A potential immune escape mechanism by melanoma cells through the activation of chemokine-induced T cell death

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The immune system attempts to prevent or limit tumor growth, yet efforts to induce responses to tumors yield minimal results, rendering tumors virtually invisible to the immune system [1]. Several mechanisms may account for this subversion, including the triggering of tolerance to tumor antigens [2, 3], TGF-α or IL-10 production, downregulation of MHC molecules, or upregulation of FasL expression [4, 5]. Melanoma cells may in some instances use FasL expression to protect themselves against tumor-infiltrating lymphocytes (TIL) [4, 5]. Here, we show another, chemokine-dependent mechanism by which melanoma tumor cells shield themselves from immune reactions. Melanoma-inducible CCL5 (RANTES) production by infiltrating CD8 cells activates an apoptotic pathway in TIL involving cytochrome c release into the cytosol and activation of caspase-9 and -3. This process, triggered by CCL5 binding to CCR5, is not mediated by TNFα, Fas, or caspase-8. The effect is not unique to CCL5, as other CCR5 ligands such as CCL3 (MIP-1α) and CCL4 (MIP-1β) also trigger TIL cell death, nor is it limited to melanoma cells, as it also operates in activated primary T lymphocytes. The model assigns a role to the CXC chemokine CXCL12 (SDF-1α) in this process, as this melanoma cell-produced chemokine upregulates CCL5 production by TIL, initiating TIL cell death.

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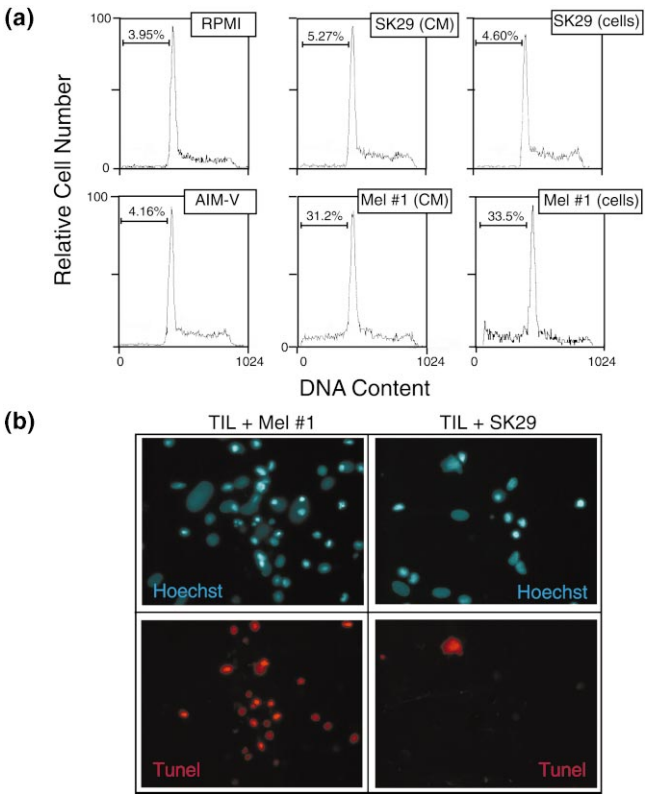
Results and discussion

Angiogenesis, migration, and host immune defences are the fundamental components on which the outcome of the metastatic process depends; all three are largely chemokine-controlled [6, 7]. Chemokines not only govern events that are critical for tumor cell migration, such as cell polarization, but also display autocrine properties [8]. By promoting the migration of appropriate cells into tissues or creating the architecture that facilitates cell inter-

Figure 1



Melanoma induces TIL cell death. **(a)** TIL were cultured in RPMI, AIM-V, or conditioned medium (CM) from SK29-MEL or MEL#1 cells or cocultured with SK29-MEL or MEL#1 melanoma cells. After 24 hr, the cell cycle stage was analyzed by PI staining and flow cytometry. Histograms show relative DNA content versus cell number; the percentage of cells in the sub-G_1 phase is shown. The figure depicts one representative experiment of the five performed. **(b)** Whole-mount TUNEL on TIL, MEL#1 cells, or the SK29-MEL cell line. Nuclear staining with Hoechst is shown in blue; apoptosis is detected as red staining.

