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Putative Target Cells for *Mycobacterium tuberculosis* Antigens MPT63 and MPT83.

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ABSTRACT

Tuberculosis – is a dangerous infectious disease. Study of diagnostic properties of Mycobacterium tuberculosis antigens important in order to clarify the molecular mechanisms of infection and for the further development of subunit vaccines and diagnostics is an urgent task of modern molecular biology, immunology and biochemistry. Our aim was to investigate the ability of antigens M. tuberculosis MPT63 and MPT83 to binding with cells of different origin and study the effect of these proteins on expression of the few activation markers of immune cell. It is shown that protein MPT63 binds to the surface of mouse spleen cells separate subpopulations and U937 cell line, whereas antigen MPT83 binds to surface of macrophage-like cell line U2149. Also, we registered increases the percentage of cells expressing markers of macrophage activation CD11b and F4/80. Our results suggest that on the surface cells of monocyte origin at a certain stage of differentiation appear molecules which specifically interacts with mycobacterium protein MPT63 and MPT83 and make that population of cells targeted by their effect. Research of target cells for MPT63 and MPT83 is an important step involvement of these proteins in the pathological process.

Keywords: Mycobacterium tuberculosis, MPT63 and MPT83, target cells, activation markers

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INTRODUCTION

Tuberculosis – is a dangerous infectious disease that causes death of approximately 2 million persons each year [1]. The ineffectiveness of current methods to control the epidemic situation of tuberculosis related not only with absence of effective diagnostics [2], but also associated with lack of understanding of the molecular mechanisms pathogenesis of mycobacterium infection. Completion of the project of the sequencing of the genome of Mycobacterium [3, 4] became the impetus toward identification and research of the specific functions and properties of new antigenic targets for creating of vaccines and diagnostic test kits [5]. Based on literature data on the structure and features of transport of antigens of M. tuberculosis MPT63 and MPT83 could be involved in molecular interactions between the pathogen and the host [6, 7]. It was shown that secretary protein of mycobacterium MPT63 was identified in the filtrate culture medium of M. tuberculosis, besides immature protein carrying NH_2-terminal peptide which is a signal to its secretion into the extracellular space [8]. Tertiary structure of MPT63 represented by two ant parallel β-sheets, arranged in immunoglobulin-like β-sandwich Along with such antigens M. tuberculosis, as CFP10 and ESAT-6, MPT63 also activate mast cells [9]. MPT83 is a lipoprotein, which is associated with cell wall of M. tuberculosis, an important class of ligands of TLR-2 [10]. It has been demonstrated that MPT83 induces the production of TNF-α, IL-6 [11] and affects the expression of matrix metalloproteinase [10, 12, and 13]. Even though until recently the main targets of mycobacterium considered macrophages, but other types of cells can be affected by them, and thus could play a significant role in the development of disease.

Control the progress of the infection, and, therefore, interact with pathogen could neutrophils [14], dendritic cells (DCs) [15], macrophages, even natural killer (NK) cells [1]. Moreover, in TB patients formation of granulomas could involve non-immune cells, such as fibroblasts and epithelial cells, which can also be target of mycobacterium antigens [16]. The aim of our work was to investigate the ability of antigens of M. tuberculosis MPT63 and MPT83 bind to cells of different origins and study the effect of these proteins in the activation of immune cells in vitro. During the implementation of work was investigated binding of fluorescently labeled derivatives of mycobacterium proteins MPT83 and MPT63 with number of lines of a target cells. We were investigated that MPT63 binds to cell surface of separate subpopulation of mouse spleen cells and U937 cell line, instead MPT83 that binds with heterogeneous macrophage-like U2149 cell line. We also established that MPT63 and MPT83 can specifically induce the activation of macrophages. Both MPT63 and MPT83 increasing the percentage of cells expressing markers of macrophage activation CD11b and F4/80, that additional information for obtained previously data concerning phagocytic activity.

MATERIALS AND METHODS

Mycobacterium tuberculosis MPT63 and MPT83 antigens and fluorescent protein mCherry gene amplification and cloning into plasmid DNA

Gene mcherry was amplified from plasmid DNA pmcherry using pair of oligonucleotides mcherry-asp CAGGAATTCGATTTGTACAGCTGTCCATGCC and mcherry-sp GAAGGATCCACCATGGTGAGCAAGGG. The product of amplification was inserting in pET28a for sites for restriction endonucleases BamHI and EcoRI. Genes encoded mpt83 and mpt63 amplified from pET24a plasmid DNA with embedded mpt63 and mpt83 sequences with a pair of primers being finished sites for restriction endonucleases EcoRI and XhoI (mpt83-sp TAGAATTCAACCAAACCCGTGTCGCAAG and mpt83-asp CTACTCGAGCTACTGTGCCGGGGGCATCAGCA for mpt83 and for mpt63 gene – mpt63-sp TAGAATTCGCTCACCACAATGATCAAGA and mpt63- asp CTACTCGAGCTACGGCTCCCAAATCAGCAGAT). The resulting PCR products and DNA-derived vector pET28a-mcherry treated with restriction enzymes EcoRI and XhoI. Vector fragment pET28a-mcherry and DNA sequence of mpt63 or mpt83 mixed in a molar ratio of 1: 3, and connected using T4 DNA ligase (Thermo Scientific, Lithuania). E.coli Rosetta cells were transformed by mixtures of plasmids construction pET28a-mcherry-mpt63 or pET28a-mcherry-mpt83.

