Increased nucleolar localization of SpiA3G in classically but not alternatively activated macrophages

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Abstract

Macrophages play a key role in innate immune response to pathogens and in tissue homeostasis, inflammation and repair. A serpin A3G (SpiA3G) is highly induced in classically activated macrophages. We show increased localization of SpiA3G in the nucleolus and co-localization with cathepsin L, upon classical, but not alternative activation of macrophages. Despite the increased expression of cathepsin L in the nuclei of classically activated macrophages, no cathepsin activity was detected. Since only pro-inflammatory, but not anti-inflammatory stimuli induce increased nucleolar localization of SpiA3G, we propose that SpiA3G translocation into the nucleolus is important in host defense against pathogens.

Keywords:
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1. Introduction

Macrophages are present in virtually all tissues and play a critical role in host defense against pathogens and in pathogenesis of autoimmune and inflammatory diseases [1]. Pathogen recognition is mediated by the so-called pattern recognition receptors, many of which belong to the Toll-like receptor family. Toll-like receptor 4 (TLR4) mediates signals generated by lipopolysaccharide (LPS), a major component of the cell walls of gram-negative microorganisms [2]. In response to LPS, mouse macrophages undergo a major change in gene expression [3]. Interferon gamma (IFN-γ) is produced by the host cells in response to intracellular pathogens, and, in combination with the TLR ligation, leads to the classical activation of macrophages [4]. Classical activation of macrophages leads to increased intracellular resistance to microbes and to increased major histocompatibility complex (MHC) class II-restricted antigen presentation [5,6]. An initial exposure of cells to a low level of LPS preceding an exposure to a higher level of LPS induces a transient state of cell refractoriness, a phenomenon known as "endotoxin tolerance" [7]. Induction of tolerance is thought to protect the host from cellular damage caused by hyperactivation of macrophages and other immune cells and likely represents a means of immune cell adaptation to a persistent bacterial infection [8]. Activated macrophages secrete also high levels of pro-inflammatory cytokines and mediators [9]. Alternatively activated macrophages develop in response to interleukin-4 (IL-4) released by basophiles and mast cells [10]. IL-4 stimulation converts macrophages into a population of cells that are programmed to promote wound healing and down regulate inflammation [4,10].

Upon the activation with IFN-γ and LPS macrophages up-regulate a variety of proteases that can degrade endocytosed pathogens and express higher levels of surface MHC class II molecules that can present the resulting pathogen peptides to the T cells [11]. A critical role in antigen processing and in the formation of peptide-receptive dimers during MHC class II-restricted antigen presentation play the endosomal cathepsins [12]. The activities of cysteine cathepsins are efficiently inhibited by their endogenous protein inhibitors cystatins [13,14], thyropins [15], and some of the serpins [16–19]. Among the cathepsin-inhibiting serpins,
serpin A3G (also called Spi2A), was first described in mouse hematopoietic progenitor cells and activated T cells [20]. In mouse fibroblasts, induction of SpiA3G expression by NF-κB extinguished cathepsin B activity in the cytosol and protected cells from the classical caspase-dependent apoptosis and caspase-independent apoptosis [21,22]. It was reported that SpiA3G is up-regulated in memory cell precursors and promotes the survival of CD8+ cytotoxic T lymphocytes, allowing them to differentiate into memory CD8+ T cells [23]. SpiA3G is also highly up-regulated in IFN-γ and LPS stimulated macrophages [24].

Initially, the aim of our work was to determine if up-regulation of SpiA3G in activated macrophages results in the inhibition of endosomal cysteine cathepsin activity. However, in classically activated macrophages, as well as upon induction of experimental endotoxin tolerance, SpiA3G did not co-localize with cysteine cathepsins in the endolysosomes. Here, we show for the first time increased localization of SpiA3G in the nucleus upon the activation of macrophages with IFN-γ and LPS. Detailed examination revealed that SpiA3G was co-localized with fibrillarin in the nucleolus of RAW 264.7 mouse macrophages. SpiA3G was found to be co-localized with cathepsin L. These results provide the first indication that SpiA3G might regulate cathepsin L activity in the nuclei of activated macrophages.

