Control of interleukin-18 secretion by dendritic cells: role of calcium influxes

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Abstract Here we show that dendritic cells accumulate the precursor form of the leaderless secretory protein interleukin-18 (pro-interleukin-18) in the cell cytosol and in organelles co-fractionating with endolysosomes. Upon antigen specific contact with T lymphocytes, particulated pro-interleukin-18 decreases rapidly, and the cytokine appears extracellularly, suggesting that exocytosis of pro-interleukin-18-containing organelles is induced. Exocytosis of secretory lysosomes is modulated by calcium: in agreement with this, calcium influx results in secretion of pro-interleukin-18. In turn, pro-interleukin-18 secretion induced by T cells is prevented by the calcium channel blocker nifedipine. Our results demonstrate a novel, calcium-mediated mechanism of post-translational regulation of secretion for interleukin-18, that allows a fast release of the cytokine.

Key words: Interleukin-18; Dendritic cell; Degradation; Leaderless secretion; Secretory lysosome; Calcium channel

1. Introduction

Interleukin (IL)-18 is a cytokine with multiple biological functions [1–3] which shares a number of features with IL-1β. Indeed, IL-18 is expressed mainly by cells of the monocyte/macrophage lineage, and possesses properties of pro-inflammatory cytokines [1–3]; similarly to IL-1β, it is synthesized as a precursor protein (pro-IL-18) of 24 kDa proteolytically processed to the 18 kDa mature form by the IL-1β-converting enzyme (ICE) [1–3]. In addition, like IL-1, IL-18 lacks a secretory signal sequence in spite of its extracellular role, raising the question of how it can be secreted by the producing cells [4]. In the case of IL-1β, we have partially characterized a secretory pathway in monocytes that avoids the classical endoplasmic reticulum (ER)–Golgi route [5], and involves translocation of the cytosolic cytokine within a specialized subset of secretory lysosomes, whose exocytosis results in extracellular release of IL-1β [6].

Like IL-1β [22], IL-18 is produced also by dendritic cells (DCs) [7,8], the professional antigen presenting cells [9]: challenge with antigen specific T lymphocytes results in a dramatic decrease of intracellular pro-IL-18, with detection of variable amounts of biologically active IL-18 in cell supernatants [8]. Here we approached the problem of how pro-IL-18 synthesis, degradation and secretion are regulated in DCs. Our data show that pro-IL-18 is a long life intracellular protein in unstimulated DCs; interaction with antigen specific T cells results in prompt release of the pro-IL-18 stored in secretory organelles belonging to the endolysosomal compartment. IL-18 secretion is modulated by calcium: the intracellular calcium rise that follows ionomycin treatment or opening of L-type calcium channels induces a rapid secretion of the cytokine; in turn, IL-18 secretion following the interaction with antigen specific T cells is prevented by blocking L-type calcium channels.

2. Materials and methods

2.1. Cells and culture conditions

DCs were obtained from adherent peripheral blood mononuclear cells (PBMC) from healthy donors cultured 8 days in RPMI medium supplemented with 10% fetal calf serum (both from Biochrome, Milan, Italy), 40 ng/ml recombinant granulocyte macrophage-colony stimulating factor and 1000 U/ml of IL-4 (both from Schering-Plough S.p.A., Milan, Italy) as described [8]. Monocytes were enriched by adherence from PBMC and activated with 10 μg/ml of lipopolysaccharide (LPS) for 1 h as described [6]. Allospecific T lymphocytes were obtained by co-culturing for 1 week purified T cells with allogenic irradiated (4000 rad) PBMC. The antigen specificity of the allogenic T cells was evaluated as described [8].

