An asymmetrically dimethylarginated nuclear 90 kDa protein (p90aDMA) induced by interleukin (IL)-2, IL-4 or IL-6 in the tumor microenvironment is selectively degraded by autophagy

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Abstract. Protein arginine methylation is a common post-translational modification resulting in the generation of asymmetric dimethylarginine (aDMA) and symmetric dimethylarginine (sDMA). Currently, the regulation of aDMA or sDMA by hypoxia, nutrient stavation or cytokines in the tumor microenvironment remains largely unknown. Here we show that p90aDMA, p70aDMA and p90sDMA, endogenous proteins containing aDMA or sDMA with mass 70 or 90 kDa, were widely and dominantly expressed in breast cancer cell lines. Notably, it was p90aDMA rather than p90sDMA that accumulated in the nucleus upon stimulation of cancer cells with interleukin (IL)-2, IL-4, IL-6 but not IL-8. In addition, the p90aDMA accumulation could be inhibited after treatment with a global methyltrasferase inhibitor, adenosine-2',3'-dialdehyde (AdOx). It seemed that some endogenous proteins in cancer cells were asymmetrically arginine-methylated upon exposure to some cytokines.. Furthermore, endogenous proteins of aDMA, such as p90aDMA and p70aDMA, were degraded in response to hypoxia, nutrient starvation and rapamycin treatment in breast and cervical cancer cells. IL-2/4/6 slightly increased basal autophagy but slightly decreased the rapamycin-induced autophagy in cancer cells, suggesting that IL-2/4/6 and autophagy inducers play distinct roles in the regulation of aDMA of proteins. Conversely, rapamycin accumulated p90sDMA in MDA-MB-231 and MCF-7 cells. Taken together, our results add a new dimension to the complexity of arginine methylated regulation in response to various stimuli and provide the first evidence that aDMA serves as one specific degradation signal of selective autophagy.

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Abbreviations: IL, interleukin; ATG, autophagy-related gene; LC3, microtubule-associated protein 1 light chain 3; PTMs, posttranslational modifications; sDMA, symmetric dimethylarginine; aDMA, asymmetric dimethylarginine; AdOx, adenosine-2',3'-dialdehyde; HBSS, Hank's balanced salt solution

Key words: interleukin, autophagy, arginine methylation, asymmetric dimethylarginine, tumor microenvironment

Introduction

The tumor microenvironment consists of extracellular matrix, stromal cells (for example, endothelial cells, fibroblasts, myofibroblasts and leukocytes), intratumoral hypoxia and nutrient starvation, and cytokines, chemokines and proteins secreted by epithelial, cancer or stromal cells (1,2). Cytokines, chemokines and growth factors, including interleukin (IL) superfamily, form complex immune signaling networks and have important roles in various aspectes of cancer initiation and progression (2-4). Several cytokines including IL-2, IL-12, TNF-α, type I IFNs and GM-CSF possess anticancer potential via intratumoral delivery (2,4,5). IL-2, which has antitumor functions that include the activation of natural killer (NK) and cytotoxic T cells, can induce durable remissions in 5-10% of patients with metastatic melanoma and renal cell carcinoma, malignancies with poor prognoses (6). During an immune response, cancer cells encounter extrinsic and intrinsic factors, including oxidative stress, nutrient availability, and inflammation, that can modulate their capacity to activate, proliferate, and survive.

Autophagy occurs at a constitutive basal level, but it can be enhanced in response to various types of stress, mainly

including oxygen tension, nutrient deprivation and chemicals such as rapamycin, all of which can inhibit mechanistic target of rapamycin (mTOR) pathway and thus initiate autophagy (7-9). Steps in the autophagy pathway involve nucleation of targeted macromolecules on the ER membrane, trafficking of autophagosomes to lysosomes and, finally, fusion of the autophagosome-lysosome, resulting in targeted protein degradation (10,11). This process is controlled by the products of numerous autophagy-specific genes (ATGs). A key regulator of autophagy is microtubule-associated protein 1 light chain 3 (LC3, a mammalian homolog of yeast Atg8), which controls major steps in the autophagic pathway including the growth of autophagic membranes, recognition of autophagic cargoes, and the fusion of autophagosomes with lysosomes (12). Recent studies demonstrate that the phosphorylation of LC3 at threonine 50 (Thr50) plays a critical role in mediating fusion of autophagosomes with lysosomes (13).

Increasing evidence shows that the posttranslational modifications (PTMs), including phosphorylation, acetylation, methylation, and ubiquitination, are important for regulating the autophagy process by providing structural and functional diversity among proteins (12,14-16). The majority of research is dedicated to the PTMs of key autophagy-related molecules containing autophagy receptors, providing a layer of regulation for the specificity and efficiency of selective autophagy. Selective autophagy refers to the selective degradation of, for instance, organelles (mitophagy and pexophagy), bacteria (xenophagy), ribosomes, macromolecular structures, specific proteins and protein aggregates (aggrephagy) by autophagy (17). Optineurin, an autophagy receptor, is phosphorylated by the protein kinase TBK1 (TANK binding kinase 1) at serine 177, which enhances the LC3 binding affinity and autophagic clearance of cytosolic *Salmonella enterica* (18). However, little is known about the PTMs of substrates determining their specific recognition by autophagy receptors. NDP52, which is an autophagy adaptor that contains an LC3-interacting region (LIR) motif (17), is induced by Nrf2 and specifically directs phosphorylated tau to the autophagic degradative pathway (19). Mad1, a member of the Myc/Max/Mad family, can also be phosphorylated at serine 145 and introduced into the autophagic degradation (20). Interestingly, EPG-11/PRMT-1 directly methylates arginines in the RGG domains of PGL-1 and PGL-3 and promotes their autophagic removal in *C. elegans* during embryogenesis (21). However, the link between arginine methylation and selective autophagy has not been clearly demonstrated in cancer.

Arginine methylation is one common PTM mainly of nuclear proteins in eukaryotic cells, and is catalyzed by a family of enzymes termed protein arginine methyltransferases (PRMTs) (22,23). Three main forms of methylarginine have been identified in eukaryotes: N^G-monomethylarginine (MMA), N^GN^G (asymmetric) dimethylarginine (aDMA), and N^GN^G (symmetric) dimethylarginine (sDMA) (24). In humans, PRMTs are classified into type I (PRMT1, PRMT2, PRMT3, PRMT4 and PRMT6), type II (PRMT5 and PRMT7) and type III (PRMT7) methyltransferases, based on their corresponding aDMA, sDMA and MMA activities, respectively. PRMT1 and PRMT5 are the major asymmetric and symmetric arginine methyltransferases, respectively (25). Arginine methylation has received increasing attention over the last years as several recent reports have illustrated a novel role for this posttranslational modification in regulating protein-protein interaction and transcriptional induction (26,27), and is often deregulated in cancer (25). However, how arginine methylation could be regulated by interleukins or autophagy in the context of the tumor microenvironment has not yet been investigated.

