operated Ca entry that regulates Ca signaling and Ca regulation of immune cell function [16].

TMB-8 has a complex pharmacological profile. It is known to inhibit Ca-induced Ca release to the cytosol, thus acting as an intracellular Ca antagonist [17, 18]. It also inhibits protein kinase C activation [17, 19].

Our results, obtained by using different concentrations of extracellular Mg, with or without calcium blockers, suggest that alterations in Mg and Ca homeostasis impact on crucial steps of the specific immune response.

METHODS

Mice

Female C3H/HeN(H-2k) mice (Charles River Laboratories, Munich, Germany), 6-8 weeks old and weighing 20-25 g, were used to isolate bone marrow cells (BMCs) from femurs and tibias. The mice were kept under special pathogen-free conditions and bred in accordance with institutional guidelines.

Ethics statement

Experimental protocols were approved by the institutional review board of 2nd Local Committee on Animal Research and Ethics (Wroclaw, Poland).

Cell culture

BMCs were aseptically flushed out with cold, complete RPMI-1640 medium using 23G needles and 5.0 mL syringes. The complete RPMI-1640 medium was prepared by adding heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 40 U/mL penicillin G, and 100 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). To obtain bone marrow-derived dendritic cells (BMDCs), BMCs were seeded into bacteriological Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in complete RPMI medium containing 20 ng/mL recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) (Life Technologies, Carlsbad, CA, USA) and 0.05 mM 2-mercaptoethanol (Sigma Aldrich) according to the protocol described by Lutz *et al.* [20]. After 10 days of culture, non-adherent cells were collected, suspended in fresh medium, and seeded onto plastic culture dishes in the presence of GM-CSF.

To generate bone marrow-derived macrophages (BMMFs), BMCs were seeded in complete RPMI-1640 medium containing 40 ng/mL M-CSF (eBioscience, San Diego, CA, USA) and 0.05 mM 2-mercaptoethanol (Sigma Aldrich). After 24 h, non-adherent cells were removed and fresh medium was added. Adherent cells were used in the experiments after 10 days of culture [21].

Mouse lymphocyte-like cell line D10.G4.1 (cat. no. TIB-224, American Type Culture Collection, Manassas, VA, USA) are Th2-helper lymphocytes established from the AKR/J(H-2k) mouse, and are extensively used to study the activation of T cells. They are specific for conalbumin in the context of I-Ak, and also respond to the monoclonal anti-receptor antibody 3D3. D10.G4.1 cells were grown in RPMI-1640 medium (Sigma Aldrich) supplemented with heat inactivated 10% FBS, 2 mM L-glutamine, 10% T-STIM (Becton Dickinson), 10 pg/mL mouse interleukin (IL)-1 α (eBioscience), 0.05 mM 2-mercaptoethanol, 40 U/mL penicillin G, and 100 µg/mL streptomycin. Actively growing cells were subcultured according to the ATCC protocol. An Mg-free medium was purchased from Invitrogen (San Giuliano M.se, Italy) and added to MgSO₄ to obtain different extracellular Mg concentrations. To prevent Ca influx from the extracellular milieu, we used verapamil, and to prevent Ca release from intracellular stores we utilized TMB-8 (Sigma Aldrich) because these compounds have similar molar mass and they are both soluble in water. The experimental strategy is schematically depicted in the figure 1.

BMDCs and BMMFs marker expression

BMDCs and BMMFs were suspended in PBS containing 10% FBS and 0.1% sodium azide, and stained with phycoerythrin (PE)-conjugated anti-CD40, anti-CD86 anti-I-Ab [major histocompatibility complex (MHC) class II], and fluorescein isothiocyanate (FITC)-conjugated anti-CD11c, anti-CD80 (former B7-1), anti-CD86 (former B7-2) and anti-F4/80 for 30 min, in the dark, at 4°C, according to the manufacturer's instruction (eBioscience). An appropriate isotype control was used for each antibody. Stained cells were evaluated by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson). Results were analyzed using the software WinMDI 2.9.

