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Expression and selective up-regulation of toxin-related mono ADP-ribosyltransferases by pathogen-associated molecular patterns in alveolar epithelial cells

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Abstract **Mono ADP-ribosyltransferases (ARTs) are a family of enzymes related to bacterial toxins that possess adenosine diphosphate ribosyltransferase activity. We have assessed that A549 constitutively expressed ART1 on the cell surface and shown that lipotheicoic acid (LTA) and flagellin, but not lipopolysaccharide (LPS), peptidoglycan (PG) and poly (I:C), up-regulate ART1 in a time and dose dependent manner. These agonists did not alter the expression of ART3 and ART5 genes. Indeed, LTA and flagellin stimulation increased the level of ART1 protein and transcript while ART4 gene was activated after stimulation of cells with LPS, LTA, PAM and PG via TLR2 and TLR4 receptors. These results show that human ARTs possess a differential capacity to respond to bacteria cell wall components and might play a crucial role in innate immune response in airways.**

Keywords: ADP-ribosylation; Alveolar epithelial cell; Ectoenzymes; Toll-like receptors

1. Introduction

Mono ADP-ribosylation is a covalent chemical modification process catalyzed by ADP-ribosyltransferases (ARTs), which attach a single moiety of ADP-ribose of NAD to specific amino-acid residues of acceptor proteins [1]. This reaction is also a widely used mechanism of bacterial pathogenesis, indeed pathogens, like *Bordetella pertussis*, *Vibrio cholerae*, *Escherichia coli*, and *Clostridium botulinum*, synthesize ADP-ribosyltransferases as exotoxins able to interrupt cellular metabolic and regulatory pathways [2]. The family of known mammalian ARTs related in structure and function to prokaryotic toxins consists of five members (ART1-5). Arginine is the amino-acid target for ART1, ART2, and ART5, while the amino-acid acceptor(s) for ART3 and ART4 have not been characterized yet. Since ART2 is not expressed in humans due to three premature stop codons [3] the only arginine-modifying ARTs expressed in humans are ART1 and ART5. It has been shown that ART1, which is constitutively expressed on the surface of cells lining the airway lumen [4], modifies the Arg-14 of α-defensin-1 (HNP-1), an antimicrobial peptide released by neutrophils, altering its biological activities [5]. Remarkably ADP-ribosylated-α-defensin-1 was purified from bronchoalveloar lavage (BAL) of smokers, showing that this modification occurs in vivo. Human Toll-like receptors (TLRs) act at the host-pathogen interface and represent the earliest surveillance system for primary infection by pathogens. So far 11 TLRs have been identified and shown to recognize a variety of pathogen associated molecular patterns (PAMPs). Although, TLRs are differentially expressed in a wide range of immune cells, several studies have provided evidence that also lung epithelial cells, which represent one of the first lines of defense against microbial attack in airways, constitutively express TLRs playing a pivotal role in innate immunity.

Therefore, given that there are a number of potential TLR agonists in the lung, we decided to evaluate whether airway epithelial cells express transferase activity and to use these cells as experimental model to determine how PAMPs affect ART activity and/or expression. Here, we report that ART1 is constitutively expressed on the surface of A549 cells, which retain features of type II alveolar epithelial cells [6]. We also established that stimulation of A549 cells with TLR agonists led to up-regulation of ART1 and expression of ART4 mRNA indicating a potentially important function for ADP-ribosylation in airway epithelial cells.

2. Materials and methods

2.1. Materials

[Adenine-U-^{14}C] NAD (274 mCi/mmol) were purchased from Amersham; Dowex AG 1-X2 from Bio-Rad; Dulbecco's modified Eagle's minimum essential medium (DMEM) was from Gibco BRL. LPS from *E. coli* serotype R515, flagellin purified from *Salmonella typhimurium* strain 14028 and Pam_3CSK_4 were from Alexis, stealth RNAi duplexes against ART1 and all the reagents for transfection are from Invitrogen, the anti TLR4 mAb HTA125 and the anti TLR2 mAb T2.5 are from eBioscience while PG and LTA from *Staphylococcus aureus* were from Sigma-Aldrich as the other reagents used in this study. The A549 cell line was purchased from ATCC while Chinese hamster fibroblast V79 cells transfected with pTet-ON-ART1 cDNA were a kind gift of Dr. J. Mac Dermot [7].

