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Running title: Caspase-8 in the macrophage differentiation.

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Research grants: This study was supported by a special grant from the Ligue Nationale Contre le Cancer to ES (équipe labélisée) and a grant from the Direction Régionale de la Recherche Clinique de l'Assistance Publique des Hôpitaux de Paris to MF. SL, SC and RF were sustained by the Ligue Nationale Contre le Cancer, CR by the Association pour la Recherche contre le Cancer and EG by the Inserm.

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Text word count: 4292
Abstract word count: 129

Scientific Heading: Hematopoiesis

Abstract

Caspases have demonstrated several non-apoptotic functions including a role in the differentiation of specific cell types. Here, we show that caspase-8 is the upstream enzyme in the proteolytic caspase cascade whose activation is required for the differentiation of peripheral blood monocytes into macrophages. Upon Macrophage Colony Stimulating Factor (M-CSF) exposure, caspase-8 associates with the adaptor protein Fas-Associated Death Domain (FADD), the serine/threonine kinase Receptor-Interacting-Protein 1 (RIP1) and the long isoform of FLICE-inhibitory protein FLIP. Overexpression of FADD accelerates the differentiation process that does not involve any death receptor. Active caspase-8 cleaves RIP1, which prevents sustained NF-κB activation, and activates downstream caspases. Altogether, these data identify a role for caspase-8 in monocytes undergoing macrophagic differentiation, i.e. the enzyme activated in an atypical complex down-regulates NF-κB activity through RIP1 cleavage.

Key words: Caspase / Differentiation / FADD adaptator protein / Macrophage / RIP threonine kinase

Introduction

A family of cysteine proteases known as caspases plays a central role in many forms of apoptosis.[1] Two main pathways of caspase activation leading to apoptosis have been described. The intrinsic pathway involves the disruption of the outer mitochondrial membrane barrier function, thus permitting the release of pro-apoptotic molecules from the mitochondria to the cytosol. These molecules include cytochrome c that, in the presence of ATP, triggers oligomerization of a platform protein named Apoptosis activating factor-1 (Apaf-1). This protein recruits and activates caspase-9 in the apoptosome. In turn, caspase-9 cleaves and activates downstream effector enzymes such as caspase-3. The extrinsic pathway starts at the level of plasma membrane by engagement of death receptors such as Fas/CD95, tumor necrosis factor receptor 1 (TNF-R1) and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5. In the presence of their respective ligand, death receptors recruit the adaptor molecule Fas-Associated Death Domain protein (FADD), which, in turn, recruits and activates an initiator enzyme, usually caspase-8, in the Death-Inducing Signaling Complex (DISC). Caspase-8 either directly activates the caspase cascade or connects the extrinsic to the intrinsic pathway through cleavage of the sentinel BH3-only protein Bid.[1,2] Additional pathways of caspase activation involve either dependence receptors in the absence of their ligand,[3] or interaction of Apaf-1-like molecules with the adaptor molecule ASC,[4] or endoplasmic reticulum stress that activates caspase-12 in mice and caspase-4 in humans.[5]

While in most cases caspase activation engage cells to die, recent evidences indicate non-apoptotic functions of these enzymes. For example, caspase-8 was involved in lymphocyte activation,[6] which might account for the combined T, B and NK cell immunodeficiency in patients with mutated caspase-8.[7] The enzymatic activity of caspase-8 is also required for fetal liver hematopoietic stem cell proliferation.[8] Signaling through caspase-8 does not always require its enzymatic activity, e.g. in cells in which signaling through

Fas/CD95 promotes survival rather than death, caspase-8 mediates NF-κB activation and its enzymatic activity is dispensable for this function. A scaffolding-related function was suggested for the enzyme that recruits FADD, the two isoforms of FLICE-inhibitory protein (FLIP) and the serine/threonine Receptor-Interacting Protein 1 (RIP1).[8-11]

Caspases were also involved in specific differentiation processes. Erythropoiesis could be regulated by a negative feedback loop in which mature erythroblasts expressing death-receptor ligands inhibit the differentiation of immature erythroblasts through caspase-8 mediated degradation of the transcription factor GATA-1,[12] whereas a transient activation of caspases that does not lead to GATA-1 cleavage is requested for erythroid differentiation.[13] Caspase activation was also demonstrated to play a role in the terminal differentiation of specific cell types that include lens epithelial cells, keratinocytes, skeletal-muscle cells, megakaryocytes, osteoblasts and drosophila spermatozoids (for review, see [14]).

We have previously reported an activation of caspase-3 and caspase-9 in human peripheral blood monocytes that differentiate into macrophages in response to Macrophage Colony-Stimulating Factor (M-CSF). This caspase activation was not related to apoptosis, nor it was observed in monocytes exposed to IL-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) that induce their differentiation into dendritic cells. By using the U937 human monocytic cell line exposed to phorbol ester as a model system, we showed that caspase activation actively contributed to the macrophagic differentiation process.[15] The role of caspase-8 in this differentiation pathway was subsequently suggested by analysis of a mouse model of conditional caspase-8 gene knockout in myeloid bone-marrow cells.[16]

The molecular pathway leading to caspase-8 activation in monocytes exposed to M-CSF, including its place in the proteolytic cascade of caspases and the functional consequences of its activation remained unknown. In the present study, we demonstrate that caspase-8 is the apical enzyme in the caspase cascade that contributes to this differentiation

pathway. In response to M-CSF, caspase-8 interacts with FADD, RIP1 and the long isoform of FLIP in the absence of any death receptor. NF-κB activation is transient along the macrophagic differentiation pathway and caspase-8-mediated RIP1 cleavage appears to prevent its sustained activation.

Materials and methods

Chemical reagents. M-CSF, GM-CSF, and IL-4 were obtained from R&D Systems and TPA from Sigma-Aldrich Laboratories. TRAIL was obtained from Alexis, the Fas agonistic antibody (clone CH11) from Biovalley Co., cycloheximide from Sigma-Aldrich, TNF-α from PeproTech and [γ-^{32}P]ATP (7000 Ci/mmol) from MP Biomedicals.

