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Research paper

A flow cytometric approach for studying alterations in the cytoplasmic concentration of calcium ions in immune cells following stimulation with thymic peptides

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ABSTRACT

$[Ca^{2+}]_i$ alterations are vital in signaling pathways of cell activation. We tried to detect such changes, in intracellular signaling pathways downstream TLR4 in immune cells, following stimulation with prothymosin alpha (proTα) and its decapeptide proTα(100–109). Human leukocytes were activated with LPS, proTα or proTα(100–109), directly or after 24 h stimulation, while neutrophils were directly challenged. Cells were loaded with Fluo-4 and cytoplasmic Ca^{2+} alterations were recorded by flow cytometry. Direct challenge with 20 μg/mL LPS induced a measurable $[Ca^{2+}]_i$ increase in macrophages and neutrophils. Monocytes and macrophages incubated for 24 h with LPS, proTα or proTα(100–109) and challenged with LPS, displayed a robust response. Lymphocytes and iDCs exhibited no alterations. Conclusively, we assessed a flow cytometry-based method for monitoring Ca^{2+} ion influx changes in immune cells. Their stimulation with proTα or proTα(100–109) generates an activating background, similar to LPS, allowing for the detection of $[Ca^{2+}]_i$ alterations induced upon subsequent challenge.

1. Introduction

The cytoplasmic concentration of calcium ions ($[Ca^{2+}]_i$) is altered when signal transduction pathways following activation of immune cells are triggered, promoting cellular processes such as oxidative burst, cytokine production, proliferation and phagocytosis [1]. To date, many methods are used to record these alterations, including confocal microscopy, spectrofluorometry and flow cytometry [2].

The principle pathway that stimulates $[Ca^{2+}]_i$ influx following cell-surface receptor triggering is initiated upon activation of phosphoinositide-specific phosphatase C (PLC), which subsequently hydrolyses phosphatidylinositol 4,5-bisphosphatate, generating inositol 1,4,5-trisphosphatate (IP_3) and diacylglycerol. IP_3 opens the endoplasmic reticulum (ER) Ca^{2+} channels, releasing Ca^{2+} in the cytoplasm. Moreover, upon Ca^{2+} depletion in the ER, the Ca^{2+} sensor stromal interaction molecule 1 (STIM1) oligomerizes and translocates to the plasma membrane, where it activates the Ca^{2+}-release-activated Ca^{2+} modulator 1 (Orai1) protein, the pore forming subunit of Ca^{2+}-release-activated Ca^{2+} channels (CRACs) on the cell membrane. In this way, $[Ca^{2+}]_i$ increase is further supplemented by Ca^{2+} entry from the extracellular space. Restoration of $[Ca^{2+}]_i$ to resting levels is mediated by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and the plasma membrane Ca^{2+} ATPase [3,4].

The PLC pathway can be activated by G-protein coupled receptors, as well as by ligand-induced endocytosis of immune receptors, such as Toll-like receptors (TLRs). For example, it has been reported that PLC activation, followed by $[Ca^{2+}]_i$ increase, mediates the translocation of the lipopolysaccharide (LPS)/CD14/TLR4 complex from the cell membrane to the endosomal network, stimulating the TIR-domain-containing adapter-inducing interferon-β

