Authors: Luz Alejandra García-Madrid, Alfonso Enrique Islas-Rodríguez.
Email: luzalejandrag@yahoo.com.mx, islas.alfonso@gmail.com
Departamento de Biología Celular y Molecular, Universidad de Guadalajara, Guadalajara, Jalisco, México.

Keratinocytes from human skin respond as typical immune cells after stimulation with *Trichophyton rubrum.*

Trichophyton rubrum is the main agent causing dermatophytosis (1). Keratinocytes are considered to be the first physical barrier of defense against pathogens (2). But not only a physical barrier. They recognize antigens through Toll like receptors (TLR) (3). The activation of this TLR, present on the surface of the keratinocytes, induce the expression of different pro-inflammatory cytokines, co-stimulatory molecules and antimicrobial peptides such as beta β-defensins (4).

The main objective of this work is to determine if lipopolysaccharides of G – bacteria (LPS), lipotheichoic acid from G+ bacteria (LTA), and conidias, isolated from *T. rubrum* were able to activate the expression of TLR2 and TLR6 on the cell surface of a primary culture of human keratinocytes through Flow cytometry. Furthermore we are looking for the presence of β-defensins 1 and 2, IL1b and IL-8 in the supernatant, of the above mentioned culture of cells, by Western blot.

From the flow cytometry data, the preliminary results showed an important dispersion in terms of proliferation, increase in size and granularity of keratinocytes, from primary cultures of skin from healthy donors, stimulated 6 hours with conidias of *T. rubrum*, and LTA, but not when non stimulated, or stimulated with LPS (Fig 1).

When keratinocytes from primary cultures of skin from healthy donors were cultivated 48 hours, it was found dispersion in terms of proliferation, increase in size and granularity when stimulated with conidias of *T. rubrum*, and LPS but not when non stimulated, or stimulated with LTA (Fig 2).

The keratinocytes expressed increased levels of TLR2 and TLR6 when were stimulated with LTA and less to *T. rubrum*, in the 6 hours cultures, but this last cells still showed increased size (Fig 3).

The Keratinocytes expressed increased levels of TLR2 in the 48 hours cultures when were stimulated with LPS and *T. rubrum.*(Fig 4)

Besides, β-defensin-2 was detected in the supernatant of cultures of keratinocytes stimulated with LPS (Fig 5).

It can preliminary be concluded that keratinocytes from primary cultures of human skin from healthy donors, are cells that respond as typical immune cells, after stimulation with *T. rubrum,* LTA and LPS in different conditions, and that this mechanism may be very important, for the protection of local environment. .

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Luz Alejandra García-Madrid, Alfonso Enrique Islas-Rodríguez

Departamento de Biología Celular y Molecular, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Jalisco, México.

Email: islas.alfonso@gmail.com

INTRODUCTION

Dermatophytosis, is a world wide distributed infection that affect the epidermal layer of the skin, is caused mainly by *Trichophyton rubrum* and constitute between 70 – 80 % of the mycoses (1, 2, 3, 4).

Keratinocytes are the main cellular population of epidermis, by its location they are considered to be the first physical barrier of defense. But not only a phyisical barrier. They are the first cells that make contact with external antigens (5) and recognize them through receptors of the Innate Immune System (IIS) called Toll-like receptor family (TLR) that recognize molecular patterns associated to a great variety of pathogens, including bacteria, viruses, fungi and protozoa (6, 7). To date, 11 members of the TLR family in mammals have been identified and several ligands for each one of them have been described. In addition, cooperation of TLR2 with TLR1 or with TLR6 for discrimination between some ligands has been reported. (8, 9, 10).

Recently, new molecules and mechanisms of action and activation of the IIS have been described, which have completely changed the perception of this response and, consequently has increased the interest in its study.

