Exosome-like extracellular vesicles from caspase-dependent cell deaths induce IL1β of bone marrow derived macrophage

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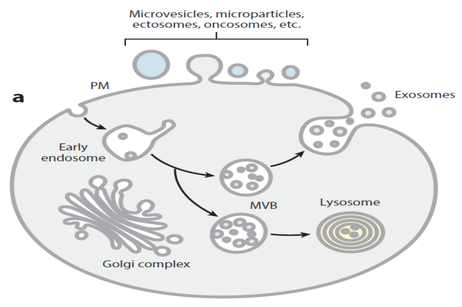
Introduction



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It has been widely accepted that apoptosis is anti-inflammatory, in contrast, necrosis is pro-inflammatory. However, our previous work has definitely shown that it is possible for pro-inflammatory damage-associated molecular patterns (DAMPs) to be released during caspase-dependent cell death. For pursuing the inflammatory mechanism of caspase-dependent cell death, therefore, we analyzed the characteristics of extracellular vesicles (EV) released from dying cells and their roles for macrophage activation.

- Extracelluar vesicle (EV)

EVs isolated from body fluid such as blood, breast milk, urine, semen, saliva, etc. These vesicles called many different names. EVs are sorted by their size, cell or tissue of origin, and their proposed function or existed outside the cells. There are three types of vesicles, which can be isolated from the cell. First, apoptotic body is secreted during the apoptosis. Second, microvesicle is generally refer to 150-1,000 nm vesicles released by budding from the plasma membrane Finally, there is an exosome.

- Exosome

Exosomes were demonstrated as a vesicle of endosomal origin. Exosomes are vesicles of 30 ~ 150 nm size, consisted of a membrane derived from endosome. Exosomes are secreted by most of cell types, the smallest extracellular vesicles secreted from cells. They seemed to be released by fusion of multivesicular late endosomes with the plasma membrane, leading to the secretion of the internal vesicles into the extra-cellular environment. As an extracellular vesicle, exosomes include proteins, lipids, and RNAs, mediating intercellular communication between different cell type in the body.

Result

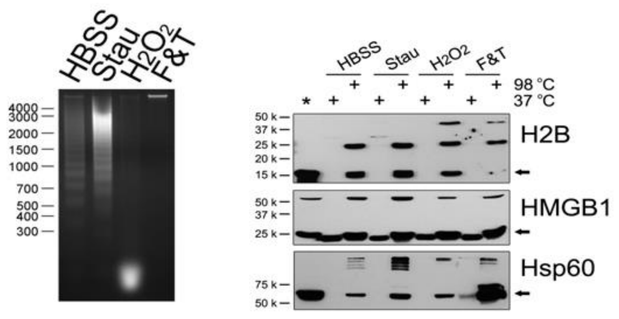


Figure1. Released genomic DNA were separated in agarose gels with molecular weight marker from the apoptotic cell deaths with amino acid deprivation (*HBSS*), and Staurosporine treatment (*Stau*), or primary necrosis with H_2O_2 treatment (H_2O_2) and freezing & thawing (*F&T*). With apoptotic conditions, DNA laddering pattern was observed, In contrast, completely degraded or intact genomic DNAs were detected with primary necrosis **(Left panel)**. The released DNA were cross-linked with proteins by the treatment of paraformaldehyde or reverted by heating at 98 °C of the cross-linked DNA, then, western-blotted for Histone H2B, HMGB1, and Hsp60. The results are showing that DNAs and their associated DAMP proteins are released during both apoptotic and necrotic conditions (**Right panel**).