Antibodies. The following mouse monoclonal antibodies were used: an anti-human HSC70 (Santa Cruz Biotechnology), an anti-human caspase-8 (MBL), an anti-TRAF2 (Stressgen), an anti-FLIP (Alexis) and anti-RIP1, anti-FADD, anti-TRADD and anti-flotillin (Pharmingen). Anti-p65, anti-Fas (Santa Cruz), anti-DR4 or DR5 (Chemicon) and anti-cleaved caspase-3 (Cell signaling) rabbit polyclonal antibodies were also used. Secondary antibodies including HRP-conjugated goat antimouse or antirabbit Abs (Jackson ImmunoResearch Laboratories), HRP-conjugated goat antimouse IgG1 and IgG2b (Southern Biotechnology). For flow cytometry experiments, we used APC-conjugated anti-CD11b or anti-CD71 or anti-CD1a, together with an APC-conjugated isotype IgG_1 matched control (Pharmingen), a FITC-conjugated CD11b together with a FITC-conjugated mouse IgG1 isotype control (Immunotech) anti-DR4, DR5, DcR1, DcR2 or TRAIL (Alexis), anti-Fas or Fas-L (Pharmingen) and an anti-cleaved caspase-8 (Cell Signaling) with its IgG_1 mouse negative control (DAKO), and 488-alexa goat antimouse or 568-alexa goat antirabbit Abs (Molecular Probes). A goat anti-caspase-8 (C-20) and a rabbit anti-$FLIP_L$ (H-150) pAb (Santa Cruz Biotechnology) were used for immunoprecipitation experiments.

Cell culture and differentiation. The human leukemic cell line U937 (CRL-1593.2, mycoplasma free and virus free; American Type Culture Collection [ATCC]), and U937

containing either pcDNA vector 3.1 or a caspase-8 dominant negative form or the cowpox virus caspase-1 and -8 inhibitor CrmA (pCDNA, C8DN, and CrmA, kindly provided by S. Grant, Medical College of Virginia, Richmond, USA), were grown in suspension in RPMI 1640 medium with glutamax-I (Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS; BioWhittaker) in an atmosphere of 95% air and 5% CO_2 at 37°C. Human peripheral blood monocytes were obtained from healthy donors with informed consent and purified using a monocyte isolation kit with a light-scattering (LS) column according to the manufacturer's instructions (Miltenyi Biotec) and exposed to appropriate cytokines. Macrophagic differentiation could be assessed by measuring the percentage of cells with a fibroblast-like shape (Station Cell Observer, Zeiss).

Flow cytometry and immunofluorescence assays. CD11b, CD71 and CD1a expression and caspase-8 cleavage were measured as previously described.[15] To detect caspase activity, we used FAM-LETD-fmk (caspase-8), FAM-DEVD-fmk (caspases-3) and FAM-LEHD-fmk (caspases-9) detection kit FLICA (Serotec) according to the manufacturer's instructions. For nuclear p65 identification, cells were fixed in 2% paraformaldehyde for 10 min at room temperature (RT) and cytospined. Cells were then permeabilized for 20 min. with 0.1% saponin and saturated 1 h with 2% FBS, before incubation overnight with the p65 Ab (C-20) in PBS containing 2% FBS. After washing, cells were incubated for 30 minutes with 568-alexa antirabbit Ab. Percentage of apoptotic cells was measured after nuclei staining with Hoechst 33342 (Sigma-Aldrich) using a fluorescence microscope (Nikon).

Vector constructs. Dominant negative FADD (FADD-DN) and wild type FADD (FADD-WT) vectors have been described previously.[17] A RIP1 mutated on the caspase cleavage site (kindly provided by Olivier Micheau) amplified by PCR was cloned into a ΔMCS lentiviral

plasmid (ZEfir without its multicloning site) downstream the EF1α promoter and upstream the GFP cassette under the control of an internal ribosomal entry sequence (IRES).

Lentivirus vector production and transduction protocol. Vector particles were produced as previously described.[17] U937 cells and primary monocytes were transduced with viral supernatants (at a multiplicity of infection (MOI) of around 100, representing 100 ng/ml of viral p24) on days 1 and 2 in RPMI containing 7.5% of BIT (BSA Insulin Transferin). For primary cells cytokines were added at the beginning of infection and differentiation was analysed at day 4. For U937 cells, EGFP-positive U937 cells were selected 7 days after infection by cell sorting using a Coulter EPICS EPS (Beckman Coulter).

siRNA transfection. Human primary monocytes were transfected with Human Monocyte Nucleofector Kit (Amaxa) according to the manufacturer instructions. Briefly 5 x 10^6 monocytes were resuspended into 100 µL of nucleofector solution with 2 µg of either caspase-8 siRNA (Fw: AGGGAACUUCAGACACCAGtt, Rev: CUGGUGUCUGAAGUUCCCUtt) (Ambion) or luciferase siRNA (Qiagen) (Fw: CUUACGCUGAGUACUUCGAtt, Rev: UCGAAGUACUCAGCGUAAGtt) before nucleofection with nucleofatcor I (Amaxa). Cells were then immediately removed and incubated overnight with 1 mL of prewarmed Monocyte Nucleofator Medium containing 2mM glutamine and 10% of FBS. Cells were then resuspended into complete RPMI medium and treated with appropriate cytokines to induce their differentiation into macrophages or dendritic cells.

Immunoprecipitation. Cells (100 x 10^6) were lysed in 1 ml lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 10% glycerol, complete protease inhibitor mixture (CPIM, Roche) for 30 min on ice. After a centrifugation at 14,000g at 4°C for 30 min, supernatants

were precleared during 2h at 4°C in the presence of 30 µl of mixed sepharose 6B (Sigma) and protein G (Amersham). After centrifugation at 1,000g for 3 min. the supernatant was incubated with anti-caspase-8 Ab (0.2 µg/mL) or anti-$FLIP_L$ Ab (10 µg/mL) at 4 °C for 20 h in the presence of 40 µl of mixed sepharose. The precipitates were washed four times in lysis buffer and analysed by immunoblotting.