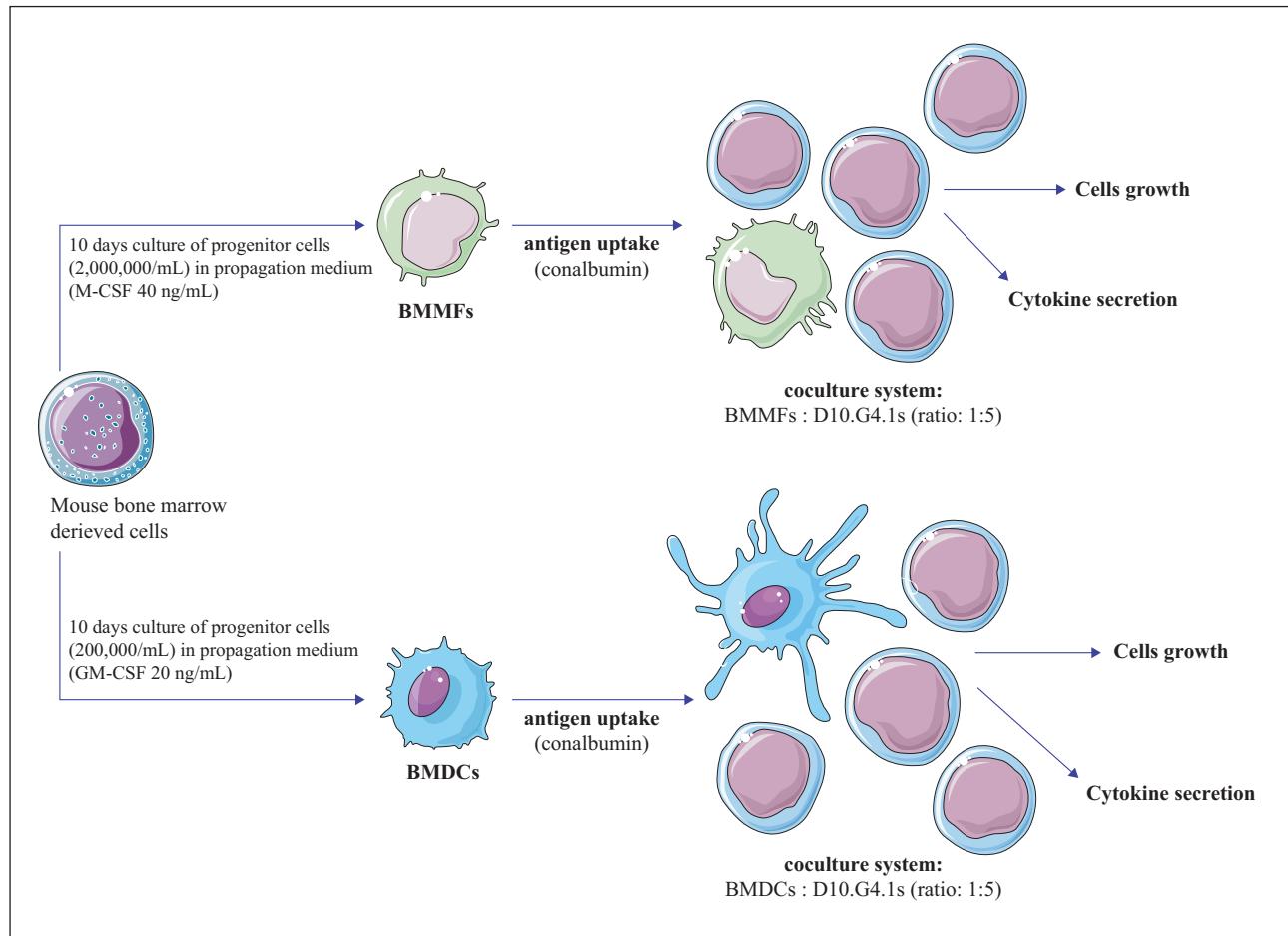


Figure 1

Experimental strategy.

Initially, primary BMDCs and BMMFs were evaluated for their capacity to take up conalbumin. Then, a stable *in vitro* co-culture model of T lymphocytes and APCs was generated to study cell growth and cytokine secretion in T lymphocytes as the result of antigen presentation. (Illustration was made on the basis of Servier Medical Art).

Endocytosis assay

To study the endocytic capacity of BMDCs and BMMFs, the uptake of soluble protein antigen (ovalbumin-FITC) (Life Technologies, Carlsbad, CA, USA) was evaluated. BMDCs/BMMFs (50,000 cells/mL) were incubated in the presence of FITC-ovalbumin (1 mg/mL), at 37°C, for 6 h, in medium with 0.5, 1.0 or 5.0 mM extracellular Mg with or without verapamil (50 μ M) or/and TMB-8 (50 μ M). The cells were then washed three times with a cold buffer (PBS containing 2% FBS, without Mg and Ca). Uptake by APCs was determined by flow cytometry. Control experiments were conducted in parallel at 4°C to determine the nonspecific adherence as the background.

Antigen presentation and cytokine levels

The capacity of APCs to present antigen to D10.G4.1 helper T cells was based on the methods of Ayala *et al.* [22] and Kwasaki *et al.* [23]. To evaluate the capacity of BMDCs/BMMFs to present antigens to lymphocyte-like D10.G4.1 cells, BMDCs/BMMFs (50,000 cells/mL) were incubated with 50 μ g/mL mitomycin C (Sigma Aldrich). After 30 minutes, the cells were washed thoroughly with cold PBS and incubated with 300 μ g/mL conalbumin (Sigma-Aldrich) for 6 h in complete medium. After three

washes with cold Mg- and Ca-free PBS containing 2% FBS, BMDCs/BMMFs were co-cultured with D10.G4.1 cells (ratio 1:5) in medium with different concentrations of extracellular Mg (from 0.5 mmol/L to 5.0 mmol/L) with verapamil (50 μ M) or/and TMB-8 (50 μ M).

To evaluate proliferation, the cells were collected at different time points, stained with a trypan blue solution (0.4%), and the viable cells were counted using an automated counter. Cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes was assessed by the MTT assay (Life Technologies).

Secreted IL-4 and IL-10 were measured by enzyme-linked immunosorbent assay kits (ELISA) (Becton Dickinson) after 24 h of co-culture, according to the manufacturer's instructions.

Statistical analysis

Statistics were calculated using *Statistica* (version 6.0; Statistica Software). Results are expressed as mean \pm SD of five separate experiments. Statistical multiple comparisons were estimated using Kruskal-Wallis test, and the comparison between two groups was estimated using the Mann-Whitney U test. In all cases, p-values lower than 0.05 were considered statistically significant.