Epidermal keratinocytes in normal conditions express TLR2, TLR3, TLR4, TLR5, TLR9 and TLR10 (6, 11). TLR2 recognize peptidoglycan and lipoteichoic acid from Gram+ bacteria, bacterial lipoproteins, mycobacterial lipoarabinomannan, glycosylphosphatidylinositol anchored proteins of *Trypanosoma cruzi*, a phenol soluble modulin produced by *Staphylococcus epidermidis*. TLR2/6 heterodimers recognize PGN and zymosan. In addition, it has been reported that TLR2 recognize mannan and Phospholipomannan of *Candida albicans*, as well as glucuronoxylomannan of *Cyptococcus neoformans* and mannan of *Saccharomyces cerevisiae*. TLR4 recognize G– component as LPS, fusion and envelop proteins of viruses, taxol of plants, and host components (8, 9, 12)..

The cell wall components of *Trichophyton rubrum* are constituted by glycoprotein (mannan) (13) that could be recognized by TLR.

After the recognition of molecular patterns associated to pathogens, the activation of these TLR induce the expression of different pro-inflammatory cytokines, co-stimulatory molecules and several antimicrobial peptides, recently recalled host defense peptides (HDP) (8, 9, 14, 15, 24).

Keratinocytes and several structures of the skin produce a great variety of HDP, β-defensins are one of the main groups (24). This peptides show antimicrobial activity predominantly against gram-negative (G–) bacteria, but also effective against gram-positive (G+) bacteria and fungi (16). β-defensin 1 is produced by keratinocytes constitutively, although some stimuli can increase their production, whereas β-defensin 2 expression is induced by several stimuli, like proinflammatory cytokines (TNF, IL-1b), LPS and bacteria (17, 18).

In addition to the antimicrobial activity, other properties of the HDP have been described. It has been verified that they are chemotactic agents for various cell types including T cells, phagocytic and mast cells, also they work as opsonization agents, stimulate the production of pro-inflammatory and anti-inflammatory substances and they favour tissue repair when promoting cell proliferation, for that reason HDP have become a target of therapeutic application (19 -23).

In spite of these evidences, at the moment few studies exist on the role of TLR and HDP in dermatophytoses. According to the above mentioned the objetive of this work is to determine if conidias isolated from *T. rubrum,* lipopolysaccharides of G– bacteria (LPS), and lipotheichoic acid from G+ bacteria, are able to activate the keratinocytes, and induce the expression of TLR2 and TLR6 on the cell surface. Furthermore we are looking if β-defensin 1 and 2, IL-1b and IL-8 are released after stimulation.

MATERIALS AND METHODS

CELL CULTURE: Primary cultures of human keratinocytes were established from skin remaining of abdomen surgery from healthy donors. Skin pieces of 1cm^2 were exposed to dispase 2.4 U/ml (Sigma) overnigth at 4°C. The epidermis was removed from the dermal layer and incubated in 0.25% EDTA-tripsin (Sigma) for 10 min at 37°C. After incubation, enzimatic activity of tripsin was stopped to adding DMEM medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco) and was homogenized by repeated aspirations. Cell suspension was centrifugated at 1500 rpm for 10 min and the pellet was resuspended in serum free medium for keratinocytes (Gibco). Cells were seeded into 75 cm^2 culture flasks and kept at 37°C and 5% CO_2. The culture medium was renewed every 3 days.

CULTURE OF FUNGI AND ISOLATION OF CONIDIAS: A clinical isolated of the fungus *T. rubrum* was cultivated in dextrose sabouraud agar at 25°C for 15 days.. Conidias were collected from the agar, transferred to sterile double distilled water and quantified in a hemocytometer.

STIMULATION: Keratinocytes were stimulated with 100µg/ml LPS (Sigma), with 100µg/ml LTA (Sigma), with *T. rubrum* conidias at a fungi cell/ human keratinocyte ratio of 10:1, and unstimulated cells for 6 or 48 hours.

