Glycoconj J (2006) 23:675–686
DOI 10.1007/s10719-006-9017-9

An unusual pro-inflammatory role of interleukin-10 induced by arabinosylated lipoarabinomannan in murine peritoneal macrophages

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Received: 22 February 2006 / Revised: 10 March 2006 / Accepted: 9 April 2006 / Published online: 18 November 2006
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Abstract Various species of *Mycobacteria* produce a major cell wall-associated lipoglycan, called Lipoarabinomannan (LAM), which is involved in the virulence of *Mycobacterial* species. In this study, we tried to establish the role of the increased IL-10 secretion under Arabinosylated-LAM (Ara-LAM) treatment, the LAM that induces apoptosis in host macrophages or PBMC. We have studied the survival and apoptotic factors by western blotting, and estimated nitrite generation by Griess reaction, quantified iNOS mRNA by semi-quantitative RT-PCR, and ultimately the fate of the cells were studied by Flow Cytometric Analysis of AnnexinV-FITC binding. As per our observations, neutralization of released IL-10 in C57BL/6 peritoneal macrophages prior to Ara-LAM treatment, as well as macrophages from IL-10 knockout (KO) mice treated with Ara-LAM, showed significant down regulation of pro-apoptotic factors and up regulation of survival factors. These effects were strikingly similar to those when peritoneal macrophages were subjected to TNF-α and IL-12 neutralization followed by Ara-LAM-treatment. However, under similar conditions virulent Mannosylated-LAM (from *Mycobacterium tuberculosis*) treatment of macrophages clearly depicts the importance of IL-10 in the maintenance of pathogenesis, proving its usual immunosuppressive role. Thus, from our detailed investigations we point out an unusual pro-inflammatory action of IL-10 in Ara-LAM treated macrophages, where it behaves in a similar manner as the known Th1 cytokines TNF- α and IL-12.

Keywords Caspases · Cytokines · Tuberculosis · Apoptosis · IL-10

Abbreviations

LAM	Lipoarabinomannan
Man-LAM	Mannosylated Lipoarabinomannan
Ara-LAM	Arabinosylated Lipoarabinomannan
IL	Interleukin
WT	Wild Type
KO	Knockout
TNF-α	Tumor Necrosis factor alpha
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
β-ME	β-Mercaptoethanol
EGTA	Ethyleneglycol-bis(b-aminoethylether) N,N,N′,N′-tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
TBS	Tris Buffered Saline

This work is financed by the Council of Scientific and Industrial Research (CSIR), Govt. of India.

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iNOS	inducible Nitric Oxide Synthase
PBMC	Peripheral Blood Mononuclear Cells
NO	Nitric Oxide
PI3K	Phosphoinositide 3′ Kinase
Ab	Antibody

Introduction

Mycobacterial pathogenesis occurs because of the ability of the pathogen to survive and expand within macrophages by subverting the host cell immune responses to infection [44, 48]. This immune subversion is established with the help of mycobacterial cell wall glycolipid Lipoarabinomannan (LAM), a chief virulence factor involved in the alteration of normal host cell signaling [5, 25, 26, 28]. Virulent strains of *M. tuberculosis* modify the non-reducing termini of the arabinofuranosyl chains with mannose cappings yielding Man-LAM, whereas rapidly growing avirulent strains such as *M. smegmatis* have no mannose cappings, yielding Ara-LAM [6, 29]. Ara-LAM treatment leads to the induction of Tumor Necrosis factor alpha (TNF-α) and Interleukin-12-mediated apoptosis of host cells [19, 50]; Man-LAM treatment results in cell survival by inhibiting *M. tuberculosis*-induced apoptosis [43], and also by activation of an altered signaling pathway involving ceramide generation via Mitogen Activated Protein Kinase (MAPK) [45], and by PI3K-Akt pathway via Bad phosphorylation [33].

Virulent pathogens are known to induce the survivability of host cells by stimulating Interleukin (IL)-10 release, a negative regulator of Th1 response [16, 37]. IL-10 inhibits Interferon gamma (IFN-γ)-mediated killing and nitrite generation during *Toxoplasma gondii* and *Schistosoma mansoni* infection [27, 38], and inhibits proliferation of co-stimulatory molecule B7 expression on antigen-presenting cells [11]. It is reported that IL-10-KO mice die rapidly due to *Toxoplasma* or *Trypanosoma cruzi* infection, due to systemic overproduction of IFN-γ, TNF-α, and IL-12 [17, 27, 31, 38]. Similarly in Leishmanial infection, IL-10 inhibits inducible Nitric Oxide Synthase (iNOS 2) expression by modulating the signaling pathways in macrophages [36]. Since Man-LAM, a cell signaling modulator of virulent *M. tuberculosis*, and Ara-LAM regulate IL-10 release differentially [8], it is proposed that survival or apoptosis of mycobacterium-infected macrophages is maintained by a balance between IL-10 and TNF-α [42].

Despite the recent studies on the role of pro-and anti-inflammatory cytokines on the regulation of cellular activities under LAM treatment, the implication of the anti-inflammatory cytokine IL-10 in avirulent Ara-LAM treated cells, where it is released in a greater amount, is yet unclear. In spite of the known action of immunosuppressive and anti-apoptotic roles of IL-10, in some cases it is known to up-regulate the pro-apoptotic, pro-inflammatory cytokine TNF-α in inducing apoptosis of T cells [35]. In this study, we have thus investigated the possibility of such an unusual pro-inflammatory role of IL-10 like TNF-α or IL-12 in Ara LAM treated-condition.

In this investigation, we demonstrate an unusual, pro-inflammatory behavior of IL-10 like TNF-α or IL-12 in inducing Ara-LAM-treated cells toward apoptosis while in Man-LAM treated-cells, IL-10 usually behaves as an anti-inflammatory cytokine.

Materials and methods

Materials

Rabbit anti p-ERK, rabbit anti-ERK, Rabbit anti PI3K, rabbit anti p-PI3K p85α, rabbit anti Akt, anti rabbit ser 473 pAkt (Santa Cruz Biotechnology, CA), rabbit cleaved anti Caspase 9, rabbit cleaved anti Caspase 3, rabbit cleaved anti PARP (Cell Signaling Tech., Beverley, MA, USA), neutralizing IL-10 antibody, neutralizing TNF-α antibody clone MP6 XT22, neutralizing IL-12 p40/p70 antibody clone C11.5 mouse IgG1 (Phar Mingen, San Diego, CA) recombinant IL-10, rTNF-α and rIL-12 (R&D Systems), TRIZOL (Sigma), MMLV reverse transcriptase (Life Technologies), Taq DNA polymerase (GIBCO BRL), iNOS2 specific primers (GenoMechanix), IL-10 ELISA assay kit (Quantikine M; R&D system, Minneapolis, Minn.), Annexin-V-FITC (Santa Cruz Biotechnology, CA).