Expression of mCherry-MPT63, mCherry-MPT83 and obtaining of fluorescent derivates with IMAC

Selected and verified clones were grown in LB medium contain 50 mg/ml kanamycin and 1% glucose at

37 °C. Target proteins were induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside 1mM) (Thermo Scientific, Lithuania) and incubated 4 h at 30 °C with intensive aeration. Fluorescent derivatives of M. tuberculosis antigens MPT63 and MPT83 were obtained by immobilized metal ion affinity chromatography (IMAC) on Ni-NTA agars (Qiagen, Germany) and were denatured in vitro step by step reducing of the concentration of urea (6, 4, 2, 1 and 0 M).

FITC labeling of M. tuberculosis proteins MPT63 and MPT83

Carried out purified recombinant proteins dialysis against carbonate buffer (0.16 M Na_2CO_3, 0, 33 M $NaHCO_3$, pH 9.5) overnight with one change of buffer. Antigens were concentrated with 10 kDa protein concentrators (Thermo Scientific, Lithuania) to a final concentration of about 1 mg/ml. Protein concentration tested densitometry. Prepared solutions of proteins mixed with a solution of FITC (Fluorescein isothiocyanate) in dimethyl sulfoxides (DMSO) (Synbias, Ukraine) (1 mg/ml) in a molar ratio of protein / FITC 1: 5, and incubated for 1 h at room temperature without light. Then conjugate (FITC-MPT63 or FITC-MPT83) dialyzed against buffer 1 mm Tris, 150 mm NaCl, pH 8,5; after dialysis was added NaN_3 0,1%.

SDS-PAGE and immune blotting assay

Protein fraction separation was performed in 10% polyacrylamide gels (PAGE), at a voltage of 10 V / cm at the denaturing condition.

Protein transfer to nitrocellulose membrane (Amersham Biosciences, United Kingdom) was performed using semi-dry Western blot in the buffer which contained 25 mm Tris-HCl, pH 8.3, 20% methanol, 192 mm glycine.

Membrane was blocked by 5% non-fat milk dissolved in PBS during 1 h at 37 °C. And incubated with anti-His-tag-HRP conjugate antibodies (1:3000). Bands have been detected by 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate.

Malignant and primary cell culture

Different origin cell lines were grown in RPMI-1640 medium with L-glutamine, 50mg/L cefalotin and with addition fetal bovine serum (FBS) to final concentration of 10% in cell culture flasks Greiner (Sartorius, Germany) and maintained at 37 ºC in a humidified incubator in 5% CO_2 atmosphere. Primary culture of peritoneal macrophages was obtained according to the methodology described in [17]. Spleen cells were obtained by mechanical rubbing of spleen and adhesion of cells to cultural flasks. Red blood cells, debris and connective tissue elements were remove by PBS. Adhesive cell lines were removed from cultural flasks with 30 mm EDTA in PBS at 15 min, 37 ºC and active mixing.

Flow cytometry. Intracellular and cell surface binding assay

For cell staining by mCherry-MPT63, mCherry-MPT83, MPT63-FITC and MPT83-FITC to 50 µl cell suspension (~ $5x10^5$ cells) were added 100 µl of buffer PBS-BSA-NaN_3 (0,8% NaCl, 0,02% HCl, 0,144% Na_2HPO_4, 0,024% KH_2PO_4, pH 7,4, 0,2% NaN_3, 2% bovine serum albumin(BSA)) containing the appropriate amount of proteins. Cells were incubated for 30 min at 4 °C, then washed twice by 1 ml of buffer PBS-BSA-NaN_3, responded in 1 ml of PBS-BSA-NaN_3 and measured fluorescence intensity using flow cytometry EPICS XL ("Beckman Coulter", USA).

For permeabilization cell precipitate were washed in BSA/PBS (0,14 M NaCl, 0,03 M KCl, 0,011 M Na_2HPO_4, 0,002 M KH_2PO_4, 1% BSA, pH 7,2). After centrifugation and sedimentation of cells supernatant was removed, and the cells were responded in 0, 25% paraformaldehyde solution in PBS (about 1 ml per 10^6 cells) aand incubated overnight at 4 °C. After fixing the cells polluted for 5 min 300 g and washed by PBS (0,14 M NaCl, 0,03 M HCl, 0,011 M Na_2HPO_4, 0,002 M KH_2PO_4, pH 7,2). Permeabilization carried out with the addition to PBS detergent Triton X-100 to a final concentration of 0, 1% and incubated for 15 min at 37 °C. The cells are then re-precipitated thoroughly washed from detergent and stained by FITC-labeled proteins or by antigens fused with red fluorescent protein mcherry at 4 °C for 30 min.

Rresearch of influencing of M. tuberculosis antigens on exposure of activation markers CD11b and F4/80

Recombinant MPT63, MPT83 and mcherry (control) were added to peritoneal macrophages obtained from mice to 0, 25 µM concentration and incubated for 24 h. Macrophages were removed from culture flasks with 10 mM EDTA in phosphate buffer during 10 min. To determine CD11b and F4/80 were used FITC-labeled monoclonal antibodies (Thermo Scientific). For labeling 50 µl cell suspension (~ $3x10^5$ cells) added 100 µl of buffer PBS-BSA-NaN_3 containing appropriate amounts of antibodies (according to the manufacturer). Stages and condition of immanency to chemistry were described above-mentioned.

Confocal microscopy

U-2149 cells were grown on glass slides to 80% confluence and washed twice with 1 ml BSA / PBS. Antigens was added in RPMI-1640 at the rate of 0, 25 µmol/ml. For nuclei staining using Hoechst 33342. The incubation was performed for 30 min at 4 °C and 5% CO2. Then the cells were washed twice in 1 ml BSA/PBS and add a solution of 0, 25% paraformaldehyde and incubated for 40 min at 4 °C. Then washed twice unbound paraformaldehyde solution by PBS and last wash by distilled water. On glass were applied 25 µl solution MOVIOL/Dabco and fixed glass with cells on the slide glass. Preparations were analyzed by confocal microscopy 510 Carl Zeiss LSM Meta.