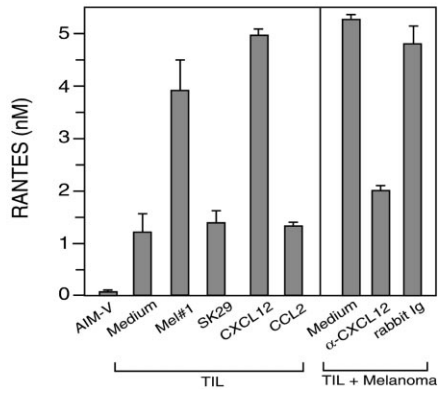
Figure 2

Chemokine expression by primary melanoma cells and TIL. **(a)** Chemokine expression in 48 hr-conditioned medium from established melanomas, melanoma cell lines, and TIL were analyzed for CCL2, 3, 5, and CXCL12 production. Results show the mean ± SD of triplicates from one representative experiment of the five performed. **(b)** CCL5 production by TIL that were stimulated 48 hr with conditioned medium from primary MEL#1 melanoma, SK29-MEL cells, or chemokines (CCl2 and CXCL12) was assessed. CCL5 concentration in AIM-V medium and CCL5 upregulation in unstimulated TIL that were cultured for 48 hr (Medium) are shown as controls. The effect of anti-CXCL12 antibody on melanoma-induced chemokine upregulation is shown.

(a)

		CCL2	CCL5	CCL3	CCL4	CXCL12 (nM)
MELANOMA	Mel #1	1.220 ± 0.093	<0.001	<0.001	<0.001	1.58 ± 0.15
	Mel #2	0.810 ± 0.058	<0.001	<0.001	<0.001	0.34 ± 0.09
	Mel #3	0.350 ± 0.046	<0.001	<0.001	<0.001	0.20 ± 0.08
	Mel #4	2.560 ± 0.139	<0.001	<0.001	<0.001	1.55 ± 0.17
	Mel #5	1.450 ± 0.035	<0.001	<0.001	<0.001	0.42 ± 0.12
	SK29-MEL	<0.001	<0.001	<0.001	<0.001	0.12 ± 0.06
	A-375	0.170 ± 0.004	<0.001	<0.001	<0.001	<0.05
TIL	TIL #1	<0.001	1.600 ± 0.080	0.230 ± 0.077	0.026 ± 0.006	<0.05
	TIL #2	<0.001	0.330 ± 0.093	0.192 ± 0.038	0.066 ± 0.013	<0.05
	TIL #3	<0.001	2.670 ± 0.186	0.449 ± 0.090	0.039 ± 0.009	<0.05

(b)



action and successful immune responses, they collaborate to establish the conditions that precede the immune response [9].

CCL5 is a chemokine that interacts with the G protein-coupled receptors (GPCR) CCR1, CCR3, CCR4, and CCR5 [11]. It is a potent chemoattractant for $CD4^+$ and $CD8^+$ lymphocytes as well as for monocytes, NK cells, and eosinophils [11]. A close relationship has been established between the induction of HIV-1-specific cytotoxicity and the CCL5 effect via CCR3 [12, 13]. In addition, using human peripheral blood lymphocytes (PBL), malignant T cells, and CD4/CXCR4 transfectants, the active role of CXCR4 has been described in the rapid, CD95-independent cell death of $CD4^+$ T cells [14]. Increased CCL5 expression by breast carcinomas correlates with enhanced tumor progression [15]. These studies suggest that chemokines also participate in activating the apoptotic cell death pathway. Here, we establish a link between the ability of chemokines to promote tumor progression and the prevention of tumor elimination by the immune system. The results show CXCL12-mediated upregulation of CCL5 production by TIL. CCL5 binding to CCR5 promotes specific CCR5 cell death via activation of a cytochrome c-dependent pathway in the infiltrating cells, the consequence of which is tumor cell escape from immune system control.