Mice

C57BL/6 mice (purchased from National Centre for Laboratory Animal Sciences, Hyderabad, India) both male and non-pregnant female, of age group 6–7 weeks, weight 18 to 20 g (for experiments with neutralizing antibody) were used. IL-10-KO mice, mutated [31], were obtained in a collaborative work with Dr. Bhaskar Saha, NCCS, Pune. For each set of experiments, 15 control C57BL/6 mice and 6 IL-10 knockout C57BL/6 mice were used.

Purification of Man-LAM and Ara-LAM

Mycobacterium tuberculosis was isolated from the blood of affected patients at the Bengal Tuberculosis Association, Kolkata, India, and cultured on L-J solid medium, prior to being cultured in Herman Kirchner's liquid medium [46, 47]. For isolation of the Lipoarabinomannan (LAM) from *M. tuberculosis*, the standard method of Hunter et al. [25] was followed.

Isolation of Ara-LAM Ara-LAM was purified according to the above method from strain LR222 of *M. smegmatis*. The bacterial cells were collected by centrifugation and the LAM was isolated [25]. All the experiments were performed with a single isolate of each culture.

LAM was isolated and roughly purified according to the process stated by Hunter et al., SDS-PAGE was performed, and the specific 37 kDa band of LAM was visible by Silver Staining, alongside the known Man-LAM isolated from *M. tuberculosis H37Rv*. Coomassie blue staining of the gel showed no bands, and protein estimation of this compound by Bio-Rad protein assay reagent also showed negligible protein content. This LAM was further purified according to the rapid and simple method stated by Hamasur et al. [23]. LPS contamination was checked by *Limulus test* and was found to be <25 ng/mg in both Man-LAM and Ara-LAM.

Isolation of murine peritoneal macrophages

Mouse macrophages were isolated by peritoneal lavage with ice-cold PBS 48 h after intraperitoneal injection of 1.0 ml of sterile 4% thioglycolate broth (Difco Laboratories, Detroit, MI). Cells were cultured as described by Fahey et al. [13]. The adherent cell population was cultured for 48 h prior to any treatment, to achieve the resting state. More than 90% of the remaining adherent cells were found to be macrophages on the basis of morphologic criteria and nonspecific esterase staining.

Treatment of macrophages

The macrophages were cultured overnight in RPMI 1640 supplemented with 10% FCS (GIBCO BRL). For neutralization of cytokine release, the cells were first pre-incubated with anti-IL-10 neutralizing antibody at a specified and standardized dose of 4 μg/ml, anti-TNF-α antibody clone MP6 XT22 (5 μg/ml) or anti-IL-12 neutralizing antibody (5 μg/ml) for 3 h, washed, treated with Man-LAM or Ara-LAM at a dose of 10 μg/ml, and then subjected to cytotoxicity assay as well as Trypan blue exclusion to ensure cell viability to be >90%. The standard dose of LAM was estimated at 10 μg/ml [9], for optimum effect.

Preparation of cell lysate

Cells were suspended in RPMI 1640 (Sigma) containing 10% Fetal Calf Serum (FCS; Gibco BRL) and cultured in 5% carbondioxide at 37°C for 24 h. The cells were pelleted by centrifugation at 400 × g for 15 min at 4°C and resuspended in ice-cold extraction buffer containing 10 mM Tris HCl (pH 7.5), 4.5 mM EGTA, 50 mM 2-mercaptoethanol, and an antiprotease mixture containing 0.33 mM leupeptin, 0.2 mM phenylmethylsulphonylflouride (PMSF), 4.8 trypsin inhibitor units of aprotinin per ml, 0.35 mM antipain [32]. The macrophage-containing suspension was sonicated at 4°C and centrifuged at 4,250 × g for 10 min at 4°C, and the supernatant was then used for experiments.

When performing western blot for detection of Caspases, cells were pelleted and subjected to freeze-thaw thrice in 20 μl cell-extraction buffer [50 mM PIPES/NaOH (pH 6.5), 2 mM EDTA, 0.1% w/v CHAPS, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml aprotinin]. The lysates were centrifuged at 15,000 rpm for 5 min at 4°C for the detection of caspases.

Electrophoresis and immunoblotting

Proteins were quantified with Bio-Rad protein assay reagent using BSA as a standard. Equal amounts of protein in each lane were subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10%PAGE) and transferred to a nitrocellulose membrane [9]. The membrane was blocked overnight with 3% bovine serum albumin in Tris-saline buffer (pH 7.5), and immunoblotting was done as described previously by Majumdar et al. [20, 21, 34].

Nitrite generation assay

Nitrite accumulation in culture was measured colorimetrically by the Griess Reaction [22, 24] using a Nitric Oxide colorimetric Assay Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). For the assay, 1×10^6 per ml concentration of cells was cultured in a 24-well tissue culture plate (Tarson). Cell-free supernatant was collected and the nitrite level was estimated as per the instructions of the manufacturer.

Isolation of RNA and RT-PCR for analyzing iNOS2 mRNA expression

Macrophages from control and IL-10-KO mice from C57BL/6 background were isolated and treated with Man-LAM or Ara-LAM. Total RNA was extracted from 3×10^6 cells using TRIZOL (SIGMA), and checked for purity by studying the OD_{260}/OD_{280} ratio, which was found to be 1.7. For cDNA synthesis, 1 μg of total RNA from each sample was incubated with random primer, 0.1 M DTT, 500 μM dNTPs, 40 U RNAse inhibitor, and 1 μl (200 U) of MMLV reverse transcriptase (Life technologies). Samples were then incubated at 37°C for 1 h followed by 5 min incubation at 95°C. cDNA from each sample was amplified with

0.5 unit Taq DNA polymerase (GIBCO BRL) in 50 μl under the following conditions: 95°C for 2 min, 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for a total of 35 cycles using Perkin Elmer Gen Amp PCR system 2400. The iNOS2 specific primer (GenoMechanix) was designed to amplify the mouse-specific iNOS2 coding regions: (Sense: 5′-AGCTCCTCCCAGGACCACAC-3′, antisense: 5′-ACGCT GAGTACCTCATTGGC-3′). PCR-amplified product was subsequently size fractioned on 1% agarose gel, stained with ethidium bromide, and visualized under UV-light. Each sample was amplified for mouse β-actin to ensure equal cDNA input. In negative control experiments with omission of the reverse transcriptase, no PCR product was detected for either set of the iNOS2 and β-actin primers (data not shown).

Measurement of IL-10 release by sandwich ELISA

The level of mouse IL-10 in the conditioned medium of macrophage culture was measured using the sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit (Quantikine M; R&D system, Minneapolis, MN.). The assay was performed as per the detailed instructions of the manufacturer. The detection limit of this assay was 15 pg/ml.