In this study, we identified for the first time that p90aDMA, which is a 90-kDa protein of aDMA in the nucleus and accumulated by IL-2, IL-4 and IL-6, can serve as a unique substrate for selective autophagy. Conversely, p90sDMA was a 90-kDa protein of sDMA and accumulated in a dose-dependent manner in response to rapamycin treatment. Taken together, our study provides evidence for immunity regulation through crosstalk between arginine methylation and selective autophagy in the tumor microenvironment by using *in vitro* models.

Materials and methods

Chemicals and reagents. Recombinant human IL-2, IL-4, IL-6 and IL-8 were purchased from Peprotech (NJ, USA). They were dissolved in water to a concentration of 5 μg/ml. 3-Methyladenine (3-MA) was purchased from Sigma (MO, USA) using as an inhibitor of autophagy. 3-MA (100 mg) was dissolved in phosphate-buffered saline (PBS) to make a 100-mM stock solution. Adenosine-2,3 dialdehyde (AdOx) was purchased from Sigma and used as an inhibitor of methyltransferase. AdOx (5 mg) was dissolved in 0.2 M HCl to make a 10-mM stock solution. Rapamycin was also purchased from Sigma.

Cell culture. Human cancer cell lines including MDA-MB-231, MDA-MB-468, MCF-7, SKBR3, T47D and HeLa cells were obtained from American Type Tissue Culture Collection (ATCC, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) containing 10% fetal bovine serum (FBS, CA, USA) and 100 U/ml penicillin-streptomycin (Gibco) in a humidified incubator of 5% CO_2 at 37˚C. The standard hypoxic conditions were 1% O_2 and 5% CO_2. Hypoxia was done in a multi-gas incubator chamber with a compact gas oxygen controller (MCO-5M, Sanyo, Osaka-SHI, Japan) to maintain oxygen concentration at 1% by injecting a gas mixture of 95% N_2 and 5% CO_2.

Small interfering RNA (siRNA) transfection. siRNA targeting ATG5 (5'-CCAUCAAUCGGAAACUCAUTT-3') and negative control siRNA (5'-UUCUCCGAACGUGUCACGUTT-3'), which was used for normalisation, were synthesised by Genepharma (Shang Hai, China). For transfection, MCF-7 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. After 24 h, cells were transfected with siRNA (50 pmol) using Lipofectamine® 2000 Reagent (Invitrogen, CA, USA) according to the instructions of the manufacturer. After 24 h of transfection, cells were treated with 100 nM rapamycin for another 24 h. Then the cells were harvested and the knockdown of ATG5 was confirmed by western blot analysis.

Western blot analysis. Monolayer cultures of respective cell lines at an 80-90% confluence were prepared with pre-cold

Table I. Primary antibodies used in western blot analysis.

Target	Source	Host	Dilution	Catalog
SYM11	Merck Millipore, MA, USA	Rabbit	1:1,000	07-413
ASYM24	Merck Millipore, MA, USA	Rabbit	1:500	07-414
LC3B	Cell Signaling Technology, Inc. (CST), MA, USA	Rabbit	1:1,000	#3868
ATG5	Cell Signaling Technology, Inc. (CST), MA, USA	Rabbit	1:1,000	#12994
TBP	Proteintech, Wu Han, China	Rabbit	1:500	22006-1-AP
β-actin	Zen Bioscience, Cheng Du, China	Mouse	1:20,000	70068

RIPA lysis and extraction buffer (Thermo Scientific, CA, USA) containing protease inhibitor cocktail (Roche, Basel, Switzerland) on ice. The total cell lysate was centrifuged and the supernatant was denatured by boiling. Protein concentrations of supernatants were analyzed by bicinchoninic acid (BCA) assay kit (Beyotime, Nan Tong, China). Equivalent amounts of total proteins (20 μg) were subjected to 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a 0.45-μM PVDF membrane (Millipore, MD, USA). The membranes were blocked for 2 h in 5% bovine serum albumin (BSA) at room temperature and incubated with specific primary antibodies at 4˚C overnight. A list of the primary antibodies used for western blot analysis are characterized in Table I. Further incubation was performed with the corresponding horseradish peroxidase-coupled secondary antibodies (1:10,000, cat. nos. sc-2004 and sc-2005; Santa Cruz, CA, USA) at room temperature for 2 h. Then the bands were detected using Super Signal® West Pico Chemiluminescent Substrate kit (Thermo Scientific), and the results were recorded using the ChemiDox™ XRS+ system. Relative protein expression was normalized with β-actin.

Nuclear cytoplasmic fractionation. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce, Thermo Fisher Scientific, CA, USA). The quality of nuclear and cytoplasmic extracts was verified by immunoblotting with protein differentially enriched in the nucleus (TBP) or the cytoplasm (β-actin).

Results

Comparison of basal sDMA or aDMA levels in breast cancer cell lines. Breast cancer is a heterogeneous group of diseases with different histological prognostic and clinical aspects (28). To investigate the preference for the substrate methylation state, we collected the lysates of five human breast cancer cell lines and evaluated the basal aDMA and sDMA using immublotting. Endogenous proteins of aDMA and sDMA can be specifically recognized by antibodies ASYM24 and SYM11, respectively. Both SYM11 and ASYM24 antibodies recognized unknown endogenous proteins containing methylated arginine in breast cancer cells with molecular mass of 90, 70, 55 and 34 kDa. Proteins of 90 and 70 kDa recognized by SYM11 were termed p90sDMA and p70sDMA. Proteins of 90, 70 and 34 kDa recognized by ASYM24 were termed p90aDMA, p70aDMA and p34aDMA. p90sDMA, p90aDMA and p70aDMA were widely and dominantly expressed in breast cancer cells but at relatively higher levels in MCF-7 cells and lower levels in the MDA-MB-231 cells (Fig. 1). Interestingly, the p90sDMA and p70aDMA proteins were predominantly expressed in breast cancer cells at relatively higher levels in the MCF-7, MDA-MB-468, T47D and SKBR3 cells but lower levels in the MDA-MB-231 cells (Fig. 1). In contrast, p90aDMA and p70sDMA proteins were constitutively expressed at a lower level in the breast cancer cell lines (Fig. 1). As a result, the ratio of p90sDMA to p70sDMA was higher than the ratio of p90aDMA to p70aDMA in each line (Fig. 1).

