厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

肝炎ウイルスの脂質二重膜を標的にした
新規抗ウイルス薬とワクチンの開発

平成２４年度　総括研究報告書

研究代表者　華山　力成

平成２５（２０１３）年　４月

別添１

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研究代表者　華山　力成　大阪大学免疫学フロンティア研究センター特任准教授

研究要旨
ウイルス性肝炎は罹患者が非常に多い疾患であるが、抗ウイルス薬の治療効果は限定的であり、C型肝炎ウイルスには有効なワクチンが存在しない。近年、肝炎ウイルスなどのエンベロープを持つウイルスの脂質二重膜には、ホスファチジルセリン(PS)と呼ばれるリン脂質が表面に露出しており、宿主の受容体と結合して感染を促進することが明らかとなっている。そこで本研究では、PSに強い結合能を持つ分子ラクトアドヘリンやそのホモログであるDel-1を用いて肝炎ウイルスのPSを覆い隠すことにより、肝炎ウイルスの宿主への結合と感染を効果的に阻害する新規抗ウイルス薬の開発を目標する。本年度私達は、ヒト・ラクトアドヘリンのPSへの結合能がマウス蛋白質に比べ低いが、ヒトDel-1はマウスラクトアドヘリンと同程度の強いPS結合能があることを見出した。更に、ヒトDel-1がエンベロープを持つウイルスの感染を培養細胞系で阻害することを明らかにした。これらの結果は今後、ヒトへの応用においてヒトDel-1組換え体蛋白質が有用であることを示している。更に、ヒトDel-1にインターフェロンを繋ぎ合わせた融合分子を作製し、肝炎ウイルス特異的にインターフェロンを運ぶ抗ウイルス薬の作製を現在行っている。

A．研究目的

肝炎ウイルスなどエンベロープを持つウイルスは、エンベロープ蛋白を介して宿主表面のウイルス受容体と結合し、エンドサイトーシスによって宿主のエンドソームに侵入し感染する。ところが最近、エンベロープウイルスの脂質二重膜の外側にはホスファチジルセリン(PS)と呼ばれるリン脂質が露出しており、これを宿主が受容体を介して結合し取り込む事によって、ウイルスの感染を強く促進している事が明らかとなった（Mercer et al., Science.（2008年）320:531-535）。実際、私達の実験においても、宿主にPSを認識する受容体TIM-4を過剰発現させると、ウイルスの感染効率が劇的に上昇する事が確認されている。

そこで本研究では、肝炎ウイルスのエンベロープ上のPSを覆い隠す事により、肝炎ウイルスの宿主への結合と感染を効果的に阻害する新規抗ウイルス薬の開発を目標にしている。私達はこれまで、PSに強い結合能を持つ分子ラクトアドヘリンを同定し、その点変異体が宿主によるPSの認識を強力に阻害する事を示した（Nature.（2002年）417:182-187）。実際、マウス・ラクトアドヘリンは、PSへの結合能が解離定数1nMと非常に高い為、レトロウイルスなどのエンベロープ・ウイルスの感染をin vitroで効率よく阻害する。一方、ヒト・ラクトアドヘリンは、PSとの結合能が解離定数10nMと比較的弱い為、ヒトへの応用を考慮した場合、更なる改変が必要である。

ヒト・ラクトアドヘリンはN末端側よりシグナル配列、2つのEGF領域、2つの血液凝固因子第8因子に相同性をもつ領域C1, C2から成り立っている。マウスのラクトアドヘリンは、3つのEGF領域を持っており、この3番目のEGF領域の存在がPSとの結合能を10倍高めることを私達は以前報告している（Nature.（2002年）417:182-187）。そこで、このマウスの蛋白質における3番目のEGF領域をヒトのラクトアドヘリンに組み込んだ組換え体蛋白質を作製し、PSとの結合能を高めることができるかを当初計画した。ところが、ヒトの蛋白質に37個とはいえマウスのアミノ酸を導入した場合、抗原性を獲得する可能性も考えられる。そこでラクトアドヘリンのホモログであるDel-1に着目した。Del-1はヒト蛋白質においてもEGF領域を3つ持っており、ヒト蛋白質をそのまま用いることができ、より安全であると考えた。

よって本研究では、ヒトDel-1が肝炎ウイルスの感染を阻害する新規抗ウイルス薬として利用可能かを検討するとともに、ヒトDel-1にインターフェロンβを繋ぎ合わせた融合分子を作製することにより、肝炎ウイルス特異的にインターフェロンを運ぶことが可能かを検討する。更に、ヒトDel-1に抗体のFc領域を繋いだ融合蛋白質を作成し、ヒトDel-1-Fcによってオプソニン化されたウイルスをマクロファージに貪食させて獲得免疫系を活性化することにより、C型肝炎ウイルスに対する効果的なワクチンの開発を目指す。

これまでウイルス感染を阻害する為、エンベロープ蛋白に対する中和抗体が数多く試みられたが、ウイルスは遺伝子変異によりエンベロープ蛋白の構造を変化させる為、顕著な効果をあげるのは困難であった。私達は、変異が生じない脂質二重膜を標的にする事により、この問題を解決し治療効果の高い新規作用機序の抗ウイルス薬を開発できると期待している。

B．研究方法

① ヒトDel-1をヒト血管内皮細胞からクローニングし、組換え体蛋白質を作製して、ヒトDel-1とPSとの結合能をELISA法にて評価する。この結合能をマウス・ラクトアドヘリンと比較する。

② エンベロープを持つウイルスの感染実験にヒトDel-1を加え、感染を阻害するかを検討する。

③ 肝炎ウイルス特異的にインターフェロンを運ぶ事とインターフェロンの副作用軽減を目的として、ヒトDel-1のN末端側にヒト・インターフェロンβを結合した融合組換え体蛋白質を作製する。