Flow cytometric analysis of cellular apoptosis by AnnexinV - FITC binding

FACS (Fluorescence Activated Cell Sorter) analysis of death of the macrophages under different treatments was done with Annexin V-FITC binding. Briefly, 1×10^6 cells per set, after the required treatment, were washed with chilled PBS, collected by repeated flushing, and pelleted down. They were then stained with 5 μl each of Annexin V-FITC, incubated for 15 min in the dark at room temperature. 400 μl binding buffer (1×) was added to each tube and analysis of the binding was done by Flow Cytometry (FACS Calibur; Recton Dickinson) [12].

Densitometric analysis

Immunoblots were analyzed using model GS-700 imaging Densitometer and Molecular Analyst version 1.5 software (Bio-Rad Laboratories, Hercules, CA.)

Statistical analysis

Results were expressed as the mean plus or minus the Standard Deviation (SD) for individual sets of experiments. Each experiment was performed at least thrice, and representative data from each set were presented in the manuscript. One or two tailed Student's *t* test for significance was performed to assess the significance of the differences between the mean values of control and experimental groups. A P value of less than 0.05 was considered significant, and less than 0.01 was considered highly significant.

Results

IL-10 is responsible for the enhanced nitrite generation, and iNOS2 mRNA expression in Ara-LAM- treated cells, but not in Man-LAM- treated cells

IL-10 secretion is induced under any pathogenic infection [16, 37]. We observed that a noncytotoxic dose of 10 μg of either Ara-LAM or Man-LAM [19], when administered to murine peritoneal macrophages, induces substantial release of IL-10 in pg/ml in 24 h. The IL-10 release in Ara-LAM-treated cells was more than two times higher than that by Man-LAM treated cells $^{**}P < 0.01$ (Fig. 1). IL-10, a known Th2 cytokine, is reported to reduce nitrite generation, a potent inhibitor of microbial pathogenesis [7, 16], so we first investigated the role of IL-10 on nitrite generation in both LAM treated macrophages. A striking observation was made: Ara-LAM-induced up-regulation of nitrite generation was significantly diminished ($^{*}P < 0.05$) both in macrophages where IL-10 release had been neutralized by anti-IL-10 Ab prior to Ara-LAM treatment, as well as in macrophages isolated from IL-10- KO mice treated with Ara-LAM (by 3 fold, $^{**}P < 0.01$) (Fig. 2a). This effect was similar to that shown by either TNF-α or IL-12 neutralization of macrophages before Ara-LAM treatment (shown as positive controls, because Ara-LAM induces TNF-α and IL-12 release

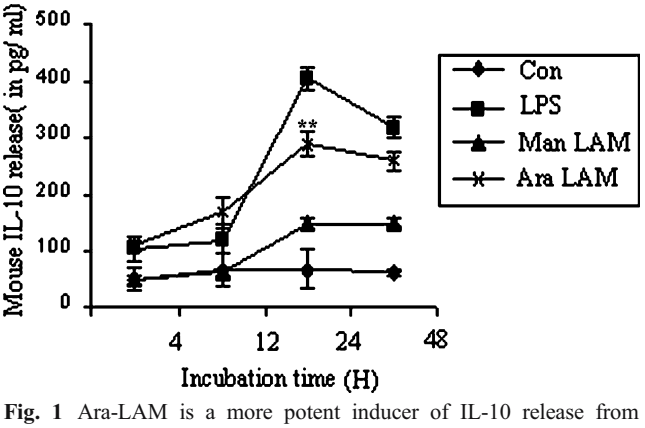

Fig. 1 Ara-LAM is a more potent inducer of IL-10 release from murine peritoneal macrophages than that by Man-LAM. Kinetics of IL-10 release (in pg/ml) by control, LPS, Man-LAM and, Ara-LAM-treated macrophages. Cell free supernatants were collected after the specified periods of incubation following treatment and sandwich ELISA performed to determine the IL-10 release. The results are expressed as mean ± SD of data from triplicate experiments, which yielded similar results. (**) indicates a significant change ($P < 0.01$)