RESULTS AND DISCUSSION

Obtaining fluorescent derivates of mycobacterium antigens fused with mCherry

The expression of fluorescent proteins as part of a single open reading frame with investigates protein is a common method of labeling that has a number of significant advantages over chemical protein modification. For our purpose was used fluorescent protein cherry.

The gene encoded mCherry was amplified by PCR and cloned by EcoRI and BamHI endonuclease restriction sites in pET28a expression vector (Fig. 1, A). Mycobacterial genes mpt63 and mpt83 (Fig. 1, B) were inserted by the restriction sites EcoRI and XhoI into pET28a-mCherry construction. Primers were selected so that the stop cordon finished the open reading frame before XhoI site, leaving only the N-terminal 6xHis tag.

E.coli Rosetta (DE3) (Novagen, Germany) cells were transformed by the resulting constructs pET28a-mCherry-mpt63 or pET28a-mCherry-mpt83 using electroporation (1400 V, pulse width ~ 4.9 ms). Cells were grown on agar LB medium and incubated at 37 °C. The obtained clones were checked for the correct insert by PCR (use pET-oligos pair).The expected length of the amplified fragments should be 1500 bp and 1600 bp for mCherry-MPT63 and mCherry-MPT83 respectively (Fig. 1, C). The vector containing the sequence only of fluorescent protein gives 1000 bp fragment so obtained products could be detect and distinguished by researchers.

Elate fractions were analyzed electro phonetically in 10% SDS-PAAG (Fig. 2, A). Also, identification of proteins was performed using anti-His tag antibodies which bound with the 6xHis tag of target proteins (Fig. 2, B).

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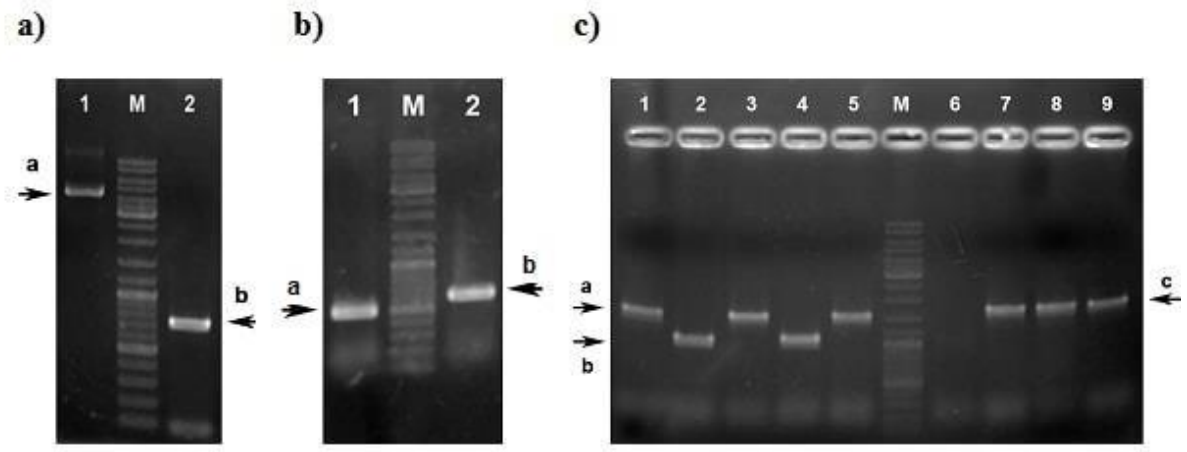

Fig 1: A. Electrophoregram of obtained pDNA pET28a (a) and mcherry gene (b). B. Results of mpt63 (a) and mpt83 (b) genes amplification. C. Electrophoregram of PCR analysis of selected clones: with mCherry gene (lanes 2, 4); mCherry-mpt63 (lanes 1, 3, 5) and mCherry-mpt83 (lanes 7, 8, 9). In verified clones by PCR electrophoregram the arrows indicated amplicon weight of about 1500 bp (a), 1600 bp (c) and 1000 bp (b).

