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Research paper

Analysis of nuclear localization of interleukin-1 family cytokines by flow cytometry

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ABSTRACT

The dual function cytokines IL-1α, IL-33 and IL-37 are members of the IL-1 cytokine family. Besides of being able to bind to their cognate receptors on target cells, they can act intracellularly in the producing cell. All three are able to translocate to the nucleus and have been discussed to affect gene expression. In order to compare and quantitate nuclear translocation of these IL-1 family members we established a robust technique which enables to measure nuclear localization on a single cell level by flow cytometry. Vectors encoding fusion proteins of different IL-1 family members with enhanced green fluorescent protein were cloned and cell lines transiently transfected with these. Fluorescent fusion proteins in intact cells or in isolated nuclei were detected subsequently by fluorescence microscopy and flow cytometry, respectively. Depending on the cellular system, cells and nuclei were distinguishable by flow cytometry in forward scatter/sideward scatter. Fluorescent fusion proteins were detectable in isolated nuclei up to three days following preparation. Signal intensity of fusion proteins of IL-33 and IL-37 in isolated nuclei but not of IL-1α, was markedly increased by fixation with paraformaldehyde, directly following cell lysis, indicating that IL-1α binds stronger to nuclear structures than IL-33 and IL-37. Nuclear translocation of fluorescent IL-37 fusion proteins in a stably transfected RAW264.7 mouse macrophage cell line required stimulation with lipopolysaccharide. Applying this method we demonstrated that a prolonged lag phase of more than 15 h before LPS-stimulated nuclear translocation was detected. In summary, we present a robust method to analyze and quantitate nuclear localization of IL-1 cytokine family members.

1. Introduction

All members of the interleukin (IL)-1 family cytokines exert their biological effects by binding to cell surface receptors. In case of the agonists, e.g. IL-1β or IL-18, commonly cause proinflammatory effects, while the antagonists, e.g. IL-1Ra or IL-37, compete for the ligand binding sites on their receptor thus abrogating signaling of the agonist. In addition, three of the eleven IL-1 family members, IL-1α, IL-33 and IL-37 are able to exert intracrine functions in the producing cells; these are therefore called dual function cytokines (reviewed in Boraschi et al., 2011; Haraldsen et al., 2009). The intracellular activity of these IL-1 family members is not fully understood, however all three are capable of translocating to the nucleus and have been discussed to affect gene expression (reviewed in Boraschi et al., 2011). Transport of proteins to the nucleus is facilitated by nuclear localization sequences (Lange et al., 2007). A classical nuclear

Abbreviations: DTAF, dichlorotriazinylaminofluorescein; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FSC, forward scatter; HMGB1 protein, high mobility group box 1 protein; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B cells; NLS, nuclear localization sequence; PFA, paraformaldehyde; SSC, sideward scatter.

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localization sequence (NLS) is found in the N-terminal region of the full length IL-1α (Wessendorf et al., 1993). Cleavage by calpain yields a C-terminal mature protein of 17 kDa (Kobayashi et al., 1990) which lacks the NLS and is unable to translocate to the nucleus (Wessendorf et al., 1993). The remaining 16 kDa N-terminal propiece still contains the NLS and can be found in the nucleus (Wessendorf et al., 1993). Full length IL-33 contains a bipartite homeodomain-like helix-loop-helix motif in the N-terminal domain, which is required for nuclear transport and binding to the heterochromatin (Carriere et al., 2007). Unlike other IL-1 family members such as IL-1β or IL-18, full length IL-33 is not processed by caspase 1 as had been reported (Schmitz et al., 2005) but rather by caspase 3 (Ali et al., 2010; Luthi et al., 2009; Talabot-Ayer et al., 2009) or caspase 7 (Luthi et al., 2009). Like with IL-1α, the N-terminal cleavage product of IL-33 (propiece) retains the ability to translocate to the cell nucleus (Ali et al., 2010; Boraschi et al., 2011). In contrast to biologically active mature IL-1β or mature IL-18, caspase-processed IL-33 is biologically inactive and does not bind to the IL-33 receptor. In IL-37 a NLS is included in exon 3, which is only used in one of five splice variants coding for isoform IL-37a (reviewed in Boraschi et al., 2011). It was demonstrated, however, that isoform IL-37b lacking this NLS translocates to the nucleus upon stimulation of RAW264.7 cells with LPS (Sharma et al., 2008). The sequence required for translocation is so far unknown. IL-37 is, like IL-1α and IL-33, truly a dual function cytokine, as the common splice variant IL-1F7b binds extracellularly to the IL-18 receptor α-chain and to the IL-18 binding protein. While the consequence of binding to the IL-18 receptor α-chain is not yet fully understood (reviewed in Boraschi et al., 2011), binding of IL-18 binding protein enhances the IL-18-neutralizing capacity of IL-18 binding protein (Bufler et al., 2002).