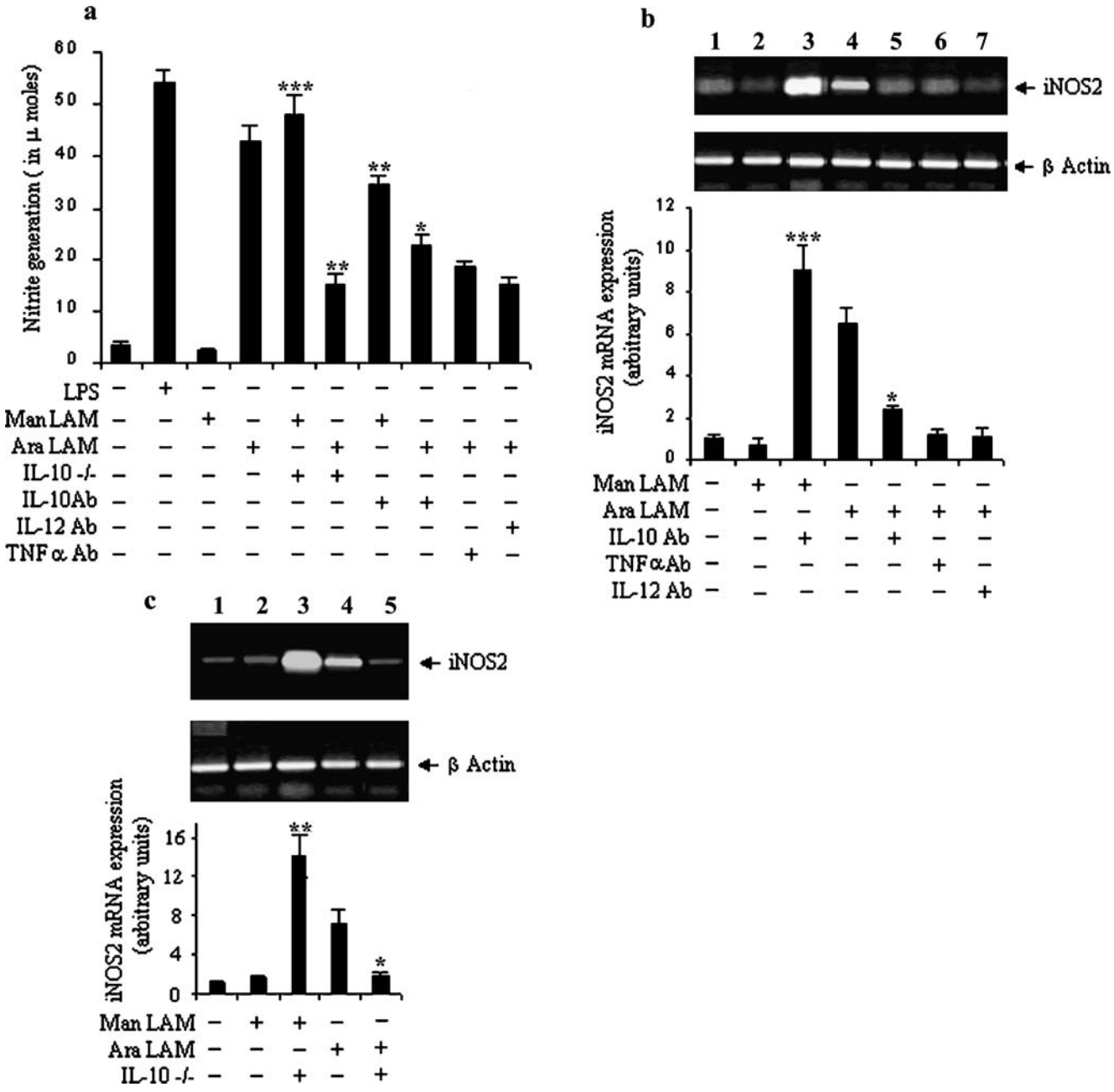

Fig. 2 IL-10 increases nitrite generation and iNOS mRNA expression in Ara-LAM-treated macrophages but not in Man-LAM-treated macrophages. WT macrophages cultured in RPMI 1640 medium (Materials and methods). In another set, macrophages from WT and IL-10- KO mice were subjected to either Man-LAM or Ara-LAM treatment. (a) Nitrite generation and the effect of IL-10 in LAM-treated cells. Cell free supernatants were collected after 48 h of incubation and nitrite generation was then measured by Griess reaction. (b) Semi-quantitative RT- PCR analysis for iNOS mRNA and β-actin were done from the macrophages pre-treated with the respective neutralizing antibodies followed by LAM-treatment and its densitometric analysis. (c) Semi-quantitative RT- PCR analysis for iNOS mRNA and β-actin were done from the macrophages isolated from WT and IL-10- KO mice treated with Man-LAM or Ara-LAM and its densitometric analysis. The RT-PCR data shown in Fig. 2(b) and (c) are the most representable of the three independent experiments performed in each case, yielding similar results, and in the nitrite generation data and the densitometric scanning analyses, the results are expressed as mean ± SD from three independent experiments, which yielded similar results. (*) , (**) and (***) indicates statistically significant change ($P < 0.05$), ($P < 0.003$) and ($P < 0.001$), respectively

and their respective mRNA expression in both macrophages and J774 cells [40, 50], which are responsible for nitrite generation [41]). On the other hand, Man-LAM treatment of macrophages does not evoke IL-12 or TNF-α generation [40], so nitrite generation is not induced. IL-10 neutralization of cells prior to Man-LAM treatment or cells from IL-10-KO mice treated with Man-LAM leads to a substantial increase in nitrite generation, showing that IL-10 plays its usual immunosuppressive role in Man-LAM-treated cells.

To further confirm our observations, we also quantified iNOS expression at the mRNA level. These observations made from the RT-PCR (Fig. 2b and c) showed significant down-regulation (*$P < 0.05$) of iNOS mRNA in Ara-LAM-treated macrophages where IL-10, TNF-α, and IL-12 releases were neutralized prior to Ara-LAM treatment compared to Ara-LAM-treated cells, but IL-10 neutralization in Man-LAM-treated cells enhanced the same (**$P < 0.01$) as to Man-LAM-treated macrophages. Our findings were confirmed by a similar finding in macrophages isolated from WT and IL-10- KO mice.

IL-10 down-regulates survival factors in Ara-LAM- treated macrophages

Since IL-10 is implicated in cellular survival during infection [16, 27, 37, 38] we investigated the effect of IL-10 on the expression of survival kinases in LAM-treated macrophages. As virulent factors like Man-LAM which evoke IL-10 release have been reported to promote cell survival by activation of ERK [4, 45] and PI3K-Akt pathway [33], we studied ERK activation in both LAM-treated cells under IL-10 neutralization. Finding no significant role of ERK in IL-10-mediated signaling in either LAM-treated macrophages (data not shown), PI3K and Akt activation profiles were studied.

Man-LAM, which is known to increase IL-10 release in both PBMC and macrophages, also induces macrophage survival via the PI3K-Akt pathway [33]. PI3K (phosphosinsositide 3′ Kinase) is in the upstream of Akt and is known to play a major role in microbial engulfment and phagocytosis [14]. As seen from the data, p-PI3K p85α fragment, which is the active portion of the kinase, is induced by both Ara-LAM and Man-LAM (Fig. 3a, lanes 2 and 4). WT macrophages where IL-10 release had been neutralized by antibody (4 μg/ml) prior to Ara-LAM treatment show significantly enhanced PI3K activity (*$P < 0.05$), like that due to TNF-α or IL-12 neutralization by respective antibodies (5 μg/ml) under similar conditions (Fig. 3a, lanes 5, 6, and 7). However, Man-LAM-treated cells if subjected to IL-10 neutralization prior to LAM treatment show highly significant inhibition of PI3K activity compared to Man-LAM treated WT cells. **$P < 0.01$ (Fig. 3a, lanes 2 and 3).

The effects of IL-10 neutralization on PI3K activity in LAM treated macrophages were confirmed by the study of Akt (Protein Kinase B) activity profile. Akt activity was examined in macrophages under IL-10 neutralization before LAM treatment as well as in macrophages from IL-10- KO mice treated with both LAM. Akt (Protein Kinase B) is an important cell-signaling factor implicated in cellular survival [10, 15]. The study of Akt profile showed similar results, but the effects were more pronounced. Ara-LAM treatment prominently inhibited Akt phosphorylation in murine macrophages (Fig. 4a, lane 4); which was signifi-

