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# ALETA™: MODE OF ACTION

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## ABSTRACT

Previous research has shown that beta-glucan has immunomodulatory effects. Paramylon is the beta-glucan found in Aleta™ and forms granules. The objective of this research was to test whether paramylon is readily phagocytosed by immune cells such as macrophages and dendritic cells (DCs). Our results showed that paramylon granules are readily phagocytosed by macrophage and dendritic cells. In addition, these activated cells then release beta glucan fragments to modulate immune responses.

***KEYWORDS*:**

Aleta™, paramylon, beta glucan, immune modulation, macrophages, dendritic cells

## MATERIALS AND METHODS:

**Products tested:** Aleta™ (Paramylon, 95% purity), Product A (70% beta 1,3/1,6 glucan product derived from the cell wall of a proprietary strain of yeast used as a supplement in human nutrition), Yeast BG. All the materials were labelled with fluorescein isothiocyanate (FITC).

**Method**: Paramylon (95% purity) and two other yeast beta glucans (Product A (~70% beta-1,3/1,6-glucan), Yeast BG) were labelled with fluorescein FITC and imaged using a fluorescent microscope. Mouse macrophages were incubated with and without paramylon-FITC at various concentrations and for various durations. After the incubation period, unbound particles were washed away and cells were incubated for 72 hours prior to staining with macrophage marker (Cd11b) and nucleus marker (4',6-diamidino-2-phenylindole, DAPI) before imaging by confocal microscopy.

Mouse dendritic cells (DCs) were incubated with and without paramylon-FITC at various concentrations and for various durations. After the incubation period, unbound particles were washed away and cells were incubated for 72 hours prior to staining with Major Histocompatibility complex (MHC II) marker and nucleus marker (DAPI) before imaging by confocal microscopy.

*Flow cytometry*: mouse macrophages and DCs were incubated with different concentrations of paramylon-FITC for different durations. The cells were harvested, washed, as well as stained for macrophage marker (Cd11b) and DC marker (Cd11c), respectively. Then the cells were analyzed by flow cytometry to quantify the percent of each cell-type population that was positive for paramylon-FITC.

*Release of beta glucan fragments*: DCs and macrophages were generated *in vitro* from bone marrow or spleen cells. These cells ($1x10^6$/ml) were mixed with FITC-paramylon (0.01 mg/ml) and were divided into groups. One group was cultured at 37°C and the other set was kept on ice (4 °C) for 12 hours. The supernatants of both groups were then collected, diluted (1:4), and examined for green fluorescence using a fluorometer.

## RESULTS AND DISCUSSION:

Paramylon (95% purity) and two other yeast beta glucans were labelled with fluorescein FITC and imaged using a fluorescent microscope (Fig. 1). The paramylon appeared as distinct granules without any apparent aggregation or clumping, whereas the purified yeast beta glucan Product A (~70% beta-1,3/1,6-glucan) contained much larger round particles or aggregates and the Yeast BG was similarly sized to paramylon but tend to clump.

