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Mycobacterial antigen(s) induce anergy by altering TCR- and TCR/CD28-induced signalling events: Insights into T-cell unresponsiveness in leprosy

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ABSTRACT

Present study investigates the role of *Mycobacterium leprae* (*M. leprae*) antigens on TCR- and TCR/CD28-induced signalling leading to T-cell activation and further correlates these early biochemical events with T-cell anergy, as prevailed in advanced stages of leprosy. We observed that both whole cell lystae (WCL) and soluble fraction of *M. leprae* sonicate (MLSA) not only inhibited TCR, thapsigargin and ionomycin induced calcium fluxes by diminishing the opening of calcium channels, but also TCR- or TCR/CD28-induced proximal signalling events like phosphorylation of Zap-70 and protein kinase-C (PKC) activity. Study of TCR- and TCR/CD28-induced downstream signals revealed that *M. leprae* antigens curtail phosphorylation of both Erk1/2 and p38MAPK, consequently altering terminal signalling events like reduced binding of NFAT on IL-2 promoter and transcription of IL-2 gene, diminished expression of activation markers (CD25 and CD69). Furthermore, *M. leprae* fractions significantly inhibited IL-2 secretion and T-cell blastogenesis in healthy individuals. Altogether, results suggest that *M. leprae* interferes with TCR/CD28-induced upstream as well as downstream signalling events resulting in reduced IL-2 production and thus inhibition in T-cell proliferation, which might be responsible for T-cell unresponsiveness leading to stage of immunosuppression and consequently, for the progression of disease.

1. Introduction

Leprosy, a neurodegenerative disease caused by obligate intracellular pathogen *Mycobacterium leprae* (*M. leprae*), exhibits clinical spectrum which is the outcome of humoral immunity and/or cell-mediated immune (CMI) response of host to infection. Individuals with tuberculoid type (TT) of leprosy, an early stage, manifest a strong delayed type of hypersensitivity (DTH) to *M. leprae* antigens but produce relatively low levels of antibody. However, lepromatous leprosy (LL) patients, the advance stage, show humoral antibody response but a progressive anergy in CMI to *M. leprae* antigens (Nath and Singh, 1980; Kaplan and Cohn, 1985). T-cells are important regulators of immune responses against intracellular pathogens hence play an important role in leprosy also.

T-cell anergy is defined as a state in which a cell is alive but fails to display certain functional responses when optimally stimulated through TCR/CD28 receptors (Crabtree, 1989). Anergic T-cells exhibit reduced IL-2 production or IL-2Rα expression or both. IL-2 is regulated at promoter level by transcription factors triggered by a cascade of signalling events, initiated by both TCR and CD28 (Schwartz, 1996). Anergic T-cell clones are reported to have multiple alterations in TCR-associated signalling (Gajewski et al., 1994). *In vivo* anergised $CD4^+$ T-cell clones show perturbed binding affinity for transcription factors to IL-2 promoter (Sundstedt et al., 1996). Altogether these reports suggest that anergy in T-cells could be due to alterations in cell signalling events, involved in IL-2 production and may be the main cause of perturbed cell cycle progression. In leprosy patients, reduced IL-2 production has been responsible for the loss of T-cell function which could be reversed by the addition of exogenous IL-2 in some cases (Mohagheghpour et al., 1985). This anergic state prevailed in LL subjects could be mainly due to defective signalling leading to improper T-cell activation and IL-2 production.

Reduced T-cell proliferation and IFN-γ production both, in healthy individuals as well as leprosy patients, are correlated with

Abbreviations: IL-2, interleukin 2; MLSA, *M. leprae* soluble antigens; WCL, whole cell lysate of *M.* leprae; $[Ca^{2+}]_i$, free intracellular calcium concentrations.