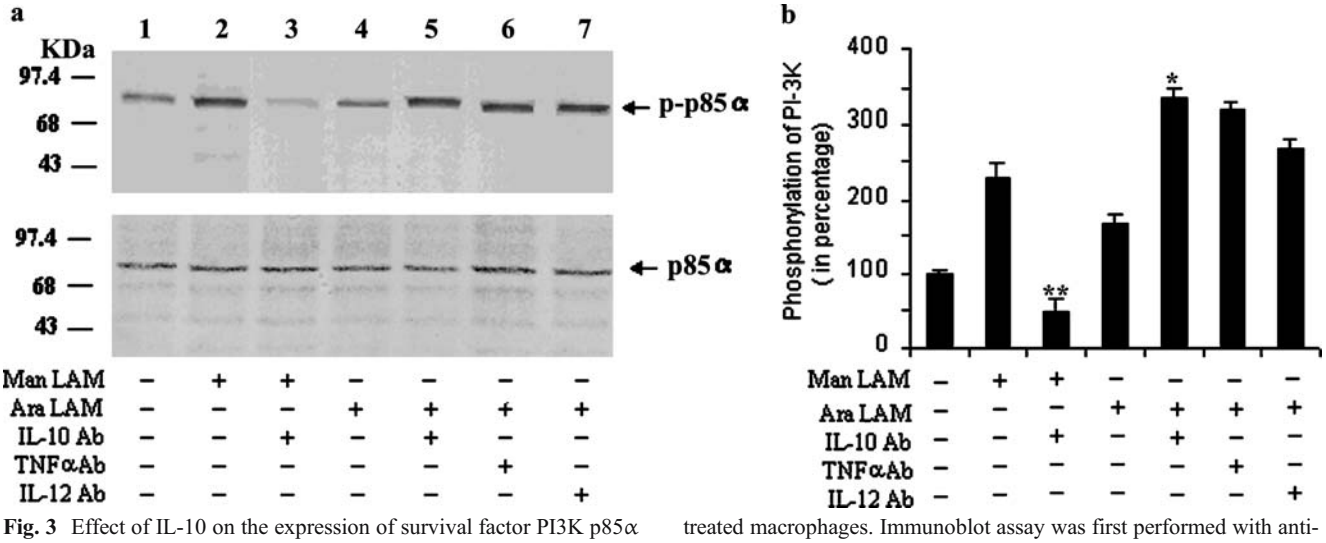

Fig. 3 Effect of IL-10 on the expression of survival factor PI3K p85α in LAM-treated macrophages. Macrophages were treated in separate sets as described earlier. After 24 h of treatment, the cell sonicate was subjected to electrophoresis and immunoblotting as described in the Materials and methods section. (a) Effect of IL-10 on the PI3K activity (expression of the active p85α) in Man-LAM- and Ara-LAM-treated macrophages. Immunoblot assay was first performed with anti-p-PI3K p85α antibody and then the blot was striped and reprobed with anti PI3K- p85α antibody. (b) densitometric scanning analysis of Fig. 3(a). The data is the most representable from three independent experiments, which yielded similar results. (**) indicates a significant change ($P < 0.01$) and (*) denotes ($P < 0.05$)

cantly enhanced if IL-10 release was neutralized prior to Ara-LAM treatment $^{**}P < 0.015$ (Fig. 4a, lane 5) or in macrophages from IL-10- KO mice when Ara-LAM was administered ($^{**}P < 0.01$) (Fig. 4c, lane 5). Same effects were exhibited by the positive controls (effect of TNF-α and IL-12 neutralization in macrophages under Ara-LAM treatment) as depicted by Fig. 4a, lanes 6 and 7. However, Man-LAM treatment of macrophages under similar conditions (either in IL-10- neutralized condition or in IL-10-KO cells) showed the natural effect of IL-10 as an immunosuppressive cytokine (Fig. 4a, lanes 2 and 3; Fig. 4c, lanes 2 and 3).

IL-10 up regulates pro-apoptotic factors in Ara-LAM- treated macrophages

After having established the unusual role of IL-10 in the down-regulation of survival factors in Ara-LAM-treated macrophages, we were further interested to investigate the status of pro-apoptotic factors like caspases 9 and 3. Caspases or Cysteine Aspartate-specific Proteases cleavage indicates apoptosis in various cell types [30, 49]. Caspase 9 is the initiator of the caspase cascade and is reported to be phosphorylated and deactivated by pAkt, leading to the inhibition of apoptosis even after cytochrome c release [3].