④ 実際に肝炎ウイルスのin vitro感染実験において、ヒトDel-1組換え体蛋白質とインターフェロンβ融合蛋白質が感染阻害効果を持つかを検討する。

⑤ ヒトDel-1に抗体のFc領域を結合させた組換え体蛋白質を作製する。hDel-1-Fcで標識されたウイルスは、免疫細胞のFc受容体を介して貪食され、ウイルスに対する獲得免疫を活性化すると予想され、C型肝炎ウイルスに対するワクチンとなりうるかを検討する。

⑥ モデル動物を用いて、肝炎ウイルスの感染阻害実験を行う。特に近年報告されたヒト化マウス(Nature（2011年）474:208-213)には免疫系が保持されており、HCV感染実験の理想的なモデル動物と考えられる為、このマウスを導入してHCVの感染実験を行い、ヒトDel-1誘導体の感染阻害効果とヒトDel-1-Fcのワクチンとしての効果を検討する。

（倫理面への配慮）

実験マウス、特にヒト化マウスを用いた実験を行うにあたっては、「動物の愛護および管理に関する法律」「実験動物の飼育及び保管並びに苦痛の軽減に関する基準」など、関連法規・指針に従った必要な措置を講じ、大阪大学動物実験規程を遵守して、動物愛護上の観点に十分配慮した環境で行う。

C．研究結果
① ヒト血管内皮細胞からRT-PCRでヒトDel-1遺伝子を単離し、C末端側にFLAG配列を付加した発現ベクターを作製した。このベクターを293T細胞に発現させ、組換え体蛋白質を作製し、培養上清から組換え体蛋白質をFLAGアフィニティーカラムを用いて精製した。精製したヒトDel-1蛋白質とＰＳとの結合能をELISAを用いて調べたところ、Kd値が約2nMと強いことが明らかになった。
② 293T細胞を用いてヒトDel-1蛋白質の大量精製を行い、エンベロープウイルスの感染阻害効果を調べた。すなわち、GFP遺伝子をコードしたモロニーマウス白血病ウイルスをNIH3T3細胞に感染させる実験系において、ヒトDel-1がウイルス感染をどの程度抑制するかを調べた。１μg/mlのヒトDel-1蛋白質をウイルスと３０分作用させたところ、感染を約７０％阻害することが明らかになった。同濃度で約９０％阻害するマウス・ラクトアドヘリンに比べれば若干落ちるが、エンベロープウイルスの感染をかなり阻害する効果があることが示された。
③ ヒトDel-1のN末端側にヒト・インターフェロンβを結合した融合組換え体蛋白質を作製した。この融合蛋白質を精製し、PSに対する結合能をELISA法にて評価したところ、野生型のヒトDel-1と変わらないことが明らかになった。

D．考察
ヒト・ラクトアドヘリンはマウス・ラクトアドヘリンが持つ３番目のEGF領域を持っておらず、この領域の有無がPSへの結合能を規定していると考えられた。そこで当初の計画では、ヒト・ラクトアドヘリンにマウス蛋白質がもつ３番目のEGF領域（３７アミノ酸）を挿入した組換え体蛋白質の作製を検討していたが、マウスのアミノ酸配列を導入することによる抗原性が懸念された。そこで更に検討を重ねたところ、ラクトアドヘリンのホモログであるDel-1はヒト蛋白質においても３番目のEGF領域をもつことが判明した為、ヒト蛋白質をそのまま用いることができ安全であると考えた。実際ヒトDel-1は、マウス・ラクトアドヘリンやマウスDel-1と同様に、PSへの結合能が解離定数1nMと非常に高いことが分かり、エンベロープ・ウイルスにin vitroで強く結合してウイルスの感染を効率よく阻害することが示され、今後生体内での感染阻害効果が期待される。

E．結論
本研究では、肝炎ウイルスのエンベロープ上のPSを覆い隠す事により、肝炎ウイルスの宿主への結合と感染を効果的に阻害する新規抗ウイルス薬の開発を目標にしている。私達はラクトアドヘリンのホモログであるDel-1がヒト蛋白質においても、PSに対して強い結合能を持つことを明らかにした。今後は、ヒトDel-1にインターフェロンや抗体のFc領域などの蛋白質を結合させた蛋白質が、抗ウイルス能を更に増強させたり、獲得免疫系を活性化してワクチンの開発に応用できるかを検討する。更にこれらヒトDel-1誘導体をモデル動物に投与する事により、肝炎ウイルスの感染を生体内で阻害し肝炎の発症を抑制できるかを検討する。

F．健康危険情報
特記すべきことはなし。

G．研究発表
１．論文発表
Toda S, Hanayama R, Nagata S. (2012) Two-step phagocytosis of apoptotic cells. Mol Cell Biol. 32(1):118-25

H．知的財産権の出願・登録状況
１．特許取得
該当なし
２．実用新案登録
該当なし
３．その他
該当なし

別添４

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
華山力成、他	Two-step phagocytosis of apoptotic cells.	Mol Cell Biol.	32(1)	118-25	2012

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Two-Step Engulfment of Apoptotic Cells

Satoshi Toda,[a] Rikinari Hanayama,[a] and Shigekazu Nagata[a,b]

Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Yoshida-konoe, Kyoto, Japan,[a] and Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kyoto, Japan[b]

