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Chapter

Molecular Mechanism Induced by Beta-Glucans from Maitake to Recover T Cell-Subpopulations during Immunosuppression

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Abstract

Breast cancer is the most frequent neoplasia in the world and one of the main causes of death among women. Some of the conventional treatments for cancer are chemotherapy or radiotherapy, knowing that both generate very toxic side effects since they usually affect all cells in active division (healthy or tumoral cells). Therefore, specific treatments are necessary, with therapies aimed at the molecular characteristics of the tumor and its microenvironment. An approach to this could be the search for natural compounds with immunopotentiating capacity and selective toxicity on tumor cells. Recently, immunomodulatory and antitumor activities have been discovered in various fungi. Among them, β-glucans of D-Fraction of *Grifola frondosa* (Maitake) can generate innate and adaptive immune responses, exerting antitumor effects. The reported therapeutic benefits of treatment of breast tumorigenesis with D-Fraction of Maitake require to deepen pharmacological and toxicological studies, in order to guarantee innocuousness and efficacy in the administration to a patient. Therefore, this chapter aimed to elucidate toxicological, pharmacodynamic, and pharmacokinetic aspects of β-glucans from D-Fraction. In this way, we hope to make a significant contribution to the pharmacological knowledge of these bioactive compounds by promoting an immunotherapeutic and antitumor strategy novel agent.

Keywords: beta-glucans, Maitake, D-fraction, toxicological, pharmacodynamic, pharmacokinetic studies, immunosuppression, T-cell lymphocyte

1. Introduction

Grifola frondosa, better known as Maitake, is an edible and medicinal fungus widely known and respected in Eastern countries for thousands of years [1]. It belongs to the *Basidiomycota* division, *Meripilaceae* family, and *Polyporales* order; it grows as a parasite on the bases of deciduous and coniferous trees in forests from Asia, Europe, and eastern North America. It has large annual basidiocarps that can measure up to 1 m in diameter and weigh 20 kg. These are branched and formed by numerous small fan-shaped hats, found themselves imbricated. At the nutritional

level, fresh specimens of *Grifola frondosa* have 80% aqueous content, while 22–27% dry weight corresponds to proteins, 50–60% of carbohydrates, and 4% of fats. It has vitamins C, D, and B (B1, B2, B6), niacin, and folic acid. Among its minerals, K, P, and Mg predominate, followed by Zn, Na, Fe, and Ca. As free sugars it has glucose, trehalose, and mannitol. In the formation of basidiocarps, mostly malic acid was identified, but also lactic acid, acetic, formic, citrus, succinic, oxalic, and pyroglutamic acids. The presence of a lectin (N-acetylgalactosamine) capable of agglutinating erythrocytes was also determined. In the early 1980s, Japanese mycologist Hiroaki Nanba identified a fraction present in its mycelium, but also in the fruiting body of *Grifola frondosa* that exhibited greater antitumor activity and significant immunomodulatory effect [2, 3] when administered orally and intravenously, which he called D-Fraction [4, 5]. Since it could be an effective immunomodulator orally, this characteristic made it extremely easy to administer compared, for example, with Shiitake (lentinan) extracts, whose effect was only optimal in intravenous administration [6]. During the 1990s, Nanba and Kubo achieved greater purification of the D-Fraction extract, achieving the MD-Fraction, with superior biological activity [5], highlighting the important antitumor effect of *Grifola frondosa* [7–10]. D-Fraction is insoluble in acid, soluble in alkali, and removable with hot water. Starting with *Grifola frondosa*, a polysaccharide formed by a main chain of branching −1-6-D-Glucoso was placed for every three residues of D-glucose. Glucans are polysaccharides made up exclusively of D-glucose units bound through glycoside bonds, with the glycine being the ones with the greatest therapeutic potential [11, 12]. The purified extract of D-Fraction has main-chain glycogen with branches of −1,3 and the main-chain glucans of the main chain −1,3 with branches −1,6; it also has high molecular weight proteins close to 1,000,000 Da [4, 5].

Numerous fungal compounds—such as D-glucans, proteoglycans, proteins—have been studied in recent years due to their ability to stimulate or inhibit specific components of the immune system [13]. The immunomodulatory effects of D-glucans on the immune system of mammals involves innate and adaptive immune responses, being able to attribute these effects to be a polysaccharide that the body cannot synthesize; the immune system recognizes it as triggering the innate and adaptive immune responses [14] that contribute to the blocking of tumor progression.

Natural killer cells, which are part of innate immunity or first line of defense at birth, are the main weapon of immunosurveillance against tumor development [1], releasing IFN-γ, TNF-α, and GM-CSF in fulfilling its role of smoothing tumor cells. Innate immunity is also composed of monocyte (macrophages) and neutrophils, which, when recognizing foreign agents and put into action various enzymes such as nitric oxide synthase (iNOS), which results in bactericidal intermediaries [13]. Macrophages secrete cytokines (IL-1, IL-6, IL-10, IL-12, TNF-α) capable of modulating innate and adaptive responses, activating, in turn, other macrophages, neutrophils, NK, and T lymphocytes. Tumor activity in NK is enhanced by IL-2, IL-12, and interferons [15]. T cells and NK lymphocytes produce IFN-γ that reinforces the activation of macrophages. The innate response induced by D-Fraction β-glucans involve activation of the protein receptor Dectin-1, which after recognizing these polysaccharides triggers events of phagocytosis and release of cytokines [16, 17]. The extract D-Fraction of Maitake activates the macrophages to secrete IL- 1 [4, 18], IL-12 [8, 17], and TNF-α [19] and stimulates the expression of nitric oxide synthase [20]. In addition to stimulating macrophages, IL-1 promotes the cytotoxicity of splenic T cells, increasing their bactericidal activity [18]. Maitake can stimulate NK cells both in vitro and in vivo, through the increase of IFN-γ and TNF-α in mice [8, 21]. In addition, in macrophages IL-12 secretion associated with NK

activation is stimulated by the increased binding to perforins in target cells, increasing tumor lysis [8, 21]. It has been suggested that NK cells are self-activated by the IFN-γ released by themselves [8]. Increased activation of NK was confirmed by increased CD69 expression—NK activation marker—present on the surface of these cells [22]. Subsequent clinical studies in cancer patients revealed that treatment with D-Fraction stimulated and maintained normal activity of NK in peripheral blood [7]. In acquired immunity, which presents as main characteristics specificity and memory [23], D-Fraction of Maitake decreases the expression of the CD69 activation marker of B lymphocytes and stimulates Th lymphocytes, specifically to the Th1 lymphocytes of lymph nodes [8, 19], presenting effects on the balance of Th1 lymphocytes/Th2 lymphocytes [19]. Carcinogenesis has been reported to imply significant imbalance Th1L/Th2L, with a progressive decrease in Th1L (main TLc inducers) and increased Th2L [24]. D-Fraction of Maitake can reverse that imbalance by polarizing the ThL response to Th1L by stimulating the secretion of IL-12 and IL-18 crucial for Th1L activation and blocking Il-1 release important for Th2L activation [19].

2. Immune-restorative capacity of Maitake Pro4X's beta-glucans in immunosuppressed BALB/c females

2.1 Quantification of 1,3-beta-glucans in Maitake Pro4X

The total content of β-1,3 glucans of the purified extract of *Grifola frondosa* (Maitake PRO4X) was quantified through a colorimetric method called Megazyme, a β-glucan commercial kit (Megazyme International Ireland, Bray, Co. Wicklow, Ireland). The samples were performed by triplicate. According to the manufacturer's instructions, 0.1 μl of Exo-1,3-glucanase (20 U/ml) was added to 0.1 ml of sample in sodium acetate buffer at 200 mM concentration and pH 5. The contents were mixed by vortex and incubated at 40°C for 60 min. Subsequently, 3 ml of Buffer GOPOD (glucose oxidase/peroxidase) was added and incubated for 20 min at 40°C. The absorbance value of all samples at 510 nm was read on a Cary 50 Agilent brand UV–Vis spectrophotometer. Finally, the following equation was applied to calculate the concentrations of 1,3-β-glucans:

$$\beta - \text{Glucan } (\%w/w) = \Delta E \times F/W \times 90$$

where.
ΔE = Absorbance value of the sample at 510 nm.
F = Conversion factor = 100.
W = Sample weight (mg).

Finally, this equation was applied to refer the values with the beta-glucan standard employed in the assay.

$$\frac{\text{Abs Standard (510nm)}}{\text{St. β-glucan}} = \frac{\text{Abs Maitake Pro4X (510nm)}}{\text{Unknown (X)}}$$

St. β-glucan = Standard β-glucan concentration
Unknown (X) = Maitake Pro4x β-glucan concentration

Then, applying the formula, using the values from **Table 1**, the concentration of 1,3-beta-glucans from Maitake Pro4X was obtained.

$$\frac{0.7937}{1.0\ \text{mg/ml}} = \frac{0.7222}{X} \Longrightarrow \boxed{X = 0.91\ \text{mg/ml 1,3 β glucan in Maitake Pro4X}}$$

2.2 Polarity and solubility of β-glucans from Maitake Pro4X

2.2.1 Polarity of Maitake extract

Evaluation of Maitake Pro4X's polarity by dissolving commercial samples into solvents of increasing polarity. It was checked whether they were soluble, highly soluble, or insoluble. The results are given in **Table 2A**. The results of **Table 2A** on

Sample	Weight (mg)	Absorbance 1 (510nm)	Absorbance 2 (510nm)	Absorbance 3 (510nm)	Average of Absorbance ± S.D	β-glucan (w/v)
Maitake Pro4X	1.00	0.7849	0.7119	0.6698	0.7222 ± 0.058	0.91 mg/ml

Total concentration of 1,3-b-glucans correspond to Maitake Pro4X commercial sample. The test was performed according to the colorimetric method of the Megazyme b-Glucan kit measuring the absorbances of the samples at 510 nm. The Absorbances values indicated in the table are the corrected values referred to the blank of reagents.

Table 1.
Colorimetric quantification of beta-glucan content in Maitake Pro4X.

A

Increase in Solvent Polarity ↓

Solvents	Maitake Pro4X
N-hexane	Insoluble
Ethyl-acetate	Insoluble
Acetone	Insoluble
Ethanol	Soluble
Methanol	Highly Soluble
Distilled water	Highly Soluble

↓ Increase in Solubility

B

Solvents	Acid/Basic	Maitake Pro4X
Distilled Water	Neutral	Highly Soluble
Hydrochloric Acid	Acid	Highly Soluble
Bicarbonate 7.5%	Basic	Soluble
Oil	---	Insoluble

(A) The solubility of the commercial versions Maitake Pro4X was verified in solvents with increasing polarity. (B) Maitake Pro4X Solubility in acidic or alkaline solvents. The results obtained were recorded as soluble, highly soluble or insoluble.