FLOW CYTOMETRY: After stimulation, cells were twice wash with PBS and incubated for 15 min with 0.025 EDTA-PBS for to detach the cells from flask surface with help of cell scraper. Cells were wash with PBS and were incubated with monoclonal antibodies against TLR2 (eBioscience) and TLR6 (Imgenex) or their respective isotypes for 45 min in the dark. After, cells were wash with PBS and then read with a flow cytometer (FACScan). Data were analysed with WinMDI version 2.8 software.

WESTERN BLOT: The supernatant of the different cultures was collected. The protein concentration in the supernatant was quantified by Bradford assay. 20µg of total proteins of supernatants was loaded onto a 16.5% SDS-PAGE. Transfer to PVDF membranes was achieved overnigth at 30 volts at 4°C with a pulse of 100 volts for 1 hour an the after day. The membrane was incubated with a policlonal goat anti-β-defensin 2 antibody (Santa Cruz Biotechnology) for 2 hours followed by incubation with anti-goat IgG-HRP antibodie for 30 min. Positive bands were visualized by quimioluminiscence reagents.

PRELIMINARY RESULTS

From the flow cytometry data, the preliminary results showed an important changes in dispersion, in terms of proliferation, increase in size and granularity of keratinocytes when it was stimulates 6 hours with conidia of *T. rubrum*, and LTA, but not when non stimulated, or stimulated with LPS (Fig 1).

When keratinocytes were stimulated 48 hours with conidia of *T. rubrum* and LPS, it was found changes in dispersion, in terms of proliferation, increased in size and granularity, but not when non stimulated, or stimulated with LTA (Fig 2).

The keratinocytes expressed increased levels of TLR2 and TLR6 when were stimulated with LTA and less to *T. rubrum* in the 6 hours cultures, but this last cells still showed increased size (Fig 3).

The keratinocyte expressed increased levels of TLR2 in the 48 hours cultures when were stimulated with LPS and *T. rubrum* (Fig 4).

Besides, β defensin-2 was detected in the supernatant of cultures stimulated with LPS (Fig 5)

It can preliminary be concluded that keratinocytes from primary cultures of human skin from healthy donors, are cells that respond as typical imune cells, after stimulation with *T. rubrum,* LTA, and LPS in different conditions, and this mechanim may be very important, for the protection of local environment.

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FIGURE LEGENDS

Figure 1.- Data from Flow Cytometry indicates the keratinocytes dispersion (scatter), after 6 hours of stimulation with; Nothing, 100 μg/ml of LPS (G−), 100 μg/ml LTA (G+), and 10 conidias of *T. rubrum* per cell. In A, it can be shown 37,705 cells when LTA was added to the culture, 20,398 cells when conidias of *T. rubrum* was added, compared to the control (15,402). In the case of LPS there were no increase. In the other culture, B, similar results were found.
Note that according to Webb A., et.al (Differentiation (2004) 72: 387-395, it is considered that when the cells in the plot show low forward scatter, indicates their small size and low side scatter means lacking in cytoplasmic complexity. In contrast, cells appear to be larger in size with greater cytoplasmic area, that exhibit more complexity in the high forward and side scatter.

Figure 2.- Here it can be shown the Keratinocytes dispersion (scatter) after 48 hours of stimulation with; Nothing, 100 μg/ml of LPS (G−), 100 μg/ml LTA (G+), and 10 conidias of *T. rubrum* per cell. It can be shown 3,81 cells when LPS was added to the culture, and 7,041 cells when conidias of *T. rubrum* was added, compared to the control (1,642). In the case of LTA there were no increase.