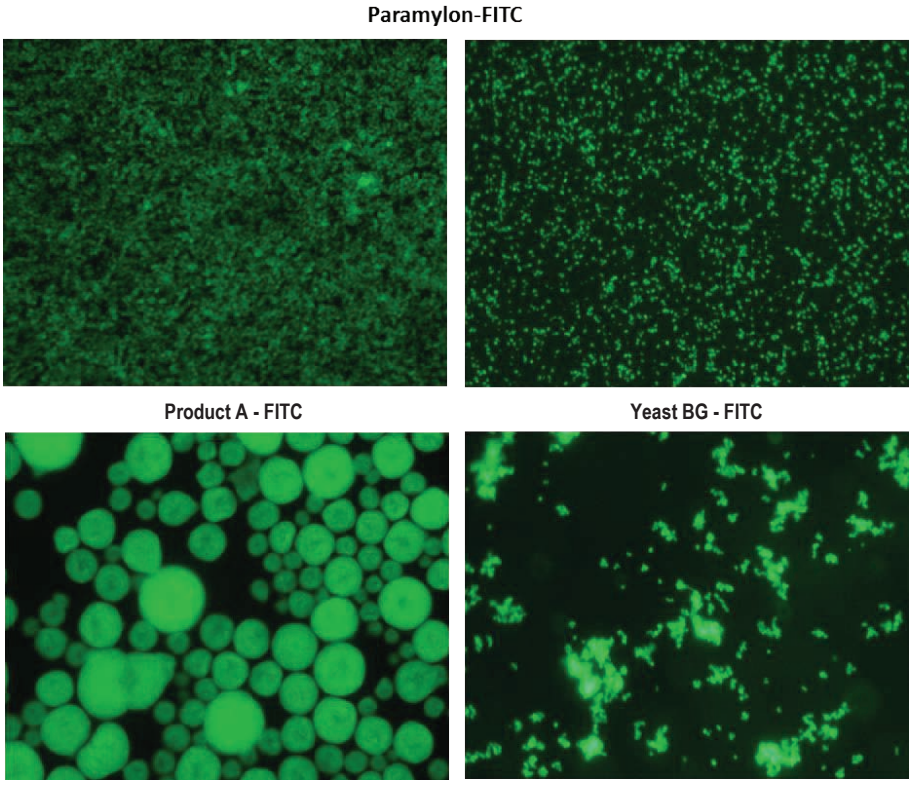


**Figure 1.** Beta glucan products labeled with fluorescein FITC.

Mouse macrophages were incubated with and without paramylon-FITC for 2 hours, unbound particles were washed away, and cells were incubated for 72 hours longer and then stained for macrophage marker (Cd11b) and nucleus marker (DAPI) before imaging by confocal microscopy (Fig. 2). These images clearly demonstrate that paramylon granules are readily phagocytosed by macrophages. This incubation was then repeated for either 1 or 6 hours to detect how quickly macrophages would phagocytose the product (Fig 3). It is evident that phagocytosis occurs rapidly and the cells continue phagocytosing granules of beta glucan as the incubation time is extended.

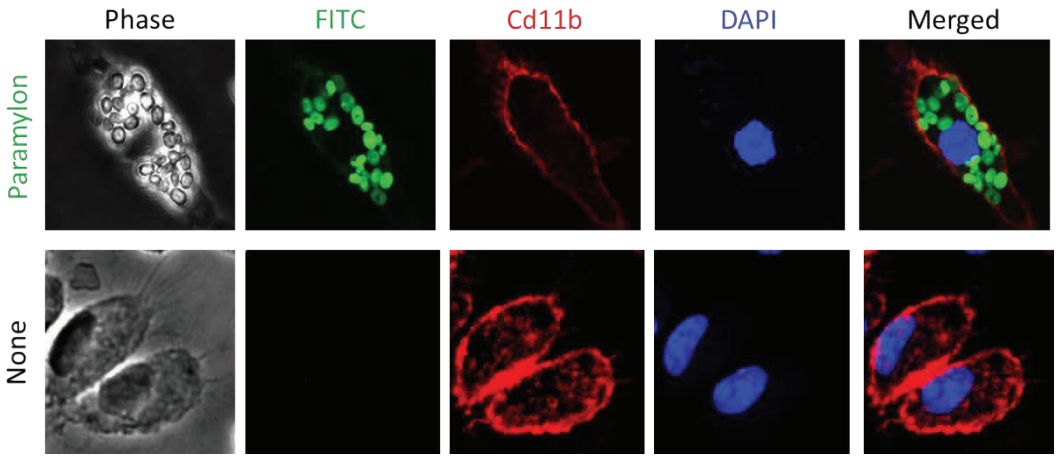


**Figure 2.** Phagocytosis of paramylon-FITC (green) by mouse macrophages. Cells were stained for macrophage marker (Cd11b, red) and nucleus marker (DAPI, blue) before imaging by confocal microscopy.