Expression of p90aDMA is specifically enhanced by IL-2, IL-4 or IL-6 but not by IL-8 in cancer cells. To determine whether the cancer immune microenvironment enriched with cytokines could mobilize arginine methylation, we treated MDA-MB-231 cells with IL-2, IL-4, IL-6 or IL-8 for 24 h because of their action on cancer cell proliferation (29). As a result, IL-2, IL-4, IL-6 but not IL-8 specifically enhanced expression of p90aDMA in the MDA-MB-231 cells (Fig. 2C and D). In accordance with Fig. 2B and D, we observed that 90aDMA was increased in a dose-dependent manner upon simulation with IL-2/IL-6 in MDA-MB-231 cells (Fig. 3A and B). However, these cytokines had no effect on the expressions of p90sDMA, p70sDMA and p70aDMA (Fig. 2).

To confirm that the methylation signals detected by the ASYM24 antibody were specific, similar experiments were performed in IL-2 or IL-6 treated MDA-MB-231 cells incubated with or without adenosine-2', 3'-dialdehyde (AdOx), a methyltransferase inhibitor. It was found that basal or IL-2/6 induced aDMA was almost blocked in the presence of AdOx (Fig. 3C and D). Of note, IL-2 or IL-6 did not alter the expression of p70aDMA (Fig. 3C and D). Therefore, it could be considered that IL-2 and IL-6 specifically induced the accumulation of p90aDMA in MDA-MB-231 cells. The reason for the specific increase in p90aDMA is unclear.

Rapamycin specifically inhibits p90aDMA expression in cancer cells independent of cytokine stimulation. Arginine methylation has been considered to be an irreversible post-translational modification until recently. However, a recent study suggested that autophagy may regulate arginine methylation (30). Then, rapamycin, an autophagy enhancer, was administrated in control or IL-2/4/6/8 treated MDA-MB-231 cells. It was the aDMA levels rather than the sDMA levels

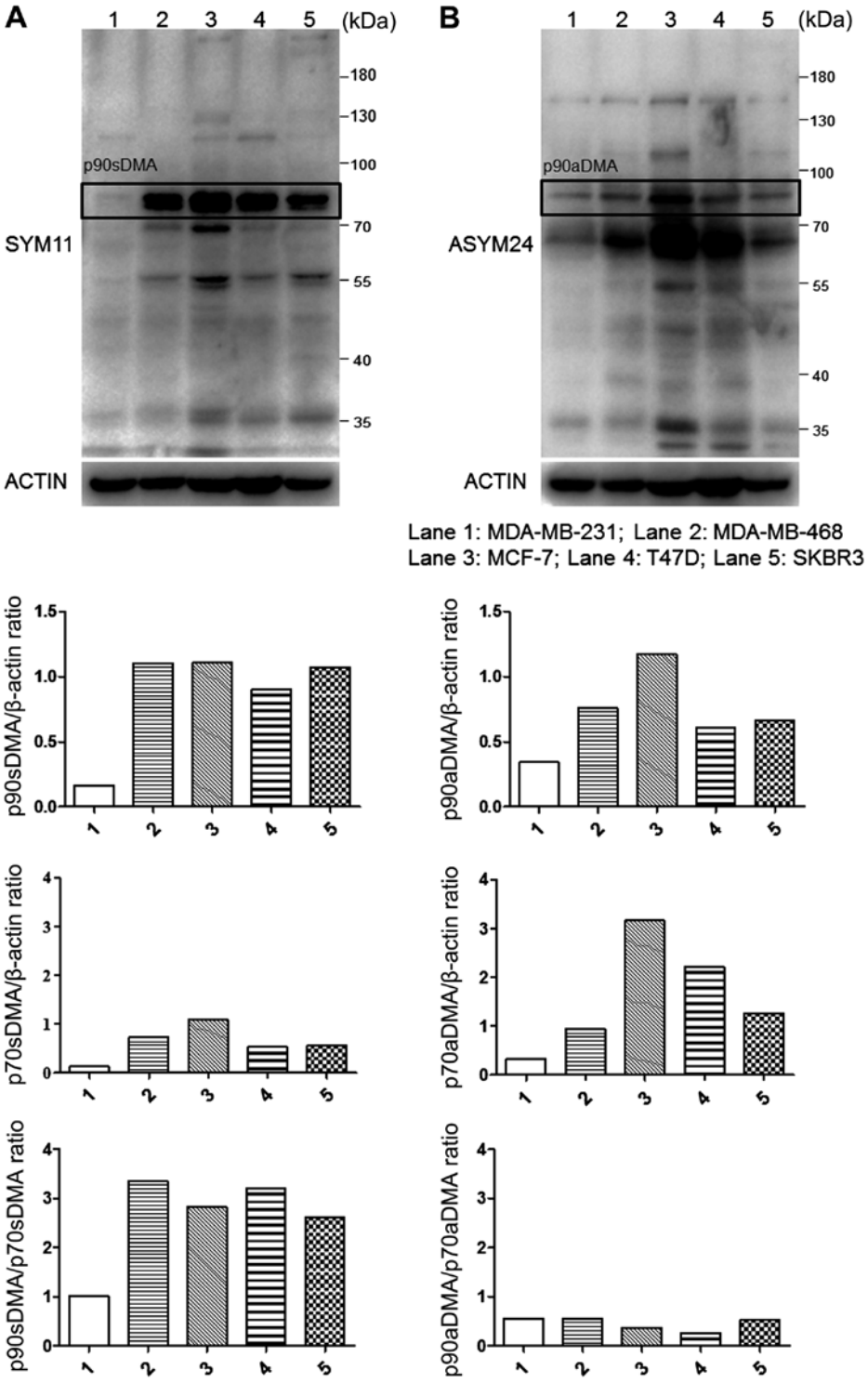

Figure 1. Constitutive expression pattern of aDMA- and sDMA-modified proteins in breast cancer cell lines. Total proteins of breast cancer cells were resolved by SDS-PAGE and probed with symmetric dimethylarginine-specific rabbit polyclonal antibodies (SYM11) or the asymmetric dimethylarginine-specific rabbit polyclonal antibodies (ASYM24). p90sDMA and p90aDMA bands are highlighted. (A) The expression of sDMA proteins in various breast cancer cell lines. (B) The expression of aDMA proteins in various breast cancer cell lines. β-actin was used as an internal control. The band intensity of p90sDMA, p70sDMA, p90aDMA or p70aDMA normalized by internal control β-actin is displayed as histograms below the western blot images, respectively.

that were significantly reduced regardless of the interleukin stimulation (Fig. 2). On the contrary, p90sDMA was increased in rapamycin treated MDA-MB-231 cells (Fig. 2A and C). These results drew our attention to the possibility that aDMA serves as a novel and specific signaling molecule that provokes selective autophagic degradation.