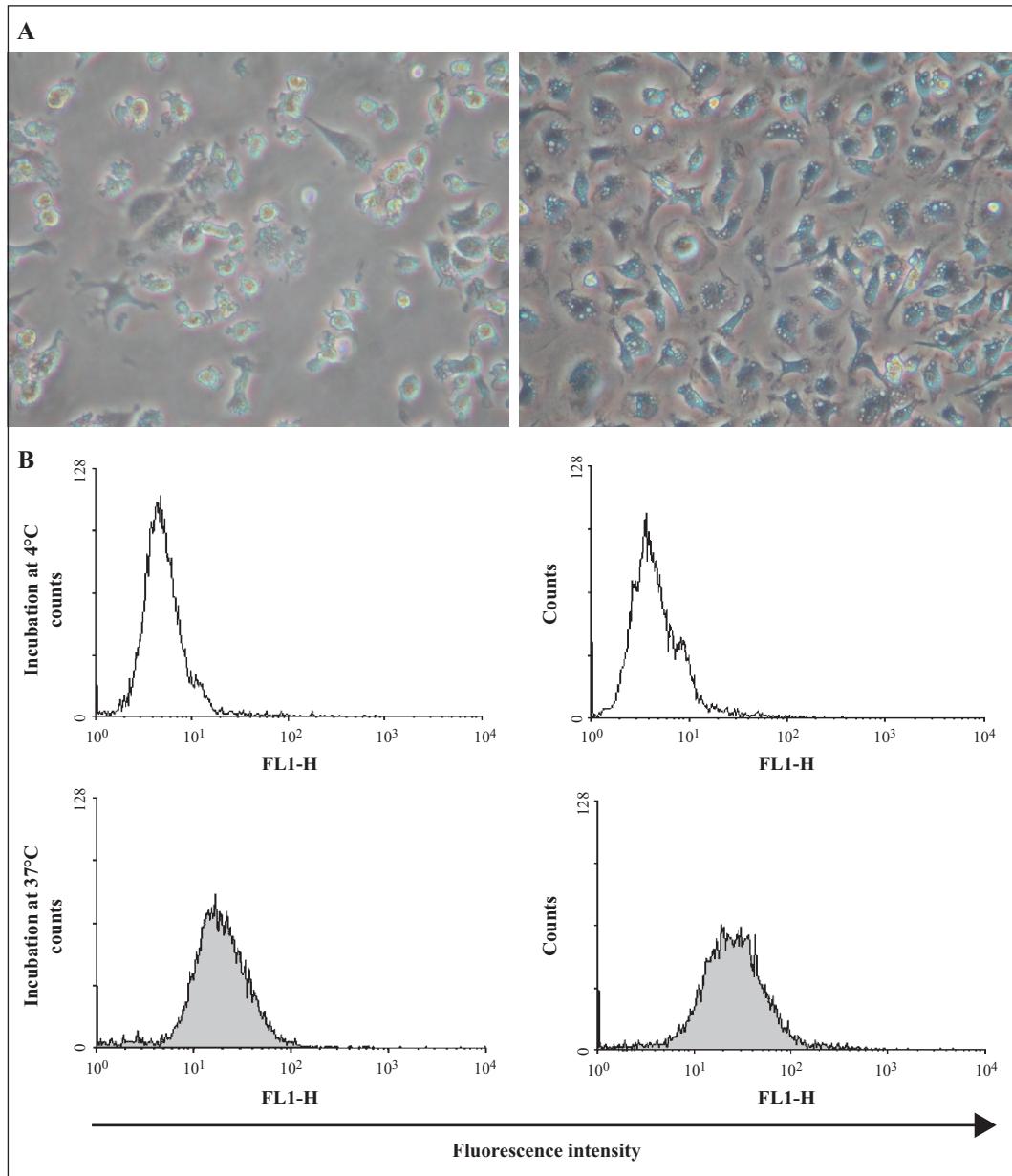


Figure 2

Characterization of primary BMDCs and BMMFs.

After isolation, the cells were cultured for 10 days as described in the Method.

A) The morphology of the cells was examined by phase-contrast microscopy (magnification 20 \times). Left panel: BMDCs; right panel: BMMFs. **B)** BMDCs (left panel) or BMMFs (right panel) were incubated with fluorescein isothiocyanate-conjugated ovalbumin (FITC-OVA) as described. Fluorescence intensity was analyzed by FACS.

RESULTS

Characterization of BMDCs and BMMFs

BMCS, isolated as described in the Methods, were cultured and exposed to GM-CSF to induce differentiation into BMDCs [20], or to M-CSF to obtain BMMFs [21]. Upon differentiation, the cells were photographed at 20 \times magnification (figure 2A). BMDCs appeared loosely adherent and irregular, with some cells forming dendritic processes. BMMFs were large with a round or oval nucleus and many cytoplasmic granules and vacuoles. Since antigen endocytosis by APCs is the initial step for the induction of the antigen-specific T-cell response, the endocytic activity of BMDCs and BMMFs was compared by measuring

the uptake of fluorescent ovalbumin, by flow cytometry. Figure 2B shows that the two cell types take up ovalbumin to a similar extent. The phenotypes of the BMCS, BMDCs and BMMFs were compared by FACS analysis. Data are summarized in table 1. BMCS show a non-differentiated phenotype when compared to BMDCs and BMMFs. CD80 and CD86 are important costimulatory molecules for T cell stimulation. While the levels of CD80 are comparable in the two cell types, BMDCs expressed lower amounts of CD86 than BMMFs. In addition, BMDCs show higher amounts of MHCII than BMMFs. F4/80, a transmembrane protein present on the cell-surface of mature macrophages [24], was detected only in BMMFs. CD11c, an integrin glycoprotein found at high levels on dendritic cells [25], was detected only on BMDCs.

Table 1

FACS analysis of marker profiles in BMCs, BMDCs and BMMFs from C3H/HeN(H2k) mice after 10 days in culture. Results are expressed as mean \pm standard deviation of five independent experiments.

	BMCs	BMDCs	BMMFs
Cell surface marker	% positive cells		
CD40	5.6 \pm 3.2	31.3 \pm 8.2	39.8 \pm 6.4
CD80	4.8 \pm 3.6	54.4 \pm 8.6	59.2 \pm 9.1
CD86	3.6 \pm 4.4	22.9 \pm 4.7	38.4 \pm 7.1
CD11c	7.4 \pm 5.6	62.9 \pm 9.2	-
F4/80	1.7 \pm 2.2	-	63.3 \pm 7.2
MHC II	9.8 \pm 4.1	64.3 \pm 8.2	55.8 \pm 10.0
MHC II ^{low}	-	34.2 \pm 5.8	35.8 \pm 7.4
MHC II ^{high}	-	33.2 \pm 4.2	19.8 \pm 4.6