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Abbreviations: ART, mono ADP-ribosyltransferase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PAM, Pam_3-CSK_4; PG, peptidoglycan; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; PAMP, pathogen associated molecular pattern; TLR, Toll-like receptor

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2.2. Cells, lysate and membrane purification

Cells cultured in DMEM containing HEPES 25 mM were trypsinized at the exponential growth phase and collected by centrifugation at $900 \times g$ for 5 min, washed three times in PBS and lysed in hypotonic lysis buffer (10 mM Tris–HCl, pH 8, and 1 mM EDTA) containing the protease cocktail of Boehringer Mannheim. To purify membranes, cell lysates were disrupted in dry ice for 10 min followed by centrifugation at $100\,000 \times g$ at 4 °C for 1 h. The supernatant was collected and the membrane fraction obtained suspended in the same lysis buffer and sonicated three times before assaying for transferase activity. V79 cells stably transfected with ART1 were cultured in selective DMEM medium containing 200 μg/ml geneticin and 100 μg/ml hygromicin. Cell-surface expression of ART1 was induced incubating cells for 48 h in the presence of 2 μg/ml of deoxycycline.

2.3. ADP-ribosyltransferase enzymatic assay

Intact A549 cells and membranes were assayed for ADP-ribosyltransferase using a standard assay [8], carried out in 0.3 ml containing 50 mM potassium phosphate, pH 7.5, 20 mM agmatine and 0.1 mM [adenine-U-^{14}C] NAD (0.05 μCi). After incubation at 30 °C, duplicate samples (100 μl) were applied to 1 ml columns of Dowex AG 1-X2. [Adenine-^{14}C] ADP-ribosylagmatine was eluted for radioassay with 5 ml of H_2O and the radioactivity counted in a Packard mod counter.

2.4. Immunoprecipitation and immunoblotting assays

Proteins resolved by SDS–PAGE (10% NuPAGE gel system using MES as running buffer) were electrophoretically transferred to nitrocellulose using a Bio-Rad apparatus. Membranes were probed with specific antibodies as specified in figure legends; bound antibodies were detected using an ECL immunoblotting detection system (Bio-Rad) according to the manufacturer's instructions. Molecular masses were estimated from calibration standard included in each gel. α-ART1 antibodies were customized by Primm srl using two ART1 specific peptides, NH_2-SQWQERQARWPEWSL-COOH and NH_2-CHLDNSAMGQSPLSA-COOH, coupled with OVA and affinity purified. Immunoprecipitation was performed using the Reversible Immunoprecipitation System from Upstate following vendor' instructions.

2.5. ART1 silencing

To silence ART1 we used three specific RNAi duplexes (shown below), selected by Invitrogen within the open reading frame of human ART1 gene.

(1) 5′-GCCUGCUAUGAUGUCUCUGCUUCUU-3′;
(2) 5′-GCAGUUUGGUGAGGACACCUUCUUC-3′;
(3) 5′-CCAUCUGGAUAAUUCAGCCAUGGGU-3′.

Scrambled RNAi duplex from Invitrogen was used as negative control. Among the three duplexes used, only RNAi duplex 3 consistently inhibited ART activity. The delivery of RNAi duplexes (30 nM) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The efficiency of transfection was assessed using the fluorescent dye Block-it (Invitrogen). Briefly, A549 cells plated onto 100 mm dishes in complete DMEM w/o antibiotics grown to 30–50% confluency were transfected with RNAi duplexes. After 48 h incubation membranes were purified from cells and assayed for transferase activity.