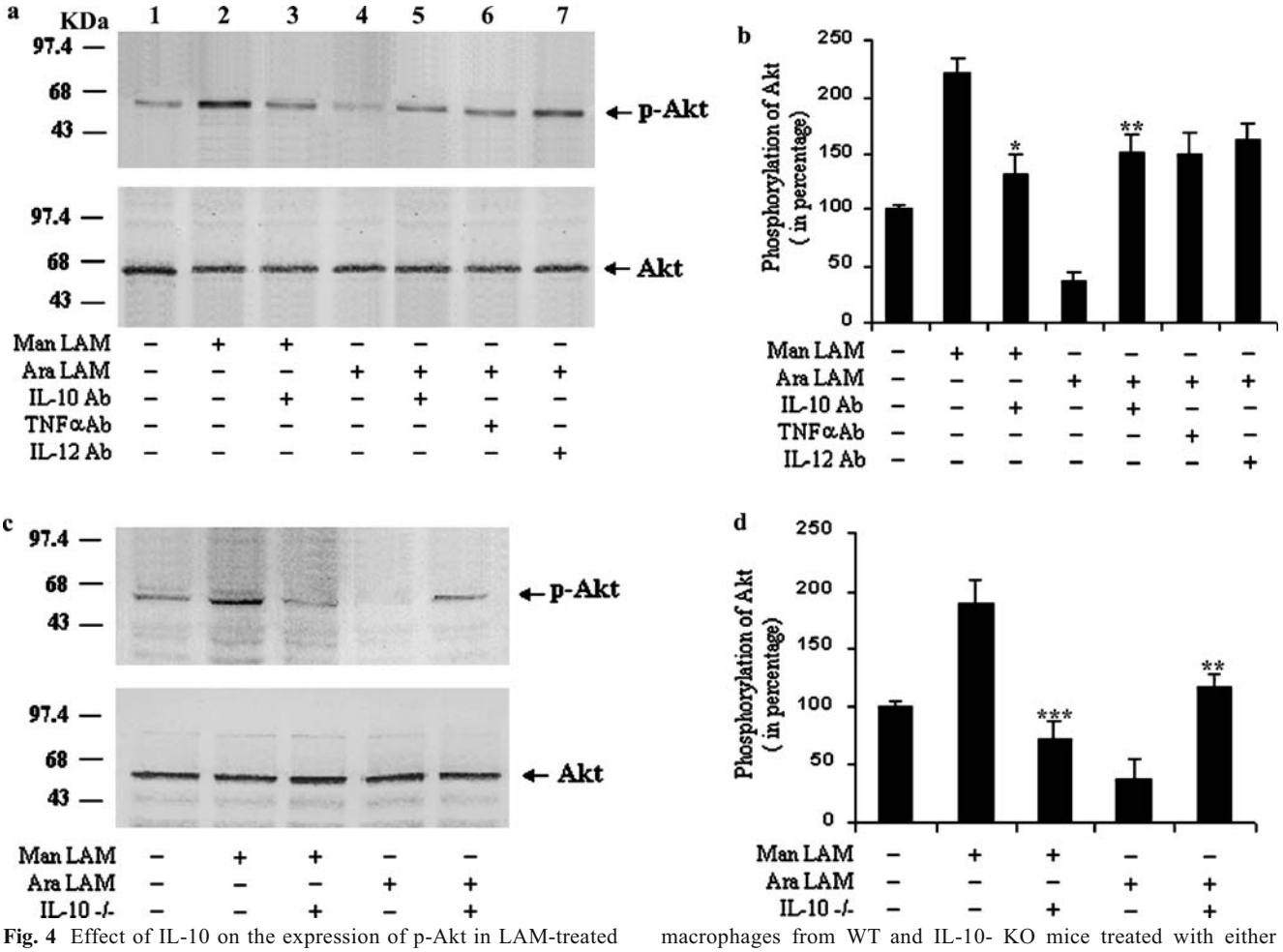

Fig. 4 Effect of IL-10 on the expression of p-Akt in LAM-treated macrophages. WT macrophages were subjected to treatment as described in the previous section. In another set, macrophages from WT and IL-10- KO mice were subjected to either Man-LAM or Ara-LAM-treatment. (a) Effect of IL-10 on p-Akt, as shown by IL-10 neutralization of either Man-LAM- or Ara-LAM-treated macrophages. (b) Densitometric analysis of Fig. 4a, showing p-Akt expression as percent of control. (*) and (**) respectively indicate significant changes $P < 0.05$ compared to Man-LAM-treated cells and $P < 0.015$ as compared to Ara-LAM-treated cells; (c) p-Akt in macrophages from WT and IL-10- KO mice treated with either Man-LAM or Ara-LAM. (d) Densitometric scanning analysis of Fig. 4c (***) and (**) indicate significant changes $P < 0.001$ and $P < 0.01$, respectively. Immunoblots were first performed with anti-p-Akt ser 473 antibody and then the blot was striped and reprobed with anti-Akt antibody. The blots shown are the most representable of four independent experiments performed in each case, which yielded similar results. The densitometric analyses of the respective blots represent the mean ± SD value from four independent experiments (in each case) giving similar observations

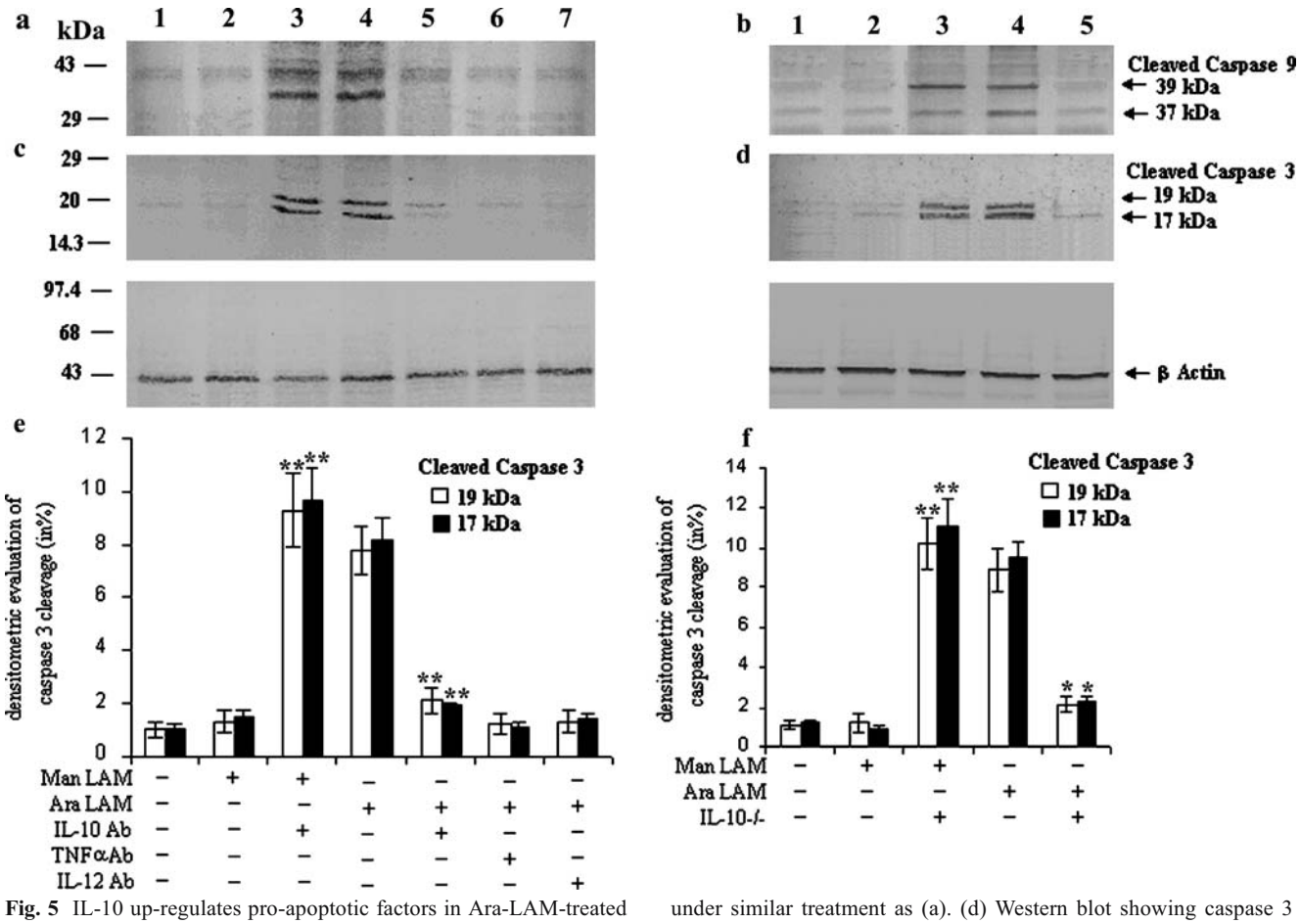

Fig. 5 IL-10 up-regulates pro-apoptotic factors in Ara-LAM-treated macrophages. Macrophages (Mϕ) were cultured and treated with neutralizing antibodies and LAM as described in the earlier sections. Following experiments were done with macrophages under the treatment of either Man-LAM or Ara-LAM. In another set, macrophages from WT and IL-10-KO mice were subjected to either Man-LAM- or Ara-LAM-treatment. 24 h post culture, the cell sonicate was subjected to electrophoresis and immunoblotting as described in the Materials and methods section. (a) Immunoblot showing caspase 9 cleaved-fragment expressions treated as above under IL-10 neutralization. (b) Immunoblot showing cleaved caspase 9 in macrophages isolated from WT and IL-10- KO mice with similar treatment. (c) Data showing caspase 3 cleavage (19 kDa and 17 kDa) under similar treatment as (a). (d) Western blot showing caspase 3 cleaved fragment expressions in macrophages isolated from WT and IL-10- KO mice under similar treatment. (e) Densitometric evaluation of (c). (**) denotes a highly significant change ($P < 0.01$ (f) Densitometric evaluation of (d). (*) denotes a change of valid significance ($P < 0.05$), and (**) depicts ($P < 0.01$). Immunoblots were first performed with cleaved caspase 9- or 3- antibody and then the blot was striped and reprobed with anti β-actin antibody to confirm equal protein loading. The blots shown are the most representable of three independent experiments performed in each case, which yielded similar results. The densitometric evaluation results shown represent the mean ± SD value from three independent experiments, yielding similar results