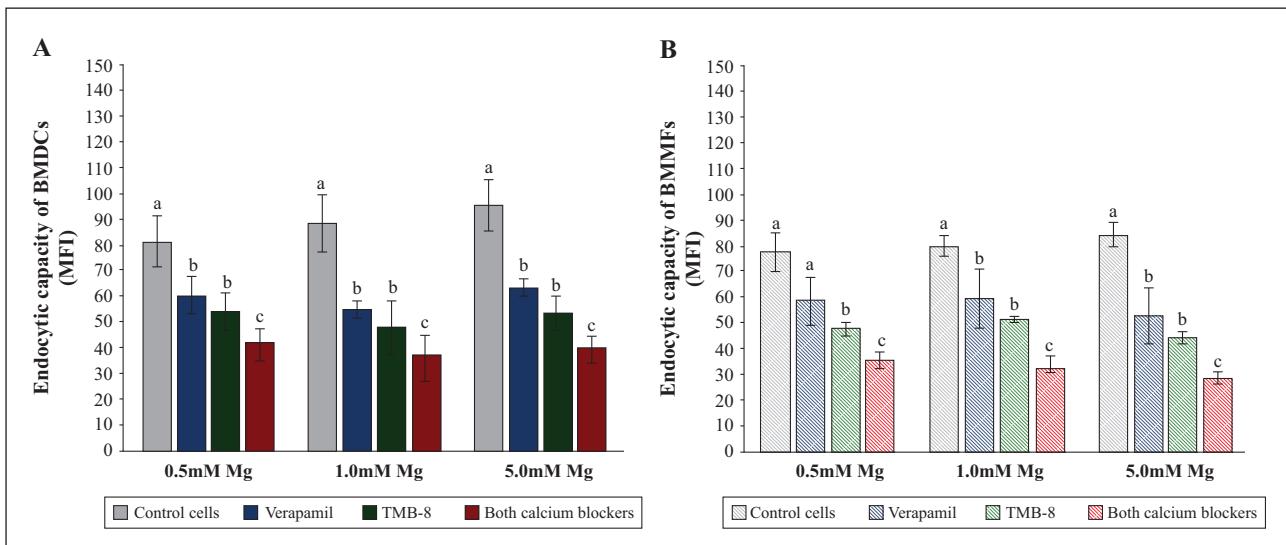
Effects of different concentrations of extracellular Mg and Ca blockers on the endocytic capacity of primary APC

To vary the extracellular availability of Mg, we used concentrations of extracellular Mg ranging from 0.5 to 5.0 mM, where 1.0 mM concentration mimics normal levels of Mg. Initially, we investigated the ability of BMDCs and BMMFs to take up antigens after culturing the cells in different concentrations of extracellular Mg, in the presence or absence of verapamil and TMB-8. It is noteworthy that 12/24 hours treatment with verapamil or/and TMB-8 had no significant impact on the expression of MHCII, CD80 or CD86 in APCs as shown by flow cytometry analysis (data not shown). To study the endocytic capacity of BMDCs and BMMFs, fluorescent ovalbumin was added for 6 h, and its uptake by bone marrow-derived APCs was measured by flow cytometry. Different concentrations of extracellular Mg did not significantly impact endocytosis by BMMFs and BMDCs. Indeed, ovalbumin uptake

was constant, between 75 and 110 MFI (median fluorescence intensity) in BMDCs, and it was between 70 and 90 MFI in BMMF (figure 3). In the presence of verapamil and TMB-8, alone or in combination, antigen uptake by BMDCs and BMMFs was significantly decreased independently from extracellular Mg concentrations. In particular, BMDC endocytosis was reduced by verapamil to 35% of the control, by TMB-8 to 40% and by the combination of the two to 55% (figure 3A). Similarly, BMMF endocytosis was decreased by verapamil to 25%, by TMB-8 to 35% and by the two Ca blockers to 60% of the control value (figure 3B).

Effects of different concentrations of extracellular Mg and Ca blockers on the cellular activity and growth of D10.G4.1 cells in co-culture with BMDCs or BMMFs

Upon initial encounter with antigen, T helper lymphocytes are activated and proliferate. To compare BMDCs and BMMFs for their capability to stimulate lymphocytes, a co-culture system of D10.G4.1 and BMDCs or BMMFs was developed. Briefly, growth-arrested BMDCs and BMMFs were challenged with conalbumin, a soluble antigen, for 6 h and then co-cultured with D10.G4.1 cells in different concentrations of extracellular Mg, with or without verapamil, and TMB-8, alone or in combination. Previous experiments had shown that verapamil or/and TMB-8 had no significant impact on the expression of CD4 and CD25 in D10.G4.1 cells (data not shown). D10.G4.1 cells were counted every 24 h for three days. Significant results were obtained after 72 h (figure 4). Different extracellular Mg concentrations did not exert any significant effect, while verapamil and TMB-8, alone or in combination, markedly inhibited T-cell growth in the two co-culture systems independent of extracellular Mg concentrations (figures 4A, C). As after 72 h the proliferation rate of T-cells is higher in the BMDC than in the BMMF co-culture system, our data suggest that BMDCs are more potent than BMMFs in activating T cells.

**Figure 3**

The effect of different concentrations of extracellular Mg and Ca blockers on endocytosis by BMDCs and BMMFs. BMDCs (A) and BMMFs (B) were cultured in 0.5, 1.0 or 5.0 mM Mg containing medium with or without verapamil or/and TMB-8 (both 25 μ M). After pre-incubation with FITC-OVA for 6 h, at 37°C, fluorescence intensity was analyzed by FACS. Results are expressed as the mean \pm SD of five separate experiments. Different letters indicate significant effect ($p < 0.05$) of treatments with Ca blockers within the same magnesium concentration used (a, b, c).