	HBSS	Staurosporine	H_2O_2	Freezing & Thawing	Media
Il10	9.30647 ± 0.68436	0.53873 ± 0.05537	3.27781 ± 0.20277	1.78707 ± 0.12949	1.0002 ± 0.04024
Il12a	0.77341 ± 0.10242	2.29612 ± 0.22359	1.16808 ± 0.0995	0.68223 ± 0.05336	1.30312 ± 0.04596
Il23a	0.74303 ± 0.19771	2.22731 ± 0.65754	0.97182 ± 0.31403	0.65648 ± 0.35131	1.25128 ± 0.22578
Il1b	245.952 ± 16.2613	593.361 ± 21.4885	18.5391 ± 0.59233	0.89825 ± 0.01976	1 ± 0.02949
Tnfa	0.44384 ± 0.01181	0.35356 ± 0.01472	4.40112 ± 0.25222	1.68783 ± 0.12972	1 ± 0.06663
Tgfb1	1.31552 ± 0.04607	1.27509 ± 0.09696	1.100233 ± 0.04856	0.85956 ± 0.03038	1 ± 0.05455
Tgfb2	0.8658 ± 0.07534	1.98733 ± 0.06346	0.41988 ± 0.05725	1.19094 ± 0.04789	1 ± 0.099599
Tgfb3	0.14702 ± 0.04106	0.44711 ± 0.04131	0.71334 ± 0.09549	0.83979 ± 0.05372	0.99998 ± 0.09238
Il6	0.69409 ± 0.03155	3.15783 ± 0.59261	17.5533 ± 1.64766	1.11933 ± 0.22032	1.0002 ± 0.17332
Nos2	2.51821 ± 1.00891	2.09435 ± 0.6051	21.0498 ± 3.56889	0.92159 ± 0.19688	0.99998 ± 0.12237
Mmp2	2.58291 ± 0.49527	2.49805 ± 0.31216	1.47026 ± 0.15163	0.67474 ± 0.04839	0.99998 ± 0.05839
Mmp9	0.52012 ± 0.03723	0.79418 ± 0.03908	9.19577 ± 0.57701	1.28435 ± 0.06074	1 ± 0.07396
Mrc1	1.26576 ± 0.01089	0.59147 ± 0.05151	1.03159 ± 0.0294	1.18477 ± 0.02917	1 ± 0.06736
Chil3	1.1274 ± 0.08281	0.20275 ± 0.0107	0.52817 ± 0.06656	0.91485 ± 0.13004	1 ± 0.0914
Retnla	0.40096 ± 0.62328	0.02793 ± 0.00579	0.34125 ± 0.05973	0.09413 ± 0.02242	1.0002 ± 0.04073
Marco	0.48494 ± 0.02968	0.33296 ± 0.08876	1.95035 ± 1.11304	17.6619 ± 1.10142	0.99998 ± 0.08039
Arg1	0.86059 ± 0.07704	5.25649 ± 0.84295	1.11814 ± 0.23799	1.56739 ± 0.20462	1.0002 ± 0.63268
Fgf2	0.32533 ± 0.15882	0.44189 ± 0.14102	0.44317 ± 0.04025	0.38077 ± 0.14294	0.32577 ± 0.08871
Figf	0.49326 ± 0.05479	1.15295 ± 0.05583	1.04194 ± 0.17364	1.04353 ± 0.19787	1.25104 ± 0.1004
Cxcl1	8.036791 ± 0.36791	14.6419 ± 0.84633	4.33981 ± 0.10063	3.77454 ± 0.1795	0.69681 ± 0.05414
Cxcl2	5.64628 ± 00.20804	31.3263 ± 0.90575	6.15546 ± 0.25161	4.60032 ± 0.22121	0.71124 ± 0.02339
Cxcl5	15.5761 ± 0.7002	5.06866 ± 0.64859	0.97081 ± 0.08006	1.11517 ± 0.23855	1.08137 ± 0.51848
Il18a	2.71955 ± 0.04494	2.68663 ± 0.06453	1.57302 ± 0.03052	1.62548 ± 0.03855	1.88993 ± 0.04414
Vegfa	1.23052 ± 0.06044	11.739 ± 0.26074	0.91842 ± 0.04651	0.97432 ± 0.01408	1.50553 ± 0.06938
Vegfb	0.83721 ± 0.0437	0.54896 ± 0.0178	0.72442 ± 0.0164	0.84561 ± 0.06585	1.13058 ± 0.04477
Vegfc	0.40606 ± 0.06333	0.38041 ± 0.10693	1.04669 ± 0.21419	1.27568 ± 0.18997	0.88334 ± 0.24763
Pdgfb	0.86924 ± 0.02511	0.64216 ± 0.02524	0.8719 ± 0.04507	0.99025 ± 0.04746	0.94888 ± 0.03541
Ifnb1	0.8005 ± 0.02879	1.80463 ± 0.12157	0.83946 ± 0.05747	1.00333 ± 0.3354	2.54042 ± 0.13415
Il1a	0.6271 ± 0.04476	1.38877 ± 0.14073	4.49545 ± 0.33543	3.73144 ± 0.2169	1.27115 ± 0.06663

Table1. Effects of Conditioned media from cell deaths on BMMQ differentiation. Conditioned media from apoptotic or necrotic cell death were treated into mouse bone marrow-derived macrophages (BMMQs), and then, BMMQ differentiation was measured by real-time PCR for various markers suggesting differentiation status of macrophages.