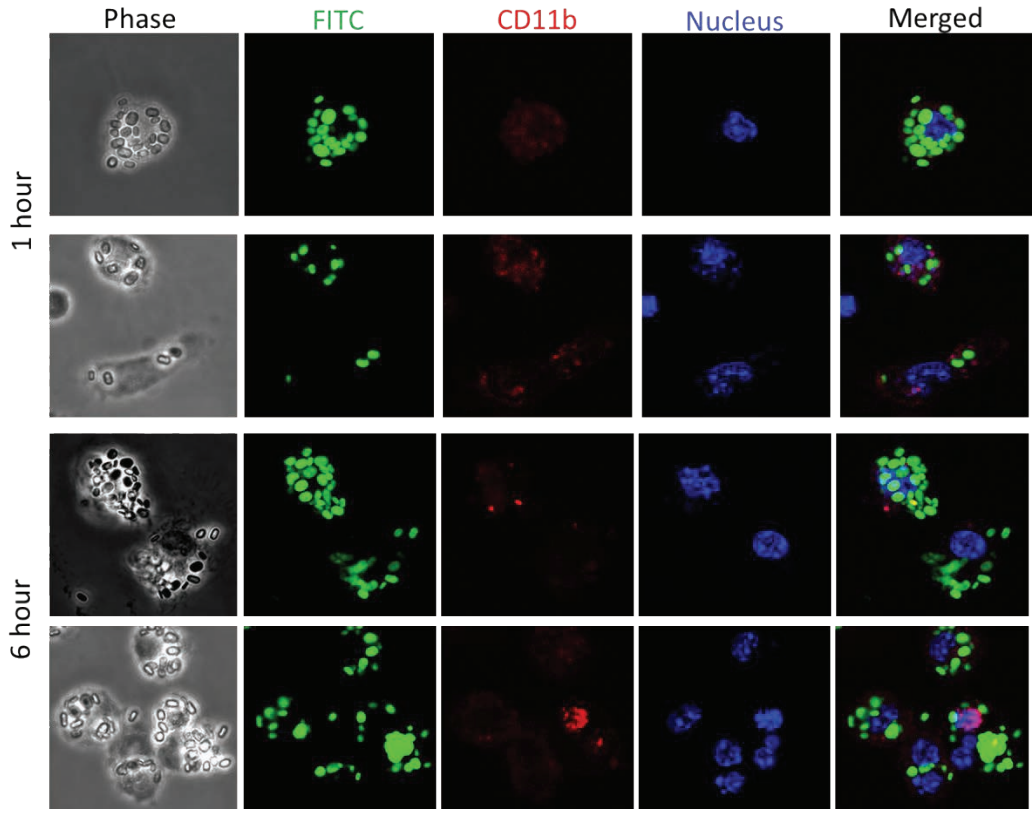


**Figure 3.** Phagocytosis of paramylon-FITC (green) by mouse macrophages for 1 or 6 hours. Cells were stained for macrophage marker (Cd11b, red) and nucleus marker (DAPI, blue) before imaging by confocal microscopy

Additional work was done to quantify the rate and extent of phagocytosis by macrophages (Fig. 4). Macrophages were incubated with different concentrations of paramylon-FITC for different durations. The cells were harvested, washed, stained for macrophage marker (Cd11b), and analyzed by flow cytometry. The percentage value given in each box corresponds to the percent of macrophages with internalized paramylon. The MFI value indicates the extent of paramylon internalization, showing a higher value corresponding to a greater number of paramylon particles internalized/bound per cell. One limitation of this experiment is that does not distinguish between paramylon particles

that are bound to the cell and ones that are internalized. However, overall the data suggests that phagocytosis is fairly rapid, with nearly 60% or 94% of cells having internalized or bound at least one particle in a culture containing 50 µg/mL within 10 min or 30 min, respectively.