DCs obtained as above were incubated in RPMI medium supplemented with 1% Nutridoma-HU (Boehringer, Mannheim, Germany) for different periods of time at 37°C in the presence or absence of allospecific T cells at a T:DC ratio of 10:1. When indicated, 100 μM cycloheximide (Sigma-Aldrich, Milan, Italy), 1 μg/ml of ionomycin (Sigma-Aldrich) or 10 μM nifedipine (NFD) or 10 μM BayK 8644 (both from Calbiochem-Inalco S.p.A., Milan, Italy) or 10 nM thapsigargin (Sigma-Aldrich) were added to the cultures. In other experiments, DCs were pretreated with 50 μM of the proteasome inhibitors MG-132 or Z-L3VS (kind gift of Drs. M. Bogyo and H. Ploegh, Boston, MA, USA) [10] or with the lysosomal protease inhibitors pepstatin (10 μM) and leupeptin (100 μM) (Sigma-Aldrich) for 6 h and co-cultured with T cells as above in the presence of the same drugs.

2.2. Subcellular fractionation and Western blot

Aliquots of Triton X-100 (Bio-Rad, Milan, Italy) cell lysates corresponding to 10^5 DCs (or 100 μg of proteins) and the correspondent trichloroacetic acid (TCA)-concentrated supernatants were solubilized in sample buffer and resolved on 12% SDS–PAGE under reducing conditions [8]. Cytosolic fraction and P1 and P2 pellets enriched in lysosomes and endosomes, respectively, were obtained as reported [11] with slight modifications [6]. Briefly, cells were washed, resuspended in

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Abbreviations: DC, dendritic cell; HRP, horseradish peroxidase; ICE, IL-1β-converting enzyme; IL, interleukin; LPS, lipopolysaccharide; NFD, nifedipine; PBMC, peripheral blood mononuclear cells; PNS, post-nuclear supernatant; TCA, trichloroacetic acid

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homogenizing buffer (250 mM sucrose, 5 mM EGTA, 20 mM HEPES-KOH, pH 7.2) at 5×10^7/ml and broken in a Dounce homogenizer. Unbroken cells, debris and nuclei were discharged by three cycles of centrifugation at 800, 1000 and $1200\times g$, and the post-nuclear supernatant (PNS) obtained was untreated or treated with 0.1 mg/ml of proteinase K (Sigma-Aldrich) for 30 min on ice followed by addition of protease inhibitors, diluted 10-fold in homogenizing buffer and centrifuged at $35\,000\times g$ for 1 min. Pellet was kept as P1, P1 supernatant was centrifuged at $50\,000\times g$ for 5 min, leading to a second pellet (P2). The P2 supernatant from undigested PNS was spun 30 min at $100\,000\times g$ to eliminate all the other organelles, and the final supernatant was concentrated by 10% TCA precipitation and used as cytosolic fraction. When indicated, P1 and P2 fractions from undigested PNS were pooled and treated with 0.1 mg/ml proteinase K for 30 min on ice in the presence or absence of 0.1% Triton X-100 (Bio-Rad, Milan, Italy) before solubilization in sample buffer. The enrichment in lysosomes and endosomes of P1 and P2 fractions was evaluated by an assay for the presence of β-hexosoaminidase activity and of cathepsin D content as described [6] (data not shown).

Western blots were performed as described [6,8]. Filters were hybridized with the anti-IL-18 monoclonal antibody (mAb) (R&D System, Milan, Italy) or with the goat anti-human ICE p20 antibody (Santa Cruz Biotechnology Inc., Segrate, Milan, Italy) followed by the relevant horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-goat anti-mouse, Dako S.p.A., Milan, Italy). When indicated, films were subjected to densitometry.