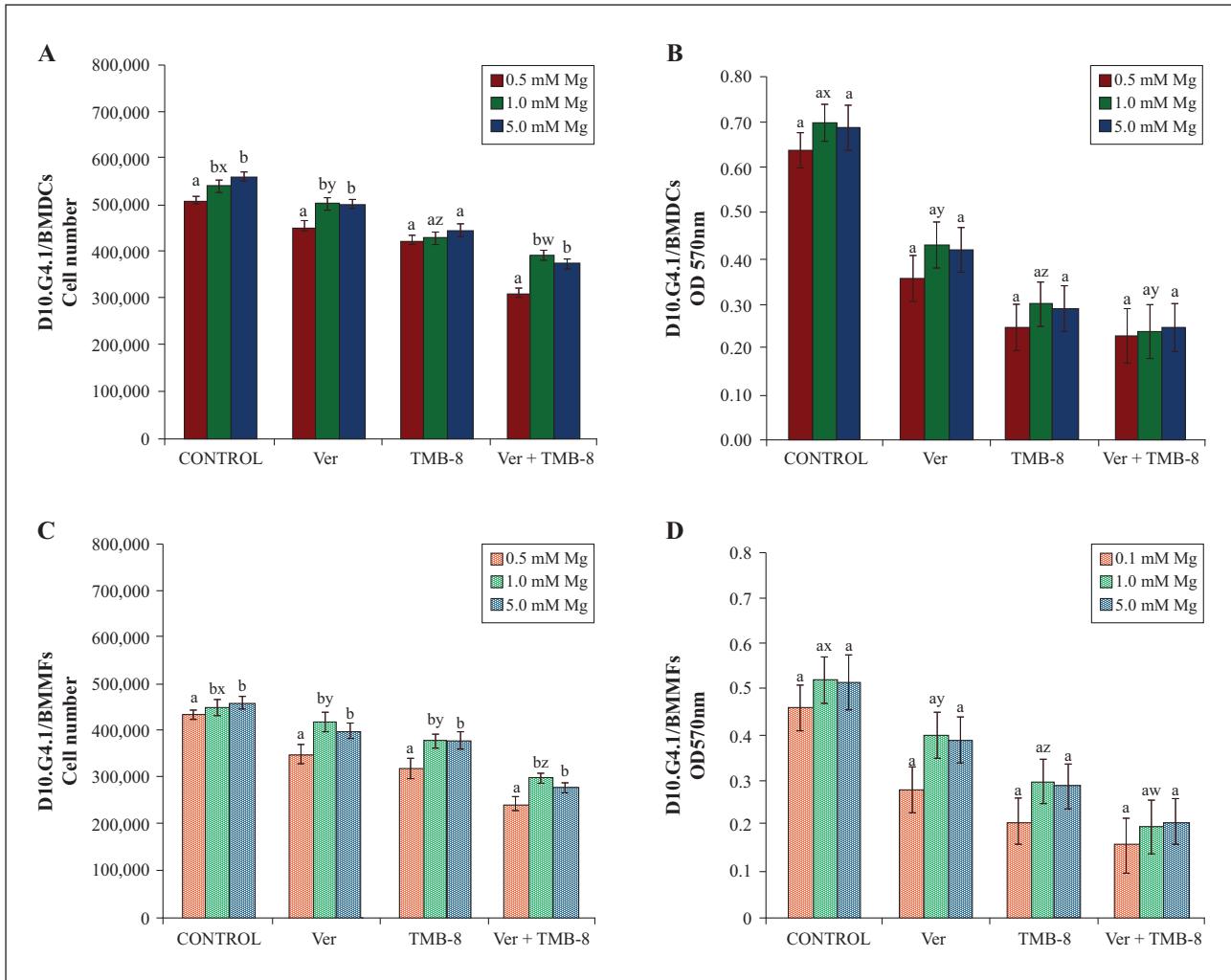


Figure 4

The effect of different concentrations of extracellular Mg and Ca blockers on the proliferation of D10.G4.1 lymphocytes co-cultured with BMDCs or BMMFs. BMDCs and BMMFs were cultured in 0.5, 1.0 or 5.0 mM Mg containing medium with or without verapamil (ver) or/and TMB-8 (both 25 μ M) for 72 h. **A, C**) cell proliferation assay on BMDCs and BMMFs, respectively. **B, D**) MTT assay on BMDCs and BMMFs, respectively.

The values are the mean \pm SD from five independent experiments. Different letters indicate significant effect ($p < 0.05$) of treatments: (a, b) effect of various Mg concentrations; (w, x, y, z) effect of Ca on cells cultured in 1 mM Mg.

We also performed MTT assays, which measure cellular metabolic activity due to NAD(P)H flux. Figures 4B, D show that verapamil and TMB-8 have an inhibitory effect that is exacerbated when the extracellular Mg concentration is low (figures 4B, D). The results obtained by MTT assay reflect the number of viable cells, and confirm the concept that BMDCs are better than BMMFs at antigen presentation.

Effects of different concentrations of extracellular Mg and Ca blockers on cytokine secretion by D10.G4.1 in co-culture with BMDCs or BMMFs

Activated Th2 lymphocytes release high amounts of IL-4 and IL-10. We therefore evaluated the secretion of these two cytokines in our co-culture system using ELISA. Our negative controls were the following: i) D10.G4.1 cells alone; ii) D10.G4.1 cells in the presence of ovalbumin; iii) D10.G4.1 cells co-cultured with either BMDCs or BMMFs.

Antigen presentation by BMDCs and BMMFs to D10.G4.1 cells stimulate IL-4 secretion (figure 5). In the two co-culture systems, 24 h culture in high extracellular Mg