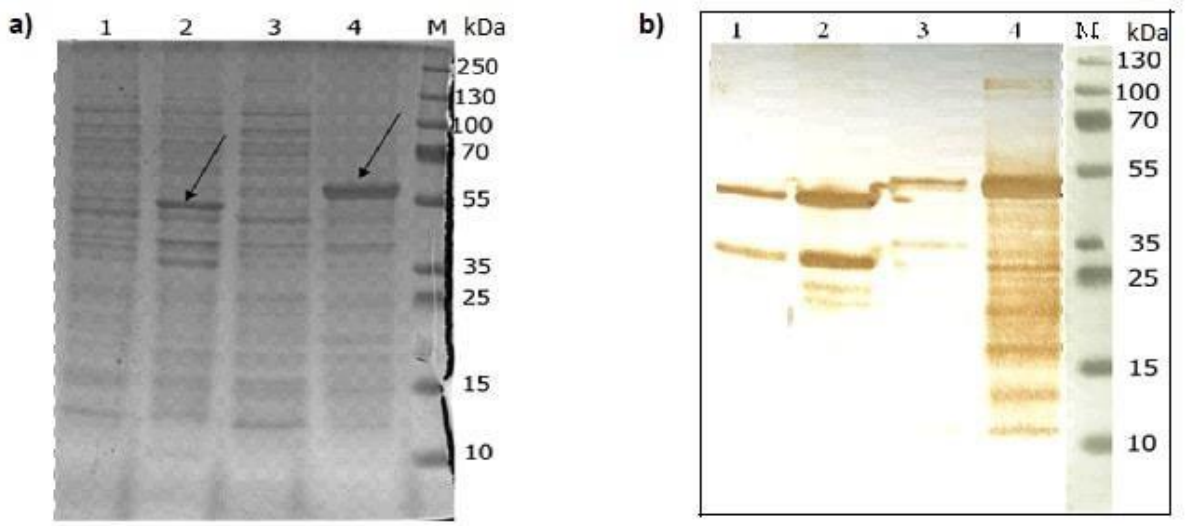

Fig 2: A. Electrophoregram of obtained mCherry-MPT63 (lane 1 - soluble fraction, lane 2 - insoluble) and mCherry-MPT83 (lane 3 - soluble protein fraction, lane 4 - insoluble). B. Western blot assay by anti-His tag conjugate. The majority of the proteins identified in the insoluble fractions (lanes 2 and 4). M - Molecular weight markers. The arrows indicated target protein mCherry-MPT63 and mCherry-MPT83.

Investigation of binding fluorescently labelled derivatives of MPT63 with different origin host cells

MPT63 is a secretary protein of Mycobacterium with unknown function yet. His alleged targets can be both immune cells and cells of other tissues and organs. As a cell models in this work has been used a number of cell lines: U937 – monocyte cells isolated from the histiocytic lymphoma; KG-1 cell line derived from a patient with bone marrow myeloid leukemia; A431 and Vero cell lines with epithelial origin derived from human adenocarcinoma and African green monkey kidney respectively; murine fibroblasts 3T3 and L929; U-2149, which were established from malignant fibrous histiocytoma, its heterogeneous cell line that presented by maprophases-like cell and fibroblasts; X63 - mouse myeloma cells and primary cultures.

Since macrophages are the main target of Mycobacterium tuberculosis, we could assume that they are the target for MPT63. We have been identified binding of MPT63 with a small subpopulation of cells U937

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(Fig. 3, A), which allows to assume that the target of MPT63 could be separate subpopulation of monocyte-derived cells. Therefore, we investigated the binding of mycobacterium proteins with a number of cell lines that are monocyte or macrophages-like cells and murine peritoneal macrophages. However, neither of murine peritoneal macrophages nor not with other cells significant binding could not be found. Based on the fact that there is a possible failures of functional characteristics in the process of accession labels to MPT63 was used two fluorescent derivatives of MPT63, a way to get them fundamentally different: fusion with cherry (MPT63 located at the C-terminus) and conjugation with FITC (at the acceding to ε-amino groups of Lys). However, in both cases, the result was similar (Fig. 3, B, C).