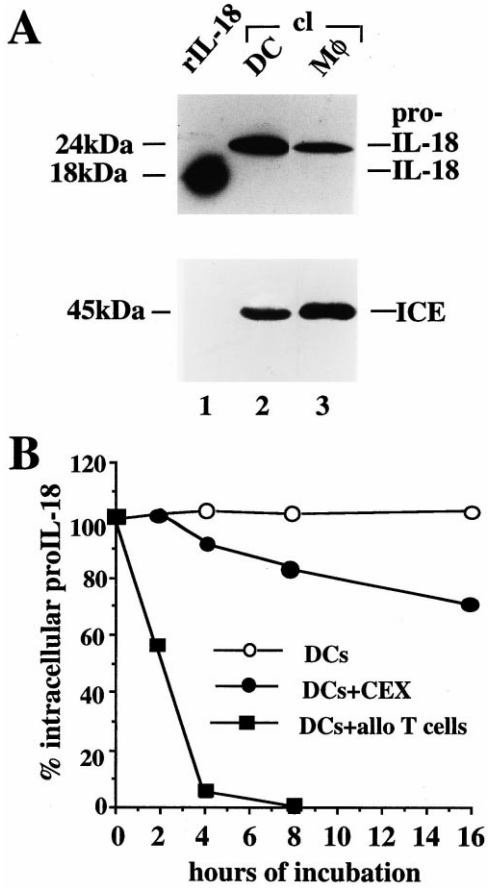

Fig. 1. Pro-IL-18 is a long life protein in DCs. A: Western blot with anti-IL-18 mAb (upper panel) or anti-ICE antiserum (lower panel) of 100 μg of cell lysates (cl) from DCs (lane 2) or LPS-activated monocytes (Mϕ; lane 3). Lane 1: recombinant human IL-18 (25 ng). One representative experiment out of 12. B: Cell lysates from DCs cultured in the absence (open circles) or presence (closed circles) of 100 μM cycloheximide (CEX) or with alloreactive T cells (squares) for 2, 4, 8 and 16 h. One representative experiment out of 10 is shown. The amount of intracellular IL-18 was analyzed by Western blot and quantified by densitometry.

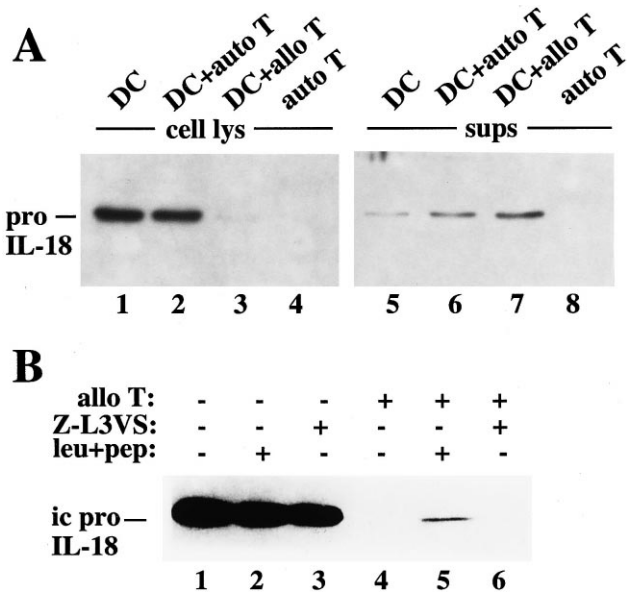

Fig. 2. The decrease of pro-IL-18 content upon DC–T cell interaction is not due to degradation. A: 2×10^5 DCs were cultured for 6 h alone (lanes 1 and 5) or with autologous activated T cells (lanes 2 and 6) or allospecific T lymphocytes (lanes 3 and 7). The cell lysates (lanes 1–3) and supernatants (lanes 5–7) from the different experiments were analyzed for their content in IL-18 by Western blot as in Fig. 1. Lanes 4 and 8: cell lysates and supernatants from 2×10^6 activated T cells as a control. One representative experiment out of 10 is shown. B: Cell lysates from DCs cultured for 6 h in the absence (lanes 1 and 4) or in the presence of L-Z3VS (lanes 3 and 6) or leupeptin and pepstatin (leu+pep, lanes 2 and 5) with (lanes 4–6) or without (lanes 1–3) alloreactive T lymphocytes (allo T) were analysed for their content in pro-IL-18 (ic pro-IL-18).