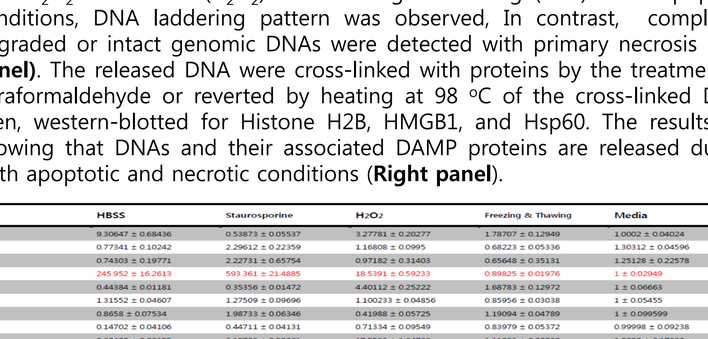


Figure 2. Cellular Death was induced by amino acid-depletion (*HBSS*), treatment with Staurosporine (*Stau*), H_2O_2, or freezing and thawing (*F&T*). Conditioned media, obtained from the supernatant, were treated into BMMQ and IL1β mRNA and its secreted protein were measured by real time-PCR and ELISA, respectively.

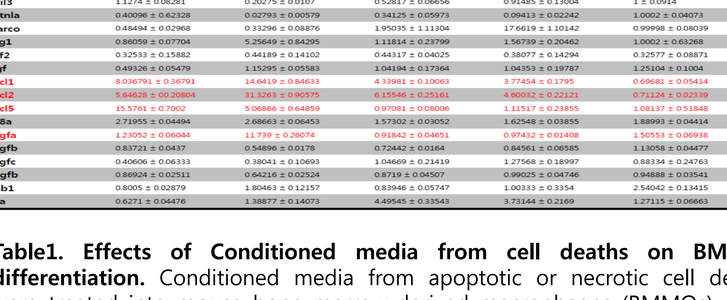


Figure 3. Extracellular vesicles were purified from the conditioned media of staurosporine-treated cells by the differential centrifugation (2000P, 14,000P, 128,000P). IL1β mRNA and protein were detected by real time-PCR and ELISA (**Left and Middle panel**). Various marker proteins for intracellular membrane organelle were confirmed by western blot (**Right panel**).

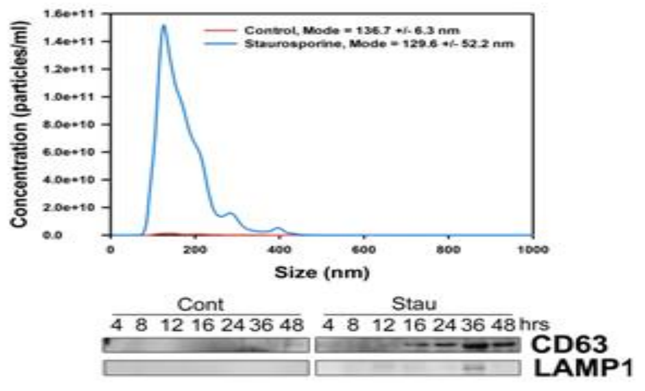


Figure 4. From supernatants of cells treated with staurosporine, extracellular vesicles were isolated. The size and concentration of the vesicles were measured by NTA (nanoparticle tracking analysis) and the expression of CD63 and LAMP1 (exosomal markers) were confirmed by western blot.

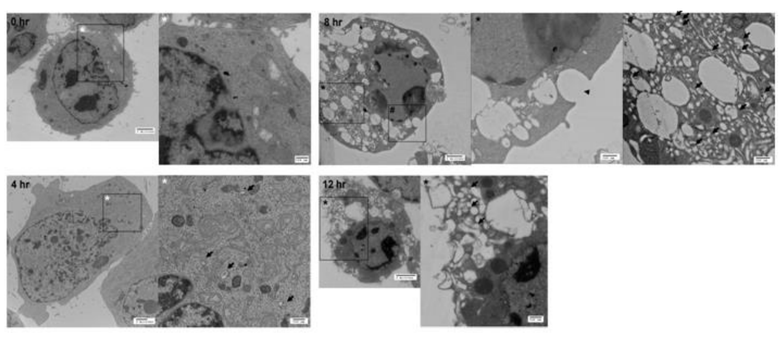


Figure 5. Transmission electron microscopy (TEM) images were taken from cells treated with staurosporine for the indicated time periods. (arrow: multivesiclular body; arrow head: remnant indicating fusion between endosome and plasma membrane)