Apoptotic cells expose phosphatidylserine on their surface as an "eat me" signal, and macrophages respond by engulfing them. Although several molecules that specifically bind phosphatidylserine have been identified, the molecular mechanism that triggers engulfment remains elusive. Here, using a mouse pro-B cell line, Ba/F3, that grows in suspension, we reconstituted the engulfment of apoptotic cells. The parental Ba/F3 cells did not engulf apoptotic cells. Ba/F3 transformants expressing T cell immunoglobulin- and mucin-domain-containing molecule 4 (Tim4), a type I membrane protein that specifically binds phosphatidylserine, efficiently bound apoptotic cells in a phosphatidylserine-dependent manner but did not engulf them. However, Ba/F3 transformants expressing both Tim4 and the integrin $\alpha_v\beta_3$ complex bound to and engulfed apoptotic cells in the presence of milk fat globule epidermal growth factor factor VIII (MFG-E8), a secreted protein that can bind phosphatidylserine and integrin $\alpha_v\beta_3$. These results indicate that the engulfment of apoptotic cells proceeds in two steps: Tim4 tethers apoptotic cells, and the integrin $\alpha_v\beta_3$ complex mediates engulfment in coordination with MFG-E8. A similar two-step engulfment of apoptotic cells was observed with mouse resident peritoneal macrophages. Furthermore, the Tim4/integrin-mediated engulfment by the Ba/F3 cells was enhanced in cells expressing Rac1 and Rab5, suggesting that this system well reproduces the engulfment of apoptotic cells by macrophages.

Every day, billions of cells that are toxic, useless, and senescent die by apoptosis and are engulfed by macrophages, presumably to prevent the release of noxious materials from the dead cells (18). The system that efficiently removes apoptotic cells from the body appears to be quite elaborate, and some details of this process are unclear (25). Apoptotic cells present an "eat me" signal to macrophages, triggering their own engulfment. Among the various molecules proposed to be involved in this process, phosphatidylserine (PS) is a strong candidate for the "eat me" signal (11). PS is transferred caspase dependently from the inner leaflet to the outer leaflet of the plasma membrane (13), and masking PS inhibits the engulfment of apoptotic cells by macrophages (2, 6, 11).

By identifying monoclonal antibodies that have positive or negative effects on the engulfment of apoptotic cells, we previously identified two molecules, milk fat globule epidermal growth factor (EGF) factor VIII (MFG-E8) and T cell immunoglobulin- and mucin-domain-containing molecule 4 (Tim4), that enhance engulfment (6, 15). MFG-E8 is a secreted protein of 75 kDa that binds to PS via its factor VIII-homologous domain. It also binds to the integrin $\alpha_v\beta_3$ complex in macrophages via an RGD motif in MFG-E8's EGF domain, thus bridging apoptotic cells and macrophages. Tim4 is a type I membrane protein of 70 kDa that binds to PS via the immunoglobulin-like domain in its extracellular region. Its cytoplasmic region is 43 amino acids long and is dispensable for engulfment (22), suggesting that Tim4 itself does not transduce the signal for engulfment. Tim4, but not MFG-E8, is expressed by resident peritoneal macrophages, and MFG-E8, but not Tim4, is expressed by thioglycolate-elicited peritoneal macrophages (15), suggesting that the two molecules function independently in the engulfment of apoptotic cells in these macrophages. On the other hand, tingible-body macrophages in the spleen express both MFG-E8 and Tim4 (7, 31), suggesting that the two molecules may cooperate in the engulfment of apoptotic cells. However, how MFG-E8 and Tim4 function in these macrophages and how Tim4 and integrins transduce the engulfment signal have not been elucidated.

Here, we established an assay system for the engulfment of apoptotic cells using a Ba/F3 suspension culture. The parental Ba/F3 cells did not engulf apoptotic cells at all. The expression of Tim4 conferred on them the ability to recognize and bind apoptotic cells but not to engulf them. When Tim4 and integrin $\alpha_v\beta_3$ complex were coexpressed, the Ba/F3 transformants efficiently engulfed apoptotic cells in the presence of MFG-E8, and this efficiency was enhanced by the expression of Rac1 and Rab5. These results indicate that Tim4 and integrin $\alpha_v\beta_3$ cooperate to mediate the engulfment of apoptotic cells: Tim4 in the tethering step and MFG-E8/integrin in the uptake step.

MATERIALS AND METHODS

Materials, cell lines, recombinant proteins, and antibodies. pHrodo succinimidyl ester (pHrodo) was purchased from Invitrogen. CellTracker Orange {CMRA; 9′-(4 [and 5]-chloromethyl-2-carboxyphenyl)-7′-chloro-6′-oxo-1,2,2,4-tetramethyl-1,2-dihydropyrido[2′,3′-6]xanthene} was obtained from Molecular Probes. A caspase inhibitor (quinolyl-valyl-*O*-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone [Q-VD-OPh]) was from R&D systems.

The mouse pro-B cell line Ba/F3 was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 45 units/ml mouse interleukin-3 (IL-3). Mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% FCS.