3. Results

3.1. Constitutive pro-IL-18 in DCs is a long life protein

As shown in Fig. 1A, both LPS-activated monocytes and monocyte-derived DCs synthesize considerable amounts of the precursor form of IL-18, that migrates as a 24 kDa band (lanes 2 and 3); the proform of the convertase ICE (p45) is also expressed by both cell types, although DCs produce more pro-IL-18 and slightly less ICE than monocytes. In spite of the expression of both pro-IL-18 and its convertase, the mature form of IL-18 (lane 1 shows the migration of the 18 kDa recombinant protein) is undetectable in both DCs and LPS-activated monocytes, at variance with cell lines co-transfected with pro-IL-18 and ICE, in which intracellular processing of the precursor was observed [12]. Secreted IL-18 is barely detectable in supernatants of monocytes [13] and DCs [8]. Kinetic analyses showed that, in DCs, pro-IL-18 is a long life protein: indeed, treatment with the protein synthesis inhibitor cycloheximide results in a slow decrease of the pro-IL-18 content, which is $>75\%$ of the control after 16 h with the drug (Fig. 1B). However, the antigen specific interaction of DCs with alloreactive T cells results in a reduction of intracellular pro-IL-18 of about 50% after 2 h of coculture, and the disappearance at 4 h (Fig. 1B). In some donors (three out of 10), the reduction was even more rapid, with lack of recovery of intracellular pro-IL-18 at 2 h ([8] and not shown).

3.2. Intracellular pro-IL-18 is not degraded upon interaction with specific T cells

The disappearance of intracellular pro-IL-18 following contact with antigen specific T lymphocytes is paralleled by secretion of small quantities of pro-IL-18 (Fig. 2A, lanes 3 and 7) in the absence of cell damage evaluated by measurement of the cytosolic enzyme lactate dehydrogenase in the supernatants (not shown). The low amounts of pro-IL-18 recovered might be due to the fact that most of the secreted cytokine is taken up by the responder T cells during the co-culture. Alternatively, pro-IL-18 may undergo degradation upon DC–T cell interaction. Treatment with the proteasome [14] inhibitors Z-L3VS (Fig. 2B, lanes 3 and 6) and MG-132 (not shown) for 6 h does not modify the amount of cytosolic IL-18 recovered from either control DCs (lane 3) or DCs interacting with alloreactive T cells (lane 6) whereas exposure to the lysosomal protease inhibitors leupeptin and pepstatin (lanes 2 and 5) results in only a little rescue of intracellular pro-IL-18 after co-culture with allospecific T cells, indicating that only a small fraction of intracellular pro-IL-18 disappears due to degradation.

3.3. Pro-IL-18 in the endolysosomal fraction is the precursor of the secreted pro-IL-18

In order to investigate the secretory mechanism of pro-IL-18, before and after interaction with T cells, PNS from DCs was subjected to sequential ultracentrifugations giving rise to a soluble fraction corresponding to the cell cytosol and two pellets, P1 and P2, enriched in lysosomes and endosomes, respectively [6,11]. Fig. 3A shows that most of pro-IL-18 is contained in the cytosol; however, a fraction (about 20%) is found in P1 and P2. Particulated pro-IL-18 is protected to protease digestion, while as expected it is degraded when the protease treatment is carried out in the presence of detergent. When the same subcellular fractionation experiment was performed after 30 min of co-incubation with alloreactive T cells, the amount of cytosolic pro-IL-18 was unchanged, whereas