Paramylon was similarly phagocytosed by bone marrow-derived dendritic cells (DCs). DCs were incubated with paramylon-FITC for 2 hours (Fig. 5), unbound particles were washed away, and then cells were incubated for additional 72 hours prior to staining with activation/maturation marker (Major Histocompatibility complex (MHC) II) and nucleus marker (DAPI) before imaging by confocal microscopy. This experiment suggests DCs readily phagocytosed paramylon but that MHC II expression is not significantly upregulated. This makes sense because MHC II molecules are typically found in antigen-presenting cells like DCs that help initiate an immune response by presenting peptides (protein fragments) from the phagocytosed particle on the cell's surface. Since paramylon is devoid of proteins (it is 95+% beta glucan), there are no peptides available for MHC II to present. This data suggests that MHC II activation is unlikely to be a major part in how paramylon can modulate immunity. Instead, cells that have phagocytosed paramylon likely activate other immune responses through cell signaling molecules called cytokines.

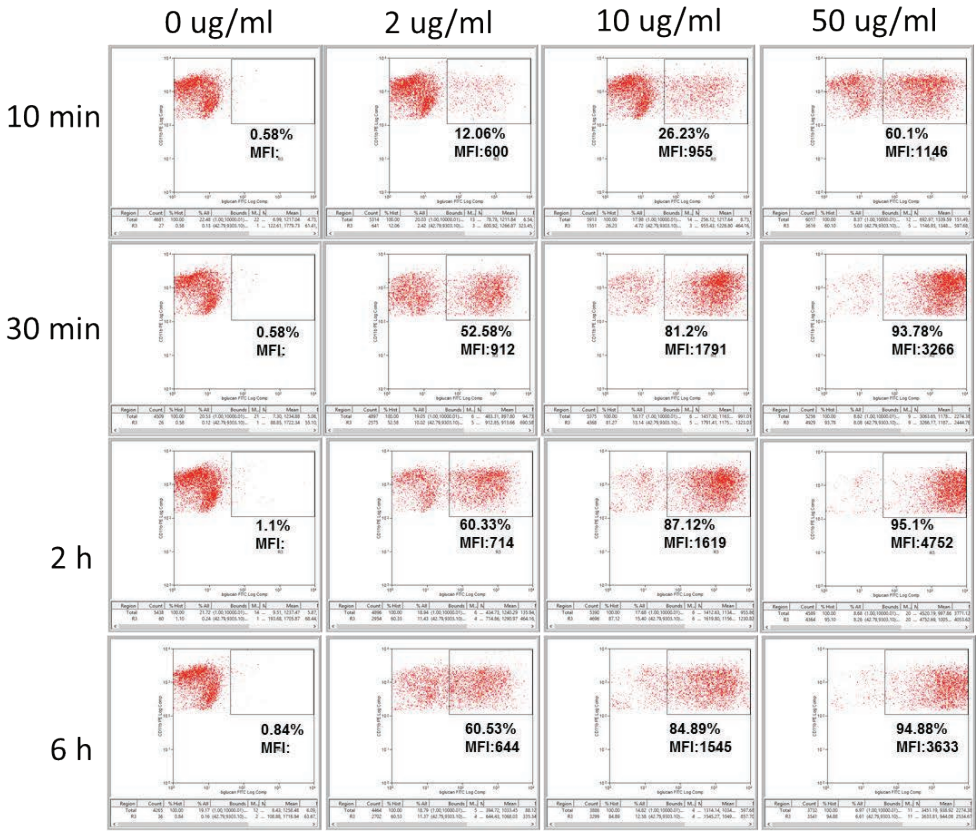


**Figure 4.** Flow cytometry analysis of paramylon internalization/binding by macrophages *in vitro*

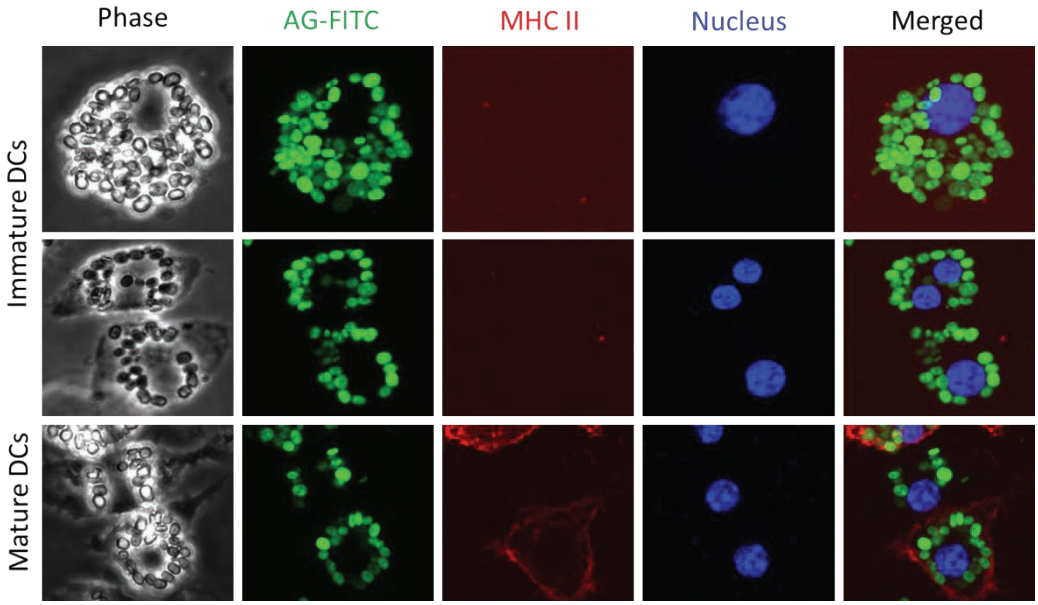


**Figure 5.** Phagocytosis of paramylon-FITC (green) by mouse dendritic cells for 2 hours. Cells were stained for dendritic cell activation/maturation marker (MHC II, red) and nucleus marker (DAPI, blue) before imaging by confocal microscopy.

Further work was done to quantify the rate and extent of phagocytosis by DCs (Fig. 6). DCs were incubated with different concentrations of paramylon-FITC for different durations. The cells were harvested, washed, stained for DC marker (Cd11c), and analyzed by flow cytometry. The percentage value given in each box corresponds to the percent of macrophages with internalized paramylon. The MFI value indicates the extent of paramylon internalization, with a higher value corresponding to a greater number of paramylon particles internalized/bound per cell. One limitation of this experiment is that does not distinguish between paramylon particles that are bound to the cell and ones that are internalized, but overall the data suggest that phagocytosis is fairly rapid, with nearly 69% or 74% of cells having internalized or bound at least one particle in a culture containing 50 µg/mL within 10 min or 30 min, respectively (Fig. 6). Compared to the data presented in Fig. 4 for macrophages, populations of DCs *in vitro* have lower percentages of cells that have internalized/ bound paramylon at the highest doses and longest durations.