Flag-tagged mouse recombinant MFG-E8 was produced in human 293T cells as described previously (6) and purified with anti-Flag-Sepharose. To prepare the leucine-zipper-tagged human Fas ligand (FasL)

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Address correspondence to Shigekazu Nagata, snagata@mfour.med.kyoto-u.ac.jp.
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(27), a FasL expression plasmid was introduced into monkey COS cells by electroporation. The COS cells were cultured in DMEM containing 1% FCS for 48 h, and the FasL that was secreted into the culture supernatant was precipitated in 60%-saturated $(NH_4)_2SO_4$ and dialyzed against phosphate-buffered saline (PBS). The cytotoxic activity of FasL was assayed with mouse WR19L cells expressing mouse Fas (W3 cell line) as described previously (29). One unit of FasL activity represented the concentration of FasL needed to produce half-maximal cytotoxicity against 5×10^4 W3 cells. Mouse recombinant IL-3 was produced by mouse C127I cells transformed with a bovine papillomavirus expression vector for mouse IL-3 as described previously (4). The biological activity of IL-3 was assayed by determining its ability to support the growth of Ba/F3 cells as described previously (4). One unit represented the concentration of IL-3 required for the half-maximal stimulation at 37°C for 48 h of 1×10^4 cells in 100 μl of medium. The hamster anti-Tim4 monoclonal antibody (MAb; clone Mat4) will be described elsewhere. The phycoerythrin (PE)-labeled rat anti-mouse integrin α_v (clone RMV-7), PE-labeled hamster anti-mouse integrin β_3 (clone 2C9.G2), and allophycocyanin (APC)-labeled streptavidin were from BD Pharmingen. The APC-labeled rat anti-mouse Mac1 (clone M1/70) was purchased from BioLegend.

Mice. The wild-type C57BL/6 mice were purchased from Japan SLC. $Tim4^{-/-}$ mice were established in the C57BL/6 background (M. Miyanishi and S. Nagata, unpublished data) and will be described elsewhere. Mice were housed in a specific-pathogen-free facility at the Kyoto University Graduate School of Medicine. All animal experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee at the Kyoto University Graduate School of Medicine.

Transformation. Mouse Tim4 and integrins were expressed in NIH 3T3 cells and Ba/F3 cells by retrovirus-mediated transformation. In brief, the recombinant retrovirus was produced in Plat-E cells (16) by transfecting them with the pMXs-puro or -neo retrovirus vector (10) carrying the cDNA sequence for green fluorescent protein (GFP), mouse Tim4 (15), mouse Rac1 or Rab5 (19), or mouse integrin α_v or integrin β_3 (6). The retrovirus was precipitated by centrifugation at 6,000 $\times$ *g* for 16 h, dissolved in medium containing 10% FCS, and used to infect NIH 3T3 cells or Ba/F3 cells in the presence of 10 μg/ml Polybrene. The transformants were selected by culturing in the presence of 1.0 μg/ml puromycin or 800 μg/ml G-418. When necessary, Ba/F3 cells expressing Tim4, integrin α_v, or integrin β_3 were sorted using a FACSAria fluorescence-activated cell sorter (FACS; BD Biosciences).

Labeling of apoptotic cells. Mouse thymocytes were labeled with pHrodo as described by Miksa et al. (14) for the engulfment assay or with CellTracker Orange for the binding assay. In brief, thymocytes (2×10^7 cells/ml) from 4- to 8-week-old C57BL/6 mice were treated at 37°C for 2 h with 70 units/ml FasL, which caused about 70% of the cells to become annexin V positive and less than 20% of them to be Sytox blue positive. The cells were washed twice with PBS and incubated at room temperature for 30 min with 0.1 μg/ml pHrodo. After stopping the reaction by adding 1 ml FCS, the cells were washed with PBS containing 10% FCS, suspended in the medium containing 10% FCS, and used for engulfment. For labeling with CellTracker, thymocytes were incubated at 37°C for 30 min in serum-free DMEM containing 10 μM CellTracker Orange, washed with DMEM containing 10% FCS, and incubated at 37°C for 2 h with FasL as described above.

Engulfment of apoptotic cells by NIH 3T3 cells. To 7.5×10^4 NIH 3T3 cells in 0.5 ml of DMEM containing 10% FCS in a 24-well microtiter plate, 1×10^6 pHrodo-labeled apoptotic cells were added, and the culture was incubated at 37°C for 90 min. The cells were detached from the plate by incubating them with 0.25% trypsin and 1 mM EDTA and collected by centrifugation. The cells were suspended in 300 μl of 20 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)–NaOH buffer (pH 9.0) containing 150 mM NaCl and 2% FCS (CHES-FACS buffer) and analyzed by flow cytometry on the FACSAria apparatus. In some cases, 6×10^4 NIH 3T3 cells were cultured in an 8-well Lab-Tek II chambered cover glass (Nalge Nunc) and incubated at 37°C for 90 min with 1×10^6 pHrodo-labeled apoptotic thymocytes in 0.8 ml DMEM containing 10% FCS. After being washed with PBS, the cells were fixed at room temperature for 10 min with 1% paraformaldehyde in PBS, immersed in CHES-FACS buffer, and observed by fluorescence microscopy (BioRevo BZ-9000; Keyence).

Engulfment of apoptotic cells with Ba/F3 cells. The GFP-expressing Ba/F3 cells (1×10^5) and 1×10^6 to 5×10^6 pHrodo-labeled apoptotic thymocytes were incubated at 37°C for 2 h in 0.6 to 3.0 ml RPMI 1640 containing 10% FCS. After the incubation, the cells were collected by centrifugation at 500 $\times$ *g* for 5 min, suspended in 300 μl of CHES-FACS buffer, and analyzed by flow cytometry as described above. For microscopic observation, the GFP-expressing Ba/F3 and pHrodo-labeled thymocytes were suspended in 100 μl of CHES-FACS buffer, transferred to the Lab-Tek II chambered cover glass, and observed by fluorescence microscopy. The numbers of GFP-positive Ba/F3 cells and pHrodo-positive thymocytes were counted using Dynamic Cell Count software, BZ-HIC (Keyence). The phagocytosis index represents the number of the engulfed apoptotic cells per Ba/F3 cell.