2.6. RNA purification and semiquantitative RT-PCR

Total RNA was purified from A549 (3×10^6 cells) using the RNAeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT-PCR, 1 μg of total RNA was reverse transcribed in cDNA at 55 °C for 2 h with Superscript III and 50 pmol each of ART1, ART3, ART4, ART5 and β-actin gene-specific primers (Table 1) using the One-step RT-PCR system from Invitrogen. The integrity of RNA and cDNA synthesis was verified by PCR measuring the amounts of the housekeeping gene β-actin. The touch-down amplification was performed directly after retro transcription with an initial denaturation step at 95 °C for 2 min, the initial annealing temperature was 70 °C for 1 min followed by 15 cycles, in which temperature decreased 1 °C per cycle. At annealing temperature of 55 °C, 25 cycles were repeated for 90 s followed by an extension phase of 90 s at 72 °C. A final extension phase at 72 °C for 15 min was also performed. PCR products were resolved on 1% Tris–acetate–EDTA (TAE)-agarose gels containing 0.5 μg/ml ethidium bromide. The forward and reverse primers all intron-spanning are shown in Table 1.

2.7. Real-time quantitative RT-PCR

The cDNA was synthesized from total RNA purified as previously described in 2.6 using the ThermoScript RT-PCR system from Invitrogen and used as template in real time PCR. The PCR mixture in a final volume of 25 μl was prepared using the iQ™SYBR® Green supermix from Bio-Rad containing the reference dye and 2 μl of cDNA template. The primer pairs for ART1 gene and actin housekeeping gene as a control reference were added at a final concentration of 200 nM/primer. Samples were run in triplicate in a Chromo-4machine (Bio-Rad) using appropriate 96-well plate. Sample mixtures underwent to the following reaction protocol: initial denaturation at 95 °C for 3 min, followed by 49 cycles of 95 °C for 15 s and 57 °C for 30 s and elongation at 72 °C for 30 s. Samples without cDNA and with water instead of template were used as controls and blanks, respectively. The primers used for ART1 and the housekeeping gene actin were:

ART1F 5′-TTGCTGCTGCTGCTCTG-3′.
ART1R 5′-AGGAATTCTTGGTTACACTGAAG-3′.
ACTF 5′-GCGAGAAGATGACCCAGATC-3′.
ACTR 5′-GGATAGCACAGCCTGGATAG-3′.

The PCR amplified product lengths are 150 and 77 bp for ART1 and actin, respectively. Efficiency and specificity of the reaction calculated using a serial dilution of cDNA was maximal for the pairs of primers used. Following PCR, the melting curve for each product was determined and the correct size calculated by agarose gel analysis. Statistical analysis for each product was performed using a Bio-Rad software based on the method as described in [9].

2.8. TLR4 and TLR2 neutralization by specific monoclonal antibodies

A549 cells cultivated in DMEM complete medium in a 6-well culture plate were pre-incubated with 20 μg/ml of anti-TLR4 (HTA125) and 50 μg/ml anti-TLR2 (T2.5) as suggested by the vendor. After 1 h we added the stimuli at the concentrations described in figure legends.

2.9. Protein assay

Protein content of the membrane preparation and cell lysates was determined using the Bradford assay (Bio-Rad) with BSA (2 mg/ml, Pierce Chemical Co.) as standard.

Table 1
ART and Actin gene-specific primers

Gene	Forward 5′–3′	Reverse 5′–3′	Product size (bp)
ART1	CAGATGCCTGCTATGATGTC	TCAAAGGAGGCCTGGACCAT	981
	AGCCAATGGCAGGAGCGTCA	TCAAAGGAGGCCTGGACCAT	735
ART3	GCAACCATGATTCTAGTGGAC	GATAAACAATGCATCAAACTACAGAGC	1083
ART4	TCT TTTGATGATCAGTACCAA	GAGAGAGATGCAATAGCTATA	722
ART5	ATCCTGCCCCTGAGCCTGGTT	GACACACAGCCACGTCTCTTC	741
	ATCCTGCCCCTGAGCCTGGTT	CGCAGTTAAAGTGGCTGCAGG	703
β-Actin	ATGGATGATGATATCGCCGCG	CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC	1003
	ATCTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGCTTGCTGATCCACATC	838

3. Results

3.1. A549 cells express an enzymatically active ART1 on the cell surface

As shown in Fig. 1, A549 intact cells express an arginine-specific ART activity. In agreement with the concept that ARTs are ecto-enzymes, this ART-activity is associated with membrane fraction. Since ART1 and ART5 are the only arginine-specific enzymes expressed in humans [10,11] we looked for their expression in A549 cells. As shown in Fig. 2a, ART1, but not ART5, mRNA was detected by RT-PCR with full-length (lane 3) and nested primers (lane 4). The amplified PCR product was shown to be ART1 by sequencing. Expression of ART1 in A549 cells was confirmed by western blot analysis of immunoprecipitates of purified membranes probed with α-ART1 polyclonal antibody (Fig. 2b) and by specific ART1 silencing (Fig. 2c).