Table 2.
Polarity/solubility of Maitake.

polarity/solubility of purified Maitake extracts in solvents of increasing polarity showed that both extracts were highly soluble in polar solvents and insoluble in nonpolar solvents. This suggests hydrophilic and polar nature of that compound. Acetone solubility, an aprotic polar solvent (without O—H, N—H links), was differential for both extracts. While Maitake Pro4X proved insoluble, the standard version was soluble. Next, to evaluation the solubility of β-glucans, a 1:4 aqueous dilution of Maitake Pro4X was performed; an aliquot of this dilution was taken in volume similar to the tested for Maitake Standard and dissolved in acetone, being soluble. Dissolution in apolar solvents (n-hexane and ethyl acetate) resulted in the formation of an insoluble upper phase of Maitake (heterogeneous system). In the case of ethanol, both extracts generated a homogeneous system.

2.2.2 Maitake PRO4X solubility

The solubility results of Maitake Pro4X in water-soluble or fat-soluble substances, of an acidic or alkaline nature, are summarized in **Table 2B**. The results of **Table 2A** regarding the solubility of Maitake Pro4X in solvents of acid or alkaline character demonstrated high solubility in acidic pH (hydrochloric acid) and neutral (distilled water) substances. Solubility was observed in bicarbonate 7.5% (m/v) (alkaline pH), with slight ionization of the extract in the form of small whitish precipitates. Maitake Pro4X was insoluble in fat-soluble substances (oil).

2.2.3 pH measurement of Maitake Pro4X

The acidic or alkaline nature of the purified Maitake D-Fraction Pro4X extract from *G. frondosa* mushroom was determined by pH measurement at two different temperatures (20 and 37°C) by the potentiometric method through a pH meter Accumet (Fisher Scientific, USA), using three calibration points and accuracy of pH 0.01. The results of pH indicated an acidic pH for Maitake Pro4X, 5.8 and 5.77, at 20°C and 37°C, respectively. Considering the oral route of administration of beta-glucan compound in mice, we have observed that the pH of Maitake Pro4X is closely related to gastrointestinal organs, the duodenum and colon.

2.3 Role of 1,3-β-glucans from Maitake Pro4X in the mechanism of cell death (apoptosis) mediated by Dectin-1 receptor activation in tumoral MCF-7 cells

2.3.1 Effect of Maitake on human tumor cells with Dectin-1 blocking

The effect of Maitake Pro4X on the feasibility and death of MCF-7 tumor cells was investigated by mediating blocking of Dectina-1 transmembrane receptors with Laminarin. Laminarin, 1,3-β-glucan extracted from *Laminaria digitata*, can specifically bind to the Dectin-1 receptor. It is considered as a Dectin-1 specific blocker. The study indicated that Laminarin connected with ovalbumin OVA could be especially recognized by the Dectin-1 receptor expressed on dendritic cells and macrophage [25]. **Figure 1** illustrates the mechanism of Laminarin blocking the Dectin-1 receptor through activation of certain proteins that inhibit the tumoral cell death induced by Maitake Pro4X alone. This figure's ideas were taken from the publication of Fesel and Zuccaro [26].

In order to study the role of Dectin-1 receptor on cell death induced by Maitake Pro4X, in vitro cultures of MCF-7 cells were treated for 24 h with β-glucans from Maitake Pro4X (367 μg/ml) in the presence and absence of Laminarin (200 μg/ml). The count of living and dead cells was performed in Neubauer's chamber, using

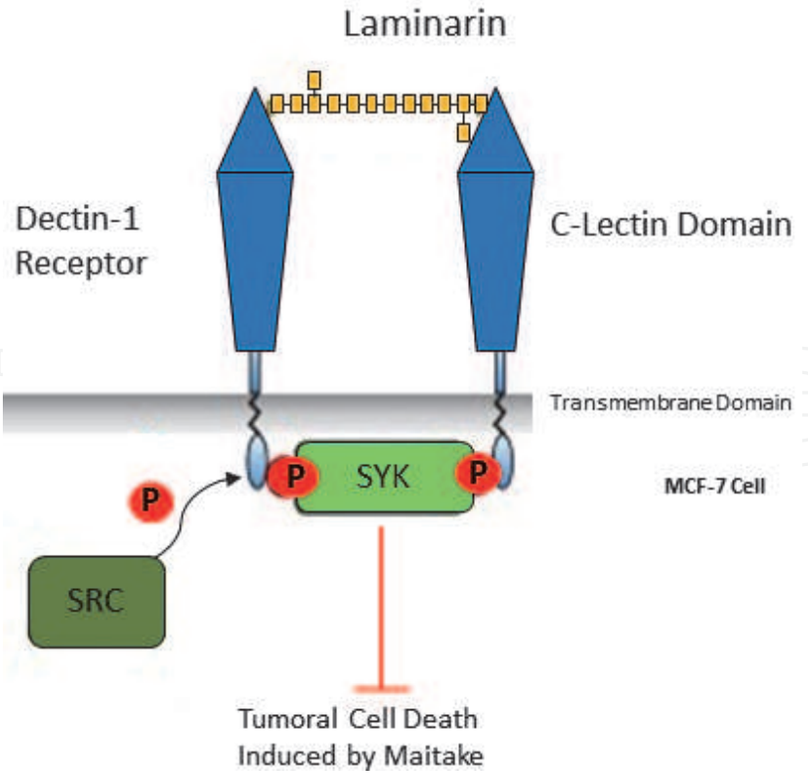

Figure 1.
β-glucan receptor and laminarin. *Upon laminarin binding it is proposed that two Dectin-1 proteins form a receptor co-motif) of both receptor molecules which is subsequently phosphorylated by SRC. Both phosphorylases (SYK, spleen tyrosine kinase) trigger downstream events to block the tumoral cell death induced by Maitake Pro4X. The idea of this graphic was taken from Fesel and Zuccaro [26].*

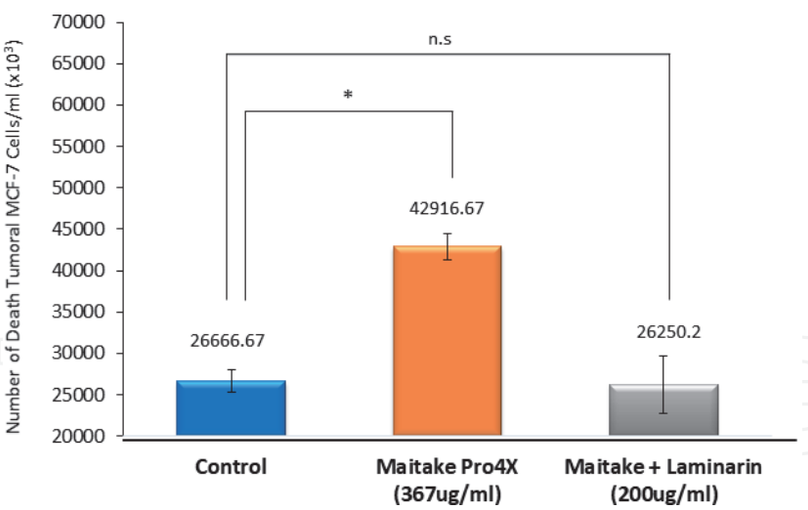

Figure 2.
Effect of tumoral MCF-7 cell death induced by Maitake and dependence of Dectin-1 receptor. *In vitro culture of MCF-7 human tumoral cells was employed to measure the effect of blocking the Dectin-1 receptor (Laminarin 10ug/ml) in the cell death mechanism induced by Maitake Pro4X (5 mg/ml). The experiment was performed by triplicate. The results correspond to mean + 2 standard deviations. In the graphic * means p < 0.05 and n.s means not statistically significant (p > 0.05).*

Trypan blue exclusion technique. The control group was used as untreated. All the experiments were performed by triplicate. The results listed in **Figure 2** about the death of MCF-7 tumor cells demonstrated that treatment with Maitake Pro4X significantly increased (*$p < 0.05$) the death of tumor MCF-7 cells (from 26,666.67 $\times 10^3$ (2.66×10^7) in the control untreated to 42,916.67 $\times 10^3$ (4.3×10^7)). A significant decrease in cell death (*$p < 0.05$) (26,3750 $\times 10^3$ (2.67×10^7)) was found when Dectina-1 receptors were blocked with Laminarin (**Figure 2**).

2.4 Biodistribution of 1,3-beta-glucans from Maitake Pro4X

2.4.1 Biodistribution in oral administration

The uptake of the 1,3-β-glucans from Maitake Pro4X in the various organs was investigated, as well as their accumulation and passage through the blood–brain barrier, after oral intake in BALBc mouse. To achieve this goal, two animals of both sexes were used for each time (n-16), which were administered orally with a single therapeutic dose of 4 **μ**l of Maitake/mouse dissolved in 8 **μ**l of distilled water, corresponding to the therapeutic dose of 5 mg of β-glucans/kg. Subsequently, the mice were treated for 30 min, 1, 2, 4, 7, 16, 24, and 30 h with oral administration with Maitake Pro4X. After that, animals from all groups were induced to death by suffocation with CO_2 in euthanasia chamber and subsequent cervical dislocation. During the autopsy, the hepatic, renal, gastrointestinal (duodenum and colon) organs, lung, and brain removed from each mouse were arranged on a metallic mesh or cell strainer moistened with cold PBS. By means of gentle circular movements with the plunger of the syringe, the organ was disaggregated in the strainer, collecting the homogenization in a falcon tube. In the case of gastrointestinal tissue, its contents were previously removed prior to processing in order to avoid cellular agglomerations. With Pasteur pipette, three washes of 1 ml each of cold PBS were performed to facilitate disintegration; the homogenate remained on ice. With 10 ml syringe and 27 G needle, several passages of aspiration expulsion of tissue filtration were performed, in order to generate complete homogenization and rupture of any cell clusters. Subsequently, it was centrifuged at 1000 rpm for 7 min at 4°C, and the supernatant was discarded. The pellet was resuspended in 4 ml of PBS + SFB 1% and preserved in the freezer at −80°C for subsequent quantification of the glycoprotein extract glucans.

Plasma quantification of 1,3-β-glucans was performed after oral administration of Maitake Pro4X in BALB/c mice. To do time-course curve, two animals were used for each time (condition), making the slaughter of them at 0.5, 1, 2, 4, 7, 10, 16, 24, and 34 h. The determination of 1,3-glucans was performed by the colorimetric test called Glucatell kit (carried out at the Cape Cod Inc. Laboratory, Maryland, USA), which allowed to obtain curves of plasma concentration (pg/ml) vs. post-administration time (h) (**Figure 3**).