2. Materials and methods

2.1. Antibodies and other reagents

Anti-cathepsin L mouse monoclonal antibody N135 was described previously [25]. Rabbit polyclonal antibodies against recombinant mouse cathepsin L were prepared by the standard immunization procedure [26]. Polyclonal anti-SpiA3G (Spi2A) antibodies (Cat.no. AP1022) were from Calbiochem (San Diego, CA) and a gift from P. Coughlin. Anti-fibrillarin antibody [38F3] [Ab4566] and anti-c-Jun [E254] antibody (ab32173) were from Abcam. Anti-Horseradish peroxidase-conjugated goat-anti-rabbit IgG antibodies, fetal calf serum (FCS), were obtained from Sigma (USA). Mouse Interferon-gamma was from Abazyme (Cat:CTK-358 ; Needham, MA), and LPS (O55:B5) was from Sigma–Aldrich (Saint Louis, MO).

2.2. Cell culture

RAW 264.7 macrophages (American Type Culture Collection (ATCC), Manassas VA) were cultured in DMEM (BioWhittaker, Gaithersburg, MD) with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM glutamine (Life Technologies, Gaithersburg, MD), and 1000 U/ml of penicillin–streptomycin at 37 °C and 5% CO2.

2.3. Preparation of cell lysates

Cell lysates were prepared as described [27]. Nuclear extracts were prepared by the method of Dignam et al. [28] with minor modifications, including the use of a protease inhibitor cocktail (Cat.No.P8340; Sigma–Aldrich) and the addition of phenylmethylsulfonyl fluoride (PMSF; Fluka Basel, Switzerland) (0.5 mM) to the resuspension and lysis buffers. The supernatants were transferred to fresh test tubes and, if not used immediately, stored at -80 °C. Total protein concentration was determined using the Bradford assay (Bio-Rad, USA).

2.4. Active site labeling of cysteine proteinases

Active site labeling experiments of cysteine proteinases were performed as previously described [29]. RAW 264.7 cells under different stimulations were labeled with 1 μM GB123 activity-based probes (ABP) in culture medium for 3 h at 37 °C and 5% CO2. Cells were washed with cold 1X PBS. Nuclear and postnuclear lysates were prepared as described above without addition of the protease inhibitor cocktail. Equal amounts of protein per lane were separated by 12.5% SDS–PAGE, and labeled proteases were visualized by scanning of the gel with a Typhoon flatbed laser scanner (Ex/ Em 532/580 nm).

2.5. Confocal microscopy

RAW 264.7 macrophages grown on cover slides, were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 10 min and permeabilized with 0.1% Triton X-100 for an additional 5 min. Cathepsin L was labeled with mouse anti-cathepsin L monoclonal antibody N135 [25] and SpiA3G with polyclonal rabbit antibodies (Calbiochem). Fluorescence microscopy was performed using Carl Zeiss LSM 510 confocal microscope. Alexa Fluor 488 or Alexa Fluor 546 and rhodamine were excited with an argon (488 nm) or He/Ne (543 nm) laser and emission was filtered using a narrow band LP 505-530 nm (green fluorescence) and 580 nm (red fluorescence) filter, respectively. Carl Zeiss LSM image software 3.0 (Correlation Plots) was used to evaluate co-localization between the two labeled proteins (i.e., between red and green fluorescence signals).