To determine if the increased nuclear p90aDMA resulting from IL-2, IL-4 or IL-6 exposure could be due to autophagy inhibition, we monitored whether it affects the ratio of LC3-II/I, a canonical hallmark of autophagy. MDA-MB-231 cells were exposed to IL-2, IL-4, IL-6 or IL-8 for 24 h, lysed and subjected to western blotting for detecting the abun-

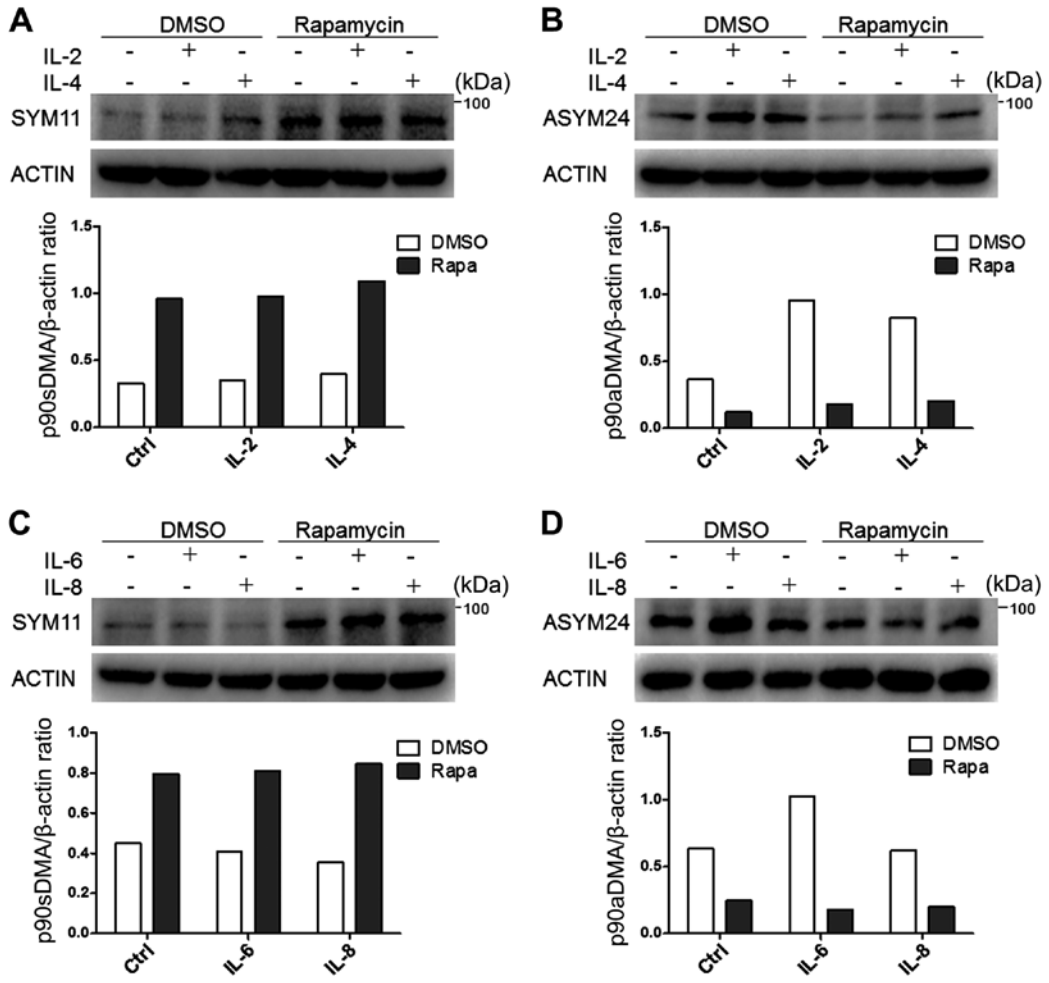

Figure 2. The p90aDMA but not p90sDMA was specifically upregulated by IL-2, IL-4 or IL-6 rather than IL-8 in cancer cells and suppressed by rapamycin. MDA-MB-231 cells were cultured in the presence or absence of 100 nM rapamycin for 24 h and the interleukins (IL-2, IL-4, IL-6 or IL-8, 25 ng/ml) were added into the cultures at the same time. Cell lysates were prepared and separated by 8% SDA-PAGE, and subjected to western blot analysis for p90sDMA (A and C) p90aDMA (B and D). The band intensity of p90sDMA or p90aDMA normalized by β-actin is displayed as histograms below the images.

dance of autophagic markers. As shown in Fig. 4A and B, IL-2, IL-4, IL-6 but not IL-8 slightly increased the ratio of LC3-II/I. To determine the dose curve of IL-2/IL-6 exposure for the expression of autophagic markers, MDA-MB-231 cells were exposed to varying doses of IL-2 or IL-6 (5, 25, 100 and 500 μM) for 24 h and assessed for expression of autophagic markers by western blotting. The ratio of LC3-II/I exhibited a similar dose-dependent trend in response to IL-2 or IL-6 with a slight increase of 20% at 25 ng/ml IL-2 and 100 ng/ml IL-6 exposure, respectively (Fig. 4C and D). We next determined whether IL-2, IL-4, IL-6 or IL-8 affects autophagy induction. We monitored rapamycin-induced autophagy by assessing conversion of LC3-I to LC3-II. IL-2, IL-4, IL-6 but not IL-8 slightly decreased conversion of LC3-I to LC3-II induced by rapamycin (Fig. 4A and B). Taken together, these findings demonstrated that IL-2, IL-4, IL-6 but not IL-8 slightly increased the basal autophagy but slightly decreased the rapamycin-induced autophagy in MDA-MB-231 cells, indicating that IL-2/IL-4/IL-6 accumulated p90aDMA in an autophagy-independent pathway.

The aDMA proteins are localized in the nucleus of cancer cells. Extensive studies have focused on autophagic turnover of cytoplasmic materials, little is known about the role of autophagy in degrading nuclear components (31). To determine the cellular localization of proteins containing aDMA, we examined p90aDMA and p70aDMA in the cytoplasm and nucleus through cellular fractionation experiments followed by western blot analysis in the IL-2 or IL-6 treated MDA-MB-231 cells, and found that both p90aDMA and p70aDMA distributed and accumulated predominantly in the nucleus regardless of cytokine stimulation, suggesting that the aDMA proteins were constitutively localized in the nucleus and might play a critical role (Fig. 5A). To monitor cytoplasmic contamination of the nuclear extracts, immunoblotting was performed using the anti-β-actin antibody and TBP (TATA box binding protein) antibody as the internal controls. The dramatic reduction of β-actin or the presence of TBP only in the nuclear fractions confirmed that cytoplasmic contamination did not occur (Fig. 5A).