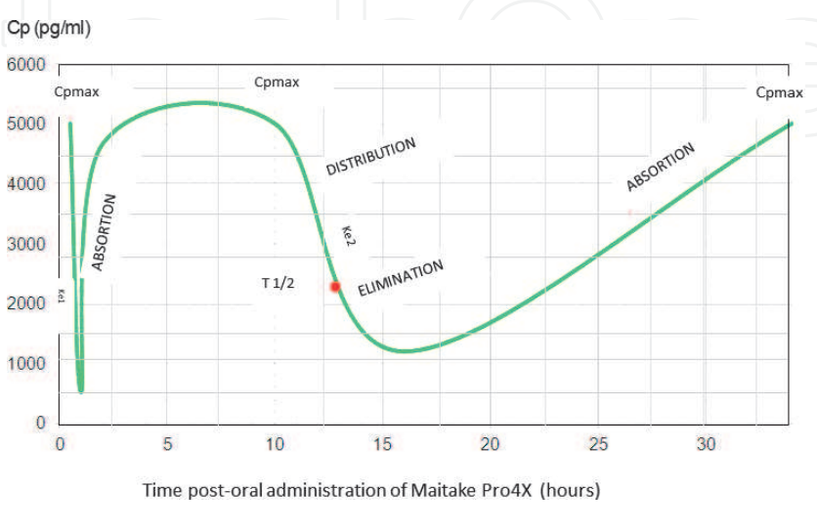

Figure 3.
Plasmatic concentration of 1,3 β -glucans vs time of postoral administration. *The blood was collected during the slaughter of BALB/c mice at pre-established times. Plasma determinations of 1,3-b-glucans were made using the colorimetric method called Glucatell kit. Three peaks of Cpmax were observed following oral administration of Maitake Pro4X (5 mg/kg).*

The pharmacokinetic parameters that were determined are: area under the curve (AUC) Cp vs. time), T 1/2 (half-life time), Cp max (maximum plasma concentration), ke (elimination constant), ka (absorption constant), Ta1/2 (absorption half-life time), Vd (apparent volume of distribution), and Clt (total systemic clearance).

1. **Maximum plasma concentration (Cpmax) and maximum time (Tmax).** The results recorded in **Figure 3** suggest that plasma levels of 1,3-glucans increased rapidly after oral administration, presenting three peaks of maximum plasma concentration:

    a. First peak Cpmax >5000 pg./ml at half an hour after oral administration of Maitake

    b. Second peak Cpmax >5000 pg./ml between 2 and 10 h of oral administration of Maitake

    c. Third peak Cpmax >5000 pg./ml at 34 h of oral administration of Maitake

After the first peak Cpmax, plasma levels of 1,3-glucans fell sharply, resulting in a new Cpmax peak between 2 and 10 h. From 10 h., there was a gradual decrease in the concentration of 1.3-β-glucans. However, after 16 h, a progressive growth of the concentration of 1,3-β-glucans began again, reaching a third peak Cpmax at 34 h after oral administration.

2. **Elimination speed constant (Ke).** The results recorded in **Figure 3** suggest that the removal of 1,3-β-glucans correspond to a first-order kinetic (mono-compartmental) model. The first Ke1 constant has a 4489/h removal speed and Ke2 has a speed of 0.236/h. This indicates a high elimination speed for the range 0.5 to 1 h and more gradual elimination between 10 and 16 h. The first elimination (Ke1) is 19 times faster than the second (Ke1 × 19 Ke2).

3. **Absorption speed constant (ka).** The ka absorption constant was determined by the residual method, assuming a kinetic model of first order with Ka1 > Ke1. First, to calculate ka1, it was drawn the Elimination line Log Cp vs. 0.86 to 0.94 h. Extrapolation was performed to the axis of the ordered, resulting in a line and −22,011x + 4.8472. The selected absorption Cps corresponded to the times 0.6 h (3850 pg./ml), 0.7 h (2300 pg./ml), and 0.8 h (1350 pg./ml). For each time, the Log Cp value was determined on the semilogarithmic elimination line and −22,011x + 4.8472, resulting in 3.53 (0.6 h), 3.31 (0.7 h), and 3.09 (0.8 h). Applying antilogarithm, the elimination Cp was obtained resulting in 3388.44 pg./ml (0.6 h), 2041.74 pg./ml (0.7 h), and 1230.27 (0.8 h). The absorption constant ka1 was calculated from the slope of the absorption line and −29,301x + 44,359, resulting in ka1 × 6.75/h. It was verified that ka1 > ke1. The second absorption constant ka2 was determined by the residual method, assuming a kinetic model of first order with Ka2 > Ke2. The elimination line Log Cp vs. time was plotted in the interval 13 to 15 h, with its corresponding extrapolation to the axis of ordered, resulting in the line and − 0.1144x + 4.83. The absorption Cp stakes chosen in the oral administration curve correspond to the times 1.2 h (1650 pg./ml), 1.4 h (2850 pg./ml), and 1.6 h (3750 pg./ml).

4. **Absorption half-life time (Ta1/2).** From both ka, the absorption half-life time (ta1/2) was established. Using the expression t1/2–0.693/ka, the ta1 1/2 ×

0.1 h was obtained for ka1, while for ka2, the ta2 1/2 × 1.9 h. These results suggest that the plasma concentration of 1,3-glucans that remains to be absorbed was halved to 0.1 and 1.9 am of oral administration.

5. **Elimination half-life time (T1/2).** From both Ke, the elimination half-life time (t1/2) was set, using the expression t1/2–0.693/Ke. For the range 0.5–1 h, t1 × 2 × 0.15 h, while between 10 h to 16 h, t1 × 2.93 h. The plasma concentration of 1,3-glucans was halved at 0.65 and 12.93 h of oral administration.

6. **Distribution volume (Vd).** The apparent volume of distribution was calculated by the expression "Dose/Cp0." The dose administered to the animals was 120 mg of β-glucans (4 ml Maitake PRO4X). Cp0 was determined by extrapolation with the axis of the ordered (ln Cp) in the range 0.5–1 h. So, for a 20 g mouse, the Vd is 2.55 l/0.02 kg, i.e., Vd is 127 ml/g (127 l/kg), this is a great value for Vd on the mouse.

7. **Clearance total (Clt).** Total systemic clearance (Clt) was determined by the expression Clt -Vd (-Ke). For Ke1, Clt-2,55 l × 4489 h-1-11.45 l/h or 190.78 ml/min), while for Ke2, its Clt is 10.03 ml/min.

8. **Area under the curve.** The area under the curve Cp vs. time reflects the amount of bioavailable compound that reaches the systemic circulation and is capable to produce an effect, as determined by the trapezoidal method, resulting in 0.118 mg.h/ml for the time interval 0–34 h.

The results shown in **Figure 4** about tissue uptake of β-glucans following oral administration suggest that the highest concentrations occurred in the stomach (5.52×10^7 pg.h/ml), duodenum (3.66×10^7 pg.h/ml), and colon (3.44×10^7 pg.h/ml). In addition, we have recorded an important level of uptake in the brain (AUC of 3.77×10^4 pg.h/ml) and lung and to a lesser extent at the liver (1.89×10^4 pg.h/ml) and renal (2.44×10^4 pg.h/ml) level. The area under the tissue biodistribution curve of 1,3-β-glucans for the different organs is indicated in **Figure 5**. The results recorded in **Figure 5A** represents the area under the stomach, duodenum, and colon uptake curve of β-glucans. The highest hepatic uptake (**Figure 5B**) occurs at 7 h (*$p < 0.05$ vs. 2 h) and the lower at 30 h (with **$p < 0.01$ vs. 2 h). On the other hand,

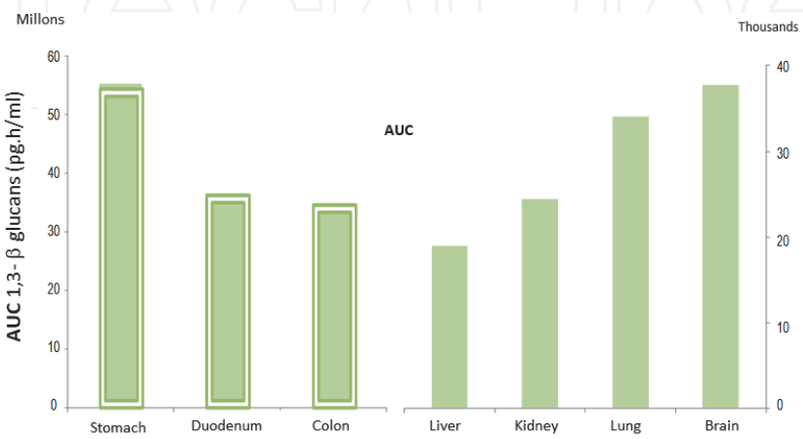

Figure 4.
Mouse tissue biodistribution of 1,3-β-glucans. The catches in the various murine organs were compared between 2 and 30 a.m. after 1,3-b-gucans Maitake Pro4X (5 mg/kg) oral administration. The determinations were made using the colorimetric method of the Glucatell kit. Biodistribution in gastrointestinal organs (stomach, duodenum, colon) was in the order of millions of pg.h/ml, while in others it was thousands of pg.h/ml.

liver uptake at 2 h is significantly higher than that which happens at an equal time in the brain (with *p < 0.05). Also, brain uptake at 30 a.m. is significantly less than that occurred at the same lung time (with **p < 0.01) (**Figure 5B**).

2.5 Molecular mechanism of dexamethasone in immunosuppression

In order to induce immunosuppression in the experimental murine model, dexamethasone was employed, a synthetic glucocorticoid class of steroid hormones with potent anti-inflammatory and immunosuppressant activities [27]. Dexamethasone-mediated T-cell suppression diminishes naïve T-cell proliferation and differentiation by attenuating the CD28 co-stimulatory pathway [28]. However, the exact molecular mechanism induced by dexamethasone in the immunosuppression is not yet known. **Figure 6** illustrated the possible molecular mechanism of glucocorticoids (GC) such as dexamethasone on immune cells; GCs