3.2. LTA and flagellin induce up-regulation of ART1

Lung epithelium represents one of the first barriers against pathogens and can be exposed to a variety of PAMPs. Since ARTs are present at the apical surface of airway epithelial cells [4] we decided to investigate if agonists of the TLR4 and TLR2 that are functionally expressed in A549 cells [12,13] affect ART activity and expression. Therefore, we stimulated A549 with LPS (TLR4 agonist), LTA (TLR2 agonist) or PG whose target are NOD proteins [14] and evaluated the membrane-associated ART-activity. As shown in Fig. 3a, arginine-specific ART-activity was enhanced 1.4- and 3.7-fold, 12 and 24 h after stimulation with LTA, while LPS and PG had no effect. When we performed a dose-response experiment, we observed that LTA induced a 1.75-fold increase over the basal level already at 0.1 μg/ml, while 10 μg/ml was as effective as 1 μg/ml inducing the transferase activity 3.6- and 3.4-fold, respectively. On the other hand the other PAMPs used, LPS and PG, failed to have similar patterns even at 1 or 10 μg/ml (Fig. 3b). Since it has been shown that A549 express also TLR5 [15] and TLR3 [16] we decided to test flagellin (TLR5 agonist) and poly(I:C) (TLR3 agonist) on ART-activity of A549 cells. Flagellin at 10 μg/ml increased transferase activity 2.7-fold over the basal

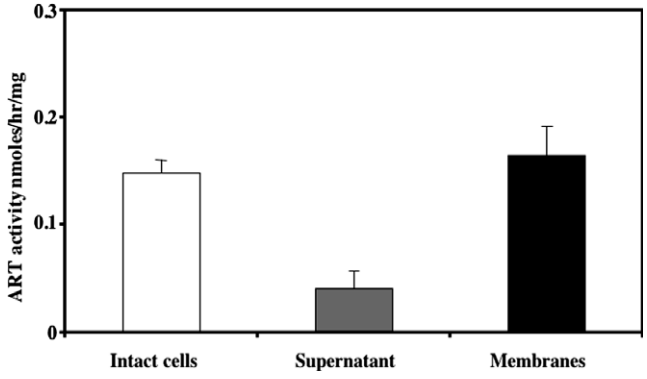

Fig. 1. A549 cells express a cell-surface arginine-specific ART. A549 cells from three 75 cm^2 flasks containing approximately 2×10^6 cells each were trypsinized and resuspended in PBS. Half of the cells were kept intact, part was lysed and membranes purified as described in Section 2.2. The arginine-specific ART-activity was assayed using the agmatine-based assay following the conditions described in Section 2.2. The values presented are the means ± S.D. of three different preparations.