Engulfment of apoptotic cells with peritoneal macrophages. Resident peritoneal cells were prepared from C57BL/6 mice at the age of 8 weeks. The peritoneal cells (1×10^5) and 1.0×10^6 pHrodo-labeled apoptotic thymocytes were incubated in suspension at 37°C for 60 min in 0.5 ml RPMI 1640 containing 10% FCS or in PBS containing 2% FCS. After the incubation, the cells were collected by centrifugation at 500 $\times$ *g* for 5 min, suspended in 300 μl of CHES-FACS buffer containing 0.67 μg/ml APC-labeled rat anti-mouse Mac1, and analyzed by flow cytometry.

Binding of apoptotic cells with Ba/F3 cells. To assay the binding of apoptotic cells to Ba/F3 cells, 1×10^5 GFP-expressing Ba/F3 cells were incubated at 37°C with 5×10^6 CellTracker Orange-labeled apoptotic thymocytes in 500 μl of PBS containing 10% FCS and subjected to FACS analysis for GFP and CellTracker Orange. The cell population that was positive for GFP and CellTracker Orange was regarded to be Ba/F3 cells to which apoptotic cells were bound. In some cases, 1×10^5 Ba/F3 cells were incubated with 1×10^6 CellTracker Orange-labeled apoptotic thymocytes at room temperature for 25 min in an 8-well Lab-Tek II chamber and then observed by fluorescence microscopy.

Binding of apoptotic cells to resident peritoneal macrophages. To assay the binding of apoptotic cells to the resident peritoneal macrophages, 1×10^5 mouse peritoneal cells were incubated in suspension at room temperature with 1×10^6 CellTracker Orange-labeled apoptotic thymocytes in 500 μl of PBS containing 10% or 2% FCS. The APC-conjugated anti-Mac1 was added to a final concentration of 0.67 μg/ml, and the cells were analyzed by flow cytometry. The cell population that was positive for APC and CellTracker Orange was regarded to be peritoneal macrophages to which apoptotic cells were bound.

RESULTS

Establishment of the assay for engulfment of apoptotic cells. Miksa et al. (14) recently developed a simple method for monitoring the engulfment of apoptotic cells by using pHrodo-labeled apoptotic cells as prey. This method permits apoptotic cells that are engulfed and transported into lysosomes to be detected by their increased light emission in the acidic lysosomal environment. We modified this method slightly. That is, since we found that the nonengulfed pHrodo-conjugated apoptotic thymocytes emit a low but significant light under neutral conditions (pH 7.4), we treated the phagocytes with basic buffer (pH 9.0) after coincubation with apoptotic cells. Furthermore, living cells were found to incorporate pHrodo to emit light even under basic conditions. Mouse thymocytes were therefore treated with a high concentration of FasL to fully induce apoptosis in all cells in a short period (2 h) before labeling them with pHrodo. Under these conditions, about 70% of the thymocytes were annexin V positive and Sytox blue negative.

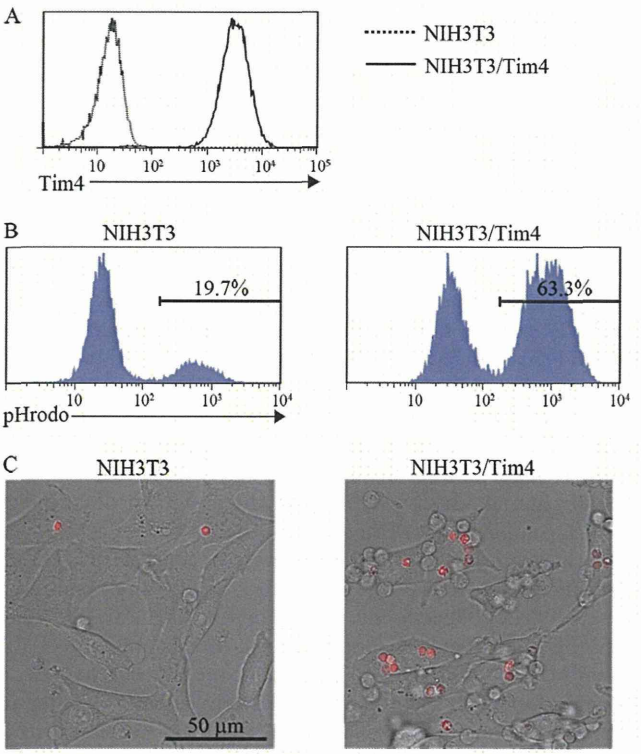

FIG 1 Effect of Tim4 on the engulfment of apoptotic cells by NIH 3T3 cells. (A) Expression of Tim4 in mouse NIH 3T3 cells. NIH 3T3 cells and transformants expressing mouse Tim4 were stained with a biotinylated anti-Tim4 MAb, followed by APC-conjugated streptavidin. The staining profile was analyzed by flow cytometry using a FACSAria apparatus. (B and C) Tim4-dependent engulfment of apoptotic cells. (B) The pHrodo-labeled apoptotic thymocytes were incubated for 90 min with NIH 3T3 cells or NIH 3T3 transformants expressing Tim4 and analyzed by flow cytometry. (C) The engulfment of pHrodo-labeled apoptotic cells was performed in a Lab-Tek chamber and observed by fluorescence microscopy.