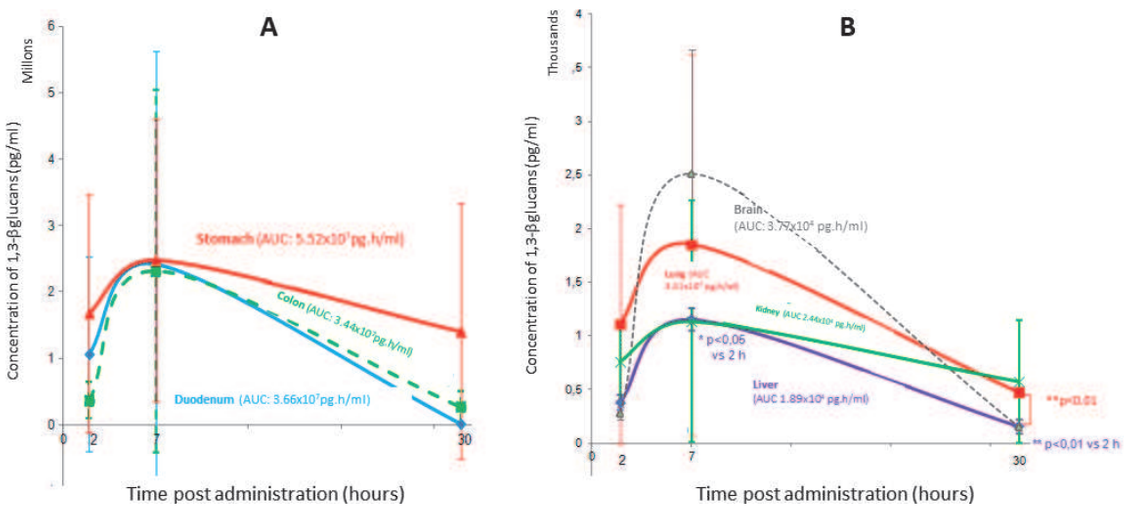

Figure 5
Tissue biodistribution curves of β -glucans. *These correspond to the area under the uptake curve of 1,3-glucans of different murine organs after oral administration of Maitake Pro4X. Mean values +2 standard deviations of: (A) gastric, colonic, and duodenal uptake in millions of pg./ml. (B) Brain, pulmonary, hepatic, and renal uptake in thousands of pg./ml. Significant differences were observed (*p < 0.05; **p < 0.01) between tissue captures and their times.*

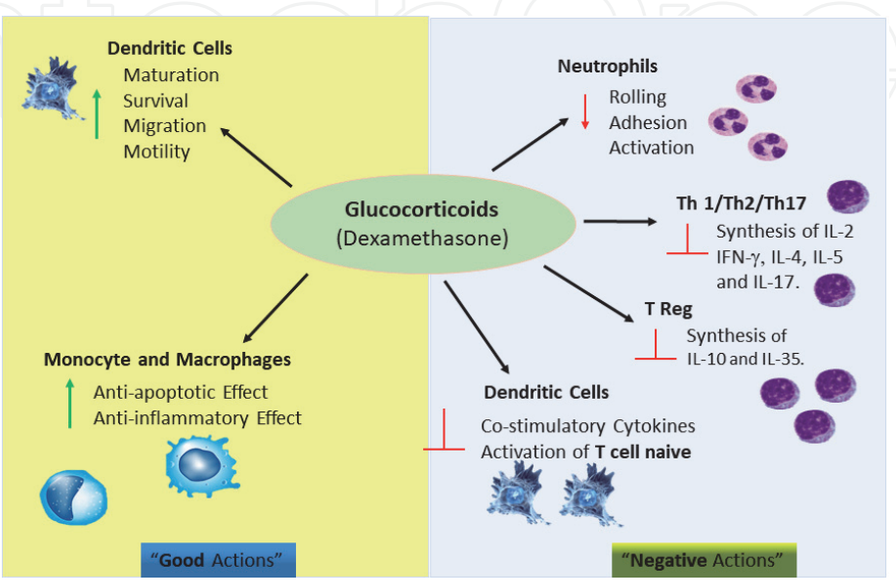

Figure 6.
Glucocorticoid (GC such as dexamethasone) activity on periphery immune cells. GCs act upon almost every immune cell type. This figure was modified from Liberman et al. [29].

promote an anti-inflammatory state on both monocytes and macrophages. GCs prevent monocytes from entering apoptosis and inhibit the liberation of pro-inflammatory mediators by both types of cells. Particularly in macrophages, GCs promote phagocytosis and motility, while they inhibit adhesion, apoptosis, and oxidative burst. They also act upon neutrophil function by inhibiting rolling, adhesion, and activation. GCs act toward dendritic cells by promoting their maturation, survival, migration, and motility, and at the same time GCs inhibit their ability to activate T cells by suppressing the production of pro-inflammatory molecules. A naïve helper T (Th) cell can differentiate into different Th lineages, and GCs exert different actions. They act upon Th1 by decreasing T-bet transcriptional activity and suppressing the production of pro-inflammatory molecules such as IL-2 and IFN-γ. They also suppress GATA3 activity in Th2 cells inhibiting the expression of IL-4 and IL-5. The action of GCs toward Th17 and regulatory T cells is not yet well understood [29] (**Figure 6**).

In order to induce BALBc mouse immunosuppression, 3 g dexamethasone/mouse was administered daily from the beginning to the end of the experimental trial, which lasted approximately for 4 weeks. A suspension of glucocorticoid 0.02% m/v was prepared, for which five tablets of a trademark 0.5 mg were bitten until completely sprayed and suspended in 12.5 ml of 10% aqueous glucose solution. The suspension was prepared weekly and kept in a refrigerator until use. From the suspension of glucocorticoid, daily dainty was administered 15 μl/mouse equivalent to 3 g/mouse/day (0.15 mg de dexamethasone/kg/day).

2.6 Effect of 1,3-β-glucans in the hematology and white blood cell formula

2.6.1 Blood microscopic analysis

Microscopic peripheral blood spread observations were performed for each of the experimental conditions. The blood was collected during the autopsy of the animals and colored with May-Grunwald-Giemsa. Mouse peripheral blood collected during the autopsy of the animals was transferred to Eppendorf tubes containing 0.5% EDTA, at room temperature. A blood smear was performed, placing a drop of blood in the center of a clean slide. A thin film of blood was obtained by means of an object cover (smear). After obtaining the blood smear, it was immediately allowed to air-dry at room temperature, to proceed to its staining. A few drops of methanol were added to fix the preparation, and after evaporation of the preparation, the entire spread was covered with a solution of the May-Grunwald dye for 2–3 min. Subsequently, and unwashed, the Giemsa dye was added for 20 min. Finally, the preparation was washed with distilled water by removing the dyes by trawling and dried at room temperature. **Figure 7** shows the following conditions:

2.6.2 Healthy control group

Normal red blood cells, clustered platelets, lymphocytes, neutrophils, and monocytes were observed (**Figure 7 Superior**). No immature cells were recorded.

2.6.3 Immunosuppressed group

This group presented in his blood abundant large cells, with prominent nucleus, and small and vacuolated cytoplasm (compatible with megakaryocytes); other mice featured megakaryocytes grouped in masse, mastocyte, and PMN basophil and also exhibited monocytes with well-condensed chromatin and megakaryocytes in apparent cell division process. Unevenly sized red blood cells (anisocytosis) were observed (**Figure 7 middle**).

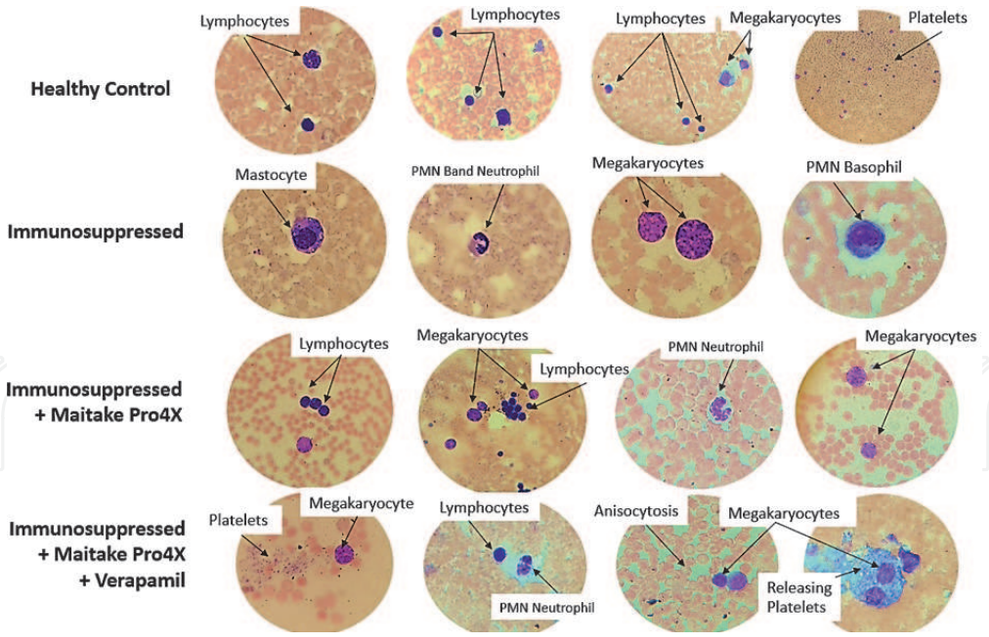

Figure 7.
Blood smear of leukocyte formula. The figure shows the microphotographs from the slides (may-Grunwald-Giemsa x40) indicated in each condition: Healthy control, immunosuppressed in the absence of Maitake Pro4X, immunosuppressed with Maitake Pro4X, and immunosuppressed in the presence of Maitake Pro4X and verapamil.

2.6.4 Immunosuppressed group treated with Maitake

It exhibited mature blood cells such as lymphocytes and polymorphonuclear granulocytes (neutrophils and basophils). In addition, some multinucleated giant cells compatible with megakaryocytes were observed (**Figure 7 middle**).

2.6.5 Immunosuppressed group treated with Maitake + verapamil

Spiculate red blood cells with hypochromia and/or loss of hemoglobin and signs of anisocytosis and hemolysis were observed. In addition, megakaryocytes were released releasing platelets and mastocyte cells in degranulation (**Figure 7 bottom**).