Abbreviations: APC–Cy7, allophycocyanin–cyanine 7 tandem dye; $[Ca^{2+}]_i$, cytoplasmic concentration of calcium ions; CRACs, Ca^{2+}-release-activated Ca^{2+} channels; FITC, fluorescein isothiocyanate; Fluo-4-AM, Fluo-4-acetoxymethyl ester; fMLP, N-formyl-methionine–leucine–phenylalanine; iDCs, immature dendritic cells; IP_3, inositol 1,4,5-trisphosphatate; LPS, lipopolysaccharide; MFI, mean/median fluorescence intensity; MyD88, myeloid differentiation primary response gene 88; Orai1, Ca^{2+}-release-activated Ca^{2+} modulator 1; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; PLC, phosphoinositide-specific phosphatase C; proTα, prothymosin alpha; proTα(100–109), carboxy-terminal decapeptide of proTα; rhGM-CSF, recombinant human granulocyte-macrophage colony-stimulating factor; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; STIM1, Ca^{2+} sensor stromal interaction molecule 1; TLR4, Toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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(TRIF)-dependent pathway [5–7]. Interestingly, TLR4 does not only recognize pathogen-associated molecular patterns, such as LPS, but also endogenous molecules acting as damage-associated molecular patterns (DAMPs), like high-mobility group protein 1 and prothymosin alpha (proTα) [8].

ProTα is a 109 amino acid long polypeptide in man, which exhibits two distinct roles depending on its localization. Inside living cells, it modulates the cell cycle, promotes proliferation and inhibits apoptosis. Extracellularly, it exerts pleiotropic immunomodulatory effects acting as DAMP, mainly *via* its immunoreactive C-terminal decapeptide proTα(100–109) [9,10]. It has been shown that proTα and proTα(100–109) bind TLR4 and induce the activation of both the myeloid differentiation primary response gene 88 (MyD88)- and TRIF-dependent pathways [11,12]. Moreover, they enhance the antigen-presenting potential of and stimulate cytokine production and release by dendritic cells (DCs) and monocytes, and induce reactive oxygen species (ROS) production by neutrophils [12,13].

In this study, we used flow cytometry to investigate whether the activation of specific immune subpopulations by proTα and proTα (100–109) is accompanied by analogous changes in $[Ca^{2+}]_i$. We also assessed whether the TLR4-mediated effect of these thymic peptides is comparable to that of the prototype TLR4 ligand, LPS.

2. Material and methods

2.1. Isolation of PBMCs and neutrophils

Buffy coats or peripheral blood was withdrawn from healthy donors. Prior to blood draw, individuals gave their informed consent according to the regulations approved by the 2nd Peripheral Blood Transfusion Unit and Hemophilia Centre, 'Laikon' General Hospital Institutional Review Board, Athens, Greece. Samples were centrifuged over Ficoll-Histopaque (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) density gradient and peripheral blood mononuclear cells (PBMCs) were isolated and washed twice with Hank's Balanced Salt Solution (HBSS; Lonza, Cologne, Germany). Neutrophils were isolated from the erythrocyte pellet, according to [13]. PBMCs and neutrophils were resuspended in complete medium, consisting of RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM Hepes, 5 μg/mL gentamycin, 100 IU/mL penicillin and 100 IU/mL streptomycin (all from Lonza) and incubated at 37 °C, in a humidified 5% CO_2 incubator. PBMCs were also cryopreserved in FBS-10% DMSO (Sigma-Aldrich) for later use.

2.2. Purification of $CD14^+$ cells

$CD14^+$ cells were separated by indirect magnetic-bead sorting using the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Briefly, PBMCs were first incubated with FcR blocking reagent for human immunoglobulins (Igs) and a cocktail of biotin-conjugated monoclonal antibodies (mAbs) against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A, and then, with microbeads conjugated to anti-biotin mAb. $CD14^+$ cells were negatively selected by separation over a magnetic column (MACS® Separation LS Columns, Miltenyi Biotec) placed in a suitable magnetic field (MACS® Separator, Miltenyi Biotec). Cell purity was assessed by flow cytometry.

2.3. Differentiation of monocytes to macrophages and immature dendritic cells

Monocytes (>90% $CD14^+$) isolated as aforementioned (Section 2.2) were cultured in six-well plates (Greiner, Bio-One, Kirchheim, Germany) at a density of $0.5–1 \times 10^6$/mL (3 mL/well) complete medium for 5 days. For differentiation to immature DCs (iDCs), 800 IU/mL recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) and 500 IU/mL rh interleukin (IL)-4 (both from R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) were added every other day and iDCs were collected on day 5 [12]. For differentiation to macrophages, 960 IU/mL rhGM-CSF were added on days 0, 2 and 4 of culture and macrophages were collected on day 5 [12]. Differentiation of monocytes to iDCs and macrophages was verified by flow cytometry.