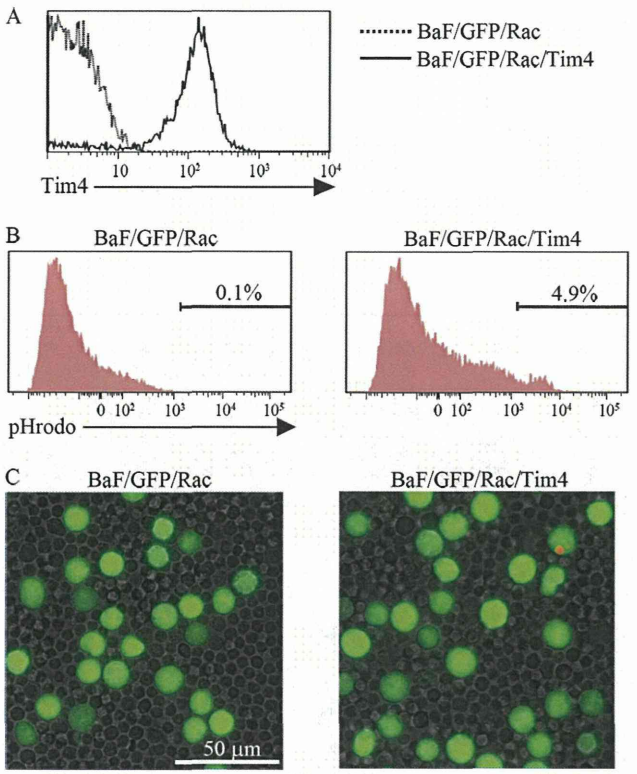

FIG 2 Effect of Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. (A) Expression of Tim4 in mouse Ba/F3 cells. The Ba/F3 cells were transformed with an expression vector for GFP and Rac1 (BaF/GFP/Rac) or with a vector for GFP, Rac1, and Tim4 (BaF/GFP/Rac/Tim4). The stable transformants were stained with a biotinylated anti-Tim4 MAb, followed by PE-conjugated streptavidin. (B and C) The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 120 min and subjected to flow cytometry. (B) The pHrodo profile of the GFP-positive population is shown. The numbers indicate the percentage of pHrodo-positive cells in the GFP-positive population. (C) The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 120 min. The cell mixture was transferred to a Lab-Tek chamber and observed by fluorescence microscopy.

We first tested the reliability of the assay by examining the Tim4-mediated engulfment of apoptotic cells by NIH 3T3 cells (15). NIH 3T3 cells, which do not normally express Tim4, were stably transformed with Tim4 to generate NIH 3T3/Tim4 cells (Fig. 1A) and incubated with pHrodo-labeled apoptotic thymocytes at a ratio of 1:13 (NIH 3T3 cells:thymocytes). An analysis by flow cytometry indicated that more than 60% of the NIH 3T3/Tim4 cells became pHrodo positive within 90 min of coincubation with the apoptotic thymocytes, while most of the cells with the parental NIH 3T3 remained pHrodo negative (Fig. 1B). Microscopic observation showed several pHrodo-positive thymocytes present inside most of the NIH 3T3/Tim4 cells, with only a small percentage of NIH 3T3 cells carrying a single pHrodo-positive thymocyte. In addition, many pHrodo-negative, unengulfed cells were associated with NIH 3T3/Tim4 cells but not with the parental NIH 3T3 cells. These results confirmed that Tim4 strongly enhances the engulfment of apoptotic cells in NIH 3T3 cells (15) and that the engulfment assay using the pHrodo-labeled apoptotic cells (14) was simple and reliable.

Binding of apoptotic cells to Tim4-expressing Ba/F3 cells without engulfment. In contrast to the NIH 3T3 cells, when the mouse pro-B cell line Ba/F3, which grows in suspension, was transformed with Tim4 (BaF/Tim4), the cells engulfed hardly any apoptotic cells (data not shown). Since a small GTPase, Rac1, enhances the engulfment of apoptotic cells (see below) (19, 30), BaF/GFP and BaF/GFP/Tim4 were transformed with Rac1. As shown in Fig. 2, very few apoptotic cells were engulfed by the BaF/GFP/Rac cells either. This assay showed that the expression of Tim4 rendered the cells able to engulf apoptotic cells, but even so, only 5% of the BaF/GFP/Rac/Tim4 transformants contained dead cells.

On the other hand, when the binding rather than engulfment of CellTracker-labeled apoptotic cells was assayed, the ability of the Ba/F3 cells to bind the apoptotic cells was clearly and strongly enhanced by Tim4 expression. That is, only 1.3% of the BaF/GFP/Rac cells bound apoptotic cells within 30 min, whereas more than 50% of the BaF/GFP/Rac/Tim4 cells bound them (Fig. 3A). Microscopic observation showed that the BaF/Tim4 cells had a tendency to aggregate (Fig. 3B), as reported previously (15). We postulated that the aggregation of the Tim4-expressing cells is due to PS-expressing exosomes (15). Apoptotic thymocytes also seemed to mediate the aggregation of BaF/GFP/Rac/Tim4 cells (Fig. 3B). The binding of apoptotic cells to BaF/GFP/Rac/Tim4 was quick,