2.7 Flow cytometry studies: expression of CD19, CD3ε, Ly6G, and CD105 molecular markers in immune cells

Flow cytometry studies were performed to analyze the immune-restorative ability of Maitake PRO4X's glucans and recover the immune cell populations with an antigenic expression of CD3ε, CD19, CD105, or Ly6G. Such studies were conducted on murine lymphoid tissues (spleen and lymph nodes) removed during the autopsy of the animals. Maitake's ability to recover immune cell populations in a murine model of immunosuppression was investigated. To do this, 29 healthy BALB/c female mice, 6–8 weeks of age with a weight between 15 and 21 g, were randomly divided into five groups:

1. Condition 1: Healthy control (n = 8)

2. Condition 2: Immunosuppressed (induced by daily administration of *dexamethasone* 0.15 mg/kg) (n = 8)

3. Condition 3: Immunosuppressed with *Maitake PRO4X* (6 mg of β-glucan/kg/Day) (n = 8)

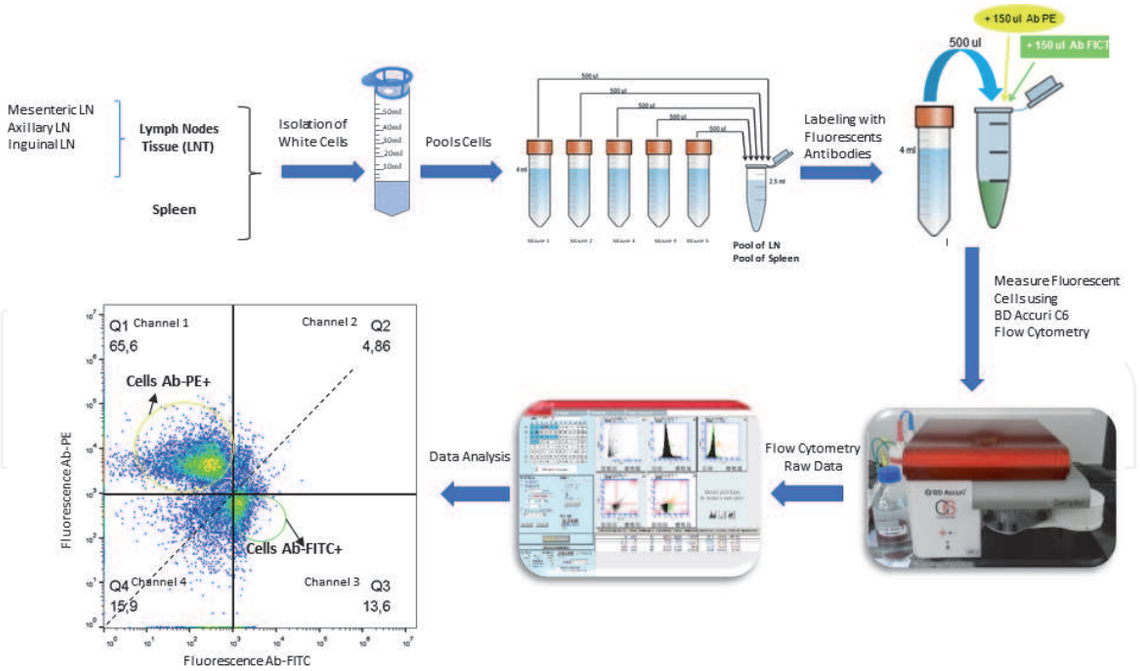

Figure 8.
Lymphoid cell collection. *Mice lymphocytes were obtained from the processing of peripheral lymphoid organs (spleen and murine lymph nodes (mesenteric, axillary, and inguinal LN). The organs were subjected to mechanical disintegration to obtain the cell suspension. After that, a pool of immune cells was performed from each condition studied, cells were stained with specific monoclonal fluorescent anti-mouse antibodies, flow cytometry was performed by triplicate in a BD Accuri C6, and data were analyzed and interpreted as is illustrated in the figure.*

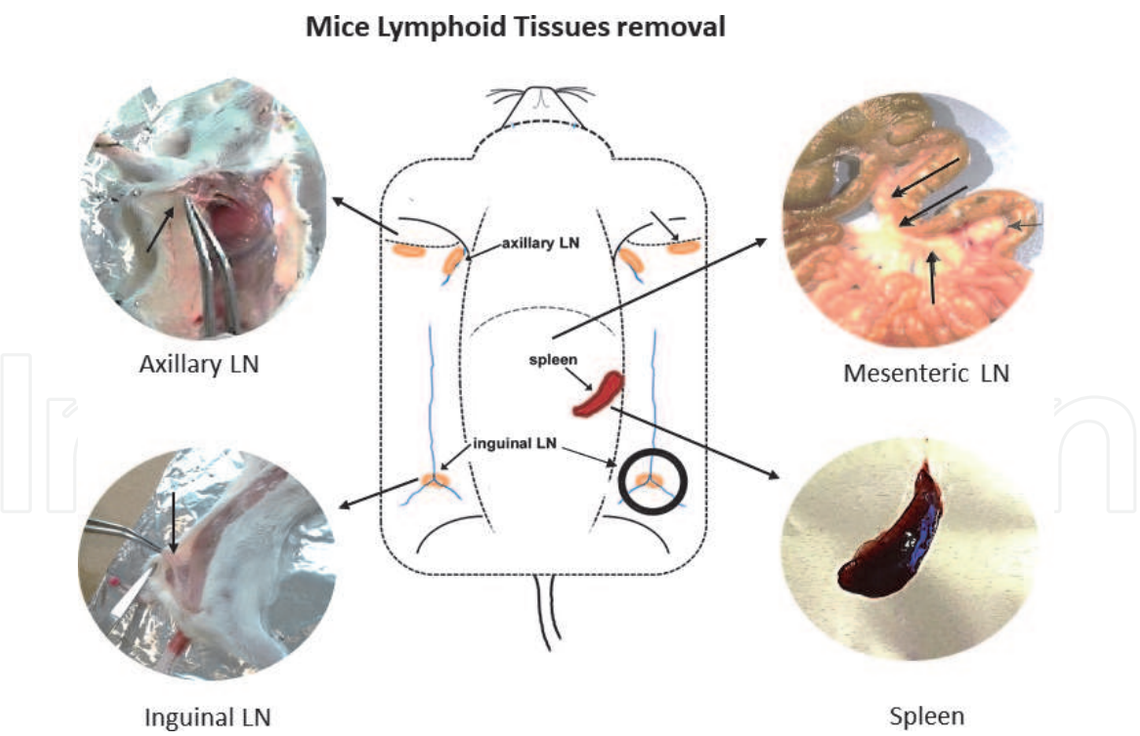

Figure 9.
Removal of peripheral lymphoid organs. *The spleen and lymph nodes (axillary, inguinal, and mesenteric LN) from the BALB/c mice were removed during the autopsy of the animals and subjected to mechanical disintegration to obtain immune cells that were subsequently labeled with the specific antibody to perform flow cytometry.*

4. Condition 4: Immunosuppressed with *Maitake PRO4X* in the presence of a calcium blockage *verapamil* (10 mg/kg/Day) (n = 5)

After 30 days of treatment, all animals were sacrificed using CO_2 asphyxiation camera, and the autopsy was performed in all of them. To perform the flow

cytometry studies, immune cells were obtained from peripheral lymphoid organs (spleen and lymph nodes), which were removed during the autopsy of the mice. These organs were placed in a strainer, located in the opening of a falcon tube, to facilitate mechanical disintegration (**Figure 8**). With the plunger of a syringe, gentle circular movements were made resulting in tissue disintegration. The homogenate was collected in the falcon tube. The processing of lymph nodes involved the removal of the inguinal, mesenteric, and axillary nodules (**Figure 9**). Inguinal nodes were observed as small white spots or hardness in the junctions of the blood vessels, while mesenteric nodules were visualized from the unrolling of the small intestine, being observed as a chain of small whitish granules located above the small intestine. From the axillary region, whitish points corresponding to the axillary nodes were removed (**Figure 9**). All three types of nodes were placed in the same strainer after removal, which was previously moistened with cold PBS. The procedure indicated in **Figure 9** allowed to obtain a ganglion homogenate pool, washed three times with 1 ml of cold PBS, and the homogenate was kept on ice during the procedure. With 10 ml syringe and 27 G needle, several suction expulsion passages of filtration were performed to generate complete homogenization and smooth any cell cumulus. Subsequently, the homogenate was centrifuged at 1000 rpm for 7 min using a temperature of 4°C; the supernatant was discarded. The pellet obtained from ganglion cells was resuspended in 4 ml of PBS with bovine fetal serum (FBS 1%), kept in ice until the time of labeled with fluorescent antibodies for flow cytometry. Immune cells were count in Neubauer's chamber before to labeled with fluorescence. In parallel the same procedure was applied with the spleen tissue.

Different populations of immune cells (T lymphocytes, B lymphocytes, natural killer cells, stem cells, PMN granulocytes, and macrophages) derived from the spleen and lymph nodes (**Table 3** and **Figure 10**) were labeled with the following monoclonal anti-mouse fluorescent antibodies:

- Anti-CD3ε-FITC
- Anti-CD19-PE

Monoclonal Antibody Anti-mouse	Clone Number	Label Used	White Cell Type Labelled
CDEε-**FITC** conjugated	145-2011	**CD3ε**	T Lymphocytes (TL) Natural Killer (NK)
CD19-**PE** conjugated	PeCa1	**C19**	B Lymphocytes (BL) Dendritic Cells (DC) Stem Cells (SC)
CD105-**Alexa Fluor** conjugated	MJ7/18	**CD105**	Macrophages Monocytes
Ly6G-**FITC** conjugated	1A8	**Ly6G**	PMN-granulocytes

The different populations of immune cells (T lymphocytes, B lymphocytes, Natural Killer Cells, Stem cells, Polymorphonuclear (PMN) granulocytes and macrophages) from splenic and ganglion tissues were labelled with fluorescent anti-mouse monoclonal antibodies, according to the detail of this table. The conjugated antibody FITC (Fluorescein isothiocyanate), has excitation and emission spectrum peak wavelengths of approximately 495 nm/519 nm, giving it a green color. PE (phycoerythrin)-conjugate fluorescence has an excitation maximum at 564 nm and an emission maximum at 574 nm, giving yellow color. Alexa Fluor (AF) conjugated antibody has the longest wavelength with excitation/emission maximum of 784/814 nm.

Table 3.
Fluorescent anti-mouse antibodies used in flow cytometry.