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presence of lipid derivatives of bacilli (Koster et al., 1987; Molloy et al., 1990). Furthermore, using phenolic glycolipid-1 antigen of leprosy bacilli, it was found that cytosolic levels of calcium, IP_3 and PKC activity was marginally increased in TT patients while LL cases showed no effect (Sharma et al., 1998). Furthermore, decreased expression of T-cell receptor zeta chain, p56lck tyrosine kinase, loss of nuclear transcription factor NF-κB and loss of Th1 DNA-binding pattern in the interferon gamma core promoter region was found in T-cells of LL cases (Zea et al., 1998). In addition, we have shown that MLSA modulates TCR-mediated calcium fluxes through store operated calcium channels, which were correlated with reduced IL-2 production (Joshi et al., 2006). Recently, reversal of T-cell anergy in LL patients was shown by presenting the *M. leprae* cell wall antigens (MLCwA) in combination with T-cell adjuvant, murabutide (active analog of muramyl' dipeptide, MDP-BE) and a Trat peptide (T-cell epitope of integral membrane protein (Trat) from *Escherichia coli*) in liposomes resulting in increased secondary messengers activity and IL-2 production (Chattree et al., 2007).

Although very few reports of alterations in signalling molecules are available so far, however, the mechanism of action of *M. leprae* on TCR/CD28-mediated signalling have not been addressed till date and hence need further investigation. Since under *in vivo* conditions, host is exposed to different antigenic components of the bacilli, it was thought worthwhile, in continuation with our report (Joshi et al., 2006) to undertake the present study to elucidate the effects of WCL and MLSA on TCR/CD28-triggered signalling which could be involved in T-cell anergy during the progression of the disease. We focused our study on Zap70 and PKC activation, calcium fluxes, activation of MAPKs, binding of NFAT on IL-2 promoter and transcription of IL-2, expression of activation markers CD25/CD69 implicated in T-cell activation. Furthermore, these findings were correlated with IL-2 production and T-cell proliferation in TCR/CD28-stimulated T-cells of healthy individuals in presence or absence of *M. leprae* antigens.

2. Materials and methods

Study was performed on Jurkat T-cell leukemic line (E6.1) (National Cell Repository, Pune, India) and peripheral blood lymphocytes (PBLs) isolated from buffy coats obtained from healthy blood donors, after their informed consent, by density centrifugation over Ficoll-HyPaque (Sigma, St. Loius, MO, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Hyclone, Utah, USA) with 2 mM L-glutamine and 1× antibiotic–antimycotic cocktail (Sigma, St. Loius, MO, USA). Cultures were maintained with 5% CO_2 at 37 °C in a humidified chamber. Experiments were performed with cell viability ≥95% as determined by Trypan blue exclusion test.

2.1. Chemicals and antigens

Phorbol 12-myristate 13-acetate (PMA), ionomycin, phytohaemagglutinin (PHA), goat anti-mouse-IgG (GAM), phenylmethylsulphonyl fluoride (PMSF), sodium orthovanadate, anti-protease cocktail and Bradford reagent were purchased from Sigma, St. Louis, MO, USA. Fura-2/AM was bought from Calbiochem, La Jolla, USA. Anti-human CD3 (clone HIT3-α), anti-human CD28 (clone CD28.2), anti-human CD25Cy5PE, anti-human CD69FITC were procured from BD Biosciences, CA, USA. Phospho-Zap-70, phospho-Erk1/2 and phospho-p38MAPK antibodies were from Cell Signalling Technology, MA, USA. Anti-Erk-2, β-actin and peroxidase-conjugated goat anti-mouse/goat anti-rabbit secondary antibodies were purchased from Santacruz Technologies, CA, USA. ECL reagents were procured from Millipore, MA, USA and tritiated thymidine was from BARC, Bombay, India. Mycobacterial antigens for this study (WCL and MLSA) were procured from Dr. P.J. Brennan, under WHO contract (NIH-N01-A1-25469), Leprosy Research Support, Colorado State University, Colorado, USA.