A major conundrum in cancer is that the presence of TIL does not result in cancer cell elimination [3]. We used a human malignant melanoma model to study the mechanisms underlying the lack of tumor rejection. Melanoma lines and infiltrating $CD8^+$ T cells from several melanoma patients were derived in vitro. TIL that were cultured in the presence of melanoma cells underwent cell death, as assessed by cellular DNA staining with the DNA intercalator propidium iodide (PI), and evaluation of the percentage of DNA in the hypodiploid subG_0/G_1 cell cycle peak (Figure 1a) or by the detection of apoptotic nuclei using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) after Hoechst staining (Figure 1b). Cell death is observed 16 hr after the initiation of coculture, reaching a maximum at 48 hr. TIL death requires the presence of freshly explanted melanomas, since culture-adapted melanoma lines, such as SK29-MEL, are unable to trigger apoptosis (Figures 1a,b and S1a). Similar results were obtained in experiments using primary melanoma cell lines derived from seven patients. To determine whether tumor-TIL cell contact was necessary, experiments were performed using conditioned medium from a melanoma cell line (SK29-MEL) or fresh melanoma cells (MEL#1) to induce TIL cell death. No significant cell death was observed when SK29-MEL-conditioned medium was used, whereas MEL#1-conditioned medium induced cell death levels similar to those

induced by TIL coculture with fresh melanoma cells (Figure 1a). No apoptosis was detected following TIL culture in fresh medium for the same period, indicating that the effect was due to a melanoma cell-released factor(s) in the medium.

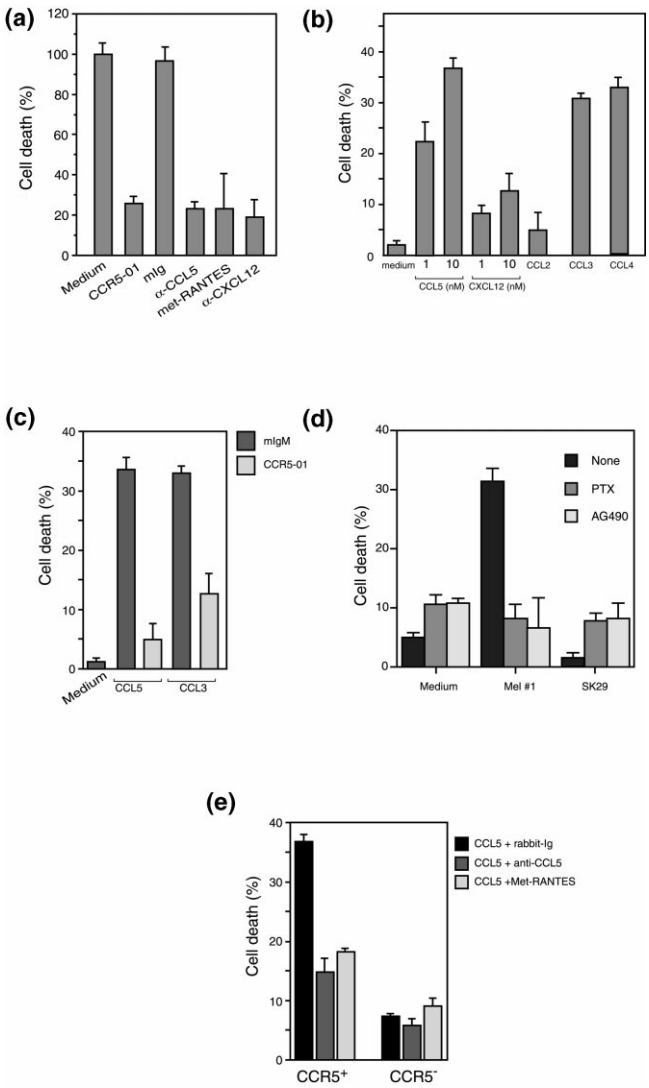
Melanoma cells trigger CCL5 production by infiltrating lymphocytes

To characterize the molecules involved in this type of cell death, we analyzed surface proteins on TIL and melanomas. Using specific antibodies in flow cytometry, we detected Fas, TNFR-II, and TNF-α levels in both cell types, whereas no significant staining was seen using TNFR-I- and FasL-specific antibodies (Figure S1b). Further characterization included chemokine receptors and melanoma-specific markers. Neither TIL nor melanoma cells express surface CCR2, but both express CXCR4, and a significant percentage of the TIL population (30%–56%) expresses CCR5 (see Supplementary material).