3. Results and discussion

3.1. SpiA3G is up-regulated in activated macrophages, but not upon the induction of experimental endotoxin tolerance or IL-4 treatment

LPS is a potent and physiologically relevant activator of macrophages. Our results confirm previous observations that the expression of SpiA3G is up-regulated upon IFN-γ and LPS treatment [24]. We have further investigated the expression of SpiA3G upon the induction of experimental endotoxin tolerance and activation of macrophages with IL-4. RAW 264.7 mouse macrophages were treated with IFN-γ, LPS and IL-4, experimental endotoxin tolerance was induced as described previously [8]. In total cell lysates, the levels of SpiA3G were found to be up-regulated in the activated macrophages, however, upon the induction of experimental endotoxin tolerance and IL-4 treatment the protein levels of SpiA3G were comparable to the levels in untreated cells (Fig. 1). Recently, several DNA microarrays studies revealed the effects of pathogens on host-cell gene expression programs in great depth and on a broad scale [3,30]. However, only little is known whether up-regulation of the specific gene expression resulted also in different localization and interactions in the cell. Greiner et al. examined the activity and distribution of cysteine cathepsins in unprimed macrophages by subcellular fractionation and the use of ABP. Active cathepsins B, H and S were found preferentially in the lysosomal fraction, while a significant amount of active cathepsin S and very little active cathepsin B and H were found in the late

![Fig. 1](image-url)
Fig. 2. LPS and IFN-γ synergistically induced changes in the localization of SpiA3G in RAW 264.7 macrophages. Cells left unstimulated (A), or were primed overnight with 300 UI/ml IFN-γ and treated with 100 ng/ml LPS for 18 h (B) or primed with 100 UI/ml IL-4. After indicated treatments, cells were stained with propidium iodide (PI) (red), fixed and labeled with SpiA3G antibodies and Alexa fluor 488 secondary antibodies (green). (D) SpiA3G co-localizes with nucleolar marker fibrillarin. Cells were primed overnight with 300 UI/ml IFN-γ and treated with 100 ng/ml LPS for 18 h, fixed and labelled with mouse anti fibrillarin antibodies and Alexa fluor 546 secondary antibodies (red), and with SpiA3G antibodies and Alexa fluor 488 secondary antibodies (green). Nucleoli were visualized also by differential interference contrast (DIC) microscopy. Arrows indicate the position of the nucleoli. Scale bar: 10 μm.

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endosomes [31]. Study by Schmidt showed that IFN-γ treatment of human monocytes resulted not only in an up-regulation of cathepsins activity but also in differential changes in cathepsin B, L and distribution in endosomes, lysosomes and whole cells [32].

3.2. LPS and IFN-γ synergistically induced changes in the localization of SpiA3G

LPS and IFN-γ, which both up-regulate SpiA3G mRNA, also up-regulate expression of several other genes in macrophages [3]. We examined whether the treatment of RAW 264.7 macrophages with these pro-inflammatory agents provokes changes in the SpiA3G localization. The classical activation of RAW 264.7 macrophages was confirmed by quantification of NO production, using Griess reagent, as described in Supplementary data (Fig. S1). In RAW 264.7 cells stimulated with IFN-γ and LPS, SpiA3G did not co-localize with endosomal/lysosomal marker Lamp1 (data not shown). Instead, macrophages activated by sequential treatment with IFN-γ and LPS, induced a relocation of the SpiA3G protein into the nucleus (Fig. 2B). In contrast, in unstimulated RAW 264.7 cells and in IL-4 stimulated RAW 264.7 cells very little SpiA3G was observed in the nucleus (Fig. 2A and C). Although nuclear localization of SpiA3G was reported more than a decade ago, its function in the nucleus has not been elucidated yet [20]. For several intracellular serpins proteinase inhibitor (PI)-9, PI-6, monocyte neutrophil elastase inhibitor (MNEI), PI-8, plasminogen activator inhibitor 2 (PAI-2) nuclear and cytoplasmic localizations were reported [33]. Although the mechanism of nucleocytoplasmic transport was described for the PI-9 [33], the signal that triggers this translocation has not been elucidated yet. We have shown that during classical activation of macrophages, that leads to development of pro-inflammatory (M1) phenotype, SpiA3G is not only up-regulated by also translocated into the nucleus (Fig. 2B). Using specific antibodies to nucleolar protein fibrillarin we confirmed localization of SpiA3G in the nucleolus (Fig. 2D). Nucleolar morphology was confirmed also with differential interference contrast (DIC) microscopy (Fig. 2D). The nucleolus is a dynamic nuclear structure involved in ribosome subunit biogenesis and mediating responses to cell stress [34,35]. The precise function of SpiA3G in the nucleolus in M1 activated macrophages is not clear yet.