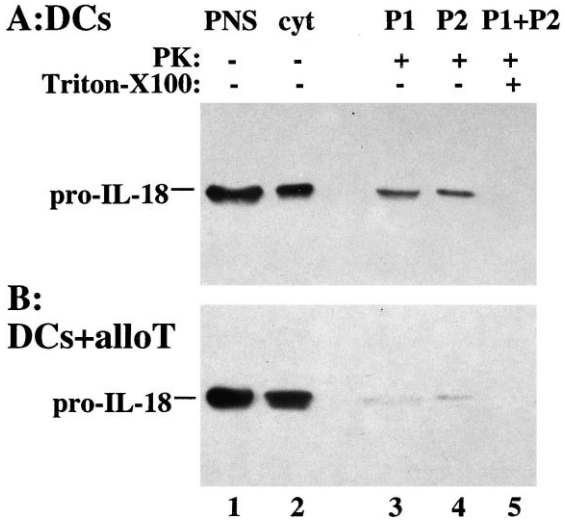

Fig. 3. Emptying of particulated pro-IL-18 upon interaction with T cells. DCs cultured for 30 min alone (A) or with alloreactive T cells (B) were subcellular fractionated [5,10] and the P1, P2 and cytosolic fractions were analyzed as for their content in IL-18. P1 and P2 were treated with proteinase K before solubilization in sample buffer (lanes 3 and 4) or treated with proteinase K in the presence of 0.1% Triton X-100 (PK+T-X100, lane 5).

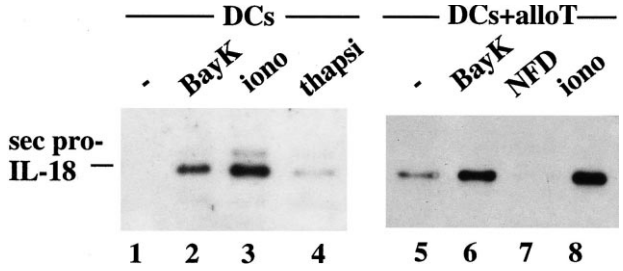

Fig. 4. Calcium modulation of pro-IL-18 secretion. Western blot of secreted IL-18. Lanes 1–4: DCs cultured for 30 min in the absence (lanes 1–3) or presence of thapsigargin (lane 4) as indicated were unstimulated (lanes 1 and 4) or stimulated for 15 min with 10 μM BayK (lane 2) or 1 μg/ml of ionomycin (lane 3). Lanes 5–8: DCs cultured for 1 h in the absence (lanes 5, 6 and 8) or presence of NFD (lane 7) were co-incubated for 6 h with alloreactive T cells and unstimulated (lanes 5 and 7) or stimulated for the last 15 min with 10 μM BayK (lane 6) or 1 μg/ml ionomycin (lane 8).

the P1 and P2 content of pro-IL-18 was decreased (Fig. 3B). At longer time of co-incubation, both the particulated and cytosolic fractions resulted depleted of pro-IL-18 (not shown and Fig. 2A).

3.4. IL-18 secretion is regulated by extracellular calcium

Cell entry of extracellular calcium mediates exocytosis of secretory vesicles [15], including secretory lysosomes [16,17] in several cell types. We then investigated whether modifications in $[Ca^{2+}]_i$ modulate IL-18 secretion. As shown in Fig. 4, 15 min exposure to the calcium ionophore ionomycin strongly induces secretion of pro-IL-18. Similarly, the L-type calcium channel agonist BayK 8644 potentiates secretion, in agreement with our previous observation that DCs express functional L-type calcium channels [18]. In contrast, increases of $[Ca^{2+}]_i$ following 30 min of thapsigargin treatment fail to induce secretion, indicating that extracellular calcium influx rather than mobilization from intracellular stores is responsible for regulated secretion. In keeping with these data, IL-18 secretion induced upon the interaction with alloreactive T cells is prevented by pretreatment of DCs for 1 h with the calcium channel antagonist NFD, whereas it is potentiated by either ionomycin or BayK 8644 (Fig. 4, lanes 5–8).