Cell lysates and immunoblotting. Whole-cell lysates were prepared as previously described [15]. Whole-cell lysates or lipid raft samples or immunoprecipitation samples were separated by SDS-PAGE, and electroblotted to nitrocellulose membrane (Schleicher and Schuell). After incubation for 2 hours at RT by 8 % nonfat milk in Tris-buffered saline (TBS)-0.1% Tween-20, membranes were incubated overnight with the primary Ab diluted in TBS-milk-Tween, washed, incubated with the secondary Ab for 30 min at RT and washed again before analysis with a chemiluminescence detection kit (Amersham).

Electrophoretic mobility shift assay (EMSA). Nuclear fractions were obtained by incubating the cells in lysis buffer (10 mM Hepes– pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.6% NP-40) in the presence of CPIM. Cell lysate was centrifuged at 1200*g* for 10 minutes and the pellet was washed once in lysis buffer and then resuspended in a buffer containing 20 mM Hepes - pH 7.8, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, in the presence of CPIM for 30 min. on ice. Nuclear extracts were cleared by centrifugation at 20 000g for 30 min, then 5 µg were incubated with 100,000 cpm of γ^{32}P-end labelled NF-κB (5'-AGTTGAGGGGCTTTCCCAGGC-3') consensus oligonucleotide (Promega) in a reaction buffer containing 5µL HNB (0.5 M Sucrose, 15 mM Tris pH 7.5, 60 mM KCl, 0.25 mM EDTA pH 8, 0.125 mM EGTA pH 5, 0.15 mM Spermin, 0.5 mM Spermidin, 1mM DTT), 2 µL MgSp (10 mM MgCl2, 80 mM Spermidin), 1.5 µL NaPi (10 mM NaPi, 1mM EDTA), 10 mM DTT and 0.2 µg poly(dI-dC). After 30 min, DNA-protein complexes were separated from

free oligonucleotides by electrophoresis in a 4% polyacrylamide gel and detected by a PhosphorImager.

Results

Caspase-8 is activated upstream of caspase-9 and -3 in monocytic cells undergoing macrophagic differentiation. We have shown previously that several caspases were activated during M-CSF-induced differentiation of human peripheral blood monocytes into macrophages as well as TPA-induced differentiation of U937 monocytic cells.[15] The present study aimed to determine how this differentiation-associated proteolytic cascade was initiated. Since caspase-8 deletion targeted in bone-marrow progenitors was shown to result in arrest of monocyte differentiation into macrophages,[16] we focused on this enzyme. Using a flow cytometry assay, we detected the time-dependent accumulation of cleaved caspase-8 in human monocytes purified from healthy donor peripheral blood and exposed to M-CSF to trigger their differentiation into macrophages. Conversely, no accumulation of cleaved caspase-8 could be detected in peripheral blood monocytes exposed to GM-CSF and IL-4 to induce their differentiation into dendritic cells (Figure 1A). The appearance of these active fragments in the cytoplasm was independent of any apoptotic feature [[15] and data not shown]. The specific activation of caspase-8 associated with macrophagic differentiation of monocytes was further suggested by using the fluorochrome inhibitor FAM-LETD-fmk (Figure 1B). siRNA-mediated down-regulation of caspase-8 expression in peripheral blood monocytes inhibited caspase-3 cleavage (Figure 1C) and negatively interfered with their differentiation into macrophages upon M-CSF exposure, as indicated by studying the expression of CD71 at the cell surface (Figure 1D) and the percentage of cells with a fibroblast-like shape (Figure 1E), without affecting their differentiation into dendritic cells upon treatment with GM-CSF and IL-4, as measured by CD1a expression (Figure 1D). Similarly, the macrophagic differentiation of U937 human leukemic cells exposed to 20 nM TPA was associated with a time-dependent increase in FAM-LETD-fmk cleavage activity (Figure 1F) and cleaved

caspase-8 (not shown). In this cell line, stable overexpression of a mutated caspase-8 that behaves as a dominant negative mutant and delays the appearance of a FAM-LETD-fmk cleavage activity (Figure 1G) inhibited the TPA-induced differentiation process, as indicated by studying the time-dependent appearance of the differentiation marker CD11b (Figure 1H), morphological changes and cell adhesion to the culture flask (not shown). This construct also prevented the differentiation-associated activation of other caspases such as those that cleave FAM-DEVD (mainly caspase-3) and FAM-LEHD (mainly caspase-9) peptides (Figure 1G). Similar results were obtained in U937 cells expressing the cowpox virus CrmA protein that inhibits caspase-1 and -8 (not shown). Altogether, these results suggested that caspase-8 was activated upstream of caspase-9 and -3 in the cascade associated with macrophagic differentiation.

Caspase-8 associates with FADD, RIP1 and FLIP isoforms along with macrophagic differentiation. Caspase-8 activation associated with the differentiation of monocytes into macrophages was further indicated by immunoblot analyses showing the appearance of a 26 kDa caspase-8 fragment in monocytes exposed for 2 days to M-CSF. This fragment was not detected in monocytes exposed for the same time to GM-CSF and IL-4, nor in untreated cells (Figure 2A, cell lysates). Immunoprecipitation of caspase-8 in these cell extracts demonstrated that this enzyme associated with the adaptor molecule FADD, the serine/threonine kinase RIP1 and $FLIP_L$ in monocytes exposed to M-CSF (Figure 2A). These observations were further confirmed in U937 cells exposed to TPA showing the time-dependent appearance of caspase-8 cleavage fragments and the association of caspase-8 with FADD, RIP1 and the two isoforms of FLIP (Figure 2B). In this model, both IP and western blots showed that the differentiation process was also associated with the time-dependent appearance of caspase-8 active fragments, as well as RIP1 and $FLIP_L$ fragments similar to

those generated by caspase-mediated cleavage.[18-20] The use of anti-FLIP antibody for immunoprecipitation confirmed the interaction of FLIP with caspase-8, FADD and RIP1 in cells undergoing macrophagic differentiation (Figure 2B).