Hypoxia and nutrient starvation disablizes nuclear p90aDMA and p70aDMA in cancer cells. Tumor cells are continually subjected to diverse stress conditions of the tumor microenvironment, including hypoxia, nutrient deprivation, oxidative or genotoxic stress (1). Among the stresses, hypoxia or starvation is a classical autophagy inducing stimulus (32,33). As shown

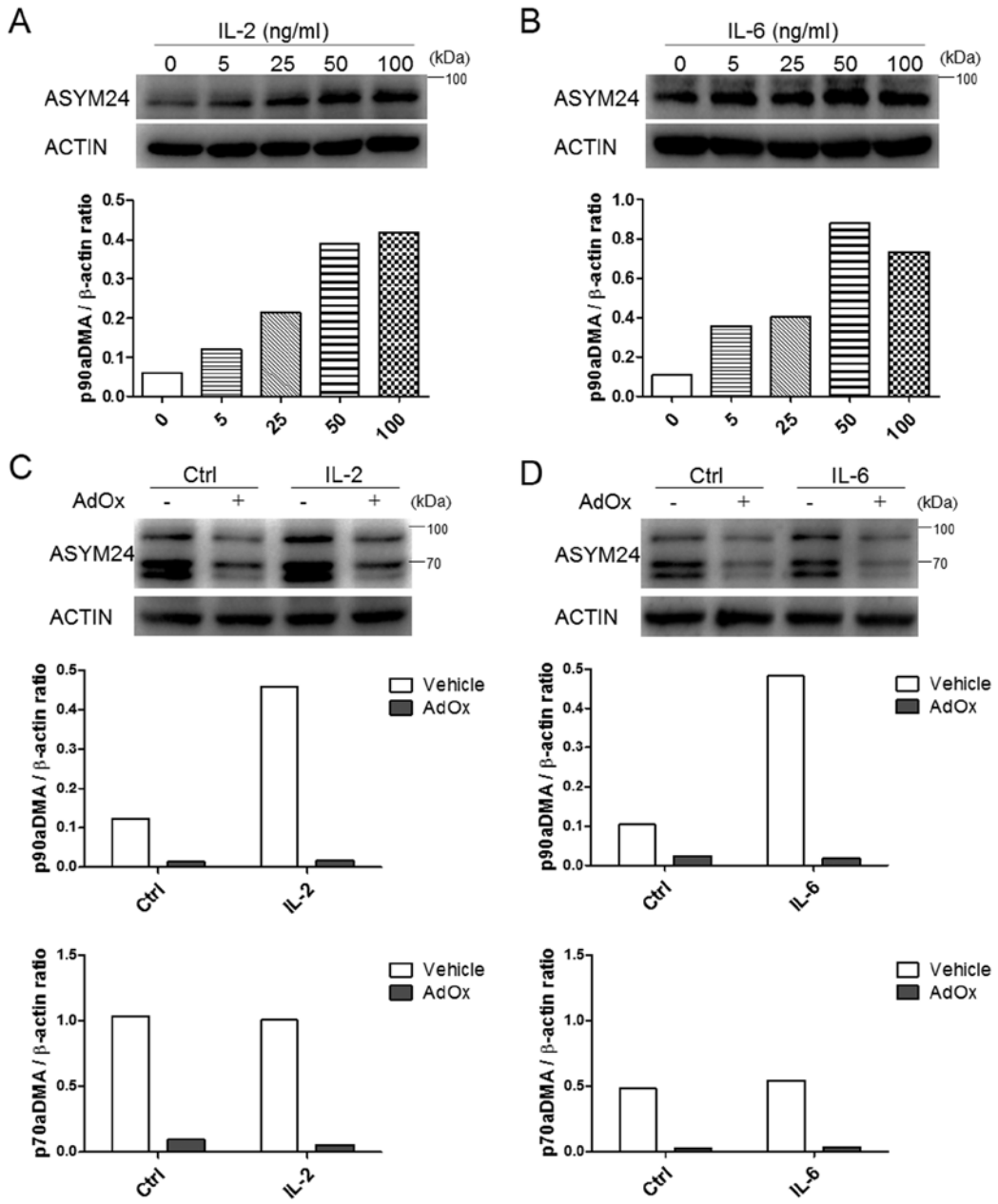

Figure 3. IL-2 or IL-6-induced p90aDMA was mediated by methyltransferases. (A and B) MDA-MB-231 cells were treated with various concentrations of IL-2 or IL-6 (0, 5, 25, 50 and 100 ng/ml) for 24 h, and the levels of p90aDMA was determined by western blot analysis. (C and D) MDA-MB-231 cells were pre-treated with 20 μM AdOx for 1 h and then treated with 25 ng/ml IL-2 or IL-6 for 24 h. The cell lysates were analyzed by immunoblotting with asymmetric dimethylarginine-specific antibodies (ASYM24). The band intensity of p90aDMA or p70aDMA normalized by β-actin is displayed as histograms.

in Fig. 5, two WB bands representing 70 kDa (p70aDMA) and 90 kDa (p90aDMA) nuclear-localized proteins, respectively, were detected in breast cancer MDA-MB-231 cells and cervical cancer HeLa cells. In accordance with the deacetylated nuclear LC3 being transported into the cytoplasm to carry out PE conjugation to pre-autophagic membranes (34), we observed that LC3 in the nucleus vanished under hypoxia or starvation (Fig. 5C and D) and aDMA proteins dominantly expressed in the nucleus were diminished synchronously (Fig. 5B, C and D).

As is well known, LC3 proteins play a key role in the selective recruitment of autophagic cargoes into autophagosomes, and serve as docking sites for adaptor proteins (12,31). Therefore, it is conceivable that proteins of aDMA including p90aDMA and p70aDMA as LC3 cargo substrates were translocated from the nucleus to the cytoplasm and tethered to the site of engulfing autophagosomes. The hypothesis needs further investigation under various stress conditions in our future work.

Autophagy inhibition reverses the degradation of p90aDMA and p70aDMA proteins in cancer cells. We first examined the effects of rapamycin treatment on LC3 protein levels in MCF-7 cells. Immunoblot analyses showed a concentration-dependent increase in the ratio of LC3-II/I, representing mounting activation of autophagy (Fig. 6A). Concomitantly with the activation of autophagy, a 90-kDa protein (p90sDMA) showed a marked dose-dependent increase in sDMA levels (Fig. 6B). Whereas the effects of rapamycin treatment on cellular aDMA in MCF-7