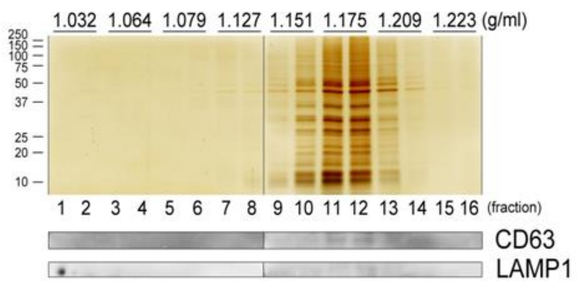


Figure 6. The vesicles described above were further separated by Optiperp density gradient centrifugation. Protein contents from each fraction were detected by silver staining and western blots for CD63 or LAMP1.

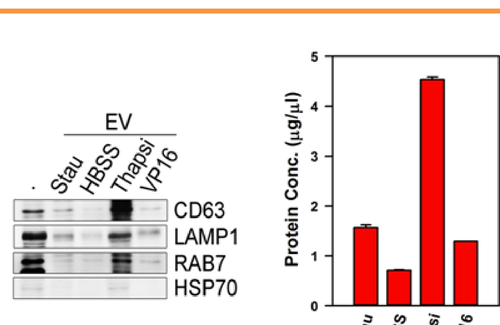


Figure 7. Extracellular vesicles were isolated from the cells treated with Staurosporine, HBSS, Thapsigargin, or VP16, expression of exosomal markers and protein contents from the released vesicles were measured by western-blot for CD63, LAMP1, RAB7, and HSP70 (**Left panel**) and Bradford assay (**Right panel**).

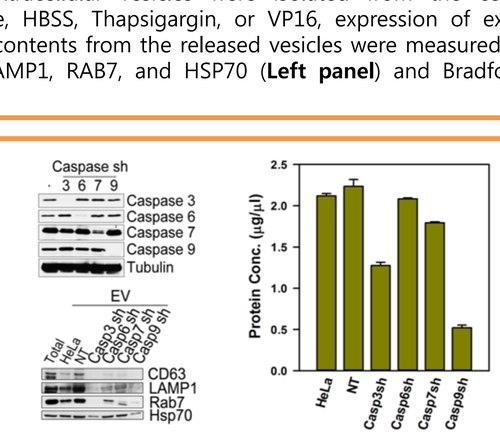
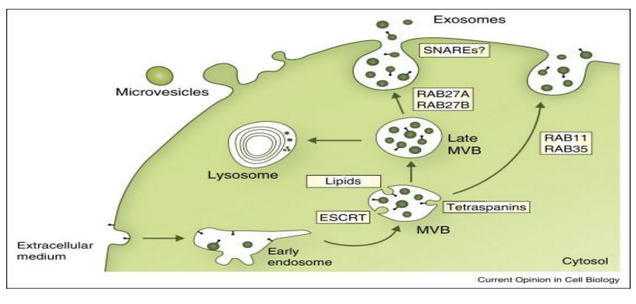


Figure 8. protein expressions were observed in the cells stably expressing shRNA for Caspase 3, 6, 7, or 9 (**Left upper**). Extracellular vesicles from stauropsorine-treated cells, were isolated and expression of exosomal markers were confirmed by western blot. (CD63, LAMP1, RAB7, or HSP70) (**Left lower**). Amount of the released protein was measured by Bradford assay (**Right**)



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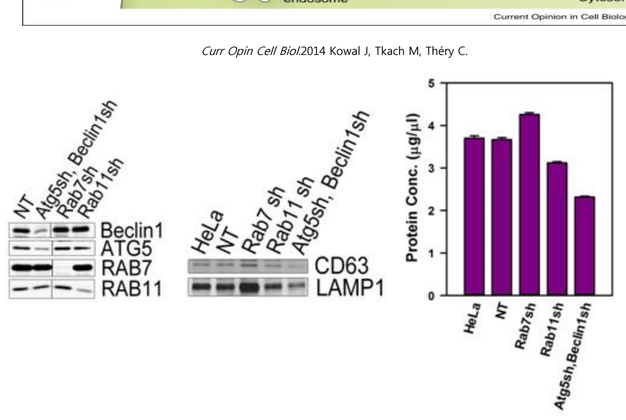