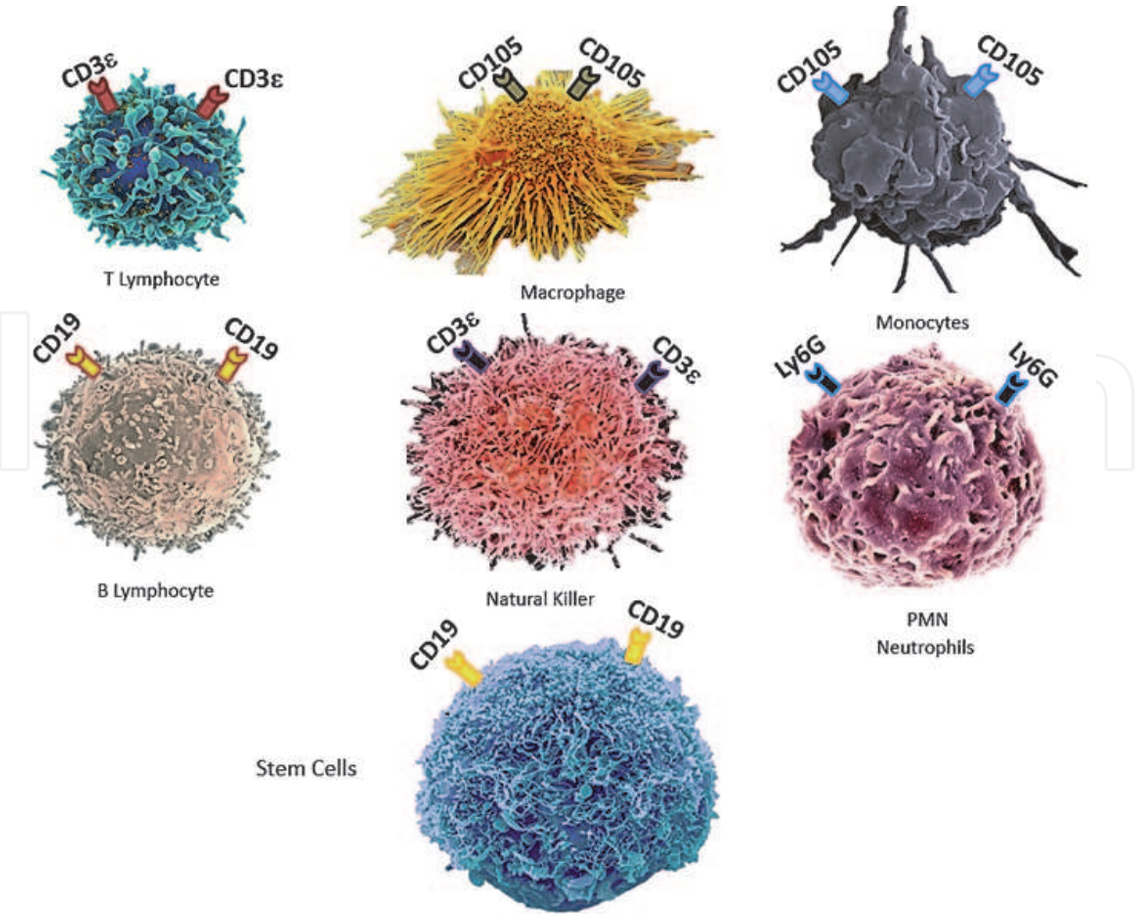

Figure 10.
Molecular markers in flow cytometry. *The immune-restorative effect of Maitake Pro4X in the recovery of immune cells with antigenic expression of CD3ε, CD19, CD105, and Ly6G of the lymph nodes and splenic organs, removed after the sacrifice of the mice, was studied.*

- Anti-CD105-AF
- Anti-Ly6G-FITC

The anti-mouse antibodies used in this study recognize specific markers (surface antigens) of murine immune cells (CD3, CD19, CD105, and Ly6G) and emit fluorescence by being found conjugated with fluorochromes (FITC, PE, and AF), which have different excitation (excitation) and emission wavelengths (Emission) (see **Table 3**). For the fluorescent labeling with the conjugated antibody with PE + FITC, 500 μl of cells from the pellet were taken from the sample pool, and added 150 μl of PE antibody (1:30) plus 150 μl of FITC antibody (1:30) were mixed and incubated in dark for 30 min at room temperature. After that time, the cells were washed twice with 500 μl of cold PBS and centrifuged for 7 min at 1000 rpm. The supernatant was discarded, the cell pellet was resuspended with 400 μl PBS + PFA (paraformaldehyde acid) 1% and kept on ice (always safe from light), until the time of reading on the cytometer, after homogenization of the samples, and according to the manufacturer's instructions. A BD Accuri C6 flow cytometer was used with a blue laser (480 nm) for the excitation wavelength and a red laser (640 nm) for the emission wavelength. The data was acquired on a logarithmic scale and analyzed with the FlowJo software (Tree Star, Ashland, OR, USA) (**Figure 8**).

The flow cytometry results of **Table 4**, **Figures 11** and **12** regarding the recovery of CD3 immune cells suggest that treatment with Maitake Pro4X allows a very significant recovery (**$p < 0.01$) of % T/natural killer lymphocytes (from

6.80 ± 7.08% to 21.09 ± 12.39%) in female BALB/c immunosuppressed with dexamethasone with respect to the immunosuppressed control (**Figures 11A–C** and **12A**). On the other hand, immune-restoration reaches normal values when Maitake treatment is adjuvant with Verapamil (from 21.09 ± 12.39% to 27.04 ± 29.96%) (**Figures 11D** and **12A**). As for the CD19 labeling, the results of

Ganglion Cells	CD3ε (%) (LT/NK)	CD19 (%) (LB/SC)
Healthy Control	51,14 ± 9,85	9,85 ± 2,62
Immunosuppressed (Dexamethasone)	6,80 ± 7,08 (**)	0,0 ± 0,00 (**)
Immunosuppressed with Maitake	21,09 ± 12,39 (**)	1,10 ± 1,67 (**)
Immunosuppressed + Maitake + Verapamil	27,04 ± 29,96 (**)	0,00 ± 0,00 (**)

The percentages obtained by flow cytometry correspond to axillary, mesenteric and inguinal lymph nodes of BALB/c females treated with Maitake Pro4X in the presence of immunological depletion and/or channel calcium blockage. The statistically very significant difference are indicated with (**).

Table 4.
Ganglion CD3ε and CD19 expression.

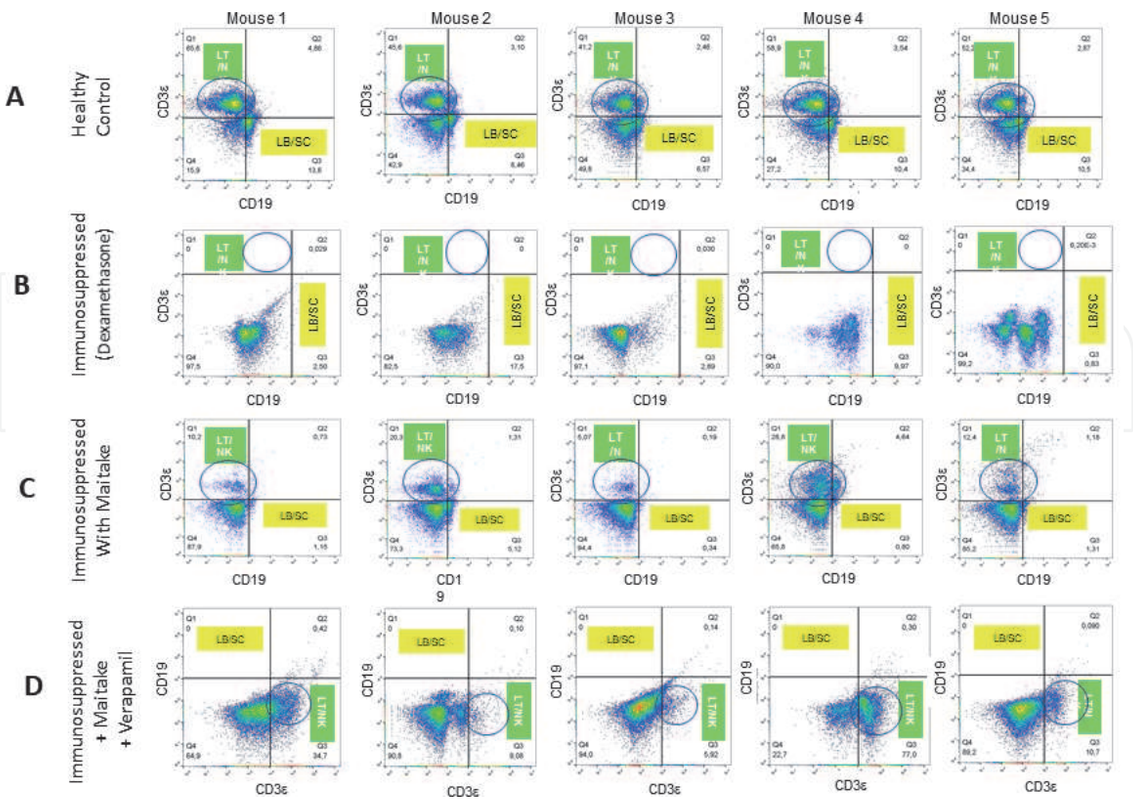

Figure 11.
Expression ganglion CD3/CD19. These correspond to axillary, mesenteric, and inguinal lymph nodes of BALB/c females of four experimental conditions: (A) healthy control group, (B) immunosuppressed condition, (C) immunosuppressed group treated with Maitake, (D) immunosuppressed group treated with Maitake + verapamil.

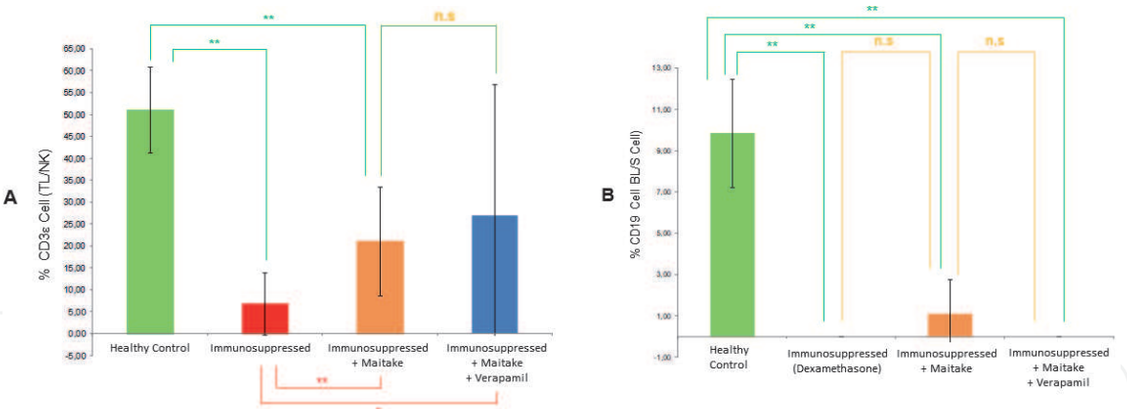

Figure 12.
Maitake's immune-restorative effect on CD3 and CD19 mouse immune ganglion cells. *The cytometric assay was performed on BALB/c females immunosuppressed with dexamethasone. The following are plotted: (A) CD3ε marking with very significant recovery of LT/NK (**p < 0.01) by Maitake effect in the immunosuppressed group and (B) CD19 marking with tendency to recover FROM LBT/SC by Maitake effect (ns, p > 0.05).*