Figure 3.- Data from Flow Cytometry indicates the number of keratinocytes that express TLR2 (A) and TLR6 (B), after 6 hours of stimulation with; Nothing, 100 μg/ml of LPS (G−), 100 μg/ml LTA (G+), and 10 conidias of *T. rubrum* per cell. In A, it can be shown 3,209 TLR2 positive cells (1,056 + 2153 cells) when LTA was added to the culture, compared to control (704 cells), and 5,470 negative to TLR2 cells that increased their size when conidias of *T. rubrum* was added, compared to the control (576 cells). In the case of LPS there were no increase. In B, it can be shown 3,223 (1139 + 2084) TLR6 positive cells when LTA was added to the culture, 1265 TLR6 positive cells stimulated with conidias of *T. rubrum* both compared to control (836 cells), and 576 TLR6 negative cells that increased their size when conidias of *T. rubrum* was added, compared to the control (250 cells). In the case of LPS there were no expression neither increase.

Figure 4.- Data from Flow Cytometry indicates the number of keratinocytes that express TLR2, after 48 hours of stimulation with; Nothing, 100 μg/ml of LPS (G−), 100 μg/ml LTA (G+), and 10 conidias of *T. rubrum* per cell. It can be shown 443 (408 + 35) TLR2 positive cells when LPS was added to the culture, 584 TLR2 positive cells stimulated with conidias of *T. rubrum,* both compared to control (283 cells), and 1,058 TLR2 negative cells that increased their size when conidias of *T. rubrum* was added, compared to the control (200 cells). In the case of LTA there were no expression neither increase.

Figure 5.- When the supernatant of a primary culture of Keratinocytes was collected after 48 hours of incubation with 100 μg/ml of LPS (G−), electrophorized, transferred and blotting with the antibody against β defensin-2, it can be seen a positive band.