3.3. SpiA3G co-localizes with cathepsin L in the nucleoli of activated macrophages

In the next step we aimed at identifying the possible target(s) of SpiA3G in the nucleoli of activated macrophages, as SpiA3G was shown to inhibit not only cathepsin B and prevent apoptosis induced by cathepsin B leaking from the lysosomes, but was also shown to inhibit cathepsin L in vitro [21]. In order to evaluate possible roles of SpiA3G in the nucleus we examined the co-localization of SpiA3G with cathepsin L in the nucleolus. Cathepsin L and SpiA3G partially co-localized in the nucleoli of RAW 264.7 cells primed with IFN-γ and stimulated with LPS (Fig. 3B), but not in unstimulated cells (Fig. 3A). Recently, three independent reports described the activity of otherwise endosomal proteinase cathepsin L in the nucleus. Cathepsin L was reported to process the CUX1 transcription factor and consequently influenced the cell cycle progression [36]. It was shown that cathepsin L deficiency causes a global rearrangement of the chromatin structure [37]. Cathepsin L cleaved histone H3.2 in the nucleus and the cleavage was relevant for the development and differentiation of mouse stem cells [38].

3.4. Increased expression of cathepsin L in the nucleus in activated RAW 264.7 macrophages did not correlate with increased activity

An increased cathepsin L expression was detected in IFN-γ primed of and LPS stimulated postnuclear cell lysates (Fig. 4A) and nuclear lysates of (Fig. 4B). Activity-based probes (ABP) can be used for monitoring enzyme activity based on their covalent reactions with active-site residues. Structure and specificity of GB 123 and GB 137 active site probes were reported before [39]. Most of the cathepsin activity in post-nuclear lysates of the RAW 264.7 macrophages could be attributed to cathepsin S, B and L activities (Fig. 5), while in the nuclear lysates of IFN-γ and LPS stimulated cells cathepsin activity could not be detected with GB 123 nor with GB 137 probe (data not shown). The myeloid and erythroid nuclear termination stage-specific protein, MENT, was the first known chromatin-associated cysteine proteinase inhibitor that interacts with chromatin and influence heterochromatin distribution [40,41]. MENT, a serpin that also inhibits cathepsin L, strongly

Fig. 3. Double labeling confocal immunofluorescent analysis for co-localization of SpiA3G and cathepsin L in the nucleoli of RAW 264.7 macrophages. Cells left unstimulated (A), or were primed overnight with 300 UI/ml IFN-γ and treated with 100 ng/ml LPS for 18 h (B). Double-labeling immunofluorescent data demonstrated that cathepsin L (red) and SpiA3G (green) were co-localized in the nucleoli of RAW 264.7 macrophages. Arrows indicate colocalization of SpiA3G and cathepsin L. Scale bar: 10 μm.
blocks cell proliferation and promotes condensation of chromatin [41]. It was shown that nuclear cathepsin L stimulates the relocation of MENT away from heterochromatin, its subsequent association with euchromatin [41]. Recently, we have shown that the nuclear catstatin, stefin B, regulates cathepsin L activity in the nucleus [42]. Although it is tempting to speculate that SpiA3G inhibits its cathepsin L activity in the nucleus, additional studies will be needed to confirm (or reject) this hypothesis. We have confirmed not only increased localization of cathepsin L into the nucleus of activated macrophages (Fig. 4B), but also its co-localization with SpiA3G in the nucleus (Fig. 2D). Since only the pro-inflammatory stimuli (IFN-γ and LPS) and not the anti-inflammatory stimuli (IL-10) blocks cell proliferation and promotes condensation of chromatin, it is not clear which protein compensates for SpiA3G deficiency in human macrophages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.febio.2010.03.031.

References