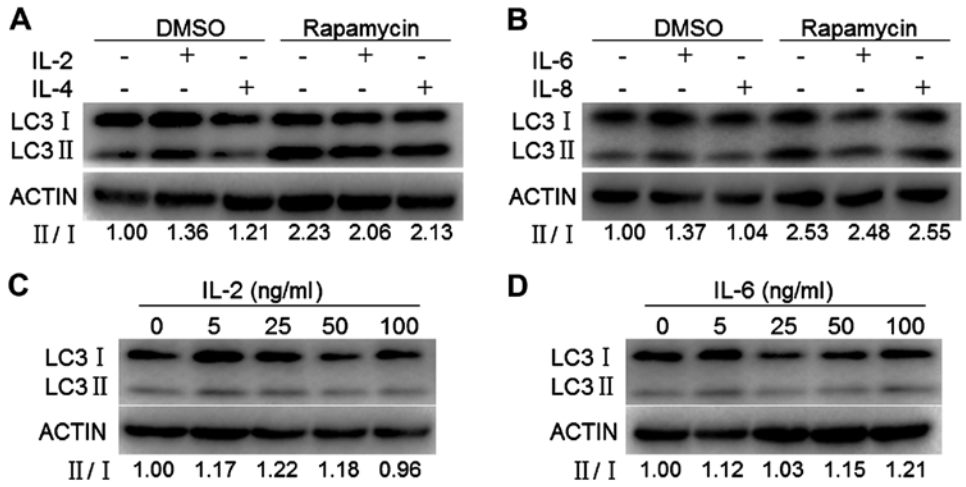

Figure 4. Autophagy is barely activated by cytokines. (A and B) MDA-MB-231 cells were treated with or without 100 nM rapamycin and the interleukins (IL-2, IL-4, IL-6 or IL-8) were added into each group. (C and D) MDA-MB-231 cells were treated with various concentrations of IL-2 or IL-6 (0, 5, 25, 50 and 100 ng/ml). After 24 h, the levels of protein expression were analyzed by western blot analysis using antibodies against LC3 and β-actin, respectively. The intensities of LC3-II/I bands were quantified using ImageJ analysis software and corrected by the band intensity of β-actin. The band intensity of LC3-II was normalized by LC3-I and the ratios of LC3-II to LC3-I was compared with that of the control.

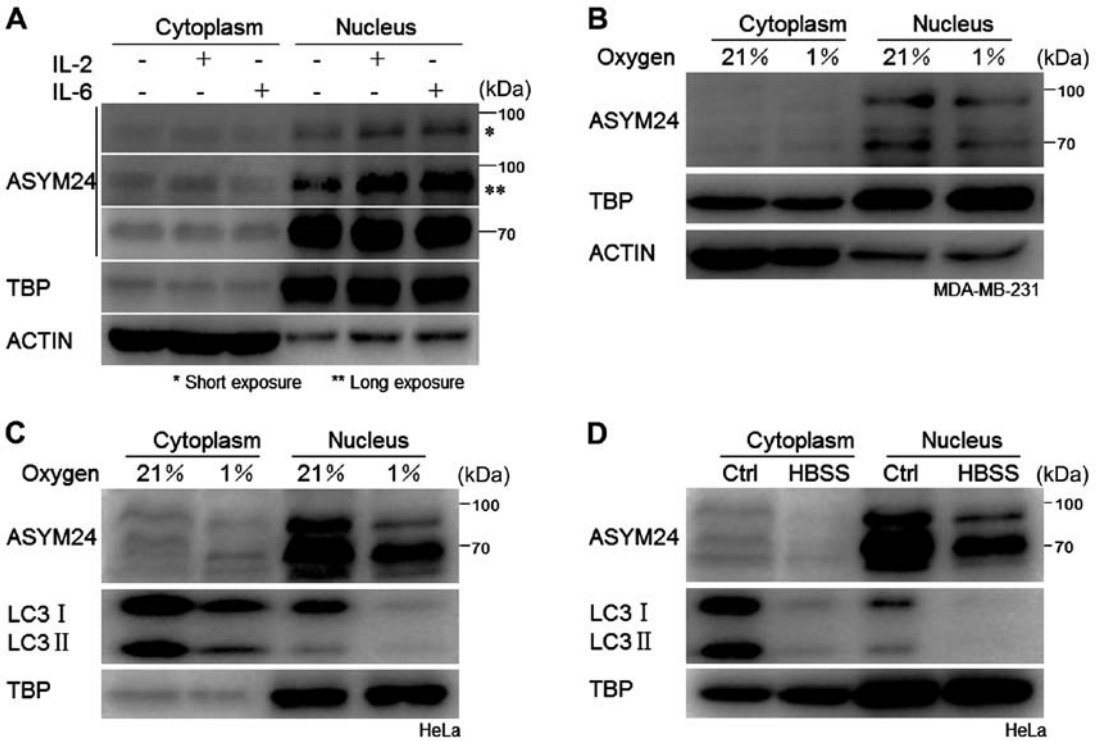

Figure 5. The p90aDMA and p70aDMA proteins accumulate in the nucleus of cancer cells and degrade in response to cellular stresses. (A) Nuclear and cytoplasmic extracts were prepared after MDA-MB-231 cells were cultured with IL-2 or IL-6 as indicated. Fractions were analyzed by western blot analysis using antibody to ASYM24. TBP was probed as a loading control. (B and C) MDA-MB-231 cells or HeLa cells were cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 24 h. (D) HeLa cells were cultured in HBSS medium for 24 h to induce autophagy or left in the complete culture medium. The cells were harvested and nuclear and cytoplasmic extracts were prepared for western blot analysis using antibodies to LC3 and ASYM24, respectively. Fractionation efficiency was determined by localization of β-actin (cytoplasm) and TBP (nucleus).

cells were tested by immunoblot using ASYM24 antibody, and exhibited to a concentration-dependent decrease in the intensity of multiple bands, including p90aDMA, p70aDMA and p34aDMA (Fig. 6C).

To determine if p90aDMA and p70aDMA degradation in rapamycin-treated MCF-7 cells was mediated by autophagy, we blocked the early stage of autophagy with 3-MA, a class III PI3K inhibitor (35), and examined its effect on p90aDMA and p70aDMA expression levels. One-hour pretreatment with 3-MA effectively blocked the rapamycin-induced decrease in the expression of p90aDMA and p70aDMA (Fig. 6D). Similarly, siRNA-mediated knockdown of ATG5 prevented