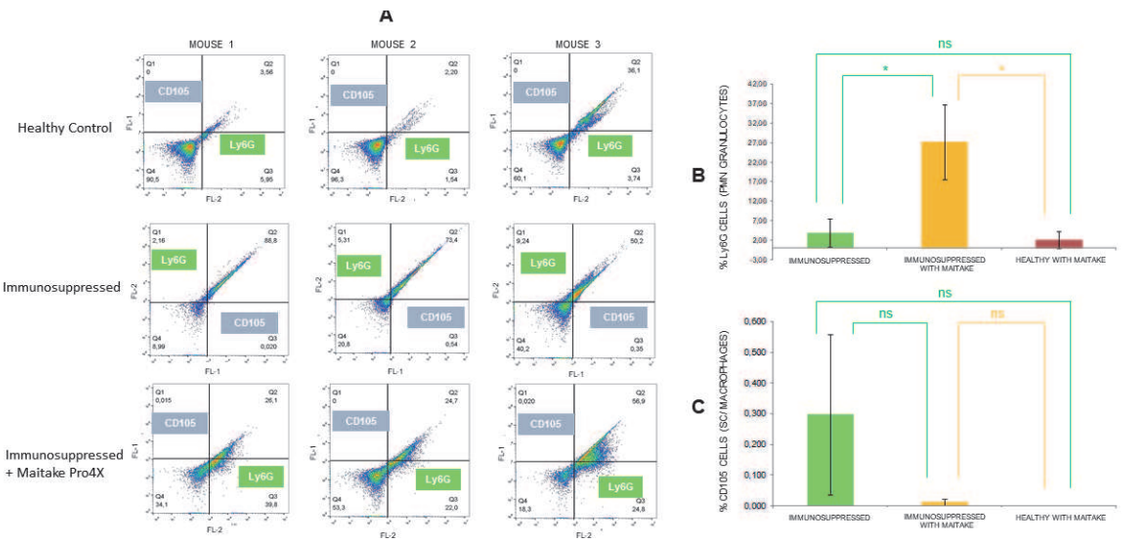

Figure 13.
Ly6G/CD105 mouse ganglion expression. *(A) Flow cytometry charts correspond to axillary, mesenteric, and inguinal lymph nodes of BALB/c females of three experimental conditions: Healthy group, immunosuppressed group alone, and treated with Maitake (B) and (C) Maitake's immune-restorative effect on Ly6G and CD105 immune ganglion cells. The cytometric assay was performed on BALB/c females immunosuppressed with dexamethasone. Graphed: (B) shows Ly6G labeling with significant differences in % of PMN granulocytes (*p < 0.05) and (C) shows the CD105 labeling cells without significant differences in the % of SC (stem cells)/ macrophages cells (ns, p > 0.05).*

Figures 11A–D and **12B** suggest that daily dexamethasone depletes the LB/SC population, while treatment with Maitake Pro4X tends to recover the LB/SC population (from 0.00 to 1.10 ± 1.67) (ns, $p > 0.05$). But this immune-restoration tends to disappear when treatment involves verapamil (from 1.10 ± 1.67 to 0.00 ± 0.00).

The flow cytometry results indicated in **Figure 13A** and **B** regarding the recovery of Ly6G ganglion immune cells suggest that treatment with Maitake Pro4X significantly increases (with *$p < 0.05$) the % polymorphonuclear granulocytes (PMNG) in the immunosuppressed mice with dexamethasone (immunosuppressed) from 3.88 ± 3.55% to 27.18 ± 9.57%, but does not increase it in healthy mice group. With respect to the CD105 cells, the results shown in **Figure 13A** and **C** suggest that dexamethasone does not induce depletion of % of stem cells (SC)/macrophages, given the not that significant differences (ns, $p > 0.05$) in the level of these cells among the experimental groups.

	CD3ε Splenic Cells (%)	CD19 Splenic Cells (%)
Healthy Control	23.47 ± 7.49	6.90 ± 2.88
Immunosuppressed	16.68 ± 9.17	0.953 ± 0.767
Immunosuppressed + Maitake Pro4X	27.79 ± 3.77	1.34 ± 0.43
	Ly6G Splenic Cells (%)	**CD105 Splenic Cells (%)**
Healthy Control	21.32 ± 15.32	0.06 ± 0.08
Immunosuppressed	18.09 ± 3.01	0.73 ± 0.72
Immunosuppressed + Maitake Pro4X	16.45 ± 4.94	0.12 ± 0.21

The percentages obtained by flow cytometry correspond to murine spleen tissue of BALB/c females treated with Maitake Pro4X in the presence of immunological depletion with Dexamethasone.

Table 5.
Splenic CD3ε, CD19, Ly6G, and CD105 cell expression.

2.7.1 Mouse splenic labeling of CD3 and CD19 cells

Flow cytometry results for splenic immune CD3 and CD19 cells' recovery are listed in **Table 5**. This trial was conducted with three experimental conditions: healthy control, immunosuppressed group alone, and immunosuppressed group treated with Maitake Pro4X. The flow cytometry results suggest that treatment with dexamethasone does not induce a significant depletion of the LT/NK population compared to the other experimental groups (ns, $p > 0.05$). There is a trend of the reduction of LT/NK % per share of dexamethasone and a trend of increased LT/NK by Maitake effect in the immunosuppressed group. Regarding the CD19 labeling (**Table 5**), we observe a significant depletion of the splenic population of LB/SC by dexamethasone (*$p < 0.05$ vs. healthy group) and a tendency to recover that population by Maitake (ns, $p > 0.05$).

As for the immune system, regulating its activation or suppression could contribute to the maintenance of good health. The use of agents that activate host defense mechanisms (immune-stimulators, immuno-suitors, or biological response modifiers) could provide an additional therapeutic tool to conventional chemotherapy [25]. For this reason, many biomedical researches are geared toward the search for new compounds capable of stimulating an immune response in immunodeficient patients, with pathologies such as HIV/AIDS, cancer, or malnutrition [30, 31]. Numerous immunostimulatory substances have been isolated from plants and superior fungi, opening the doors for the development of new drugs [30]. This provides an effective alternative for the treatment of conditions that alter the normal balance of the body's immune response [32, 33].

Within this context, we have investigated the immune-restorative capacity of Maitake Pro4X's glucans in BALB/c females immunosuppressed with dexamethasone. In parallel to immunosuppressive treatment, we treat mice with Maitake glycoprotein extract; it is important to note that no animals perished as a result of immunosuppressive treatment and that clinical signs of toxicity or disease were also not observed.

3. Discussion and conclusions

The antitumor and immunosurveillance properties of D-Fraction of Maitake have been little explored for the application in breast tumor or clinical

immunosuppression pathologies. For this reason, this chapter aims to deepen research on the pharmacokinetic and pharmacodynamic aspects of β-glucans from Maitake D-Fraction, as well as their therapeutic effects on immune recovery of specific lymphocyte populations. This is in order to make a significant contribution to the development of new preventive and therapeutic strategies to optimize the pharmacological use of Maitake's glucans.

As part of our experimental studies, we have observed in physicochemical characterization assays that the pH value of purified Maitake extracts measured at 20°C (ambient temperature) and 37°C (body temperature) resulted to be acidic with pH 5.8 for Maitake Pro4X. Moreover, this product was soluble in polar solvents (water, methanol, ethanol) and insoluble in apolar solvents (n-hexane, ethyl acetate), demonstrating its hydrophilic and polar nature. We also observe an inverse relationship between the proteoglycan concentration and its solubility in polar solvents (such as acetone), resulting in insoluble in acetone. The above suggests that D-Fraction of Maitake has hydrophilic and polar nature. The pH similarity of the product to the gastrointestinal pH suggests a good absorption at that level, which is associated with the biodistribution results shown here for oral administration.

The high molecular weight of β-glucans from Maitake extract suggests the great difficulty of these molecules in crossing biological membranes by simple diffusion, requiring a specific receptor after entering the systemic circulation, in order to trigger their molecular action mediated by second intracellular messengers. Nakashima et al. [34] in agreement with Brown et al. [35, 36] had reported that the Dectina-1 membrane receptor recognizes "glucans" with links "β-1,3" and/or "β-1.6" present on the fungal walls, proposing that receptor as a new receptor and as a therapeutic target for the immunomodulatory effects of glucan compounds.

Another of our in vitro assays showed that the treatment of MCF-7 breast tumor cells with Maitake + laminarin (Dectin-1 inhibitor) significantly reduced the effect of cell death triggered by Maitake ($p < 0.05$). These results suggest a greater affinity of Dectina-1 receptors with laminarin than Maitake's glucans. In addition, a likely laminarin-Dectin-1 complex would prevent the binding of β-glucans to the tumor receptor by blocking the mechanism that leads to programmed cell death.

As for our pharmacokinetic parameter studies in oral administration, we have observed three peaks of plasma concentration of 1.3-β-glucans with a maximum peak that was recorded half an hour after administration (followed by an abrupt decline). The second peak was recorded between 2 and 10 h thereafter and the last at 34 h after oral administration of Maitake Pro4X-glucans (5 mg/Kg). The results of our trials suggest that the plasma removal (clearance) of 1.3-β-glucans corresponds to a first-order kinetic model (mono-compartmental), obtaining a very rapid first plasma disappearance initiated at half an hour and a second more gradual elimination at 10 h later. Hong et al. [37] have reported that orally administered β-1,3-glucans are transported by macrophages to the spleen, lymph nodes, and bone marrow, implying a possible plasma reduction of the compound. For our part, we have observed that the absorption of the compound was higher than the elimination of the compound and that the volume of the distribution obtained was very high (127 ml/g). These results would indicate the extensive distribution of glucans in tissues and poor plasma protein binding. So that probably the 1,3-β-glucans could be found mostly in their "free" form in blood circulation, being able to diffuse the extravascular compartments, to interact with their receptors, and to trigger a biological response.

Moreover, in the hematological analysis of mouse peripheral blood in various experimental conditions, we have observed that the dexamethasone (immunodepleted) group had many giant cells of immature appearance compatible with megakaryocytes, while the condition with Maitake + dexamethasone group presented low number of immature cells, in addition to increasing number of lymphocytes, PMN neutrophils, basophils, and some megakaryocytes. In turn, the

group dexamethasone + verapamil + Maitake exhibited megakaryocytes and mast cells in degranulation [38]. The healthy control group did not have immature cells, with clustered platelets, lymphocytes, neutrophils, and monocytes observed. Our microscopic observations suggest that as a result of immunodepletion in mice by dexamethasone, large immature cells are recruited into the bloodstream, while concomitant treatment with Maitake could contribute to cell maturation, corroborated by the presence of mature basophil cells, lymphocytes, and polymorphonuclear granulocytes. These results suggest that Maitake's glucans could induce cellular maturation leading to immune-restoration. However, when treatment with Maitake involves calcium receptor blockage, its immunosurveillance capacity would be reduced, highlighting the dependence of extracellular calcium. Estrada et al. [39] have reported that the "glucans" increase the proliferative capacity of cultured lymphocytes treated with dexamethasone, reversing the immunosuppressive effect of glucocorticoid, suggesting the ability of the glucans to restore significantly specific and non-specific immune parameters in both cell cultures and animals treated with dexamethasone. For their part, Kotthoff et al. [40] have reported that antigen-presenting cells generated in the presence of dexamethasone have reduced the ability to stimulate the proliferation of T cells, while treatment with β-glucans induces the expression CD69 T-cell maturation markers and promotes Syk and STAT3 phosphorylation, with increased IL-10 secretion, while reducing the production of IL-12, IL-23, and TNF-A. Masuda et al. [41] reported, in murine models of tumorigenesis, that MD-Fraction of *Grifola frondosa* could generate systemic immune response, directly inducing the maturation of dendritic cells through a Dectin-1 pathway of the receptor Dectin-1 lectin type C. The therapeutic response of orally administered MD-Fraction was associated with specific T-cell responses of induced systemic tumor antigen through Dectin-1-dependent activation of dendritic cells, (ii) increased T-cell infiltration, and (iii) decreased number of immunosuppressive cells caused by tumors, such as regulatory T cells and myeloid-derived suppressive cells [41]. For our part, the studies of flow cytometry in dyed lymph nodes with CD3ε (LT, NK)/CD19 (LB, SC) suggested that treatment with Maitake allows a very significant recovery ($^{**}p < 0.01$) of the T/natural killer lymphocyte population of immunosuppressed animals with dexamethasone. In turn, concomitant treatment with verapamil would not affect the described immune-restoration but would instead contribute to lymphocyte recovery reaching normal values. So Maitake-induced cellular maturation could be affected by extracellular calcium blockage based on our microscopic results, but recovery of T/natural killer lymphocytes would not be influenced by the ion sequestration. With respect to the LB/SC population, we note that Maitake Pro4X tended to recover that lymphocyte population, but without statistical significance, while joint treatment with verapamil inhibited this trend. The results described support our hypothesis that extracellular calcium sequestration affects some of the functionalities of Maitake's glucans. On another hand, the molecular marker CD105 (for stem cells/macrophages) was found no significant differences in the cell population between the different experimental conditions. Similar results were obtained for ganglion molecular marker CD19 (stem cells/LB) with no significant differences between animal groups. Regarding Ly6G ganglion molecular marker (polymorphonuclear granulocytes), we observe that treatment with Maitake significantly increased the percentage of the cell population in immunosuppressed mice ($^{*}p < 0.05$) but not in healthy animals.