Death receptors are not associated with caspase-8 in cells undergoing macrophagic differentiation. We then analysed whether caspase-8 activation and the recruitment of FADD, RIP1 and FLIP isoforms could involve a death receptor. Blocking antibodies targeting TNF-α, TNFR1, Fas and TRAIL did not affect TPA-induced differentiation in U937 cells [[15] and data not shown]. As signaling through death receptors can occur in a ligand-independent manner,[21] we checked the effect of differentiation on the expression of death receptors at the cell surface and their distribution in the plasma membrane lipid rafts. M-CSF induced macrophagic differentiation of monocytes and TPA-induced differentiation of U937 cells (not shown) did not significantly modify the expression of Fas, TRAIL receptor DR4 and TRAIL decoy receptors DcR1 and DcR2 at the cell surface. The only change identified in death receptor expression along macrophagic differentiation was an increase in DR5 expression at the cell surface. TPA treatment did not induce any redistribution of the death receptors Fas and DR5 in plasma membrane rafts. Similarly, neither FADD nor RIP1 nor procaspase-8 were significantly redistributed in the rafts along the TPA-induced differentiation of U937 cells (see supplementary information).

When analysed by immunoblot in cell lysates, DR5 protein level slightly increased whereas expression of DR4, Fas, and the adaptor molecules TRAF2 and TRADD involved in TNFR1-mediated apoptosis[18] remained unchanged in U937 cells undergoing TPA-induced differentiation (Figure 3). Co-immunoprecipitation experiments using an anti-caspase-8 antibody were repeated in U937 cells undergoing TPA-induced differentiation and compared to those performed in U937 cells exposed to either TRAIL (Figure 3A) or CH11 anti-Fas

agonistic antibody (Figure 3B) or TNFα (Figure 3C). All these treatments induced interaction of caspase-8 with specific receptor (TRAIL and Fas pathways) or adaptor (TNF pathway) molecules (Figure 3) as well as with FADD and FLIP isoforms. In response to TRAIL and TNFα, caspase-8 also interacted with RIP1 (Figure 3). In U937 cells exposed to TPA, these experiments confirmed the recruitment of FADD, RIP1 and FLIP isoforms whereas neither DR4, nor DR5, nor Fas, nor TRAF2, nor TRADD were associated with caspase-8 (Figure 3A to C).

A role for FADD in macrophagic differentiation. We then used a lentiviral construct to introduce either wild-type or a FADD dominant negative mutant deleted of most of the death effector domain in U937 cells and their expression was checked by western blotting (Figure 4A). Wild-type FADD expression increased the apoptotic response of U937 cells to CH11 anti-Fas antibody whereas the dominant-negative construct protected the cells from Fas-mediated cell death (Figure 4A). Wild-type FADD overexpression enhanced the kinetics of TPA-induced macrophagic differentiation in U937 cells, as indicated by studying the expression of CD11b at the cell surface (Figure 4B) and the cell adhesion to plastic flasks (not shown). FADD wild-type overexpression in peripheral blood monocytes also accelerated their differentiation into macrophages upon M-CSF exposure, without affecting their differentiation into dendritic cells upon exposure to GM-CSF and IL-4 (Figure 4C). On the other hand, expression of the FADD mutated construct did not affect TPA-induced differentiation in U937 cells (Figure 4B). Altogether, these observations suggested that the role of FADD in macrophagic differentiation was independent of its interaction with death receptors.

A role for caspase-8-induced RIP1 cleavage in macrophagic differentiation. We then analyzed whether RIP1 cleavage played a role in macrophagic differentiation. This cleavage

as well as the proteolytic cleavage of $FLIP_L$, were specifically observed in monocytes induced to differentiate into macrophages under M-CSF exposure (Figure 5A). RIP1 and c-$FLIP_L$ cleavage require caspase-8 activation as these cleavage are strongly delayed in U937 cells stably transfected with a caspase-8 dominant negative mutant exposed to TPA (Figure 5B). Overexpression of the baculovirus p35 or the cowpox virus CrmA caspase inhibitory proteins also prevented RIP1 and c-$FLIP_L$ cleavage in TPA-treated U937 cells (data not shown). In these cells, lentivirus-mediated expression of a mutated RIP1 construct, in which the caspase-mediated cleavage site had been modified, decreased the cleavage of RIP1 (Figure 5C) and negatively interfered with the differentiation process (Figure 5D).

Caspase-mediated RIP1 cleavage is required for NF-κB activity modulation. The family of Rel/NF-κB transcription factors plays an essential role in macrophagic and myeloid dendritic differentiation,[22,23] and caspase-mediated cleavage of RIP1 was shown to negatively regulate NF-κB activation.[19] By using an electrophoretic mobility shift assay, we observed, in accordance with previously published observations,[22] that NF-κB DNA binding activity was transiently increased in monocytes undergoing macrophagic differentiation whereas this increase was sustained in cells undergoing dendritic differentiation (Figure 6A). These results were further confirmed by showing that nuclear expression of p65 was higher at day 4 and 6 of M-CSF treatment when caspase-8 was down-regulated by specific siRNA (Figure 6B). A transient activation of NF-κB activity was also observed in U937 cells treated with TPA, reaching a maximum 24 hours after the beginning of TPA treatment, then decreasing at 48 hours (Figure 6C and 6E). The supershift obtained with antibodies against p50 and p65/RelA NF-κB subunits confirmed the previously reported activation of a p50/p65 NF-κB complex (data not shown). In U937 cells stably expressing the caspase-8 dominant negative mutant, NF-κB activation did not decrease after 24 hours of TPA treatment (Figure 6C). Similar

results were obtained in cells expressing CrmA, p35 (not shown) and in those expressing a non cleavable RIP1 (Figure 6E). These results were also confirmed by studying p65 nuclear expression. Expression of caspase-8 dominant negative (Figure 6D) or non cleavable RIP1 (Figure 6F) mutants in U937 cells induced a higher nuclear expression of p65 along the macrophagic differentiation as compared to corresponding empty vectors. Altogether, these results suggested that caspase-8 mediated RIP1 cleavage was required to down-regulate NF-κB activation during the macrophagic differentiation pathway.