Figure 9. Hela cells stably transfected with NT, Beclin1 and Atg5, RAB7, or RAB11 shRNAs were examined for expressions of proteins by western blot (**Left**). Extracellular vesicles from stauropsorine-treated cells, were isolated and expression of exosomal markers were confirmed by western blot. (CD63, LAMP1) (**Middle**). Amount of the released protein was measured by Bradford assay (**Right**).

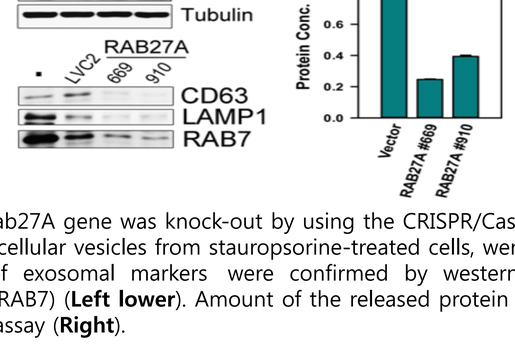


Figure 10. Rab27A gene was knock-out by using the CRISPR/Cas9 system (**Left upper**). Extracellular vesicles from stauropsorine-treated cells, were isolated and expression of exosomal markers were confirmed by western blot. (CD63, LAMP1, and RAB7) (**Left lower**). Amount of the released protein was measured by Bradford assay (**Right**).

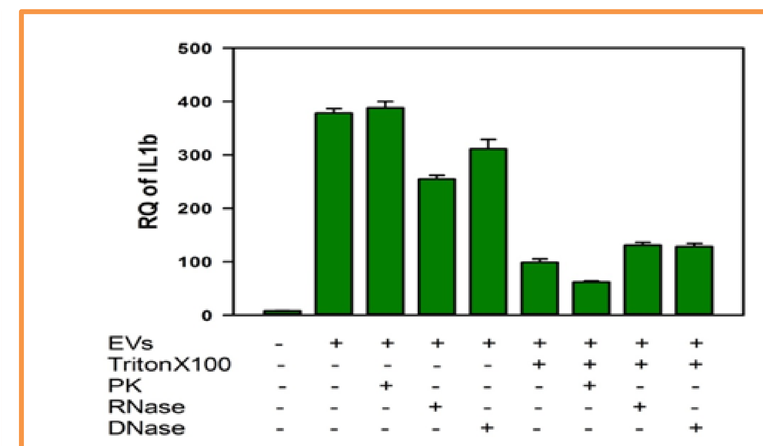


Figure 11. The isolated extracellular vesicles, after treated with Triton X 100, Proteinase K, RNase and/or DNase, were treated into bone marrow-derived macrophages. Il1β mRNA expression was analyzed by real-time PCR from the macrophage.

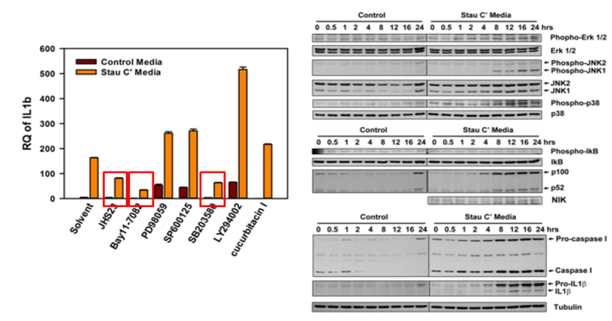


Figure 12. mRNA expression of Il1β is measured by real-time PCR from the BMMQ stimulated with the conditioned media in the presence of IkB, NF-kB, MAPK, PI3K or STAT3 inhibitors (**Left**) Western blots from the BMMQ treated with media control or the conditioned media, showing activation of MAPK, NF-kB, and caspase 1 pathway, and induction and maturation of pro-Il1 β (**Right**).