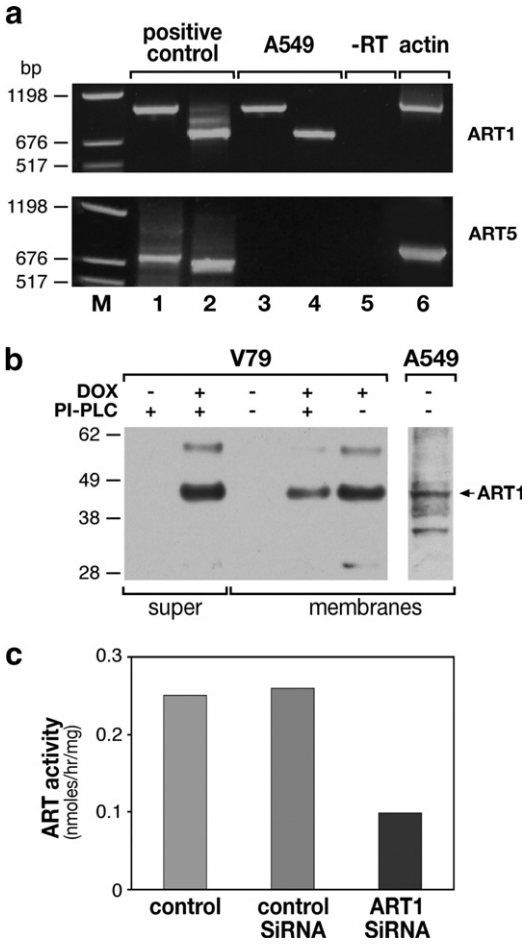

Fig. 2. Expression of ART1 in A549 cells. (a) Total RNA extracted from A549 (1 μg) was reverse transcribed following the procedure explained in Section 2.6. PCR was carried out with ART1 full-length (lane 3) and nested (lane 4) primers or with ART5 full length (lane 3) and nested (lane 4) primers. As positive control for ART1 and ART5 we used 0.5 μg of skeletal muscle and testis RNA, respectively, amplified with full-length (lane 1) and nested (lane 2) primers. In lane 5, PCR was performed with ART1 and ART5 full-length primers without previous RT. β-Actin was amplified as positive control for A549 (lane 6). PCR products were 981 and 735 bp for ART1, 741 and 703 for ART5, 1003 and 838 for β-actin. Positions of molecular mass markers (M) are on the left. (b) Immuno-precipitates from membranes purified from A549 cells (20 μg/lane) were analyzed by immunoblot with α-ART1 rabbit polyclonal antibody. Positive controls were immuno-precipitates from membranes purified from ART1-transfected V79 cells incubated with doxycycline (Dox) to induce ART1 expression treated or not with 1 (U/ml) of PI-PLC for 1 h at 37 °C to release ART1. Immuno-precipitates were also obtained from the supernatant of ART1-transfected V79 cells treated with PI-PLC. Negative controls were immunoprecipitates obtained from membranes and supernatant of V79 cells untreated with Dox. One representative experiment out of three is shown. (c) Untransfected A549 cells (control) or cells transfected with 30 nM of scrambled SiRNA (control SiRNA) or SiRNA targeting ART1 (ART1 SiRNA) were analyzed for transferase activity. After 48 h from transfection, membranes were purified and used to measure the arginine-specific transferase activity. Data show one representative experiment out of two.

level after 24 h of stimulation, while poly(I:C) had no effect (Fig. 4a). To check if the up-regulation of ART-activity is due to an increase in ART1 protein expression, membranes used for the enzymatic assay were immuno-precipitated as described in methods, resolved by electrophoresis, and blotted.

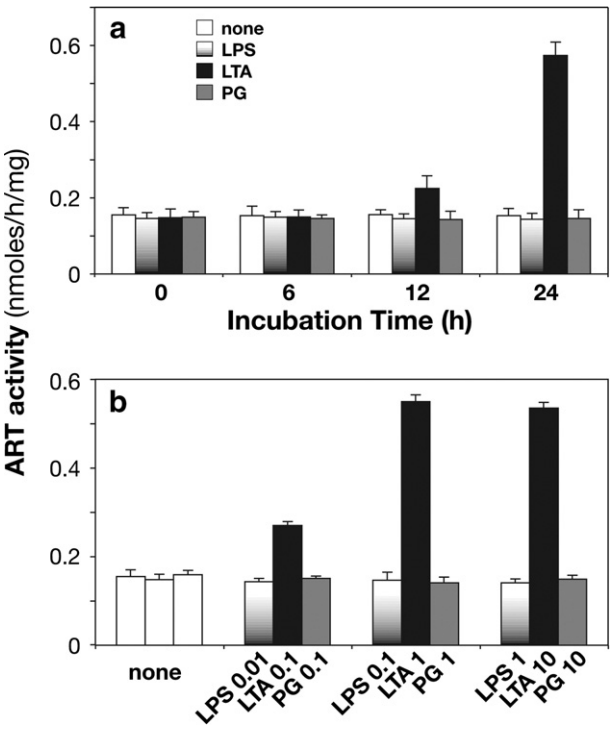

Fig. 3. The arginine-specific ART activity is increased by LTA. A549 were left untreated (none), stimulated with LPS (0.1 μg/ml), LTA (1 μg/ml) or PG (1 μg/ml) for 6, 12 and 24 h (a) or with different doses of LPS (0.01, 0.1, 1 μg/ml), LTA (0.1, 1, and 10 μg/ml) and PG (0.1, 1, and 10 μg/ml) for 24 h (b). Membranes of the stimulated A549 cells were prepared as described in methods and the arginine-specific ART activity assayed with the agmatine-standard assay. Data are means ± S.D. of a representative experiment performed in triplicate.