This receptor expression pattern prompted analysis of the chemokines produced by both TIL and melanoma cells, as chemokines have been implicated in tumor growth [16]. Primary melanoma cells from several patients, but not the established melanoma cell lines SK-MEL-28, SK29-MEL, or A-375, produced elevated CCL2 (MCP-1) and CXCL12 levels, whereas TIL produced CCL3, 4, and 5 (Figure 2a). This data was supported by the finding that TIL cocultures with fresh melanoma cells, but not with SK29-MEL or its conditioned medium, upregulated TIL CCL5 production (Figure 2b). In determining whether the melanoma-produced chemokines were responsible for this effect, we found that CXCL12 (5 nM), but not CCL2 (5 nM), also upregulates CCL5 production by TIL (Figure 2b). This finding concurs with the blocking effect of anti-CXCL12 antibodies on MEL#1-promoted CCL5 upregulation (Figure 2b). All together, these data suggest that melanoma-secreted CXCL12 is responsible for the upregulation of CCL5 production and subsequent effects.

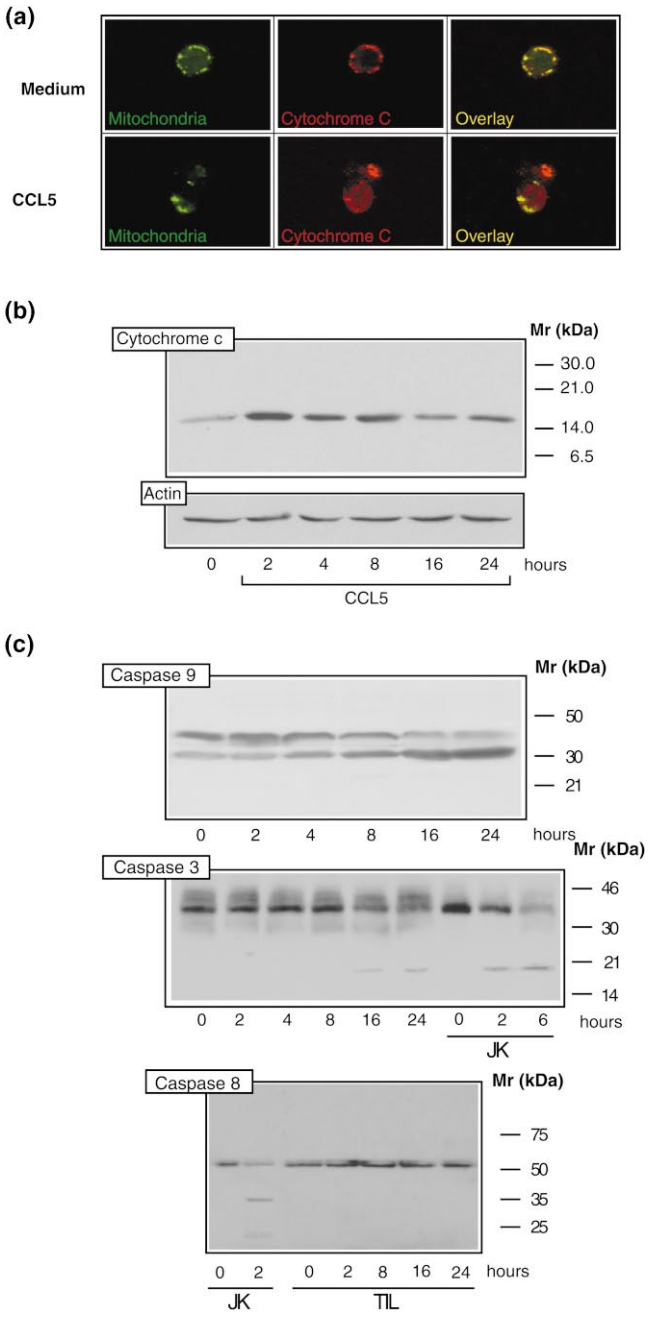
As chemokines may have a role similar to that described for CCL5-induced cell death in HIV-1-infected T cells [12, 13], we attempted to identify the mechanisms by which melanoma cells trigger TIL death. Melanoma-conditioned, medium-induced TIL death was blocked by a CCL5 antagonist (met-RANTES, 10 nM) by neutralizing anti-CCL5 (10 μg/ml) or anti-CCR5 (CCR5–01, 20 μg/ml) mAb, but not by isotype-matched control mAb (Figure 3a). Neutralizing anti-CXCL12 antibody also blocks TIL death, indicating a CXCL12-induced, CCR5-mediated mechanism compatible with CXCL12-induced CCL5 upregulation. To further correlate cell death with chemokine production, TIL were incubated (24 hr) with several concentrations (1–10 nM) of CXCL12 or CCL2, 3, 4, or 5. Whereas CCL2 did not promote cell death, incubation for 24 hr with the CCR5-related chemokines CCL3, 4, or 5 induced cell death (Figure 3b), which is blocked by neutralizing anti-CCR5 mAb (Figure 3c). CXCL12 induced moderate TIL death (10%–15%) at 24 hr, which increased thereafter (Figure 3b). Finally, the cell death