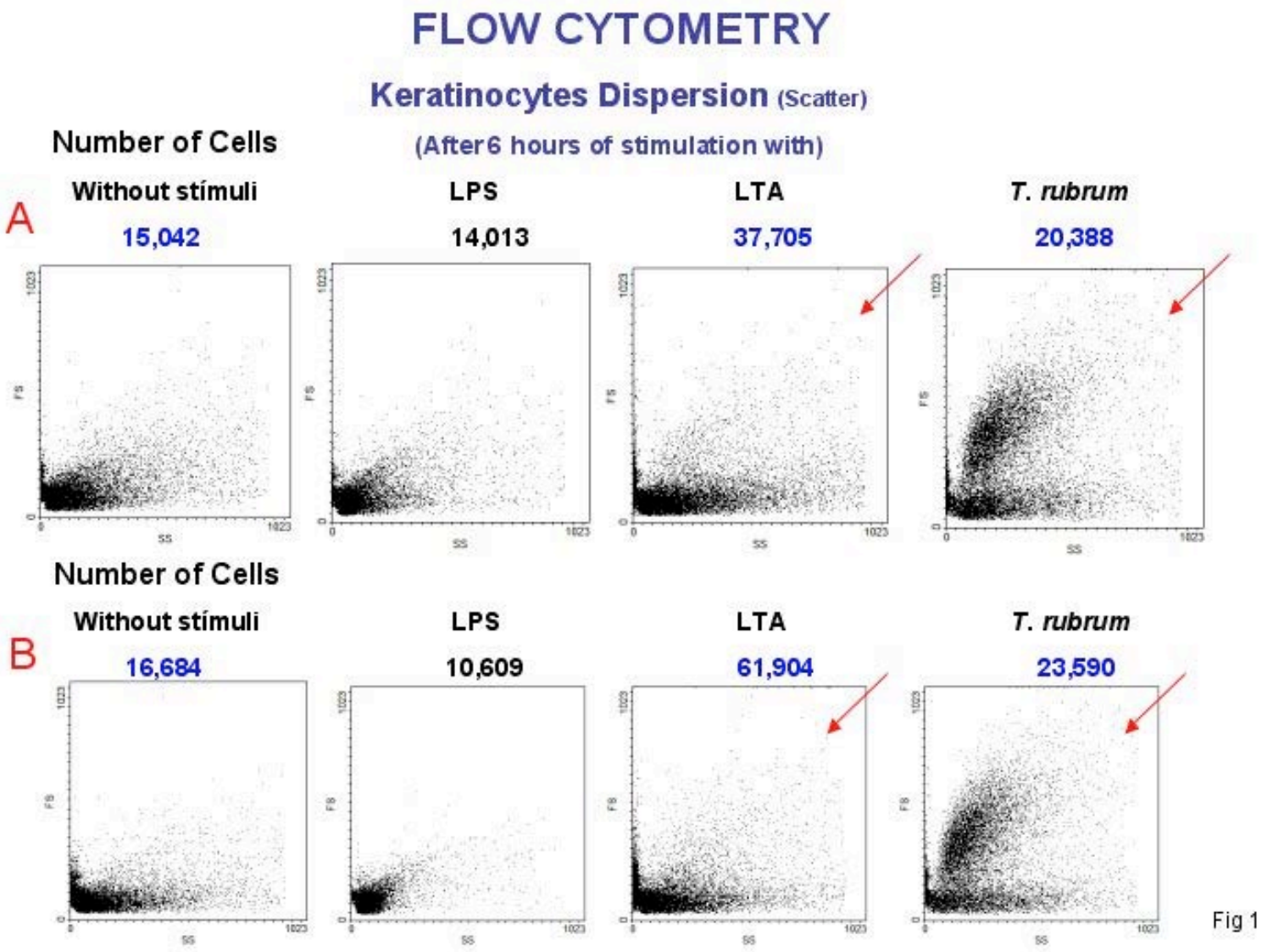

FLOW CYTOMETRY
Keratinocytes Dispersion (Scatter)
(After 6 hours of stimulation with)
A
Number of Cells
Without stímuli
15,042
LPS
14,013
LTA
37,705
T. rubrum
20,388
B
Number of Cells
Without stímuli
16,684
LPS
10,609
LTA
61,904
T. rubrum
23,590
FS
SS
0
1023

Fig 1

FLOW CYTOMETRY

Keratinocytes Dispersion (Scatter)

(After 48 hours of stimulation with)