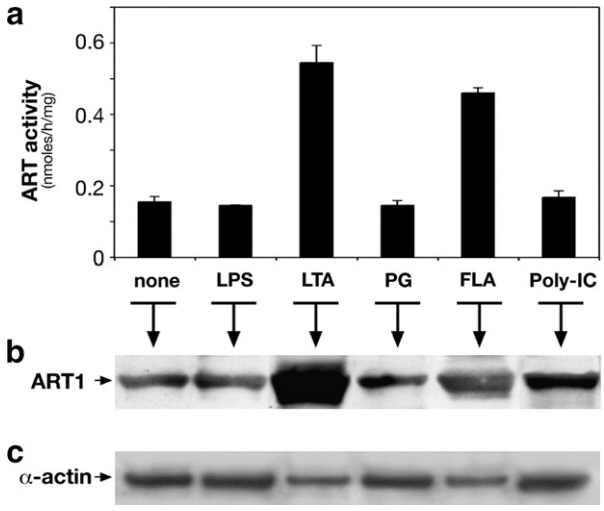

Fig. 4. LTA and flagellin increase ADP-ribosyltransferase activity and ART1 expression in A549 cells. Membranes were obtained from cells treated with LPS (0.1 μg/ml), LTA (1 μg/ml), PG (1 μg/ml), flagellin (10 μg/ml) or poly-IC (10 μg/ml) for 24 h and assayed for ADP-ribosyltransferase activity using agmatine as ADP-ribose acceptor as described in Section 2.3. Results are expressed as the means ± S.D. ($n = 3$) (a). Immunoprecipitates (20 μg) from membranes used for activity were electrophoresed and blotted, ART1 expression was determined by immunoblotting with α-ART1 specific antibodies diluted 1:1000. Data are representative of three independent experiments, each of which exhibited similar results (b). Equal gel loading was confirmed by reprobing stripped membranes with α-actin antibody diluted 1:5000 (c).

Membranes were probed using α-ART1 polyclonal antibodies. As shown in Fig. 4b, both LTA and flagellin significantly up-regulated ART1 expression, compared to un-stimulated control cells.

3.3. Differential regulation of ART1 and ART4 mRNA by bacterial products

Since there are no enzymatic assays to detect the activity of ART3 and ART4 that lack the R-S-EXE motif typical of arginine-specific ARTs [17] and likely possess a different amino-acid specificity, we evaluated by RT-PCR the effects of TLRs agonists on ART gene expression. Culturing A549 for 24 h with LPS, LTA, PG, flagellin or poly(I:C) did not induce the expression of either ART3 or ART5 mRNA, while a strong stimulatory effect on ART4 mRNA expression was found after stimulation of cells with LPS, LTA and PG but not flagellin and poly(I:C) (Fig. 5a). The nucleotide sequencing identified the amplified PCR product of 722 bp as ART4 mRNA. We then repeated the stimulation with LPS, LTA, PG, adding also the synthetic TLR2 agonist Pam_3CSK_4 (PAM). As shown in Fig. 5b, the synthetic TLR2 agonist was as effective as LTA to induce ART4. A pretreatment of cells with anti-TLR4 and anti-TLR2 that functionally block TLR4 and TLR2 receptors, respectively, before stimulation, inhibited ART4 activation indicating TLR4 and TLR2 involved in the mediation of ART4 induction (Fig. 5b). The presence of anti-TLR2 did not completely block the activation of the ART4 gene after stimulation with PG. To validate more accurately the results observed in Fig. 5a regarding the effects of LTA and flagellin on ART1 we performed a qRT-PCR to measure ART1 specific mRNA levels. A549 cells were stimulated as above described including PAM. The results of the relative expression of ART1 relative to the actin housekeeping signal are shown in Fig. 5c. This profile substantiates more clearly the profile shown in Fig. 5a, confirming the up-regulation exerted on ART1 gene by LTA but not by PAM, and flagellin. The incubation with TLR2 antagonist specifically blocks the ART1 up-regulation by LTA indicating that the effect is mediated by this receptor.