Discussion

Previous studies had suggested a role for caspase-8 in cytokine-induced proliferation of hematopoietic progenitors,[8] and in M-CSF-induced differentiation of myeloid precursors.[16] However, the molecular pathway leading to caspase-8 activation in myeloid precursors upon cytokine exposure remained unidentified. The present study suggests that, in the proteolytic caspase cascade that mediates monocyte differentiation into macrophages, caspase-8 occupies an apical position. Its activation involves a multiprotein complex that includes the adaptor molecule FADD, the serine / threonine kinase RIP1 and the two FLIP isoforms. Overexpression of FADD accelerates the differentiation process whereas a FADD mutant that prevents Fas-mediated apoptosis does not interfere with macrophagic differentiation. Together with the lack of detection of any interaction of caspase-8 with a death receptor, these observations suggest that death receptors, which in other circumstances are required for inducing FADD interaction with caspase-8,[11] may not play a role in differentiation-associated caspase-8 activation. Active caspase-8 appears to cleave RIP1 that, in turn, down regulates NF-κB, which may favor the macrophagic differentiation process. A schematic model of this pathway is proposed in Figure 7.

How caspases are activated to fulfill non-apoptotic functions remains poorly known.[14] Here, we show that caspase-8, which, in the setting of apoptosis, behaves as either an upstream initiator [24] or a downstream, effector [25] enzyme, is activated upstream of caspase-9 and –3 in monocytes undergoing macrophagic differentiation. The observation that caspase-8 behaves as an upstream enzyme in this setting is in accordance with the specific inhibition of macrophagic differentiation observed in a mouse model of conditional caspase-8 gene knockout in bone-marrow cells.[16] In mice, deletion of *bid* gene, which encodes a BH3-only protein connecting death-receptor mediated activation of caspase-8 to the mitochondrial pathway of cell death,[15] provokes accumulation of monocytes in the peripheral blood and

spleen, thus mimicking a human disease known as chronic myelomonocytic leukemia.[26] Whereas this observation was related to a default in monocyte death, another interpretation is a decreased differentiation leading to cell accumulation. Interestingly, all the differentiation-associated caspase cascades may not be initiated in the same way as caspase-8 is not activated in erythroid cells whose differentiation involves caspase-3 activation.[13,27]

Caspase-8 recruitment by adaptor molecules induces its oligomerization and its subsequent activation, either through its full processing or not.[28] Upon death receptor engagement, caspase-8 is recruited by FADD through interaction between the death effector domain of each protein. Here, we show that, in monocytic cells undergoing macrophagic differentiation, caspase-8 interacts with FADD. Whereas a strong overexpression of FADD induces cell death ([29] and unpublished data), a limited increase in wild-type FADD expression, as obtained by lentivirus-mediated transfer,[17] accelerates the macrophagic differentiation of monocytic cells. Interestingly, a FADD-DN construct lacking most of the death effector domain competitively inhibits Fas-mediated caspase-8 activation and cell death [17] without affecting the macrophagic differentiation. In this latter setting, the FADD mutant may not compete with the formation of the caspase-8 activating platform, suggesting that FADD-mediated recruitment of caspase-8 is independent of its death domain-mediated interaction with a death receptor. Accordingly we did not identify any interaction of caspase-8 with Fas, DR4 and DR5, nor with TRADD and TRAF2, two molecules that are involved in the formation of the soluble complex II that recruits caspase-8 upon TNF-mediated death signaling.[18]

A critical question raised by the participation of active caspases in non-apoptotic functions is how cells survive after activating these enzymes.[14] Various explanations have been proposed such as activation of a protective signaling pathway involving NF-κB.[30,31] Activation of this transcription factor is transient when monocytes differentiate into

macrophages and sustained when they differentiate into dendritc cells.[22,23] Because si-RNA mediated caspase-8 downregulation into macrophages or expression of a dominant negative mutant of caspase-8, or CrmA, or p35 in U937 cells maintain a high level of nuclear p65 and NF-κB activity, caspase-8 activation may be necessary to regulate NF-κB activity along the macrophagic differentiation pathway.

Our data suggest that NF-κB regulation involves caspase-8-mediated RIP1 cleavage. A dual role was assigned to RIP1 in cell death, i.e. survival through NF-κB activation and apoptosis induction. The kinase can be cleaved by caspase-8 at Asp^{324} to generate a C-terminal cleavage product that blocks NF-κB activation, thus promoting cell death,[19,32,33] its subcellular localization can change, depending on the molecular complex it is associated with [18] and the protein can be degraded by the proteasome machinery through ubiquitination by the A20 NF-κB inhibitory molecule.[34,35] The present study shows that caspase-8 mediated cleavage of RIP1 modulates NF-κB activity in cells undergoing macrophagic differentiation. Interestingly, another RIP kinase, RIP4, was recently shown to be involved in keratinocyte differentiation as RIP-4 null mice demonstrate a severely affected keratinocyte differentiation associated with a complete absence of cornified layer.[36] Similar to RIP1, the pro-NF-κB activity of RIP4 is inhibited by caspase-mediated cleavage, most probably generating a dominant negative C-terminal, ankyrin-containing fragment.[37] Since caspases have been reported to play a role in keratinocyte differentiation, one could speculate that caspase-mediated cleavage of RIP4 may be one of the consequences of caspase activation that is required for appropriate differentiation in these cells.