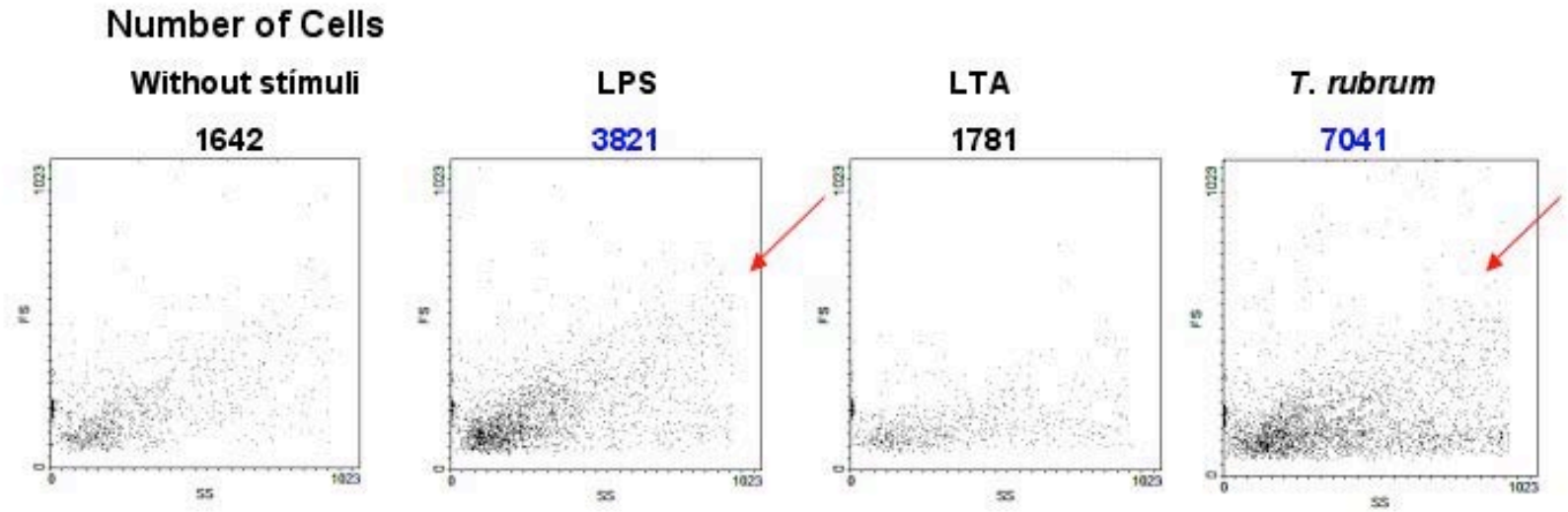


Fig 2

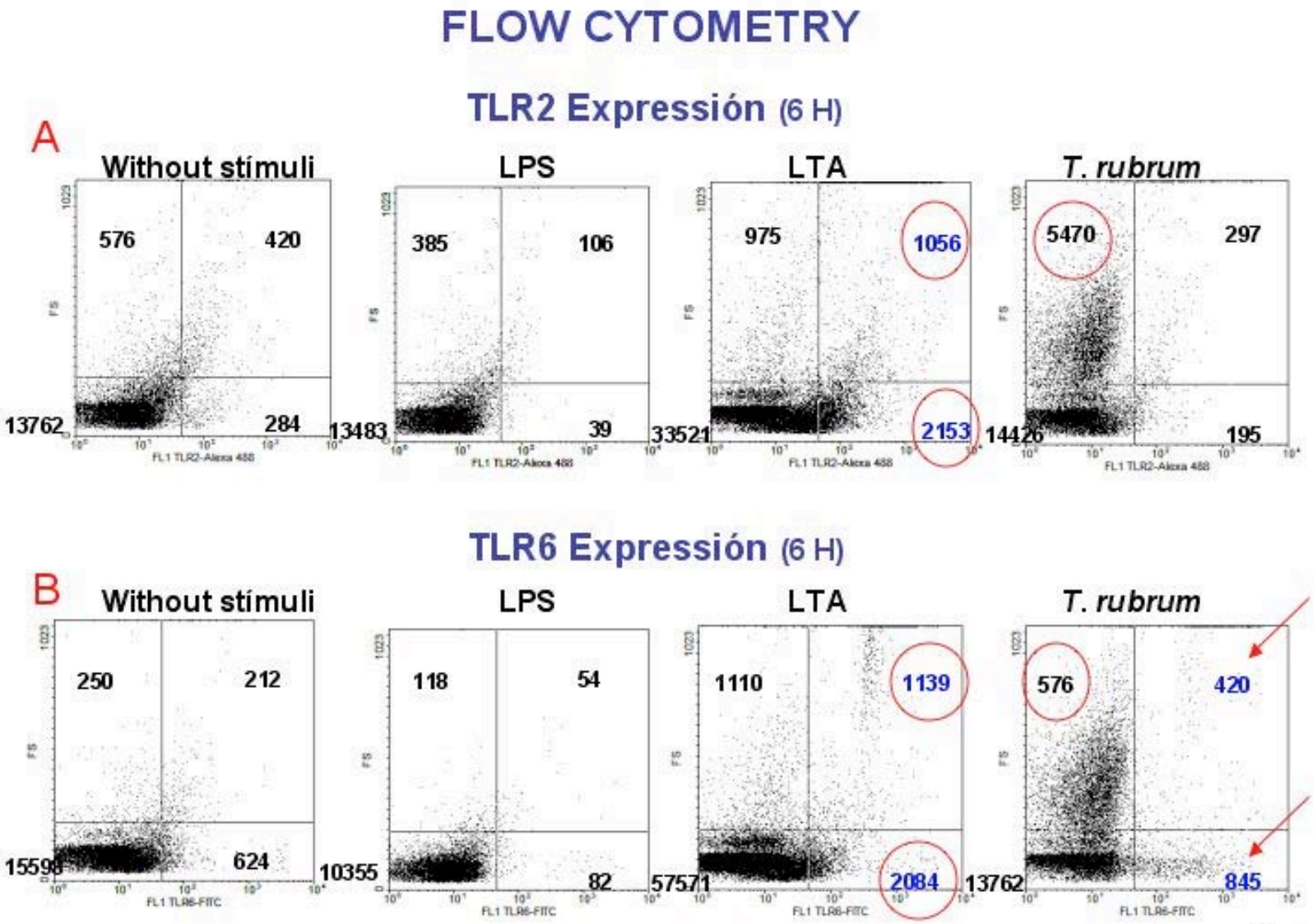

FLOW CYTOMETRY
TLR2 Expression (6 H)
A
Without stimuli
576
420
13762
284
LPS
385
106
13483
39
LTA
975
1056
33521
2153
T. rubrum
5470
297
14426
195
FL1 TLR2-Alexa 488
TLR6 Expression (6 H)
B
Without stimuli
250
212
15598
624
LPS
118
54
10355
82
LTA
1110
1139
57571
2084
T. rubrum
576
420
13762
845
FL1 TLR6-FITC