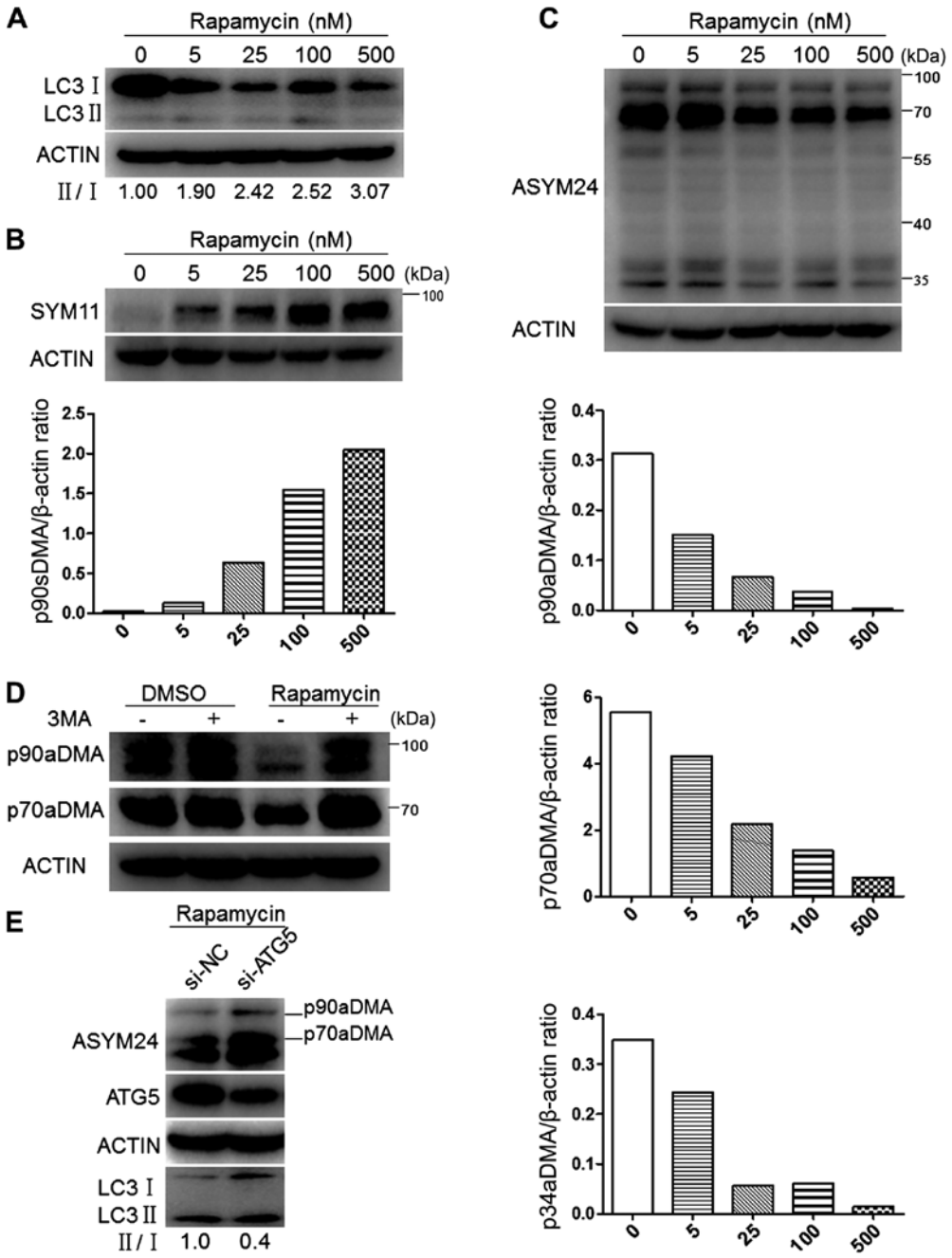

Figure 6. Autophagy selectively degrades nuclear aDMA proteins in cancer cells. (A) MCF-7 cells were treated with various concentrations of rapamycin (0, 5, 25, 100 or 500 nM) for 24 h. The induction of autophagy by rapamycin treatment was determined using immunoblotting assay with antibody to LC3. The intensities of LC3-II/I bands were quantified using ImageJ analysis software and corrected by the band intensity of β-actin. The ratios were normalized to those of control samples and shown below the figure. (B and C) MCF-7 cells were treated with rapamycin at the indicated concentration for 24 h. Cell lysates were prepared and separated by 8% SDA-PAGE. The dose-dependent expression of proteins containing sDMA and aDMA was determined using immunoblotting. The band intensity of p90sDMA, p90aDMA or p70aDMA normalized by β-actin was displayed as histograms below (B) (p90sDMA) or (C) (p90aDMA, p70aDMA and p34aDMA). (D) MCF-7 cells were exposed to rapamycin, 3-MA alone or in combination for 24 h. Whole-cell lysates were prepared and analyzed by western blot analysis using antibody to ASYM24. (E) MCF-7 cells were transfected with control siRNA or ATG5-specific siRNA for 48 h and then treated with rapamycin (100 nM) for another 24 h. Levels of protein expression were measured by western blot analysis using antibodies to ASYM24, ATG5, LC3 and β-actin.

the rapamycin mediated decrease in p90aDMA and p70aDMA expression levels, whereas the non-targeting control siRNA had no effect (Fig. 6E). Therefore, we conclude that rapamycin triggers p90aDMA and p70aDMA degradation through the autophagy pathway and aDMA serves as a specific degradation signal for autophagy.

Discussion

We demonstrate for the first time that IL-2, IL-4 and IL-6 specifically promote nuclear accumulation of p90aDMA, which is abrogated by AdOx, confirming that IL-2/4/6 treatment can effectively increase asymmetric dimethylarginine

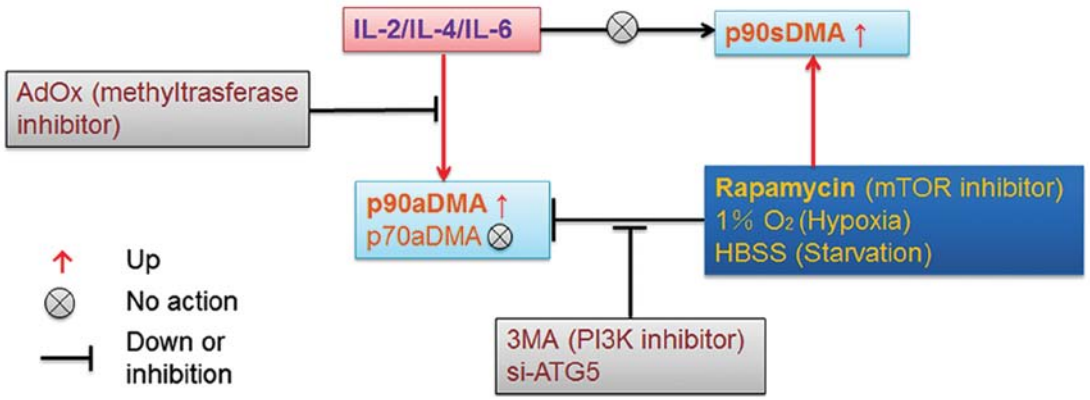

Figure 7. A schematic model for the interactions between cytokines, aDMA proteins and autophagy. Autophagy differentially regulates aDMA and sDMA proteins, promoting p90sDMA but decreasing p90aDMA and p70aDMA. The p90aDMA is specifically upregulated by IL-2, IL-4 or IL-6 but not IL-8 in the nucleus of cancer cells, which is recognized by autophagy, entering into the autophagic degradation process.

proteins (Figs. 2, 3 and 5A). In addition, the interleukins slightly increased basal autophagy but had an opposite effect on rapamycin-induced autophagy, which may decrease p90aDMA expression (Figs. 3 and 4). It can be concluded that IL-2/4/6 stimulated p90aDMA expression is not caused by inhibiting autophagy. Moreover, indirect evidence suggests that p90aDMA, p70aDMA and even p34aDMA could be degraded by LC3-mediated selective autophagy in response to hypoxia, starvation and rapamycin (Figs. 2B and D, 5B-D and 6C), whereas rapamycin can accumulate p90sDMA in a dose-dependent manner (Fig. 6B). It is obvious that the aDMA induced by IL-2, IL-4 or IL-6 determines a highly processive initial reaction on the substrate for specific autophagic degradation. Our findings reveal a novel role for selective autophagy in the regulation of immunologic responses and highlight the role of the posttranslational modification of aDMA in controlling nuclear protein aggregation induced by interleukins and selective autophagy of ubiquitinated proteins (Fig. 7). One fundamental question not addressed here is which endogenous proteins are methylated at arginine sites and translocated into the nucleus in response to IL-2/4/6 treatment.