4. Discussion

Recent studies in human monocytes, have demonstrated that PAMPs modulate ART expression [18], however, ART regulation by bacterial products remained largely unexplored particularly in airway epithelial cells. Since we have previously shown that ART1, ART3 and ART4 are expressed in intermediate and ciliated epithelial cells purified from bronchoalveolar lavage (BAL) of healthy volunteers [4] we evaluated whether PAMPs can modulate ARTs in A549 cells, a type II lung epithelial cell line.

In this report, we provide evidence that A549 cells constitutively express an enzimatically active ART1 on the cell surface. Remarkably, ART1 was up-regulated both at mRNA and at protein level by LTA and flagellin but not by LPS, PAM, PG, and poly(I:C). ART4 mRNA not constitutively expressed by A549 was induced by LPS, LTA, PAM and PG but not by flagellin or poly(I:C).

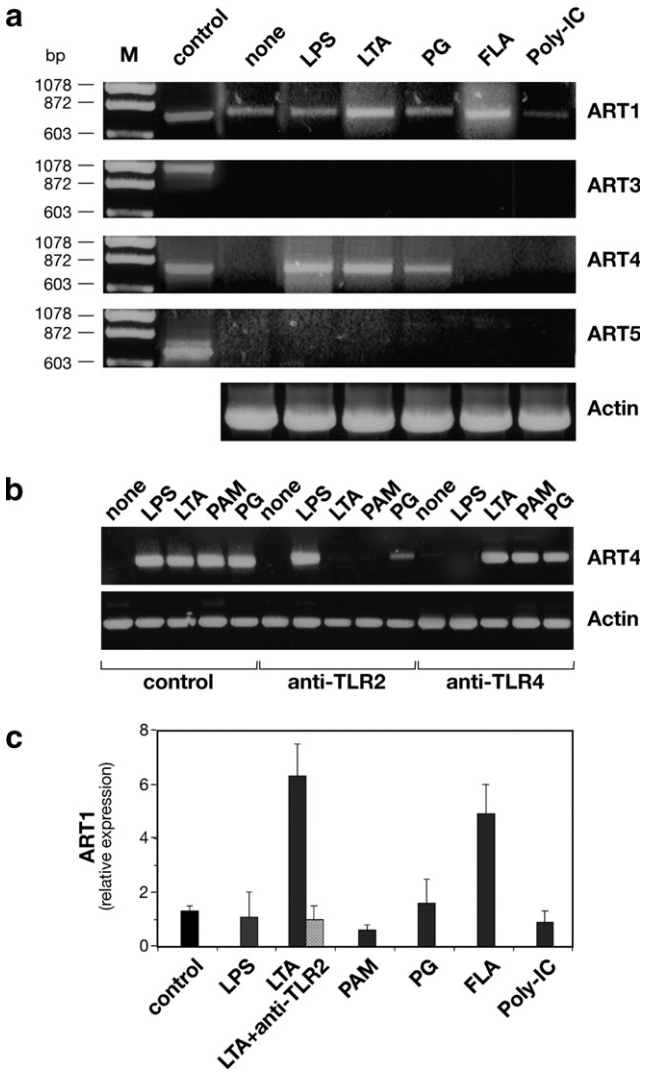

Fig. 5. Effect of different TLR agonists on ART gene expression (a) A549 cells were treated with LPS (0.1 μg/ml), LTA (1 μg/ml), PG (1 μg/ml), flagellin (10 μg/ml) or poly(I:C) (10 μg/ml) for 24 h. RNA was extracted and ARTs mRNA levels assessed by RT-PCR amplification as described in Section 2. Control RT-PCR was carried out with human skeletal muscle (ART1), human testis (ART3), human spleen (ART4) and human testis (ART5) RNA. Molecular mass markers (M) were used to confirm the size of the PCR products. The results presented in this panel were confirmed in two independent experiments using different A549 preparations (a). (b) A549 cells were treated as above described including PAM (1 μg/ml). In other experiments cells were pre-incubated with 20 μg/ml of anti-TLR4 (HTA125) or 50 μg/ml of anti-TLR2 (T2.5) for 1 h at 37 °C before adding PAMPs as above described. Total RNA was purified after 24 h of incubation and processed as before to assess the levels of ART4 mRNA. (c) Real-time quantitative RT-PCR analysis of ART1 transcript in A549 after stimulation of cells with PAMPs using the same conditions as described in (a). Data shown are relative expression of ART1 mRNA with respect to the actin housekeeping signal. Data are means ± S.D. of a representative experiment performed in triplicate.

The ART family is a group of proteins structurally related to ADP-ribosylating toxins. ART1 and ART5 that transfer the ADP-ribose moiety to arginine are likely candidates for the ART activity found in these cells. Identification of ART1 transcripts and specific silencing indicated ART1 responsible for the activity. Immunostaining with α-ART1 polyclonal antibodies confirmed the presence of the protein associated to the cell membrane and localization experiments performed with intact cells, showed that the catalytic site of this enzyme is located on the outer side of the cell membrane, the same orientation found for ART1 on polarized and intermediate epithelial cells purified from bronchoalveolar lavage [4].