4. Discussion

In this paper, we describe a novel way of control of secretion for IL-18 in DCs, mediated by secretory lysosomes and regulated by extracellular calcium influx. DC pro-IL-18 is a long life protein, which in line with its lack of signal sequence [4] accumulates in the cell cytosol: however, a consistent portion co-fractionates with organelles belonging to the endolysosomal compartment. We have previously proposed that interaction of DCs with alloreactive T lymphocytes results in a secretory switch of the cytokine: however, whereas the intracellular pro-IL-18 content is dramatically decreased, only minute amounts of pro-IL-18 are detected in the supernatants [8]. The present findings that treatment with specific proteasome or lysosomal protease inhibitors does not rescue intracellular pro-IL-18 indicate that the rapid disappearance of the cytoplasmic pro-IL-18 is independent of the activation of degradative processes, and support the hypothesis that the low recovery of secreted IL-18 is due to the uptake by activated T cells, which bear the IL-18 receptor [3]. Interestingly, anti-

gen specific T cells trigger the secretion of the pro-IL-18 contained into the endolysosomal fraction, as indicated by the decrease in the content of particulated pro-IL-18 shortly after DC–T cell interaction. Longer interactions between DCs and T lymphocytes lead to the emptying of the cytosol also: whether this is due to increased translocation of cytosolic pro-IL-18 into secretory lysosomes or to a direct secretion across the plasma membrane remains to be established. In any case, like for IL-1β [5], we can rule out secretion along the ER–Golgi route, as pro-IL-18 secretion is not inhibited by blockers of classical secretion such as brefeldin A (not shown).

The release of inflammatory mediators as well as the secretion of lysosomal hydrolases from many cell types occurs by a regulated exocytotic process of lysosomes which behave as secretory organelles [19]. Similarly, IL-18 could use secretory lysosomes to reach the extracellular space: indeed, we have recently shown that also IL-1β secretion involves exocytosis of endolysosomal related vesicles [6]. As both IL-1β and IL-18 accumulate in the cytosol of the producing cell, their release through secretory lysosomes would imply a translocation step across the membrane of lysosomes or their precursor organelles. A pathway of translocation for cytosolic proteins to lysosomes under stress conditions has recently been characterized [20]. In that case, however, the fate of the translocated proteins is degradation. In contrast, in *Dictyostelium discoideum*, the transport of a leaderless adhesion protein from the cytosol to contractile vacuoles (acidic organelles similar to lysosomes) results in expression of this protein on the cell surface upon exocytosis of contractile vacuoles modulated by extracellular osmotic conditions [21]. Thus, a mechanism of export of leaderless secretory proteins that uses intracellular acidic vesicles as a vehicle to the extracellular space might have been conserved along evolution. In monocytes, exocytosis of IL-1β-containing endolysosomes resulting in IL-1β secretion is triggered by extracellular ATP [6]. In contrast, secretion of pro-IL-18 by DCs is unaffected by ATP, but is induced by calcium influx. This is in agreement with a number of reports showing a calcium-regulated exocytosis of secretory lysosomes in various cell types [16,17]. Whereas calcium ionophores inducing extracellular calcium entry promote pro-IL-18 secretion, mobilization of calcium from intracellular stores does not. Moreover, treatment of DCs with BayK 8644, a dihydropyridine derivative that binds to L-type calcium channels inducing a calcium influx, results in pro-IL-18 secretion; in turn, pre-exposure of DCs to the calcium channel blocker NFD inhibits the release of pro-IL-18 induced by T cells. These data confirm the presence of functional L-type calcium channels on DCs [18] and provide the evidence of an additional DC function, namely IL-18 secretion, mediated by the activation of these structures. This novel mechanism of secretion for IL-18, involving regulated exocytosis of secretory endolysosomes, allows an early secretion of this cytokine by DCs during the secondary immune response, which can provide a first warning to the immune system of the presence of potentially dangerous pathogens. The rapid shut-down of intracellular IL-18 after interaction with specific T cells might represent a mechanism of negative feed back aimed to focus the early phases of the response on antigen specific lymphocytes, preventing an uncontrolled amplification of the immune reaction.

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