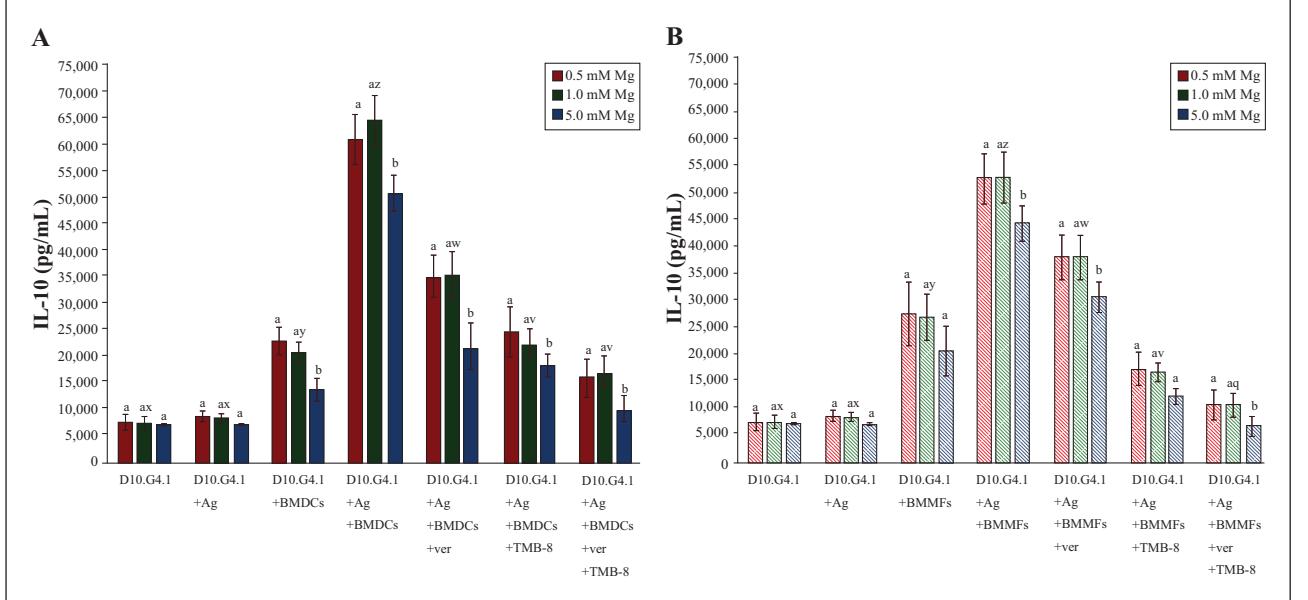
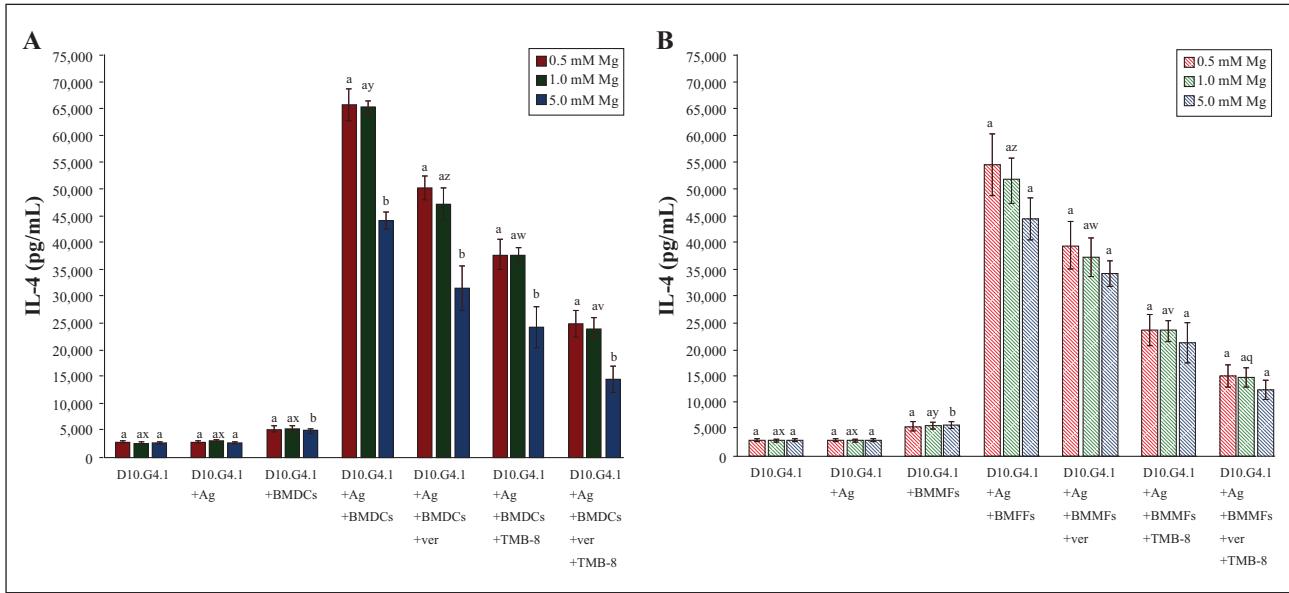
significantly decreased IL-4 secretion and exacerbated the inhibitory effect of verapamil and TMB-8, alone or in combination (figure 5). The inhibitory effect of high concentrations of extracellular Mg is more pronounced in BMDCs than in BMMFs.

Similarly, high Mg reduced IL-10 secretion in the two systems (figure 6). After 24 h exposure, verapamil and TMB-8, alone or in combination, inhibited IL-10. A high Mg concentration (5.0 mM) enhanced the inhibitory effect of Ca blockers, and this effect was more pronounced in BMDCs than in BMMFs.

The concentrations of secreted INF- γ and IL-17 were below the ELISA detection threshold. IL-2 was not measured because the D10.G4.1 cells require the addition of 2 ng/mL IL-2 to the medium for growth to occur (data not shown).

DISCUSSION

The availability of Ca and the possibility of mobilizing it from intracellular stores are crucial for the activation of various players in immune reactions, including T cells,



The effect of different concentrations of extracellular Mg and Ca blockers on IL-10 secretion by D10.G4.1 lymphocytes co-cultured with BMDCs or BMMFs.

BMDCs (A) and BMMFs (B) were cultured in 0.5, 1.0 or 5.0 mM Mg with or without verapamil or/and TMB-8 (both 25 μ M) for 24 h. IL-10 levels were measured by ELISA.

The values are the mean \pm SD from five independent experiments. Different letters indicate significant effect ($p < 0.05$) of treatments: (a, b) effect of various Mg concentrations; (w, x, y, z, v, q) effect of Ca blockers on cells cultured in the control Mg concentration (1.0 mM).

macrophages, PMNs and mast cells. Accordingly, Ca channel blockers can affect the activation of cells involved in the innate immune response [26, 27]. Less is known about the contribution of Mg and, to our knowledge, almost no data are available about the effects of altering both Ca and Mg on immune cells. Our interest in the effects of Mg stems from the evidence of the well-known relationship between Mg and inflammation [28]. At the cellular level, low Mg activates phagocytic cells, and in

rats Mg deficiency renders macrophages hyper-responsive to immune stimuli [29]. This is probably due to the increase in intracellular Ca which follows Mg deprivation so that, upon activation, the cells release higher amounts of Ca from the intracellular stores than controls, thus exacerbating their response [30]. Interestingly, a study performed on Mg-deficient mice has shown that nifedipine is able to reverse some inflammatory symptoms and, accordingly, high concentrations of extracellular Mg reduce the

response of phagocytic cells to the immune stimuli [31]. In particular, Lin *et al.* have demonstrated that increasing concentrations of extracellular Mg significantly mitigated LPS-induced up-regulation of inflammatory molecules, and inhibited endotoxin-induced NF- κ B activation in a murine macrophage-like RAW264.7 cell line [32]. Moreover, high extracellular Mg decreases TLR-stimulated proinflammatory cytokine production by human primary monocytes through the induction of constitutive I κ B α levels [33].