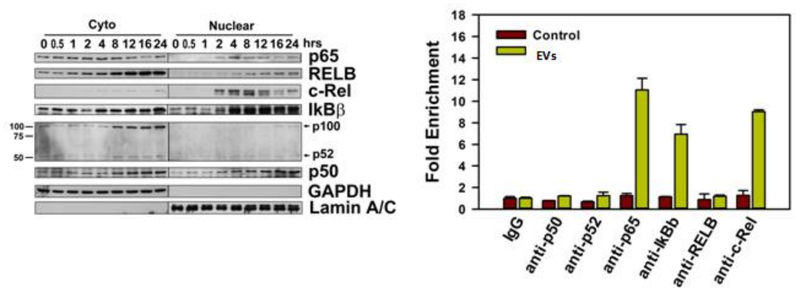
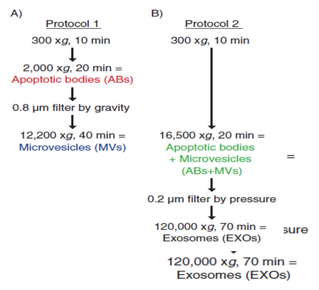


Figure 13. To confirm activation of NF-kB pathway, nuclear and cytoplasmic fractionation were done with the BMMQ stimulated with the vesicles. Western blots for NF-kB subunits were executed (**Left**). ChIP-qPCR assay for illuminating the recruitment of NFkB subunits to Il1β promoter regions showed that p65, C-Rel and IkBβ bound to Il1β promoter sequence (**Right**).

method

Extracellular vesicle isolation.



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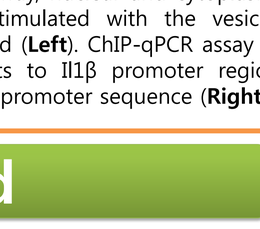
- Extracellular vesicles were collected from the supernatant of dying cells through differential centrifugation.

Characterization of the vesicle released by dying cell.

- Western blot for exosome markers such as CD63, LAMP1, Hsp60 and Rab7.
- NTA (Nanoparticle Tracking Analysis) for the concentration and size of released vesicles.
- OptiPrep density gradient centrifugation (Sigma-Aldrich) to determine the density of fractions.



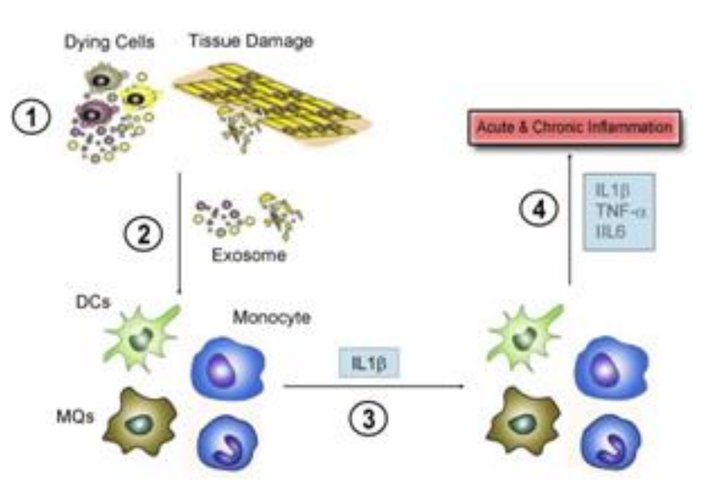
Expression constructs and lentiviral transfections.

- Lentiviral constructs expressing shRNAs for caspase 3, 6, 7, 9, Atg5, Beciln1, Rab5 and Rab11.
- Lentiviral constructs expressing gene knock-out using the CRISPR/Cas9 system for Rab27A and Rab27B

Preparation, culture and treatments of BMMQ.

- BMMQ is isolated from 6-week-old female BALB/c mice, and the resulting bone-marrow cells were re-suspended in RPMI 1640 containing 10% (v/v) fetal bovine serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 100 U ml^{-1} recombinant M-CSF.

conclusion



① When cells are dying in caspase-dependent manner, they release extracellular vesicles.

Release of vesicles are definitely associated with activation of Caspase 3 and 9, and regulated Rab protein such as RAB7 RAb11 and RAB27A.

② The extracellular vesicles from dying cells have characteristics of exosome in the aspects of their protein expression, size and density, and induce Il1β from the BMMQ, dependent of p38 MAPK and NF-kB signaling including p65, c-Rel and Ikbβ

③ ④ further study