The intracellular functions of the dual cytokines IL-1α, IL-33 and IL-37 are still under investigation. The best characterized molecule is IL-1α, due to the fact that it was the first IL-1 family member to be found in the nucleus (Grenfell et al., 1989; Mizel et al., 1987). Processes affected by intracellular IL-1α include regulation of cell proliferation, migration and senescence (Werman et al., 2004). Binding of the N-terminal propiece of IL-1α to several nuclear proteins was demonstrated (Buryskova et al., 2004; Hu et al., 2003), and may affect transcription (Werman et al., 2004). IL-33, in fact, was originally identified as a nuclear factor (NF-HEV, Baekkevold et al., 2003) before it became clear in 2005 that it is an IL-1 family member (Schmitz et al., 2005) and IL-33 was found to associate with heterochromatin (Carriere et al., 2007; Roussel et al., 2008). Recently we demonstrated that full length IL-33 binds to the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) p65, thereby dampening gene expression induced by NF-κB (Ali et al., 2011). IL-37 may affect gene expression as well, since stably transfected RAW macrophages expressing full length IL-37, expressed markedly reduced levels of several cytokines following LPS stimulation as compared with LPS-stimulated cells transfected with empty plasmids (Sharma et al., 2008). How IL-37 achieves this, remains to be investigated.

Moreover, dual function cytokines may serve as alarmins. The term 'alarmin' was invented by Joost Oppenheim to address endogenous molecules that indicate tissue and cell damage (Bianchi, 2007). Set free from destructed cells, they provide alarm signals to the immune system, as demonstrated for high mobility group box 1 (HMGB1) protein (Scaffidi et al., 2002). Thus, it may be necessary to monitor not only accumulation of dual function cytokines in cell nuclei but as well their release from cell nuclei.

We applied flow cytometry to detect transiently expressed fluorescent fusion proteins of IL-1 cytokine family members in isolated cell nuclei and obtained quantitative data on a single cell level. Depending on the cytokine, paraformaldehyde (PFA) fixation was necessary to retain the fluorescent fusion proteins in nuclei during preparation.

2. Materials and methods

2.1. Cell culture

HEK293RI cells and HeLa cells were maintained in DMEM (PAA Laboratories, Coelbe, Germany) with 10% fetal calf serum (PAA Laboratories) in a humified incubator at 10% CO_2, 37 °C and passaged every 2–3 days. HEK293RI cells were kindly provided by Z.D. Cao (Tularik, South San Francisco, CA; Cao et al., 1996). HeLa cells (Scherer et al., 1953) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells stably expressing an EYFP fusion protein of IL-37 splice variant IL-1F7b were generated and cultured as described (Sharma et al., 2008).

2.2. Plasmids

The coding sequences of full length proteins of IL-1α, IL-1β, IL-18 and IL-33 with N-terminal myc-tags were subcloned into pEGFP-N1 (Takara Bio Europe/Clontech, Saint Germain en Laye, France) to express enhanced green fluorescent protein (EGFP) fusion proteins. pEGFP-C1 (Takara Bio Europe/Clontech) was used to express EGFP. IL-1F7b-YFP (Sharma et al., 2008), based on vector pEYFP-N1 (Takara Bio Europe/Clontech), was used to express IL-37/EYFP fusion proteins.

2.3. Preparation of cell nuclei and flow cytometry

HeLa cells and HEK293RI cells were transfected by a polyethylenimine (Sigma-Aldrich, Munich, Germany) transfection method (Ehrhardt et al., 2006) and harvested the following day. Stably transfected RAW264.7 cell lines were used following LPS-stimulation as indicated. The nuclei were prepared as described (Rosner and Hengstschlaeger, 2008). Briefly, the cells were washed twice with PBS, detached from the culture plate with 0.05% trypsin/0.02% EDTA (PAA Laboratories), washed twice in PBS and resuspended in buffer F1 (20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM $MgCl_2$, Rosner and Hengstschlaeger, 2008). After 2 min of incubation at room temperature and subsequent incubation for 10 min on ice, detergent IGEPAL CA-630 (Sigma-Aldrich) was added to a final concentration of 0.5% and the cells were homogenized by passing through a 20 G needle for three times. The nuclei were harvested by centrifugation for 5 min at 600 ×*g* at 4 °C and washed three times in buffer F1 supplemented with 0.5% IGEPAL CA-630. FLAG epitopes were detected with 5 μg/ml