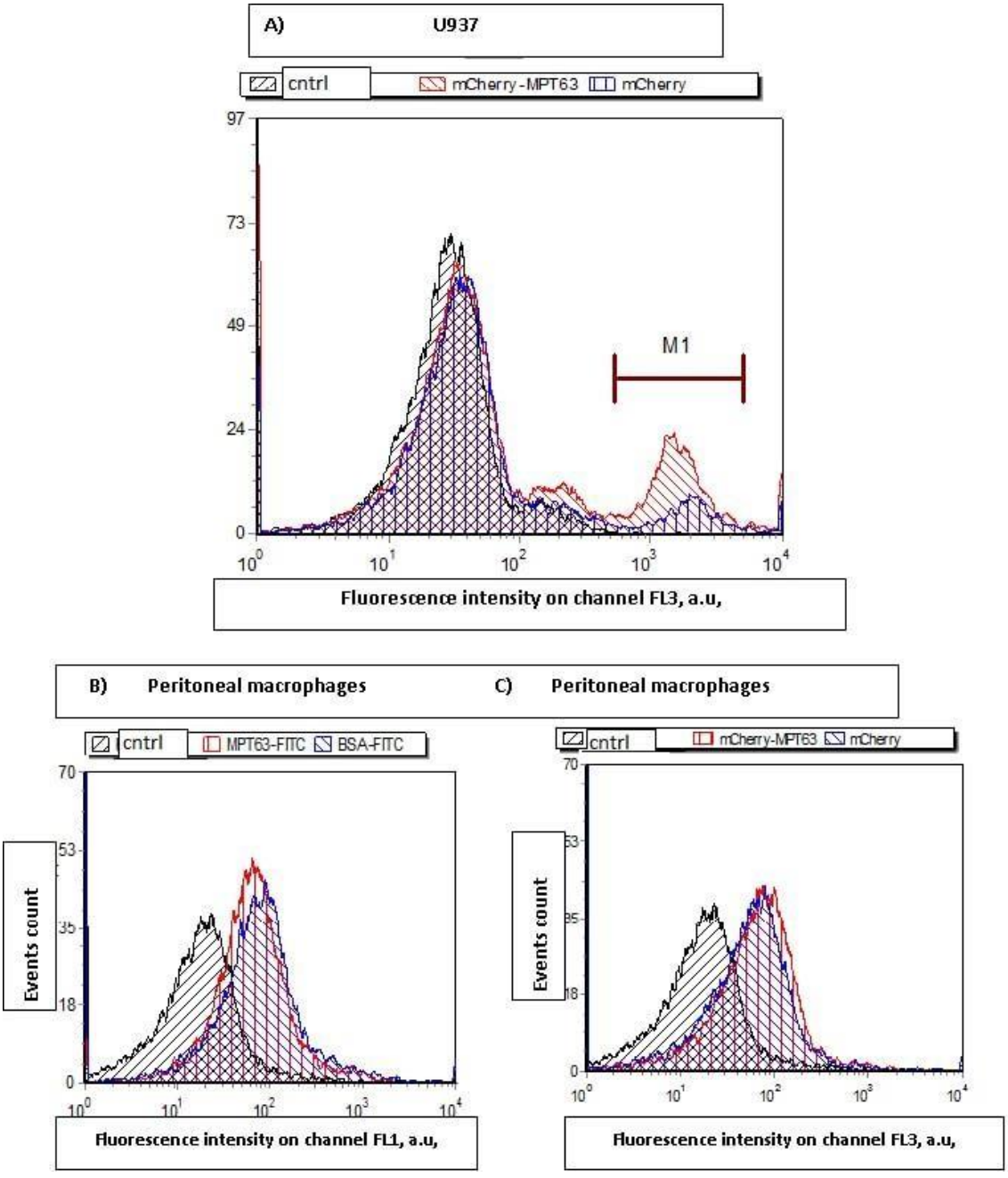

Fig 3: Comparative histograms of fluorescence intensity of U937 cells (A) and peritoneal macrophages (B, C), which were stained with recombinant fluorescent proteins (mCherry-MPT63, cherry), FITC-labeling proteins (MPT63-FITC, BSA-FITC) or were unstained (control).

Thus, none of the models used to detect the binding failed, we repeated the experiment with total mouse spleen cells (Fig. 4, A). In this case, very clearly identified subpopulation that stained with MPT63.

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However, in the spleen are various cells, including lymphocytes, macrophages, and dendritic cells at various stages of differentiation and stoma cells that do not belong to the immune system. Therefore, we also investigated the binding of MPT63 with cell lines that are models of lymphocytes, fibroblasts and epithelial cells. But with 3T3 cells lines that represent fibroblasts and A431 that represent epithelial cells significant binding was not detected (Fig. 4, C, D).Likewise, the binding was not observed with X63 cells that represent lymphocytes (Fig. 4, B).

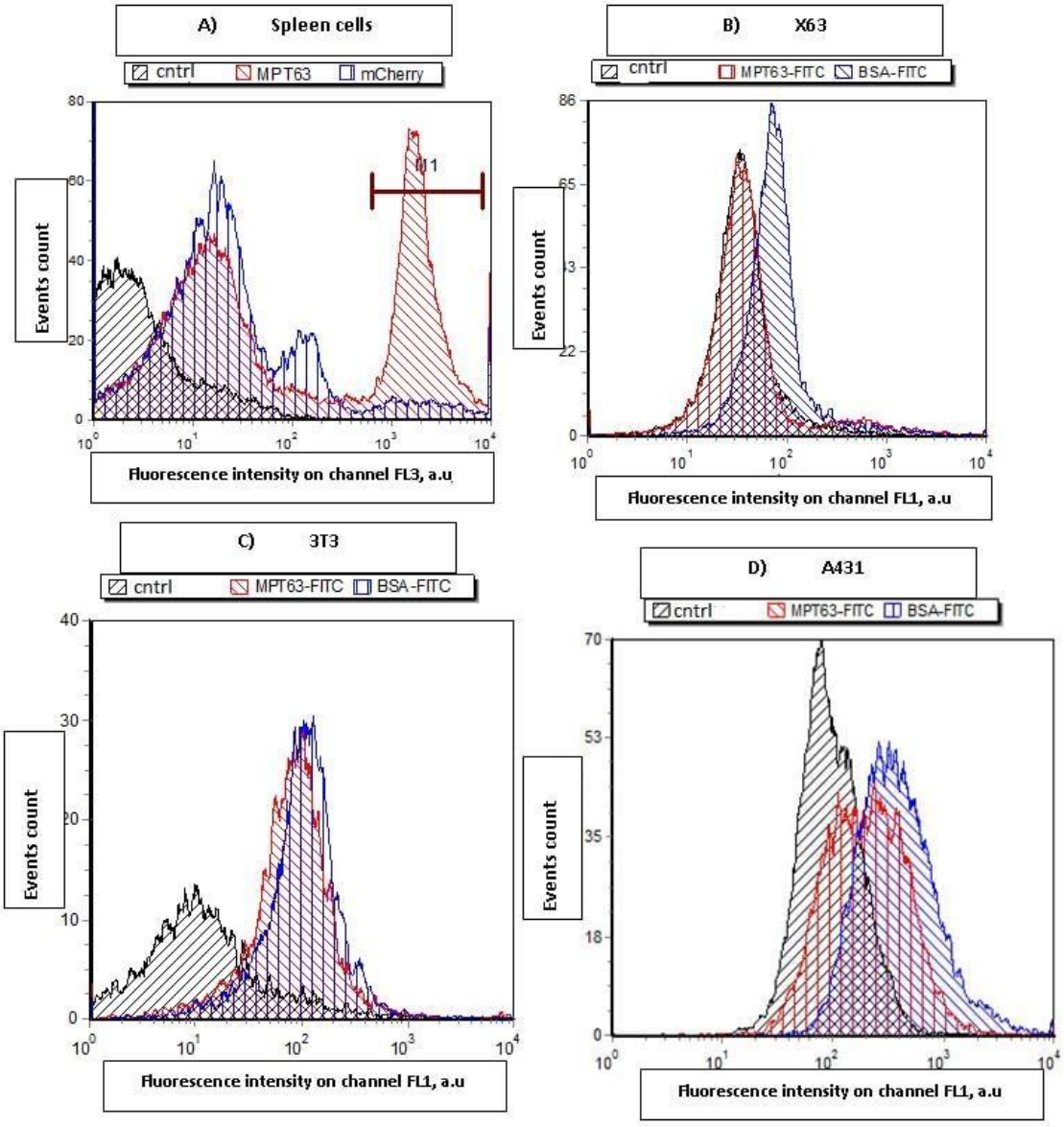

Fig 4: Comparative histograms of fluorescence intensity of common population of spleen cells (A), X63 cell line (B), 3T3 cells (C) and epithelial A431 cell lines (D) which were stained with recombinant fluorescent proteins (mCherry-MPT63, cherry), FITC-labeling proteins (MPT63-FITC, BSA-FITC) or were unstained (control).