A549 produce β-defensins in response to LTA [13] and this effect is sustained over the 24 h time period [19]. Defensins exhibit a wide range of antimicrobial activities but are also cytotoxic for several types of mammalian cells including A549 [20]. Interestingly, α-defensins are substrate of ART1 in bronchoalveolar lavage fluids of habitual smokers and it has been shown that while ADP-ribosylated-defensins retained their chemoattractant activity they lost cytotoxic activity [5]. Defensins reach cytotoxic concentrations during lung inflammation determining severe damage to epithelial cells [21]. Enhancement of ART1 activity and expression could cause an increase in ADP-ribosylated defensins and this might protect cells lining the airway lumen from negative effects of cytotoxic concentrations of antimicrobial peptides. As lung epithelia is particularly exposed to inflammation this observation might be of clinical relevance.

While we failed to induce expression of ART3 and ART5 mRNA with the TLR agonists used, our results show that LTA, LPS, PAM and PG exerted a strong activation of ART4 gene expression. Inhibition exerted by functional antibodies to TLR2 and TLR4 indicates that this effect is mediated by TLR2 and TLR4 receptors. These results confirm that, as others reported in monocytes, ART4 regulation at least at mRNA level, is a more general intracellular event related to cell-activation by a variety of extracellular stimuli [18]. The immune response to bacterial flagellin is mediated by TLR5 [22] and likely this receptor mediates the ART1 up-regulation. On the contrary while PAM the synthetic TLR2 ligand does not up-regulate ART1 transcript, TLR2 is involved in LTA regulation. This discrepancy as above for PG activation of ART4 could be explained by the presence of contaminants in purified PAMPs and not in synthetic agonists, which can activate different TLRs [14]. Overall the above observations lead to a differential response of the ARTs to the TLR agonists. These effects may be only partially explained by the heterogeneity in expression of TLR4 and TLR2 in these cells. TLR4 is not surface expressed in A549 [12], but the intracellular receptor is functionally responsive to LPS [12] and in A549 mediates ART4 activation as our results evidence. It has been shown recently that non-hematopoietic cells including epithelial cells play a crucial role for NOD-1 innate immune recognition of PG [23]. Given their large surface area, lung epithelial cells interacting with pathogens might play a primary role in inflammatory response recruiting immunological cells, therefore our studies highlighting the stimulatory effect elicited by TLR agonists specifically mediated by TLR2, TLR4 and TLR5 receptors on ARTs are relevant to support the involvement of mono ADP-ribosylation in immune response in airways. Although ARTs are structurally and functionally related proteins, they might follow different pathway of activation in response to PAMPs, suggesting different physiological roles and indicating that the challenge of future studies will be exploring the specific role of ART1 and ART4 in inflammatory disorders.

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