c-$FLIP_L$, which acts either as a competitive inhibitor for caspase-8 recruitment [38] or associates with and activates caspase-8 [39] in death receptor-mediated signaling, was shown to stimulate T cell proliferation by associating with RIP1 and caspase-8 when overexpressed in T cells. In turn, caspase-8 cleaves c-$FLIP_L$ to a p43 form that recruits more efficiently RIP1

than full length c-FLIP$_L$ [20,40] and activates rather than inhibits NF-κB.[41] Thus, caspase-8 mediated FLIP$_L$ cleavage identified in monocytes undergoing macrophagic differentiation could potentially contribute to NF-κB activity regulation together with RIP1 fragment. Caspase-8 also activates downstream caspases such as caspase-9 and caspase-3 whose targets may also play a role in the macrophagic differentiation pathway.[42] Further studies will determine the connection between caspase-8 and other caspases and the deregulation of these pathways in human monocytic diseases such as chronic myelomonocytic leukemia. If a default in caspase activation is observed in M-CSF-treated monocytes from patients with this later disease, then a limited activation of these enzymes could possibly restore there differentiation, thus preventing monocyte accumulation.

Acknowledgments : We thank Arlette Hammann, Anabelle Legrand, André Bouchot and Jean Christophe Deschemin for technical assistance.

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Figure legends

Figure 1. Caspase-8 involvement in monocyte differentiation into macrophages. A, B) Human monocytes (Mo) were purified from healthy donor peripheral blood and exposed for indicated times (d, days) to 100 ng/mL M-CSF to trigger their macrophagic differentiation (Mac) or 100 ng/mL GM-CSF plus 10 ng/mL IL-4 and 50 µM β-mercaptoethanol for inducing their differentiation into dendritic cells (DC) before flow cytometry analysis of **A)** cleaved caspase-8 expression and **B)** FAM-LETD cleavage activity. **C, D & E)** Monocytes were transfected with either Luciferase (siLuc) or caspase-8 si-RNA (siC8); **C)** Expression of caspase-8 and cleavage fragments and cleaved caspase-3 was analyzed by immunoblotting. Hsc 70: loading control. Molecular weights are indicated in kDa. * indicates cleavage fragments. Caspase-8 (black bars) and cleavage fragments (grey bars) were normalized to Hsc70 expression and represented as fold decreases; **D)** Expression of CD71 or CD1a was studied by FACS analysis. Results are normalized to values obtained in cells transfected with luciferase si-RNA; black bars: siLuc; white bars: siC8; **E)** Percentages of cells with a fibroblastic-like shape, indicating macrophagic differentiation, as observed microscopically

(mean +/- SD of three measurements); **F)** U937 cells were treated with 20 nM TPA for indicated times (h, hours) before flow cytometry analysis of FAM-LETD cleavage activity. **G & H)** U937 cells were stably transfected with an empty vector or a vector encoding a caspase-8 dominant negative mutant (C8-DN) before flow cytometry analysis of **G)** FAM-LETD, FAM-DEVD and FAM-LEHD cleavage activities (gray curves, control vector; white curves, C8-DN) and **H)** CD11b expression at the cell surface (open circles, control vector; full circles, C8-DN). One representative of at least three independent experiments is shown or mean +/- SD of three independent experiments. * $p<0.05$; ***p<0;005.

Figure 2. Caspase-8 association with FADD, RIP1 and FLIP proteins in cells undergoing macrophagic differentiation. A) Peripheral blood monocytes (Mo) were treated for 2 days as in Figure 1 to induce their differentiation into macrophages (Mac) or dendritic cells (DC) before lysis. These lysates were used for immunoblotting before (Lysates) or after (IP:caspase-8) immunoprecipitation with an anti-caspase-8 antibody. **B)** U937 cells were exposed to 20 nM TPA for indicated times (hours) before analysis as in panel **a** or using an anti-FLIP antibody (IP:FLIP) for immunoprecipitation. Molecular weight are in kDa. * indicates cleavage products. Beads are a negative control without antibody for IP. One representative of at least three independent experiments is shown.

Figure 3. Caspase-8 does not associate with death receptors in cells undergoing macrophagic differentiation. U937 cells were exposed to 20 nM TPA for indicated times (hours) before lysis. These lysates were used for immunoblotting before (Lysates) or after (IP:casp-8) immunoprecipitation with an anti-caspase-8 antibody. As positive controls, U937 cells were treated with 500 ng/mL of TRAIL for 30 minutes **(A)** or 100 ng/mL of CH11 Fas antibody plus 0.8 µg/mL of CHX for 30 minutes **(B)** or 500 ng/mL of TNF-α for 3 hours **(C)**.

Molecular weight are in kDa. * indicates cleavage products. Beads are negative controls without antibody for IP. One representative experiment is shown.

Figure 4. Influence of FADD constructs on the differentiation pathway. A, B) U937 cells were infected with lentiviral constructs encoding either EGFP alone (T or open circles) or wild-type FADD (WT or full squares) or mutated FADD in which the death effector domain has been partly deleted (DN or full circles), then selected on EGFP expression by cell sorting. **A)** Expression of FADD (24kDa) and FADD-DN (17kDa) was analysed by immunoblotting . Hsc 70: loading control. Molecular weight are in kDa. Cells were either left untreated or treated with 100 ng/mL of CH11 Fas antibody plus 0.8 µg/mL of CHX for 6 hours before measuring the percentage of apoptotic cells after Hoechst 33352 staining of the nuclear chromatin; **B)** Cells were exposed to 20 nM TPA for indicated times before measuring CD11b expression by flow cytometry. **C)** Monocytes were infected with lentiviral constructs encoding either EGFP alone (black bars) or wild-type FADD (white bars) and treated for 4 days as in Figure 1 to induce their differentiation into macrophages (Mac) or dendritic cells (DC) before flow cytometry analysis of the cell surface expression of CD71 or CD1a. Results are normalized to EGFP infected monocytes. Results are the mean +/- SD of at least three independent experiments. **p<0.01; ***p<0;005, NS, non significant.