Thus, according to the obtained data MPT63 was bond with a certain population of cells of the spleen and a small subpopulation of U937 cells, but the binding of the cells, which is a model of fibroblasts, epithelial and lymphocytes was not fixed. Just cannot detect the binding of the studied monocyte- and macrophages-like cell lines. Therefore, the target cells could be not studied populations (e.g. dendritic cells) or macrophages at a certain stage of differentiation (for which indirectly indicates a small part of the binding of U937 cells).

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In addition, recently Mustafa and colleagues [18] had shown that MPT63 as some other secretary proteins of mycobacterium can be found in inflammatory cells in the lungs of TB patients. Obviously, these proteins accumulate in infected cells as a result of life Mycobacterium. However, it is probable that MPT63 may also have intracellular target by acting directly on the infected cells. Therefore, we also tested the binding of MPT63 on permeabilized macrophages to investigate the possible presence of intracellular targets. However, as seen in Fig. 5, A, differences in binding of MPT63 and control antigen was not observed. During the labeling of permeabilized macrophages in inner cells space under the microscope were notable characteristic structure, but these structures were found in both samples stained by MPT63 and control samples of intact cells (Fig. 5, B, C).

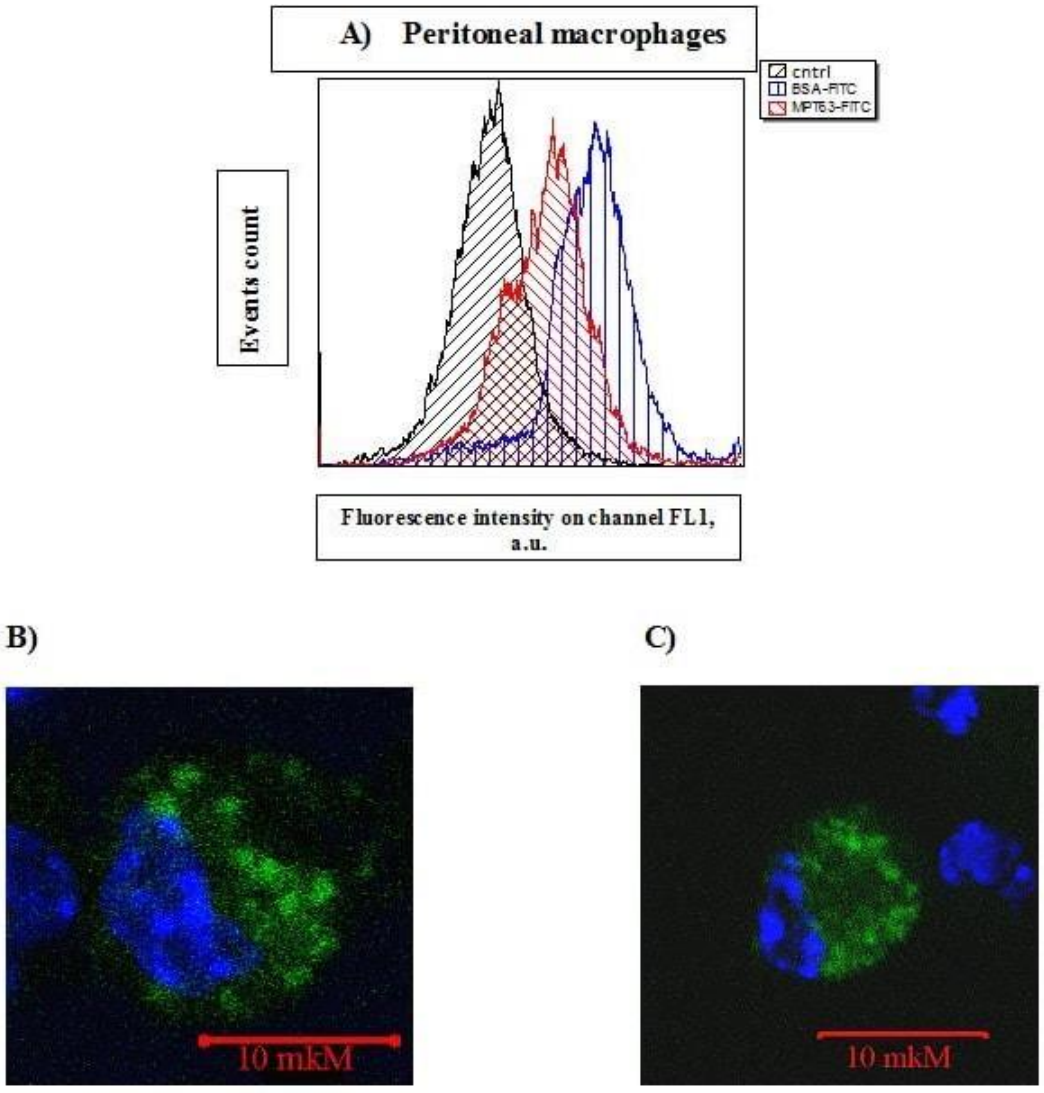

Fig 5: A. Search of potential inner cell targets for MPT63 used as a model peritoneal macrophages. B, C. Confocal microscopy of potential inner cell targets for MPT63: B - peritoneal macrophages labeling with MPT63- FITC, C - intact (unstained) peritoneal macrophages