2.4. Cell stimulation for Ca^{2+} influx determination

Following dose and time kinetic studies ([12,13] and data not shown), PBMCs, monocytes, monocyte-derived macrophages and iDCs were stimulated for 24 h at 37 °C with 1 μg/mL LPS (Sigma Chemical Co; TLR ligand-tested), 200 ng/mL proTα or 25 ng/mL proTα(100–109) (isolated and synthesized, respectively as described in [14]). Neutrophils were not stimulated, due to their short lifespan in culture. Following staining with Fluo-4-acetoxymethyl ester (Fluo-4-AM; Molecular Probes, Life Technologies GmbH Darmstadt Germany), cells were challenged with the ionophore A23187 (2 μM; Life Technologies) as a positive Ca^{2+} influx stimulus, LPS as a model ligand of TLR4, proTα or proTα (100–109). Neutrophils were additionally challenged with the chemotactic peptide N-formyl-methionine–leucine–phenylalanine (fMLP; Sigma) (Table 1). All cell populations were analyzed by flow cytometry.

2.5. Flow cytometry analysis

Purity of magnetically-sorted $CD14^+$ cells was tested by staining with saturating concentration of anti-human IgG1 anti-CD14-fluorescein isothiocyanate (FITC) mAb (BioLegend Inc., CA, USA) for 30 min on ice. The phenotype of macrophages, iDCs and neutrophils was verified upon staining with mAbs for CD206, CD86 and CD11b, respectively. Staining was performed using the anti-human IgG1 mAbs anti-CD206-allophycocyanin–cyanine 7 tandem dye (APC–Cy7) (BioLegend), anti-CD86-FITC and anti-CD11b-phycoerythrin (PE) (both from BD Pharmingen, Franklin Lakes, NJ, USA) at saturating concentrations for 30 min on ice. As isotype controls, anti-human IgG1 mAbs (BD Biosciences), labeled with the same fluorochromes were used. Fluorescence was measured in a BD FACSCanto II flow cytometer and data analysis was conducted using FACSDiva software. For detecting fluorescence intensity, 530/30(FITC), 585/42(PE) and 780/60(APC–Cy7) filters were used.

To monitor alterations in $[Ca^{2+}]_i$, harvested cells were washed, re-suspended at $0.5–1 \times 10^6$ cells/mL in HBSS supplemented with 10% FBS, and 1 mL suspensions were distributed into FACS tubes (BD Biosciences). Cells were incubated with 5 μM Fluo-4-AM for 60 min at 37 °C, washed, re-suspended in HBSS and incubated for 30 min at 37 °C, so as to allow intracellular esterases to cleave the AM of Fluo-4, generating the Ca^{2+}-sensitive form of the dye. For each sample, we recorded baseline Ca^{2+} levels (referred to as background) for 30 s. We then challenged the cells with the ionophore A23187 (2 μM), LPS (10 ng/mL to 40 μg/mL), proTα (200 ng/mL) or proTα(100–109) (25 ng/mL). Neutrophils were also challenged with fMLP (1 μM). Analysis was conducted using FACSDiva software in dot-plots of Fluo-4 fluorescence intensity over time. Gates were set in forward scattered light (FSC) and side scattered light (SSC) dot-plots, to exclude non-viable or disrupted cells. Further gates were set in PBMCs to analyze distinct subpopulations. For the analysis of samples challenged with the ionophore A23187, Fluo-4 fluorescence intensity was depicted on a logarithmic scale, whereas in all other sample groups, on a linear scale. Generated graphs are based on Fluo-4 Mean/Median Fluorescence