Caspase 3 is in the downstream of precursor caspase 9, and it is known that *M. tuberculosis* promotes apoptosis in human neutrophils by activating caspase 3 [39]. Here we observed exactly the opposite effects as those shown by survival factors. As seen from the results, control WT macrophages do not show detectable caspase 9 (Fig. 5a, lane 1 and Fig. 5b, lane 1) or caspase 3 cleavage (Fig. 5c, lane 1; Fig. 5d lane 1), whereas Ara-LAM-treated macrophages undergo apoptosis, thereby showing increased fragmentation of both caspase 9 (Fig. 5a, lane 4, and Fig. 5b., lane 4) and caspase 3 (Fig. 5c, lane 4 and Fig. 5d, lane 4). WT macrophages where IL-10, IL-12 or TNF-α releases had been neutralized by respective neutralizing antibodies prior to Ara-LAM treatment show markedly diminished cleavage of either caspase 9 (Fig. 5a, lanes 5, 6, and 7) or caspase 3 (Fig. 5c, lanes 5, 6, and 7). Prominent decrease in caspase 9 (Fig. 5b, lane 5) and caspase 3 cleavage (Fig. 5d, lane 5) have also been seen in macrophages from IL-10- KO mice upon treatment with Ara-LAM. Exactly the opposite effect was seen in case of Man-LAM-treated macrophages under similar conditions (Fig. 5a-d, lane 3).

The effects of IL-10 on caspases 9 and 3 were confirmed by PARP cleavage status in cells treated with both LAM. PARP (Poly Adeno Ribose Polymerase) is the enzyme essential for DNA repair, and its cleavage leads to DNA degradation and apoptosis [18]. The expression of cleaved

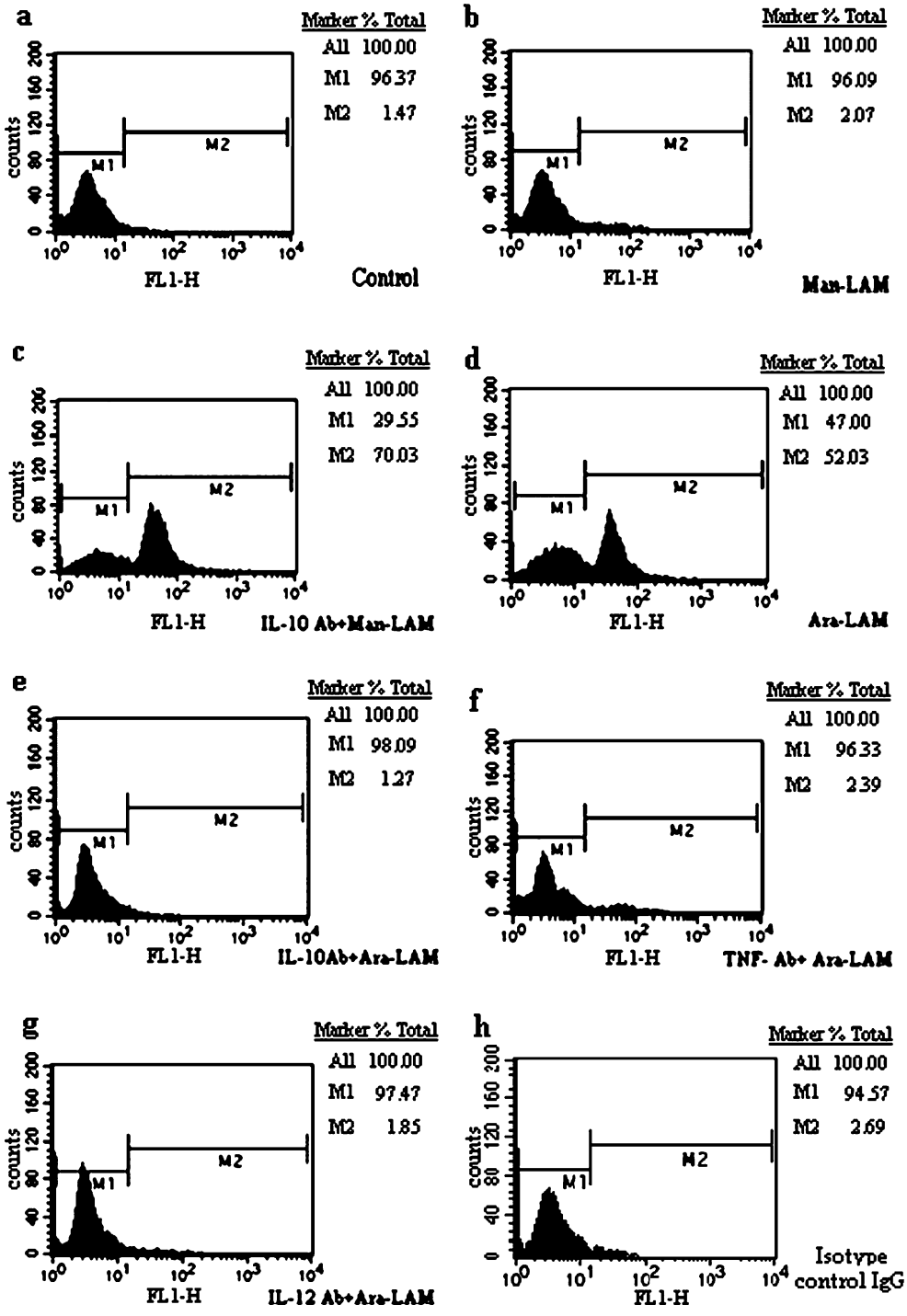

Fig. 6 Flow Cytometric Analysis of apoptosis in LAM-treated murine macrophages and effect of IL-10. Murine peritoneal macrophages (1×10^6) were cultured overnight as described clearly in the methods sections as well as earlier in this section. They were incubated for 24 h as specified in the figure. After the incubation time, the cells were stained with Annexin-V-FITC to assess binding by Flow Cytometry. (a) Control macrophages. (b) Man-LAM (10 μg) treated macrophages. (c) WT cells pre-incubated with IL-10 neutralizing antibody (4 μg/ml) prior to Man-LAM-treatment. (d) WT macrophages treated with Ara-LAM (10 μg). (e) Macrophages subjected to IL-10 neutralization before Ara-LAM-treatment, (f) Effect of TNF-α neutralization by anti TNF-α antibody (5 μg /ml) on Ara-LAM-treated cells. (g) Effect of IL-12 neutralization by antibody (5 μg/ml) on Ara-LAM-treated macrophages. (h) Control IgG matched sera M1 and M2 indicate the percentage of viable (live cells) and apoptotic cells respectively, as shown by the level of Annexin V-FITC binding. The percentage of cells in M1 and M2 in the data are shown in the top right of the panel for each treatment

PARP (89 kDa) fragment also showed similar results (data not shown). These findings suggest that IL-10 inhibits apoptosis in Man-LAM-treated macrophages, facilitating cell survival, but in Ara-LAM-treated cells it up-regulates the expression of pro-apoptotic factors.