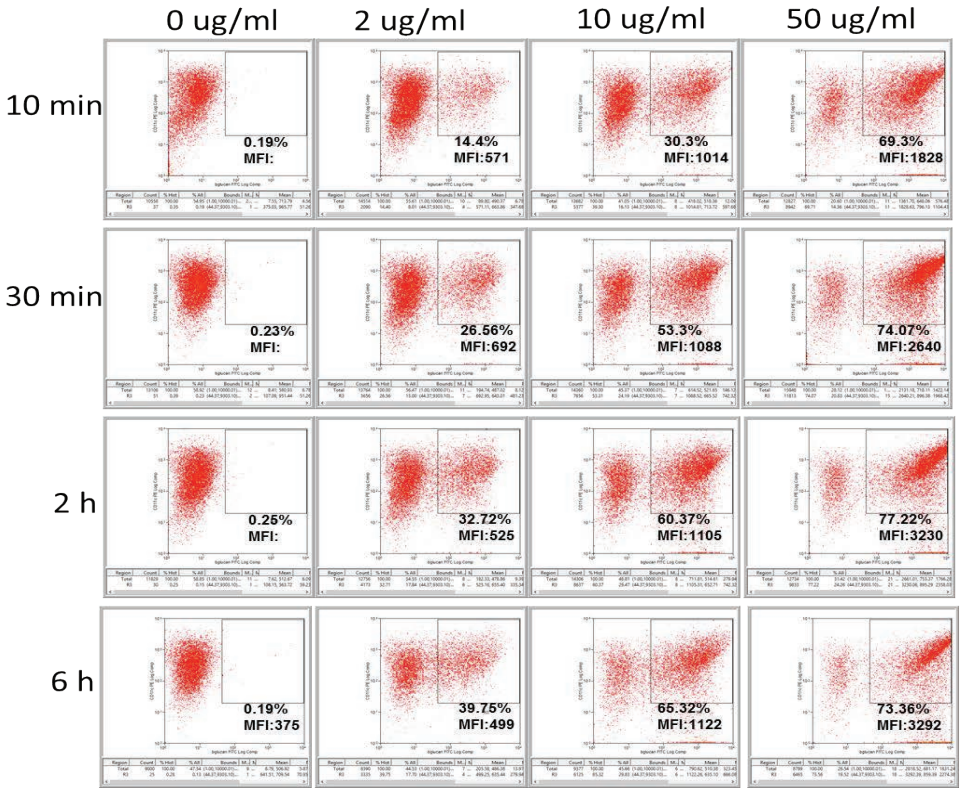


**Figure 6.** Flow cytometry analysis of paramylon internalization/binding by dendritic cells (DC) *in vitro*

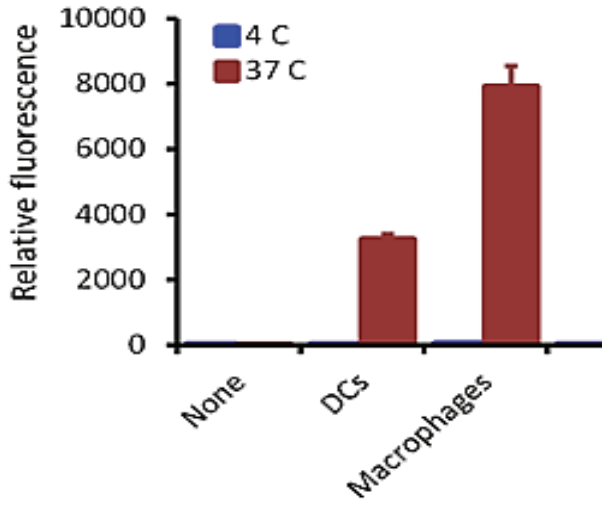


**Figure 7.** Relative fluorescence (times of florescence intensity as compared to the blank control) in the supernatant of cells cultured at 37°C or 4°C

To examine whether macrophages and DCs, release fragments of beta glucan after phagocytosing whole particles, cells were mixed with paramylon-FITC and then divided into two groups. One group was cultured at 37°C and the other set was kept on ice (4 °C) for 12 hours. The supernatants of both groups were then collected, diluted, and examined for green fluorescence using a fluorometer. As apparent in Fig. 7, macrophages appear to release FITC-containing

fragments more efficiently than DCs. The supernatants from cells kept on ice showed no fluorescence, indicating that released FITC is, in fact, caused by cellular activity and not as the result of passive release from the labelled paramylon. These data suggest that macrophages, and to a lesser extent DCs, can process paramylon granules and release shorter, smaller fragments of beta glucan into their surroundings. Further work should be done to detect the presence of beta glucan in the supernatant of similar cell cultures and identify its degree of polymerization to better understand how polymer size can affect activity.

## CONCLUSION

This work has demonstrated that paramylon was readily phagocytosed by macrophages and dendritic cells. Macrophages and DCs most likely release small fragments of beta glucan into their surroundings, which can then activate other cells throughout the body.

## REFERENCES

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