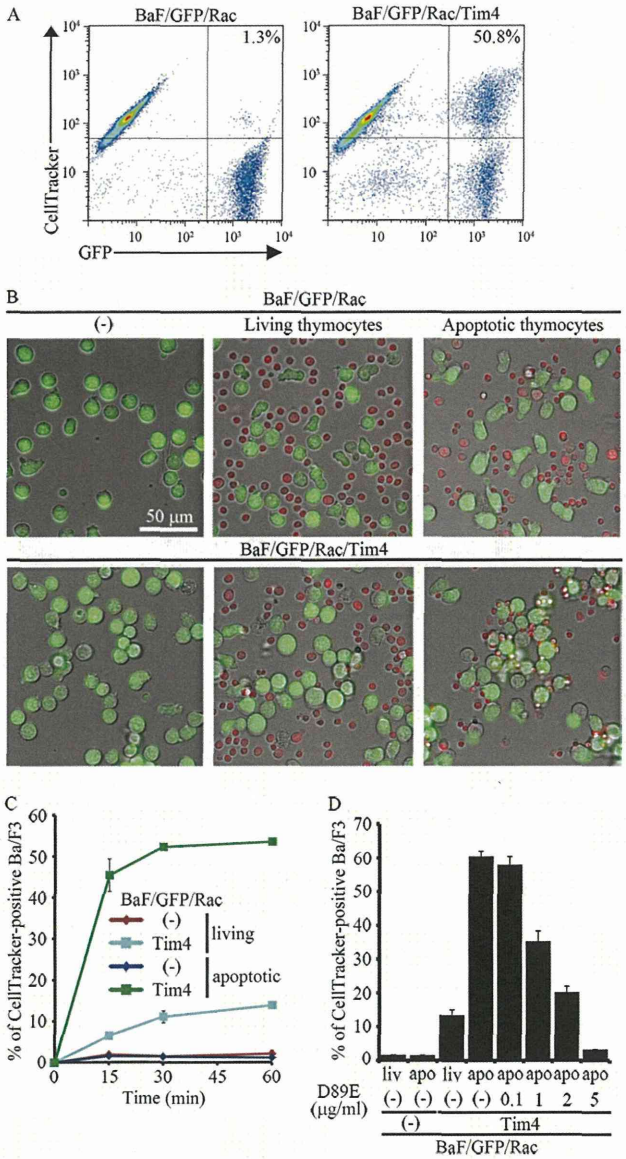

FIG 3 Tim4-dependent binding of apoptotic cells to Ba/F3 cells. (A) FACS analysis of the binding of apoptotic cells to Ba/F3 cells. Ba/F3 cells expressing GFP and Rac1 (BaF/GFP/Rac) or GFP, Rac1, and Tim4 (BaF/GFP/Rac/Tim4) were incubated at 37°C for 30 min with CellTracker Orange-labeled apoptotic thymocytes and subjected to flow cytometry for GFP and CellTracker Orange. The numbers indicate the percentage of the CellTracker-positive cells in the GFP-positive population. (B) Microscopic analysis of the binding of apoptotic cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated for 25 min at room temperature with CellTracker Orange-labeled living or apoptotic thymocytes in a Lab-Tek chamber and observed by fluorescence microscopy. (C) Time course of the binding of apoptotic cells to Tim4-expressing cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with CellTracker Orange-labeled living or apoptotic thymocytes at 37°C for the indicated periods of time. The percentage of GFP-positive Ba/F3 cells that bound apoptotic cells was determined by flow cytometry as described above. The experiments were done in triplicate, and the average values are plotted (bars indicate SDs). (D) Phosphatidylserine-dependent binding of apoptotic cells to Tim4-expressing Ba/F3 cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with CellTracker Orange-labeled living or apoptotic thymocytes at 37°C for 30 min in the presence of the indicated amount of MFG-E8 D89E. The cell mixture was subjected to FACS analysis, and the percentage of GFP-positive Ba/F3 cells that bound apoptotic cells was determined as described above. The experiments were done in triplicate, and the average values are plotted (bars indicate SDs).

with near maximum binding observed within 15 min at 37°C (Fig. 3C). The D89E mutant of MFG-E8, which can mask PS (6), dose dependently inhibited the binding of apoptotic cells to the BaF/GFP/Rac/Tim4 cells (Fig. 3D), confirming that the apoptotic cells bound to Tim4-expressing cells in a PS-dependent manner.

Stimulation of engulfment of apoptotic cells by integrin $\alpha_v\beta_3$. The above-described results indicated that Tim4 recognized apoptotic cells and tethered them to phagocytes but did not mediate their engulfment. The $\alpha_v\beta_3$ integrin complex, which is the vitronectin receptor, is known to transduce a signal for engulfment (1, 6, 26). Macrophages express the integrin $\alpha_v\beta_3$ complex, but Ba/F3 cells do not. We therefore expressed integrin $\alpha_v\beta_3$ in BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells (see Fig. S1 in the supplemental material). A real-time reverse transcription-PCR analysis indicated that the expression levels of Tim4 and integrin α_v mRNA in the Ba/F3 transformants were comparable to those in mouse resident peritoneal macrophages (see Fig. S2 in the supplemental material). As shown in Fig. 4A, the expression of the integrin $\alpha_v\beta_3$ complex alone in these Ba/F3 cells had little effect on their ability to engulf apoptotic cells. However, the addition of MFG-E8 to the integrin $\alpha_v\beta_3$-expressing cells ($\alpha\beta$ cells) strongly enhanced the engulfment. Specifically, about 25% of the BaF/GFP/Rac/Tim4/$\alpha\beta$ cells engulfed apoptotic cells in the presence of 0.1 μg/ml MFG-E8. Observation by microscope showed that only a few apoptotic cells were inside Ba/F3 cells expressing integrin $\alpha_v\beta_3$, but several dead cells were found in the Ba/F3 cells expressing integrin $\alpha_v\beta_3$ and Tim4 (Fig. 4B). Thus, the phagocytic index (the number of engulfed dead cells per phagocyte) of the BaF/Rac/Tim4 cells increased to 0.4 with the expression of integrin $\alpha_v\beta_3$ (Fig. 4C). The increase in the engulfment of apoptotic cells by Ba/F3 cells expressing integrin $\alpha_v\beta_3$ was not due to a more efficient recruitment of apoptotic cells; as shown in Fig. 4D, apoptotic cells did not bind to the integrin $\alpha_v\beta_3$-expressing Ba/F3 cells, even in the presence of MFG-E8, unless Tim4 was also expressed. These results indicated that Tim4 and integrin $\alpha_v\beta_3$/MFG-E8 function in different steps of the engulfment process for apoptotic cells.