Investigation of binding fluorescently labelled derivatives of MPT83 with heterogenic cell line U2149

Aattempts to registrant binding of MPT83 with peritoneal macrophages or cell line U937, which shows the expression of TLR2 [19] failed. Obviously, this is due to low expression of TLR2. MPT83 binding with target cells has been demonstrated only in HEK293 cells transected with TLR2 [11]. But we found that MPT83

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binding to the surface of U2149 cells (Fig. 6, A). Results obtained by flow cytometry also been confirmed help by microscopic pictures: MPT83 specifically bind to the surface of cells (Fig. 6, B).

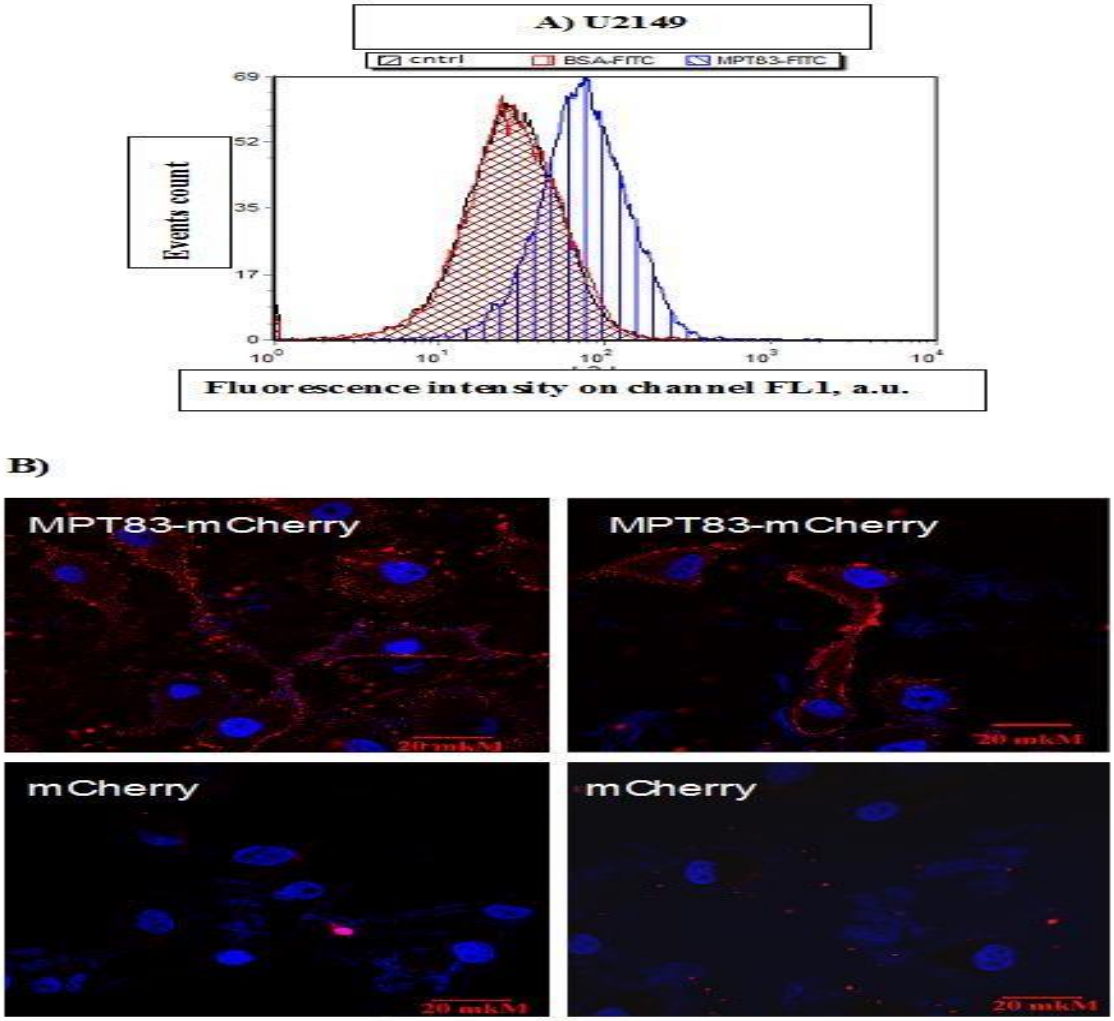

Fig 6: A. Fluorescence intensity of U2149 macrophage-like cells using FITC-MPT83. B. Confocal microscopy of U2149 cell stained with mCherry (control) and MPT83 fused with mCherry.

U2149 cell line was described Greenberg et al in 1989 [20]. In particular, they had shown that it includes a macrophage-like- and fibroblast cells. MPT83 not bind with a separate population within the line as we would expect. Overall, TLR2 is not considered as a one possible target for MPT83. In particular, the TLR2 knockout murine macrophages by stimulation with MPT83 observed a significant increase in TNF-a, IL-6, and IL-12, although lower than for wild-type macrophages of mice [11]. So, MPT83 mechanisms of interaction with host cells are not fully understood. The fact that cell lines U2149 so strongly bind with MPT83 makes it very attractive model for further studies of the mechanisms of those antigen of Mycobacterium.