Lin et al. [42] in previous studies reported the ability of Maitake's glucans to activate the biological response in hematopoiesis, promoting bone marrow recovery after injury and stimulating activation of the Maitake forming unit granulocyte and monocyte colonies (GM-CFU), while Kodama et al. [43] have reported that D-Fraction derived from *Grifola frondosa* is able to activate immunocompetent cells

such as macrophages and T cells, with modulation of the balance between T-helper lymphocytes 1 and 2. Continuing with our studies at the splenic level, we do not find in the spleen more significant effect of immune-restoration than that described for lymph nodes, prompting the tendency to recover immune populations by Maitake effect. In splenic tissue the CD19 (LB/SC) molecular marker labeling was significantly depleted during treatment with dexamethasone compared to the healthy group treated with Maitake Pro4X ($^*p < 0.05$), while treatment with Maitake alone induced only trend in the recovery of B lymphocytes ($p > 0.05$). In another sense, dexamethasone did not induce significant depletion of the splenic CD3 cell population (LT/NK) with respect to the healthy group treated with Maitake, although it tended to reduce LT/NKs, while concomitant treatment with Maitake tended to increase this population. Dexamethasone also did not significantly deplete the splenic Ly6G cell population (PMN granulocytes) and CD105 (SC/macrophages) for the healthy group treated with Maitake. In the splenic CD105 (SC/macrophages), dexamethasone tended to increase that population compared to the healthy group treated with Maitake, while joint treatment with Maitake tended to reduce it. These effects were like those observed for SC/macrophages at the ganglion level. These results suggest greater relevance of the immune-restorative effect of Maitake and immunosuppressant effect of dexamethasone at the ganglion level rather than splenic. Kay and Czop [44] previously reported that dexamethasone stimulates monocyte gluconic receptors and promotes phagocytosis by macrophages of particles of β-glucans. In vivo studies in murine models of immunosuppression showed that dexamethasone (immunosuppressant) promotes the presence of immature immune cells in circulation, while concomitant treatment with Maitake stimulates maturation and cell differentiation (with the presence of polymorphonuclear granulocytes, lymphocytes, and basophilic cells). Extracellular calcium blockage (Maitake + verapamil adjuvant) partially affects the immune-restorative effect for certain lymphocyte populations. Flow cytometry studies demonstrated a significant immune-restorative effect of Maitake at the ganglion level, with recovery from polymorphonuclear granulocytes and LT/NK, while concomitant treatment with verapamil further increased the recovery of LT/NK.

In conclusion, the in vivo studies in murine models of immunosuppression showed that dexamethasone (immunosuppressant) promotes the presence of the high number of immature immune cells in circulation, while the concomitant treatment with Maitake stimulates maturation and cell differentiation (with the presence of polymorphonuclear granulocytes, lymphocytes, and basophilic cells). The extracellular calcium blockage (Maitake + verapamil adjuvant) partially affects the immune-restorative effect for certain lymphocyte populations. Flow cytometry studies demonstrated a significant immune-restorative effect of Maitake at the ganglion level, with recovery from polymorphonuclear granulocytes and LT/NK, while concomitant treatment with verapamil further increased the recovery of LT/NK.

From the in vivo biodistribution studies in murine models, we have concluded that after oral administration, there was increased uptake of the gastrointestinal compound with the predominance of gastric uptake but also important uptake in the duodenum and colon (in the order of millions of pg.h/ml). The presence of β-glucans in the brain allows Maitake's ability to pass through the blood–brain barrier (BHE). The lower relative uptake recorded at the hepatorenal level allows us to conclude a lower rate of inactivation and excretion of the compound, evidenced by the longer circulation time of the compound in the body after a single administration.

In resume, based on the results obtain in this work, we can propose the following putative molecular mechanism on mice (**Figure 14**): After oral administration of 1,3-1,6-β-glucans from Maitake Pro4X, due to its hydrophilic nature and high solubility in the stomach/duodenum with similar Ph, the complex 1,3-1,6-β-glucans are captured

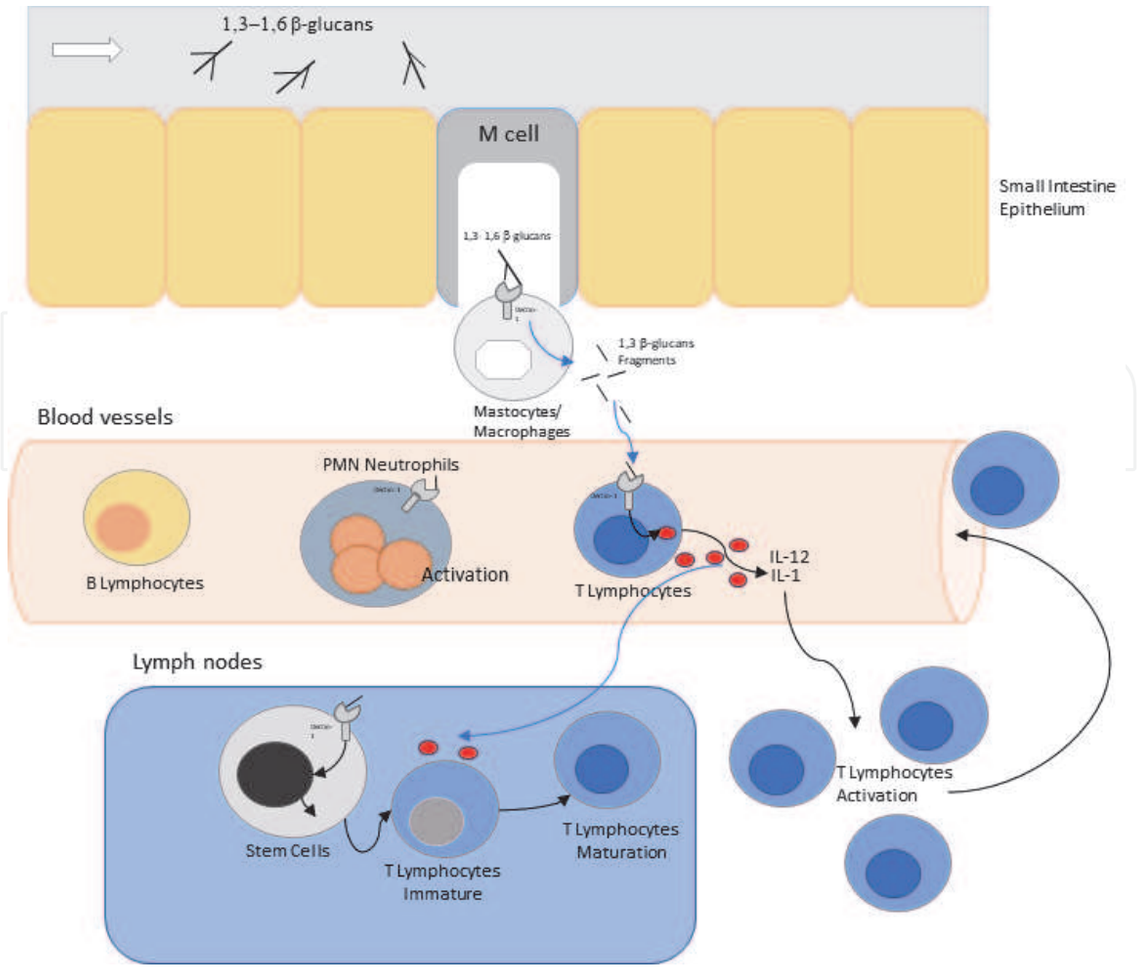

Figure 14.
Putative molecular mechanism induced by 1,3-b-glucans from Maitake Pro4X to revert the immunosuppression situation. *We have demonstrated Maitake's D-fraction's ability to restore specific lymphocyte populations in lymph nodes, including polymorphonuclear granulocytes and LT/NK, as well as induce maturation of T cells on bone marrow and lymph nodes.*

by mastocytes/macrophages through Dectin-1 receptors in M cells in the epithelium from the small intestine; after that, beta-1,3-glucan fragments (are broken in pieces) pass through the blood vessels' endothelium impacting the receptor Dectin-1 on T-lymphocyte cells, stimulating their expression of lymphokines such as IL-12 and IL-1, attracting more T lymphocytes, and helping in the recovery of T-cell population during immunosuppression. On lymph nodes (ganglion cells), β-1,3-glucan fragments stimulated the stem cells to differentiate into TL and mature to reestablish the TL cells on blood circulation due to dexamethasone effect on immunosuppression. The most spectacular action of beta-glucans is the effect on PMN granulocytes especially on neutrophils (the first barrier of defense in the body) (**Figure 14**). In conclusion we can be optimistic to see how promising the beta-1,3-glucan natural compound can be to be applied in immunodepleted situations helping the T cells and PMN granulocytes recover its population through stem cell differentiation, maturation, and activation in order to reestablish a new immune barrier ready to defend the body.

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Conflict of interest

The authors declare no conflict of interest.

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Abbreviations

ThL	T-helper lymphocyte
NK	natural killer cells
IFN-γ	interferon gamma
IL-	interleukin
TNF-α	tumor necrosis factor alpha
iNOS	nitric oxide synthase
GM-CSF	granulocyte macrophage colony-stimulating factor
BL	B lymphocytes
TL	T lymphocytes

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