Ara-LAM- induced IL-10 facilitates apoptosis in murine macrophages: An unusual pro-inflammatory role

After studying the differential role of IL-10 on various pro-apoptotic factors in Man-LAM- and Ara-LAM-treated macrophages, we directly investigated the percentage of apoptotic cells by analyzing the Annexin V-FITC binding of the cells under treatment. Double staining with Annexin-V-FITC-Propidium Iodide was not performed, as study of necrosis was not essential in our case. As expected from our previous findings, Man-LAM-treated cells showed hardly any Annexin-V-FITC binding like the control cells (Fig. 6a and b), while in cells where IL-10 release had been neutralized by antibody prior to Man-LAM treatment showed a higher percentage of early apoptotic cells (Fig. 6c). However, in Ara-LAM-treated cells, the majority of the cell population was pro-apoptotic to apoptotic, as depicted by a considerable Annexin V-FITC binding (Fig. 6d), while such population of apoptotic cells was significantly decreased if macrophages were subjected to IL-10, IL-12, and TNF-α neutralization with respective antibodies (Fig. 6e, f, and g). Figure 6h showed the effect of Isotype matched IgG on macrophages.

Discussion

The present investigation was aimed at determining the role of IL-10, the release of which was induced by Lipoarabinomannan isolated from non-pathogenic *Mycobacterium smegmatis*. According to background reports, Man-LAM, which is one of the main factors involved in survival of the virulent *M. tuberculosis* and establishment of pathogenesis [33, 45], as well as Ara-LAM (isolated from avirulent *M. smegmatis*) which facilitates cellular apoptosis [19], both induce the release of Interleukin-10 [8]. It is known that the Ara-LAM exerts its cytotoxic effects due to strong induction of pro-inflammatory cytokines TNF-α and IL-12 [40, 50]. However, it is a surprising observation that the release of IL-10 is more than two times greater in Ara-LAM-treated macrophages than that in Man-LAM-treated macrophages (Fig. 1), consistent with the background reports in PBMC [8]. Though IL-10 is a known Th2 cytokine responsible for immunosuppressive properties [2], like inhibition of Interferon gamma (IFN-γ)-mediated killing and nitrite generation during various infections [7, 16], however, in some cases, it is reported to induce T-cell apoptosis by augmenting the role of pro-inflammatory TNF-α [35].

Nitrite generation is one of the primary responses to pathogenic invasion, which is known to be inhibited by IL-10 for establishment of pathogenesis [7, 16]. We noted for the first time, the ambiguity in the usual functions of IL-10 from nitrite generation level itself. Man-LAM is known not to evoke NO release, while Ara-LAM up-regulates the same via induction of Th1 cytokines [41]. It was observed that neutralization of IL-10 release in macrophages prior to Man-LAM treatment showed increase in NO generation, and its mRNA expression. This observation was rational as far as the usual functioning of IL-10; but neutralization of IL-10 prior to Ara-LAM treatment showed an effect similar to that when IL-12 or TNF-α release had been neutralized before Ara-LAM treatment. Such observations were confirmed even in macrophages isolated from IL-10- KO mice treated with both LAM (Fig. 2a). Such results gave a clue that Ara-LAM-induced IL-10 probably functions differently from the usual Th2 cytokine.

Since IL-10 is involved in cellular survival during various infections including Leishmaniasis, Tuberculosis, Toxoplasmosis, or Schistosoma infection, its effect on various survival factors was studied in LAM treated macrophages. Man-LAM has been shown to induce survival in PBMC via MAPK pathway through ceramide generation [45], and also via PI3K-Akt pathway through Bad phosphorylation in J774 macrophages [33]. ERK, also a known regulator of nitrite release in host cells [1, 21], was found not to be involved in IL-10-mediated signaling in both LAM-treated cells (data not shown). Our studies regarding the status of survival factors PI3K and Akt showed a similar profile; Man-LAM- and Ara-LAM-treatment of macrophages both led to up-regulation of PI3K activity. However, Man-LAM showed an increase in Akt activity, while Ara-LAM treatment showed a very significant down-regulation of the same. We made a striking observation at this level: Ara-LAM treatment of macrophages after neutralizing the IL-10 release in macrophages, (also confirmed in macrophages from IL-10- KO mice) exhibited a significant increase in both PI3K with $P < 0.05$ (Fig. 3a and b) and Akt activities (Fig. 4a-d), the inductive effect on Akt activity being more prominent. These findings coincided with the effects shown by the neutralization of the known Th1 cytokines in Ara-LAM-treated cells, suggesting that IL-10 probably functions similar to that of the Th1 cytokines in inhibiting the expression of survival factors, in other words, hindering cell survival in Ara-LAM-treated cells. To further confirm the above statement, the profile of apoptotic factors caspase 9, caspase 3 cleaved-fragments were also investigated. The observations were exactly the opposite as seen in the case

of survival factors; Ara-LAM treatment of macrophages exhibited increased caspases 9 and 3 cleavage, indicating apoptosis, whereas Man-LAM-treated cells do not. Surprisingly, WT macrophages in IL-10 neutralized condition or macrophages from IL-10- KO mice when treated with Ara-LAM showed a very significant inhibition of caspase 9 and 3 cleavage, whereas Man-LAM treatment of cells under similar conditions showed an increase in caspase cleavage. Our observations at this level could definitely prove the unusual Th1 like function of IL-10 in Ara-LAM-treated cells, where it not only hindered cell survival but also was directly involved in facilitating apoptosis, like TNF-α and IL-12.

Finally the study of the fate of LAM-treated macrophage by Flow cytometric analysis confirms our hypothesis (Fig. 6). Thus, our findings hint at the possibility of such an unusual pro-inflammatory role of IL-10 in Ara-LAM-treated cells, seeming that Ara-LAM might be able to reverse Man-LAM-mediated pathogenesis (unpublished data). To our knowledge, this is the first report that could show, with direct evidence, that the Ara-LAM-mediated cytotoxicity in host cells might be attributed to such a pro-inflammatory and pro-apoptotic role of the Th2 cytokine IL-10, along with the Th1 effects of TNF-α and IL-12. This property of Ara-LAM could emerge as a devise for developing potential therapeutic strategies against Man-LAM-mediated pathogenesis.

Acknowledgments We express our deep gratitude to Prof. M. Siddiqi, Director, Bose Institute, and Prof. G.C. Misra, Director, National Centre for Cell Science (NCCS), Pune. We are also grateful to Dr. C.B. Bhattacharyya, the chief pathologist, the superintendent, and the staff of Bengal Tuberculosis Association (B.T.A), Kolkata, India, for valuable assistance. We also acknowledge Dr. Sujoy K. Dasgupta (Dept. of Microbiology, Bose Institute) for his kind gift of purified Man-LAM isolated from *M. tuberculosis H37Rv.*

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