Our results raise the fundamental questions how IL-2/IL-4/IL-6 regulates the biogenesis of p90aDMA and whether aDMA takes place in the cytoplasm or the nucleus of cancer cells (Figs. 2B and D and 3A and B). Protein arginine methylation is a common posttranslational modification in higher eukaryotes, but its precise role in providing structural and functional diversity among proteins is not well understood. Arginine methylation has been shown to affect several cellular processes, including intracellular localization, protein-protein interactions as well as transcription (36,37). Signal transducers and activators of transcription (STAT) proteins, for example, are a family of latent cytoplasmic transcription factors which mediate interferons (IFNs), interleukins, and some growth factors and peptide hormone signaling in cells (38). Once tyrosine phosphorylated, STAT proteins form homo- or heterodimers, which are actively imported into the nucleus and bind to DNA consensus motifs to elicit specific transcriptional responses (38-40). However, arginine 31 methylation of STAT1 enhances its DNA binding by reducing association with the specific inhibitor PIAS1, thus intensifying the growth-restraining activities of the interferons (41). In addition, arginine methylation of STAT1 controls the rate of STAT1 dephosphorylation by modulating its interaction with PIAS1 and the nuclear tyrosine phosphatase TcPTP (42). STAT1 and STAT2 have a structural arginine/lysine-rich element involved in IFN-induced nuclear import (43). Considering: i) interleukins mainly activate STAT signaling pathways (39,40), and ii) p90aDMA derived from endogenous proteins has a similar molecular mass to the 90 kDa and nuclear location of activated STAT proteins (39,40), it can be deduced that p90aDMA may be one or several proteins of the STATs family that could be dimethylated by IL-2/4/6 commonly or differentially and promote its or their translocation into the nucleus. Further research involves the use of ASYM24 antibody to proteins of 90 kDa containing aDMA for immunoprecipitation (IP) experiments. The purified proteins are then digested and subjected to immublotting or mass spectrometric analysis to confirm our hypothesis.

Selective autophagy is a degradative pathway that controls the quality and abundance of proteins and cellular organelles and is mediated by autophagy receptors that simultaneously bind the designated target and LC3/GABARAP proteins on autophagosomal membranes (11). The autophagic/signaling adaptor LC3 is known to exert its functions through multiple domains containing a ubiquitin core with two α helices, α1 and α2 attached at its N-terminus, which is utilized as the interaction site with its target proteins (44). Physical linkages between autophagy adaptor proteins via polyubiquitin chains are required for autophagy flux. A recent research demonstrates that the deacetylated nuclear LC3 is transported into the cytoplasm to carry out PE conjugation to pre-autophagic membranes by sequential interaction with Atg7 and Atg3 (34). Especially, nuclear lamina protein lamin B1 degradation is achieved by nucleus to cytoplasm transport degradation that delivers lamin B1 to the lysosome via LC3-lamin B1 interaction in the nucleus (31). The reduction in p90aDMA, p70aDMA and p34aDMA levels implicates the specificity and apparent affinity of aDMA for autophagy receptors (Figs. 5B-D and 6C-E). Based on the above reports and results that p90aDMA and p70aDMA are dominantly expressed in

the nucleus, we may deduce that selective autophagy via LC3 is required for the translocation to the autophagosomes and degradation of p90aDMA and p70aDMA. Once autophagy is stimulated in response to stress such as hypoxia, starvation or rapamycin, proteins undergoing aDMA can be transported to autophagosomes by deacetylated LC3 and suffer from autophagic degradation specifically. Based on our findings that p90aDMA and p70aDMA, dominantly expressed in the nucleus, are recognized and bound by LC3 translocating to the cytoplasm (Figs. 2, 3, 5 and 6A and C), we conclude that aDMA as a signal is required for LC3-mediatad selective autophagy traffic.

As a stress integrator pathway, autophagy is a major mechanism that mediates protein and organelle degradation in response to external and internal signals. Additionally, autophagy has been verified in different contexts to regulate immune responses to various stimuli (45-50). In addition to the role of p62, NDP52 and optineurin as adaptors in selective autophagy, these proteins have also recently been shown to regulate innate immunity signaling pathways and, thus, were suggested to represent a new class of pattern recognition receptors, the sequestosome-1-like receptors (SLRs) (51,52). When IL-2 and IL-6 accumulate p90aDMA, which may be a STAT protein, to activate some signaling pathways or transcription, external stresses such as hypoxia, starvation or rapamycin antagonize the signaling transduction via autophagic degradation of p90aDMA (Fig. 2B and D). A growing body of evidence indicates that similar elimination of signaling molecules play key roles in autophagy-regulated immune responses (53-57). For instance, microglial autophagy plays an important role in the clearance of extracellular Aβ (β-amyloid) fibrils and the regulation of Aβ-induced inflammation, thereby affecting neuronal viability (53). One the other hand, emerging data have suggested that additional mechanisms involved in cancer-related inflammation (CRI) are induction of angiogenesis, metastasis, invasion of surrounding tissues and genetic instability by inflammatory mediators, leading to accumulation of random genetic alterations in cancer cells (29,58,59). Based on the above, the possibility is raised that a negative feedback via the targeted regulation of p90aDMA is established in our study bridging immune microenvironment and selective autophagy that may have a potentially pivotal role in shaping the oncogenesis, immunogenic cell death and even heterogeneity in response to dynamic changes in a cancer cell metabolic, environmental, or developmental status.

In conclusion, our data support that a model for the link between arginine methylation and selective autophagy in the immune microenvironment could be proposed for further investigation. Additionally, it remains unclear how aDMA (or sDMA) is regulated by interleukins (or rapamycin) and their function need to be further explored. Collectively, our study expands what is known about the tumor microenvironment and supports the idea of the regulation of arginine methylation as a new immune-therapeutic method in the future.

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