Fig 3

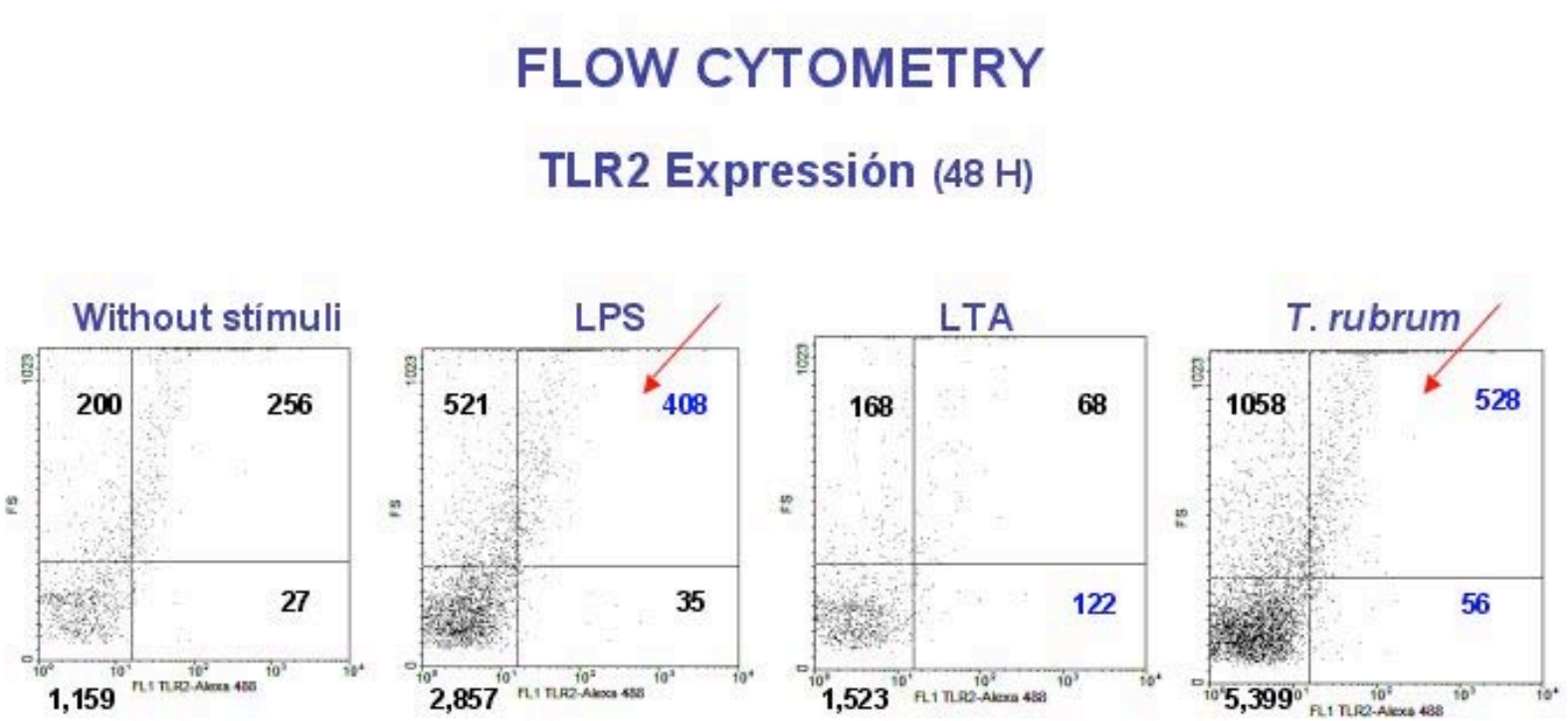
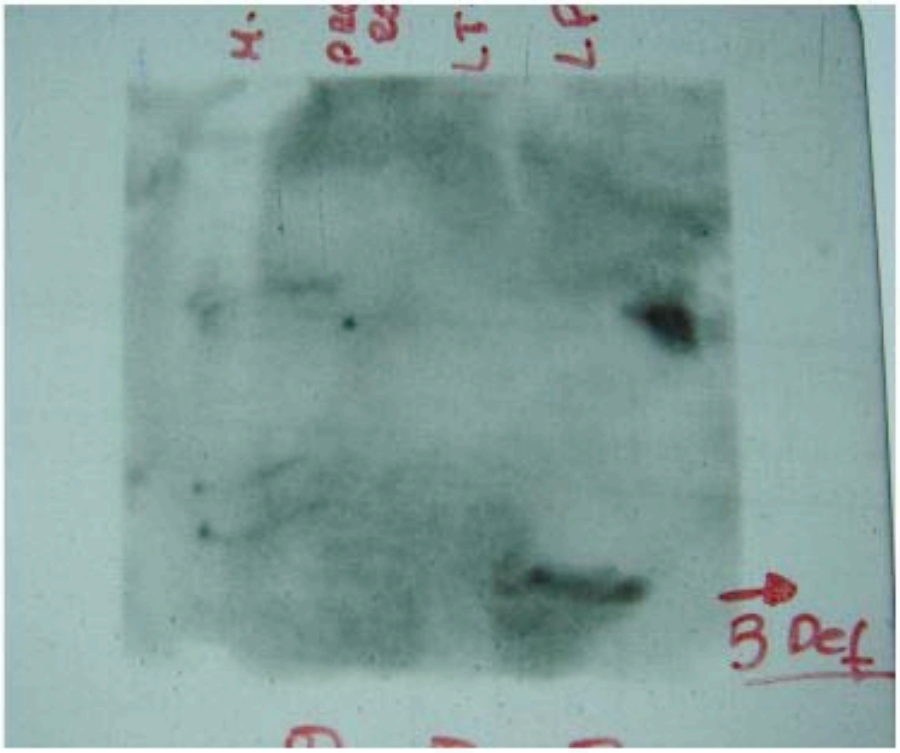

FLOW CYTOMETRY
TLR2 Expressión (48 H)
Without stímuli
200
256
27
1,159
FL1 TLR2-Alexa 488
LPS
521
408
35
2,857
FL1 TLR2-Alexa 488
LTA
168
68
122
1,523
FL1 TLR2-Alexa 488
T. rubrum
1058
528
56
5,399
FL1 TLR2-Alexa 488

Fig 4

Western Blot



M R LTA LPS NS

Fig 5