The aim of this study was to decipher how altering both Mg and Ca homeostasis impacts on some aspects of the cascade of events involved in antigen presentation. In particular, we focused our attention on (i) antigen uptake by APCs as the initial step of the induction of antigen specific Th2-cell response and (ii) subsequent Th2-cell activation and response evaluated in terms of T cell proliferation and cytokine secretion. For this reason we developed an experimental model in which we interfere with the homeostasis of both Ca and Mg by culturing the cells in different concentrations of Mg, based on the evidence that Mg is a natural Ca antagonist [4]. We also used verapamil and TMB-8 to impact on Ca influx and Ca release from intracellular stores, respectively [14-19]. Our results indicate that different concentrations of extracellular Mg have no significant impact on antigen uptake by the two types of APCs we examined, while interfering with Ca availability impairs the endocytic capacity of APCs by more than 50%. It is noteworthy that verapamil and TMB-8 have additive effects *in vitro*, indicating that both Ca entry and Ca release from intracellular stores are required for optimal endocytic activity. When D10.G4.1 cells were co-cultured with BMDCs or BMMFs, the total cell number was not affected by extracellular Mg, while it was reduced by verapamil and TMB-8. In agreement with previous studies [3], these results suggest that Ca is necessary for antigen processing in order to activate T cells. However, it should be recalled that Gomes *et al.* [34] indicated that the Ca antagonist nicardipine did not affect the APC signalling capacity to prime naive T cells or to induce Th2 proliferation, while this compound altered Ca signalling in T cells. This discrepancy can be ascribed to the fact that different cells and different culture conditions have been used.

We also found that high extracellular Mg alone or in combination with TMB-8 and verapamil significantly decreased IL-4 and IL-10 secretion in the T cell-BMDC and -BMMF co-culture systems. Since Ca is crucial for the secretory machinery, we argue that high extracellular Mg concentrations act to antagonize Ca entry. Indeed, Mg inhibits Ca influx through L-type voltage-dependent Ca channels [35], and it is known that Th2 cells express these channels which can be effectively blocked by L-type Ca channel blockers [36]. Exposure to verapamil and culture in high concentrations of Mg yield similar results. We argue that high extracellular Mg and verapamil prevent the Ca transients required for IL-4 and IL-10 secretion. Also, the inhibition of Ca release from intracellular stores determined by TMB-8, reduces the secretion of the two cytokines, thus indicating that both Ca entry and intracellular Ca release contribute to the regulation of the secretory pathways. Surprisingly, the Mg-dependent decrease in IL-4 and IL-10 does not result in significant alterations of D10G4 cell

proliferation. We hypothesize that, because of the reduced release of IL-10, the production from macrophages and DCs of cytokines and mediators is reprogrammed, thus altering the cytokine network and generating a microenvironment that, according to our data, does not impact on D10G4 cell proliferation. It is also possible that high extracellular Mg does not antagonize Ca as much as Ca blockers. Indeed, the scenario is different in the presence of Ca blockers, which jeopardize Ca transients known to be fundamental in the regulation of cell growth.

Overall, our results are in agreement with previous studies in other cell systems showing that high extracellular Mg impairs antigen presentation. Treatment with Mg significantly reduced the capacity of human epidermal Langerhans cells to activate T cells in a mixed epidermal lymphocyte reaction with allogenic, naïve, resting T cells as responders [11]. In addition, Mg suppressed Langerhans cell function when added to epidermal cell suspensions *in vitro* [11]. These results were associated with the reduced expression of HLA-DR, co-stimulatory CD80/86 molecules, by Langerhans cells and with the suppression of the constitutive TNF α production by epidermal cells *in vitro*. Moreover, nifedipine and verapamil significantly suppressed the antigen-presenting capacity of Langerhans cells in a dose-dependent manner, reduced the percentage of class II MHC antigen-positive epidermal cells, and significantly suppressed class II MHC and CD80 levels in Langerhans cells [37]. The present study was designed to assess specifically antigen presentation and the subsequent Th2 cell response. Our previous work has shown alterations of the delayed hypersensitivity response in Mg-deficient and Mg-supplemented mice, suggesting that the Th1 response is modulated by the Mg status [38]. Because of the relevance of the balance between the Th1 and Th2 cytokine response in immunity, more studies are necessary on Th1 cells to obtain an integrated view of the contribution of Ca and Mg to immune function.

CONCLUSION

Our results suggest that changes to Mg and Ca homeostasis play a role in some crucial steps of the specific immune response. The additive effect of high Mg and Ca blockers might be of interest for modulating innate immune responses in various clinical settings.

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REFERENCES

1. Trombetta ES, Mellman I. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu Rev Immunol* 2005; 23: 975-1028.
2. Koski GK, Schwartz GN, Weng DE, *et al.* Calcium mobilization in human myeloid cells results in acquisition of individual dendritic cell-like characteristics through discrete signaling pathways. *J Immunol* 1999; 163: 82-92.
3. Delvig AA, Robinson JH. The role of calcium homeostasis and flux during bacterial antigen processing in murine macrophages. *Eur J Immunol* 1999; 29: 2414-9.