Figure 3



CCL5 induces cell death in TIL and PBL through a CCR5- and G protein-dependent mechanism. **(a)** Melanoma-conditioned medium-induced TIL death is blocked by preincubation with met-RANTES or with neutralizing anti-CCR5 (CCR5–01), -CCL5, or -CXCL12 mAb. **(b)** TIL were incubated with the indicated chemokine (24 hr, 37°C), and the percentage of cell death was analyzed. **(c)** CCL5- and CCL3-induced TIL cell death is blocked by incubation with neutralizing anti-CCR5 mAb (CCR5–01). **(d)** TIL were treated with PTx or AG-490 prior to incubation with conditioned medium from primary melanoma or SK29-MEL cells. (a–d) show the mean ± SD of the percentage of cells in the sub-G_1 phase. **(e)** The effect of CCL5 treatment on cell death in $CCR5^+$-activated PBL, determined by CCR5/annexin V double staining in flow cytometry. The figure shows the percentage of annexin V-positive cells in $CCR5^+$ or $CCR5^-$ PBL after CCL5 stimulation (10 nM, 24 hr), alone or in the presence of antagonists (anti-CCL5 mAb or met-RANTES).

Figure 4



CCL5 induces cytochrome c release and caspase-3 and -9 activity. **(a)** TIL, unstimulated or stimulated with CCL5 (10 nM, 24 hr), were fixed, permeabilized, and stained with anti-cytochrome c or anti-mitochondria antibodies (see Supplementary material). The figure shows specific staining of mitochondria (green), cytochrome c (red), and a computer overlay of both images (yellow). **(b)** TIL were CCL5-stimulated (10 nM) for the times indicated. After washing, mitochondria-free cellular extracts were prepared as indicated (see Materials and methods), electrophoresed, transferred to nitrocellulose membranes, and the Western blot was analyzed with anti-cytochrome c mAb. The membrane was stripped and reprobed with anti-actin antibody as a protein loading control. **(c)** TIL as in (b) were analyzed in Western blot with anti-caspase-9, -3, or -8 antibodies. The loading equivalence was controlled using a protein detection kit. As a positive control in the anti-caspase-3 and -8 Western blots, Jurkat cells were stimulated with anti-human Fas mAb (CH11), and cell lysates were analyzed as indicated above.

triggered by MEL#1 and TIL coculture is blocked by anti-CXCL12 antibody and reconstituted by exogenous CCL5 addition (data not shown).

CCL5 triggers an apoptotic pathway involving cytochrome c release from mitochondria as well as caspase-8 and -3 activation

CCL5 signaling is mediated by triggering receptor homodimerization, which recruits the JAK1 tyrosine kinase, and subsequent association of the $G\alpha_i$ protein to the receptor complex [17]. Blocking this signal by treatment of TIL with pertussis toxin (PTx, which arrests $G\alpha_i$ protein activation) or AG-490 (which inhibits JAK activation) prevents cell death induced by CCL5 or by melanoma-conditioned medium (Figure 3d). This indicates that both $G\alpha_i$ protein and JAK kinase activation are required to induce specific CCL5-mediated cell death in TIL, as neither PTx nor AG-490 block Fas ligation-induced cell death (data not shown). We thus conclude that the melanoma induces death in TIL by activating CCL5 production; this chemokine triggers an apoptotic pathway that requires chemokine receptor activation. This pathway is not exclusive to TIL but can also be triggered in PBL, in which CCL5 promotes specific depletion of CCR5-expressing cells, as determined by CCR5/annexin V staining (Figure 3e). This depletion is blocked by neutralizing anti-CCL5 Ab, anti-CCR5 mAb, or met-RANTES (Figure 3e).