Figure 5. Caspase-mediated RIP1 and FLIP cleavage in cells undergoing macrophagic differentiation. A) Peripheral blood monocytes (Mo) were treated as in Figure 1 for indicated times (days) to trigger their differentiation into macrophages (Mac) or dendritic cells (DC) before analyzing the expression of indicated proteins by immunoblotting. **B)** U937 cells transfected with either an empty vector (Co) or a mutated caspase-8 expressing vector (C8-

DN) were treated with 20 nM TPA for indicated times (hours) before immunoblot analysis of indicated proteins. **C, D)** U937 cells transduced with a lentivirus encoding an empty vector (ΔMCS or open circles) or RIP1 mutated on the caspase cleavage site (RIPm or full losanges) were exposed to TPA as in panel b before **C)** immunoblot analysis of indicated proteins. RIP1 cleavage fragment was normalized to FADD expression (fold increase). **D)** CD11b expression measured by FACS analysis. One representative of at least three independent experiments is shown or mean +/- SD of three independent experiments. **p<0;01, ***p<0;005.

Figure 6. Caspase-8 activity is required for modulation of NF-κB activation. A) Primary monocytes (Mo) were exposed for indicated times (d, days) to 100 ng/mL M-CSF to trigger their macrophagic differentiation (Mac) or 100 ng/mL GM-CSF plus 10 ng/mL IL-4 for inducing their differentiation into dendritic cells (DC). NF-κB DNA-binding activity was assessed by EMSA (upper panel) and the results were analysed using a phosphorimager and expressed relative to untreated cells (lower panel). **B)** Mo transfected with either luciferase (siLuc) or caspase-8 si-RNA (siC8) were treated for 4 (d4) or 6 (d6) days with M-CSF before quantifying nuclear p65 positive cells by immunofluorescence; **C, D)** U937 cells stably transfected with a caspase-8 dominant negative mutant (C8-DN) or the corresponding empty vector (Co) were exposed to 20 nM TPA for indicated times (hours). NF-κB DNA-binding activity was assessed by EMSA as in A, and the percentage of cells with nuclear p65 was determined by immunofluorescence as in B. **E, F)** U937 cells stably transfected with a lentivirus encoding RIP1 mutated on its caspase cleavage site (RIPm) or the corresponding empty vector (ΔMCS) were exposed to 20 nM TPA for indicated times (hours). NF-κB DNA-binding activity was assessed by EMSA as in A, and the percentage of cells with nuclear p65 was determined by immunofluorescence as in B; One representative of at least

three independent experiments or mean +/- SD of 3 independent experiments. * p<0.05; ***p<0;005.

Figure 7. Proposed role for caspase-8 in monocytes undergoing macrophagic differentiation. Exposure of primary monocytes to M-CSF or U937 cells to TPA triggers caspase-8 interaction with FADD, FLIP and RIP1. Caspase-8 is activated in this multimolecular platform, which induces the cleavage of RIP1. In turn, RIP1 cleavage fragment could down-regulate NF-κB activity. Caspase-8 may also activate downstream caspases that could further contribute to the differentiation pathway by cleaving other cellular targets.