4. Lewis RS, Cahalan MD. Potassium and calcium channels in lymphocytes. *Annu Rev Immunol* 1995; 13: 623-53.
5. Jähnen-Dechent W, MK W. Magnesium basics. *Clin Kidney J* 2012; 5: i3-14.
6. Iseri LT, French JH. Magnesium: nature's physiologic calcium blocker. *Am Heart J* 1984; 108: 188-93.
7. Simchowitz L, Foy MA, Cragoe Jr. EJ. A role for $\text{Na}^+/\text{Ca}^{2+}$ exchange in the generation of superoxide radicals by human neutrophils. *J Biol Chem* 1990; 265: 13449-56.
8. Mak IT, Kramer JH, Chmielinska JJ, Khalid MH, Landgraf KM, Weglicki WB. Inhibition of neutral endopeptidase potentiates neutrophil activation during Mg-deficiency in the rat. *Inflamm Res* 2008; 57: 300-5.
9. Bussière FI, Mazur A, Fauquert JL, Labbe A, Rayssiguier Y, Tridon A. High magnesium concentration in vitro decreases human leukocyte activation. *Magnes Res* 2002; 15: 43-8.
10. Tintinger G, Steel HC, Anderson R. Taming the neutrophil: calcium clearance and influx mechanisms as novel targets for pharmacological control. *Clin Exp Immunol* 2005; 141: 191-200.
11. Schempp CM, Dittmar HC, Hummeler D, et al. Magnesium ions inhibit the antigen-presenting function of human epidermal Langerhans cells in vivo and in vitro. Involvement of ATPase, HLA-DR, B7 molecules, and cytokines. *J Invest Dermatol* 2000; 115: 680-6.
12. Weglicki WB, Dickens BF, Wagner TL, Chmielinska JJ, Phillips TM. Immunoregulation by neuropeptides in magnesium deficiency: ex vivo effect of enhanced substance P production on circulating T lymphocytes from magnesium-deficient mice. *Magnes Res* 1996; 9: 3-11.
13. Li FY, Chaigne-Delalande B, Kanellopoulou C, et al. Second messenger role for Mg^{2+} revealed by human T-cell immunodeficiency. *Nature* 2011; 475: 471-6.
14. Lee KS, Tsien RW. Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nifedipine in single dialysed heart cells. *Nature* 1983; 302: 790-4.
15. Striessnj J. Ca^{2+} channel blockers. In: Offermanns S, Rosenthal W, eds. *Encyclopedic reference of molecular pharmacology*. Berlin, Germany: Springer, 2004, p. 201-7.
16. Suzuki Y, Inoue T, Ra C. L-type Ca^{2+} channels: a new player in the regulation of Ca^{2+} signaling, cell activation and cell survival in immune cells. *Mol Immunol*. 2010; 47: 640-8.
17. Kumar S, Chakrabarti R. [8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate, HCl], the inhibitor of intracellular calcium mobilization, blocked mitogen-induced T cell proliferation by interfering with the sustained phase of protein kinase C activation. *J Cell Biochem* 2000; 76: 539-47.
18. Chiou CY, Malagodi MH. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br J Pharmacol* 1975; 53: 279-85.
19. Sawamura M. Inhibition of protein kinase C activation by 8-(N,N-diethylamino)-octyl-3, 4, 5-trimethoxybenzoate (TMB-8), an intracellular Ca^{2+} antagonist. *Kobe J Med Sci* 1985; 31: 221-32.
20. Lutz MB, Kukutsch N, Ogilvie AL, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; 223: 77-92.
21. Weischenfeldt J, Porse B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc 2008; pdb prot5080.
22. Ayala A, Ertel W, Chaudry IH. Trauma-induced suppression of antigen presentation and expression of major histocompatibility class II antigen complex in leukocytes. *Shock* 1996; 5: 79-90.
23. Kawasaki T, Choudhry MA, Schwacha MG, Bland KI, Chaudry IH. Effect of interleukin-15 on depressed splenic dendritic cell functions following trauma-hemorrhage. *Am J Physiol Cell Physiol* 2009; 296: C124-30.
24. Leenen PJ, de Brujin MF, Voerman JS, Campbell PA, van Ewijk W. Markers of mouse macrophage development detected by monoclonal antibodies. *J Immunol Methods* 1994; 174: 5-19.
25. Sadhu C, Ting HJ, Lipsky B, et al. CD11c/CD18: novel ligands and a role in delayed-type hypersensitivity. *J Leukoc Biol* 2007; 81: 1395-403.
26. McMillen MA, Lewis T, Jaffe BM, Wait RB. Verapamil inhibition of lymphocyte proliferation and function in vitro. *J Surg Res* 1985; 39: 76-80.
27. Matzner N, Zemtsova IM, Nguyen TX, Duszenko M, Shumilina E, Lang F. Ion channels modulating mouse dendritic cell functions. *J Immunol* 2008; 181: 6803-9.
28. Mazur A, Maier JA, Rock E, Gueux E, Nowacki W, Rayssiguier Y. Magnesium and the inflammatory response: potential physiopathological implications. *Arch Biochem Biophys* 2007; 458: 48-56.
29. Malpuech-Brugere C, Nowacki W, Daveau M, et al. Inflammatory response following acute magnesium deficiency in the rat. *Biochim Biophys Acta* 2000; 1501: 91-8.
30. Malpuech-Brugere C, Rock E, Astier C, Nowacki W, Mazur A, Rayssiguier Y. Exacerbated immune stress response during experimental magnesium deficiency results from abnormal cell calcium homeostasis. *Life Sci* 1998; 63: 1815-22.
31. Sakaguchi H, Sakaguchi R, Ishiguro S, Nishio A. Beneficial effects of a calcium antagonist, nifedipine, on death and cardiovascular calcinosis induced by dietary magnesium deficiency in adult mice. *Magnes Res* 1992; 5: 121-5.
32. Lin CY, Tsai PS, Hung YC, Huang CJ. L-type calcium channels are involved in mediating the anti-inflammatory effects of magnesium sulphate. *Br J Anaesth* 2010; 104: 44-51.
33. Sugimoto J, Romani AM, Valentín-Torres AM, et al. Magnesium decreases inflammatory cytokine production: a novel innate immunomodulatory mechanism. *J Immunol* 2012; 188: 6338-46.
34. Gomes B, Cabral MD, Gallard A, et al. Calcium channel blocker prevents T helper type 2 cell-mediated airway inflammation. *Am J Respir Crit Care Med* 2007; 175: 1117-24.
35. Sonna LA, Hirshman CA, Croxton TL. Role of calcium channel blockade in relaxation of tracheal smooth muscle by extracellular Mg^{2+} . *Am J Physiol* 1996; 271: L251-257.
36. Savignac M, Gomes B, Gallard A, et al. Dihydropyridine receptors are selective markers of Th2 cells and can be targeted to prevent Th2-dependent immunopathological disorders. *J Immunol* 2004; 172: 5206-12.
37. Katoh N, Hirano S, Kishimoto S, Yasuno H. Calcium channel blockers suppress the contact hypersensitivity reaction (CHR) by inhibiting antigen transport and presentation by epidermal Langerhans cells in mice. *Clin Exp Immunol* 1997; 108: 302-8.
38. Ryazanova LV, Rondon LJ, Zierler S, et al. TRPM7 is essential for Mg^{2+} homeostasis in mammals. *Nat Commun* 2010; 1: 109.