2.2. Measurement of Ca^{2+} mobilization

Jurkat T-cells (2×10^6/ml) were washed with phosphate buffered saline (PBS), pH 7.4. Cells were incubated with Fura-2/AM at 1 μM for 30 min at 37 °C in loading buffer [(in mM): NaCl, 110; KCl, 5.4; $NaHCO_3$, 25; $MgCl_2$, 0.8; KH_2PO_4, 0.4; HEPES, 20; Na_2HPO_4, 0.33; $CaCl_2$, 1.2. pH 7.4]. After loading, cells were washed three times ($500 \times g$ for 5 min) and remained suspended in the identical buffer. $[Ca^{2+}]_i$ was measured as reported elsewhere (Grynkiewicz et al., 1985; Joshi et al., 2006). Fluorescence intensities were measured in ratio mode using Varian ECLIPSE spectrofluorometer equipped with Fast filter accessory (Varian Incorporation, St. Helens, Australia) at 340 nm and 380 nm (excitation filters) and 510 nm (emission filter). Cells were stirred continuously throughout the experiment. Test molecules were added into the cuvettes in small volumes with no interruptions in recordings. The intracellular concentrations of free Ca^{2+} $[Ca^{2+}]_i$, were calculated by using the following equation: $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (Sf2/Sb2)$. A value of 224 nM for K_d was added into the calculations. R_{max} and R_{min} values were obtained by addition of ionomycin (5 μM) and $MnCl_2$ (2 mM), respectively. All experiments were performed at 37 °C.

2.3. Treatment of cells and Western blot analysis of Zap-70, MAPK activation and estimation of PKC activity

Cells were stimulated essentially as described (Kim and White, 2006). Briefly, 5×10^6/ml serum starved Jurkat T-cells were treated or not with 10 μg/ml of WCL or MLSA for 2 h, then stimulated or not with anti-CD3 antibodies (10 μg/ml) alone or with CD28 (5 μg/ml) at 4 °C for 15 min. After washing once with chilled PBS, prewarmed PBS (37 °C) containing GAM (5 μg/ml) was added and cells were further incubated at 37 °C for 10 min. Reaction was stopped with chilled PBS and Jurkat T-cells were lysed with 50 μl of buffer (HEPES, 20 mM pH 7.3; EDTA, 1 mM; EGTA, 1 mM; NaCl, 0.15 mM; Triton X-100, 1%; glycerol, 10%; PMSF, 1 mM; sodium orthovanadate, 2 mM; anti-protease cocktail). After centrifugation ($13{,}000 \times g$ for 5 min), cells lysates were used immediately or stored at −20 °C. The protein contents were determined with Bradford reagent. Denatured proteins (30 μg) were separated by SDS-PAGE (10%) and transferred to polyvinylidine difluoride (PVDF) membranes. Immunodetection of phosphorylated forms of Zap70, Erk1/2 and p38MAPK was performed using 2 μg/ml of phospho-specific antibodies in TBS with 2.5% BSA with overnight incubation at 4 °C. After washing with TBST (TBS + 0.05% Tween-20), PVDF membranes were treated with peroxidase-conjugated goat anti-mouse/anti-rabbit secondary antibodies, peroxidase activity was detected with ECL reagents. Equal loading of the proteins was confirmed after striping the blot and reprobing for total forms of Erk-2/β-actin. Densitometric analysis of bands was performed using Quantity One™ software (Bio-Rad, Hercules, USA), whereas PKC activity in the cytosolic fractions was measured in duplicate using nonradioactive protein kinase assay kit according to manufacturer's instructions (Calbiochem, La Jolla, USA). Optical densities of the reactions were directly proportional to enzymatic activity of PKCs.

2.4. Purification of nuclear proteins for EMSA

DNA–protein binding assays were carried out with nuclear extract from serum starved 5×10^6/ml Jurkat T-cells treated or not with *M. leprae* antigens for 2 h, then stimulated with PMA (5 nM) plus ionomycin (2 μM) for 2 h as described elsewhere (Matata and