Figure 1

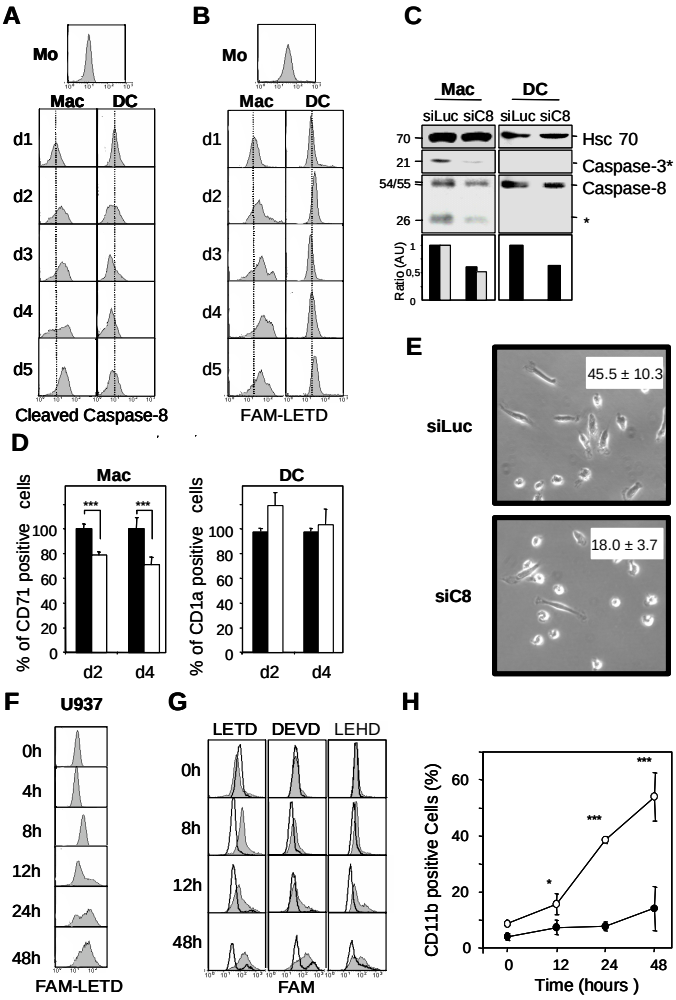

Figure 2

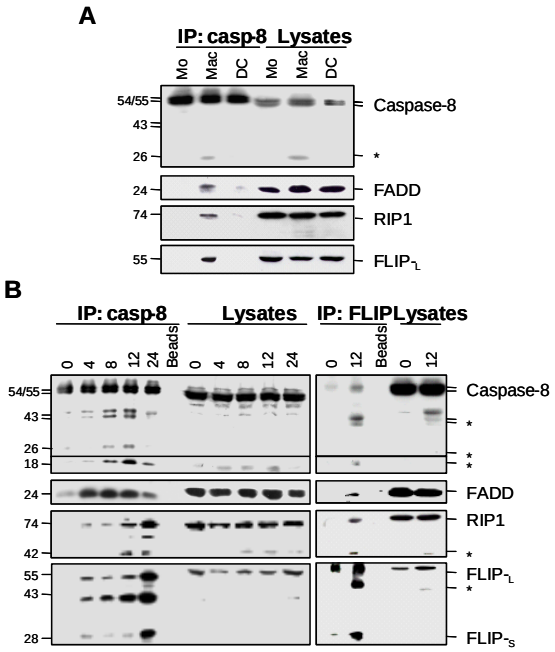

Figure 3

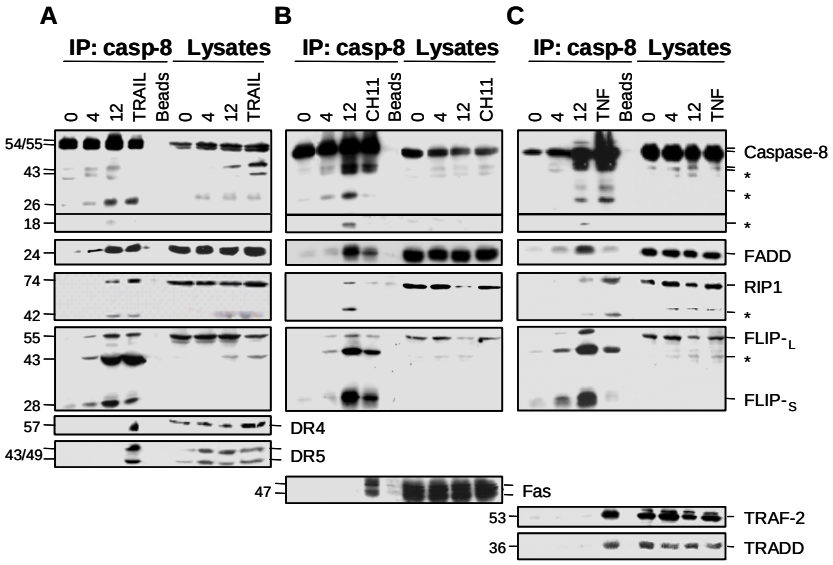

Figure 4

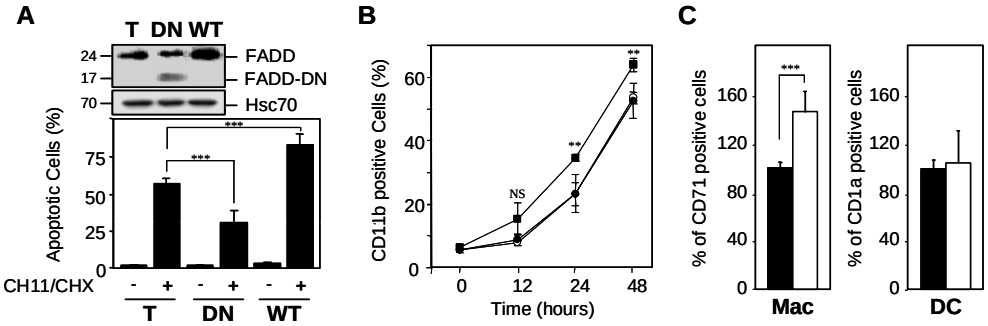

Figure 5

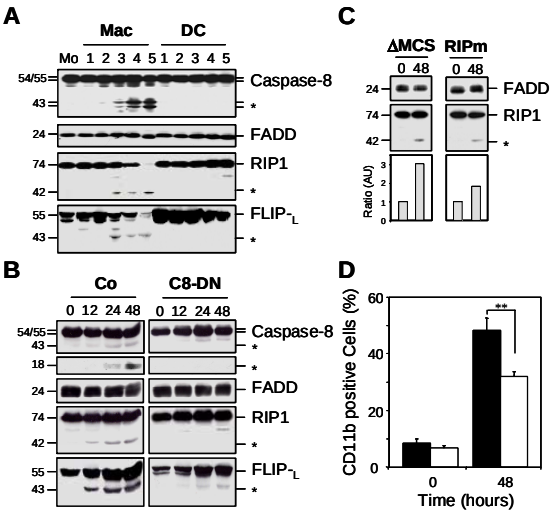

Figure 6

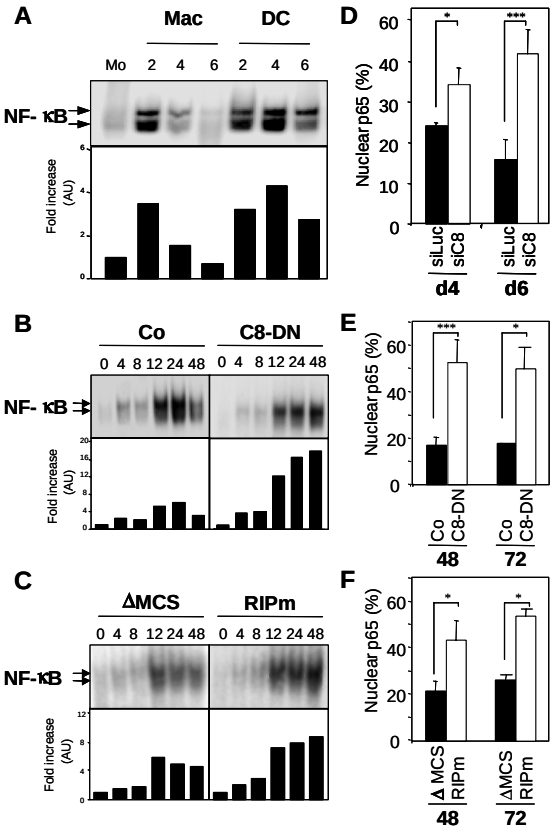

Figure 7