Two main mechanisms have been proposed for apoptosis triggering; first, caspase-8 recruitment to death receptors (Fas or TNF) and second, cytochrome c release from mitochondria to the cytosol, followed by Apaf-1 recruitment, which activates caspase-9 and leads to cell death [18]. When TIL were maintained in culture, cytochrome c was localized in mitochondria (Figure 4a), whereas when these cells were incubated with CCL5 or melanoma-conditioned medium, cytochrome c was released to the cytosol, as detected by immunofluorescence (Figure 4a) or Western blot (Figure 4b). As expected in the cytochrome c pathway, caspase-9 was activated, which is shown by the presence of the cleaved 30 kDa form (Figure 4c, upper panel), as was caspase 3, which is shown by the presence of the 17 kDa cleaved form (Figure 4c, middle panel). Caspase-8 activation was not detected in CCL5-induced cell death (Figure 4c, lower panel). We thus conclude that, in TIL, CCL5 activates an apoptotic pathway triggered by cytochrome c release from mitochondria, which involves caspase-9 and -3 activation. This concurs with results in apoptotic pathway activation via another chemokine receptor, CXCR4 [14].

Here, we describe a mechanism that operates through caspase-9 and -3, is independent of caspase-8 activation, and is engaged by melanoma cells to escape immune rejection. This pathway appears to be triggered by cytochrome c disruption of the ternary complex formed by Apaf-1, Bcl-2, and caspase-9, resulting in caspase-9 release and activation [19]. This mechanism drives an interplay between the cascade of chemokines produced by tumor cells (CXCL12) and infiltrating leukocytes (CCL5), leading ultimately to autocrine and/or paracrine death of the infiltrating cells via the CCR5 receptor (Figure S1c).

These studies, in conjunction with those of other laboratories, illustrate that chemokines form part of an autocrine/paracrine loop that prevents tumor rejection, which may be a general mechanism for neoplastic cell growth. Chemokines also regulate the other arms of this system by coordinating cell proliferation, metastatic capacity, and angiogenesis, a critical triad in tumor progression [20, 21]. Detailed study of this autocrine/paracrine chemokine circuit may thus improve understanding of the onset of tumor metastasis as well as provide new tools for clinical intervention. Our results illustrate that pharmacological products that neutralize these chemokines may help break immunological unresponsiveness to melanoma, providing a complementary approach in malignant melanoma therapy.

Materials and methods

Cell culture

Melanoma cells and their respective TIL isolated from patients were kindly donated by Dr. Paloma S.-Mateos. Melanoma cells were characterized by flow cytometry using specific anti-Melan-A-103 [22] and anti-melanoma-associated antigen ME I-14 (Novocastra) mAb [23]. Established melanoma cell lines SK-MEL-28 (ATCC HTB-72) and A-375 (ATCC CRL-1619) as well as Jurkat cells (ATCC TIB-152) were from the American Type Culture Collection (ATCC). SK29-MEL cells [24] were kindly donated by Dr. M. Serrano, DIO/CNB, Madrid, Spain. When indicated, cells were treated with pertussis toxin (PTx; Sigma) or AG-490 (Calbiochem) as described [25]. For cocultures, melanomas were plated in AIM-V medium and incubated 24–36 hr, after which TIL were added. Human PBL from healthy donors were purified on Ficoll-Paque (Amersham Pharmacia).

Assessment of apoptotic cell death

Following stimulation with chemokines (10 nM) or conditioned medium, apoptosis was evaluated by staining cellular DNA with PI and TUNEL.

Chemokine determination

Melanoma cells (0.5×10^6 cells/ml) and TIL (1×10^6 cells/ml) were cultured for 48 hr, the supernatant was collected (conditioned medium), and the chemokine levels were measured using immunoassay kits.

Subcellular fractionation

TIL cells were washed once in PBS, resuspended in isotonic buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 250 mM sucrose, and homogenized in a Teflon homogenizer. Nuclei and unbroken cells were separated (120×g, 5 min), and the supernatant was centrifuged (10,000×g) to collect the mitochondrial pellet and analyzed for cytochrome c. The mitochondrial pellet was analyzed as a positive control of subcellular fractionation (data not shown).

Supplementary material

Detailed information on antibodies employed, flow cytometry, immunofluorescence, Western blot analysis, and other techniques can be found at http://images.cellpress.com/supmat/supmatin.htm.

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