Long-term influence of MPT63 and MPT83 on expression of cell surface activation markers of immune cells

CD11b (β-chain of Mac-1, also known as receptor complement CR3) is integrins, which in large quantities represented on activated macrophages and dendritic cells, and directly involved in the induction of cellular immune responses, in particular the processes of phagocytosis, extracellular cytotoxicity and migration [21-23]. Furthermore it is known that CR3 has an important role in the capture of mycobacterium by macrophages both antibody-dependent and by antibody-independent mechanism [24].

Since the results of the study MPT63 interaction with phagocytic cells allowed to assume that this interaction occurs, we explored how induction MPT63 influence on the expression of CD11b. To do this, as a

model were used peritoneal mouse macrophages. It was found that after incubation with MPT63 within 24 h the percentage of cells with high expression of CD11b increased. As a control protein used cherry, which is the same as MPT63 was expressed in E.coli cells, and not least, was isolated and purified according to the same protocol. Both proteins are used in concentrations of 0.25 mM, and the amount of protein that was added was about the same. This allowed us to test the possible effect of different bacterial contaminants that could get into the sample during the protein obtaining. However, in three independent experiments pre-incubation with MPT63 increased the percentage of cells with high expression of CD11b, and cells which were pre-incubation with cherry little different from intact (Fig. 7). However, if the incubation time was reduced to 30 min and 8 h the effect is not observed. Changes in the exposure of CD11b on the surface may occur within a few minutes after stimulation [25], which is due to intracellular pool. Whereas after stimulation by MPT63 to change the level of representation of CD11b was required time, we suggest the process is likely due to changes in gene expression of CD11b.

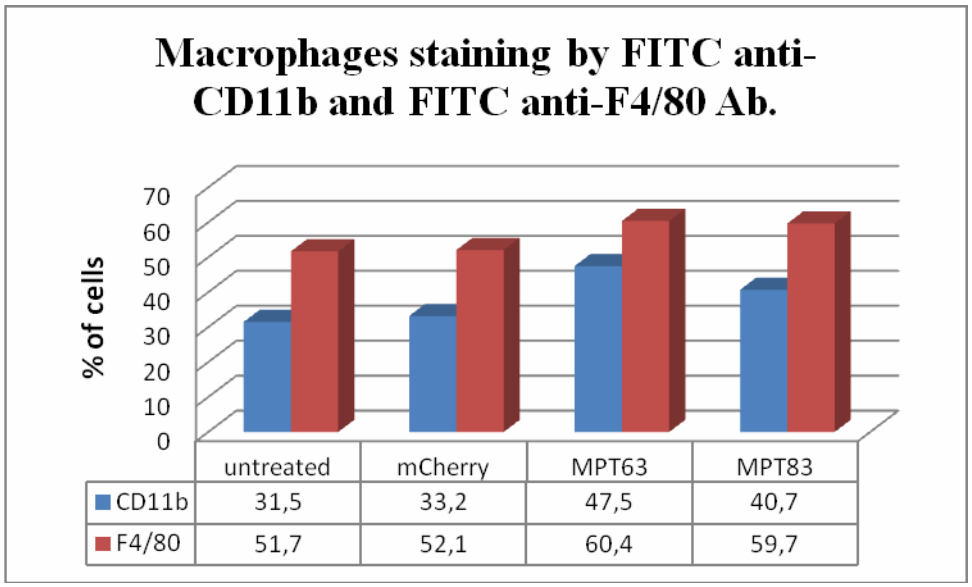

Fig 7: Comparison chart affect of mycobacterium proteins to the exposure of activation markers of mature mouse macrophages CD11b and F4/80.

An analogous experiment was conducted with MPT83 and we were obtained essentially similar results too. Just as in the case MPT63 and MPT83 of incubation for 30 min or 8 h the effect is not observed. Although after incubation with MPT63 level of cells with high expression of CD11b was higher than after incubation with MPT83, in general, likely in both cases increased expression of CD11b is a consequence of activation of macrophages caused by stimulation MPT63 and MPT83.

Further, we investigated that stimulation with MPT63 and MPT83 lead to increase the percentage of cells with high expression of F4/80 (Fig. 7) which is a marker of mature macrophages.

Thus, both MPT83 and MPT63 stimulated macrophages activation. For MPT83 this effect is fully predictable and can be explained by stimulation through interaction with TLR2. However, the results obtained shows that MPT63 just able to specifically induce macrophage activation too and, therefore, there must be a specific mechanism of MPT63 effect on macrophage cells.

CONCLUSIONS

1. It was obtained clones-producers of fluorescent derived mycobacterium proteins MPT63 and MPT83 fused with mcherry.
2. It was shown that about 16% of the target proteins are synthesized in soluble form, and 84% - in the form of inclusion bodies. The percentage of recombinant protein of total protein pool of E.coli was 33.8% for mCherry-MPT83 and 28.3% for mCherry-MPT63. The yield of mCherry-MPT83 was 75 mg per 1 liter of E.coli culture and mCherry-MPT63 - 36 mg.

3. We found that MPT63 specifically binds to subpopulation of histiocytic lymphoma human cell line U937 are likely to represent a population of cells differentiated aside macrophage phenotype.
4. It is shown that MPT63 capable of binding to mouse spleen cells, characterized by a high level of granularity and probably belonging to a particular subpopulation of macrophages.
5. Used flow cytometry and confocal microscopy methods we showed that the protein of M. tuberculosis MPT83 binds to the surface of macrophage-like cells of U2149 cell line.
6. We registered an increase the percentage of cells of peritoneal macrophages from mice that expressed markers of macrophage activation after MPT63 or MPT83 long-term treatment.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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