To examine whether macrophages engulf apoptotic cells in two steps, we analyzed the engulfment of apoptotic cells with mouse resident peritoneal macrophages that strongly express Tim4 (15). As shown in Fig. 4E, the wild-type peritoneal macrophages efficiently engulfed apoptotic cells, but this ability was lost in Tim4-deficient peritoneal macrophages, because they could not tether apoptotic cells. Binding of Tim4 to phosphatidylserine does not require Ca^{2+} (15), while the integrin signaling is known to require Ca^{2+} (28). Accordingly, apoptotic cells efficiently bound to the Tim4-expressing peritoneal macrophages in the absence or presence of Ca^{2+} (Fig. 4F). On the other hand, the engulfment of apoptotic cells by the peritoneal macrophages did not occur in the absence of Ca^{2+}. These results indicated that the engulfment of apoptotic cells by mouse resident peritoneal macrophages proceeds in two steps: Ca^{2+}-independent tethering and Ca^{2+}-dependent uptake steps.

Effect of small GTPases Rac1 and Rab5 on engulfment by Ba/F3 cells. The Rho and Rab family GTPases regulate the engulfment of apoptotic cells by macrophages and immature dendritic cells. That is, to form the phagocytic cup to engulf apoptotic cells, the integrin $\alpha_v\beta_3$ expressed by macrophages activates Rac1 of the Rho family to induce actin polymerization (1). Rab5, a member of the Rab family, regulates the fusion of phagosomes with endosomes (3). As shown in Fig. 5, the overexpression of Rac1 in BaF/

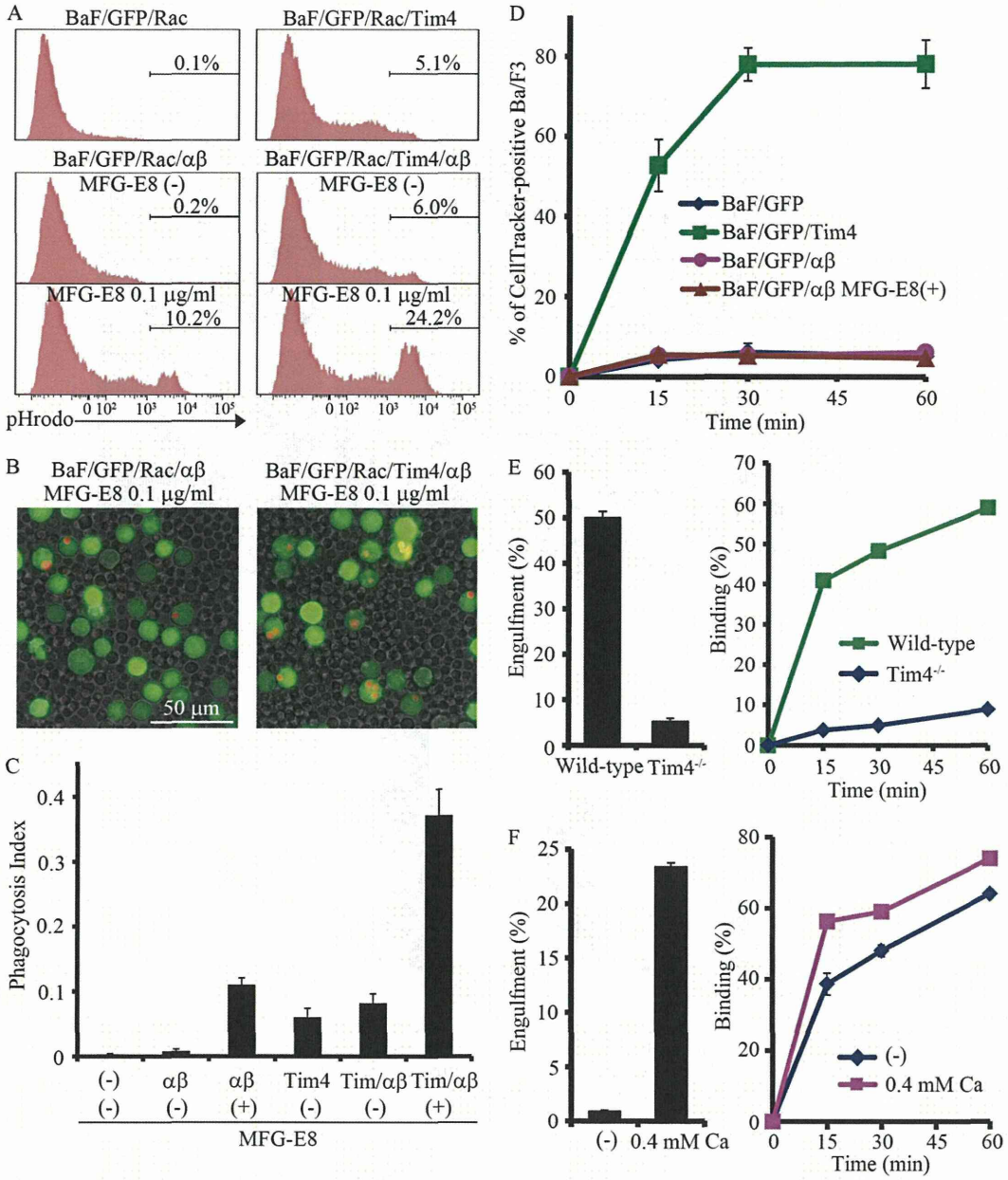

FIG 4 Effect of integrins/MFG-E8 and Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. (A) Effect of integrin $\alpha_v\beta_3$/MFG-E8 and Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. BaF/GFP/Rac, BaF/GFP/Rac/$\alpha\beta$, BaF/GFP/Rac/Tim4, or BaF/GFP/Rac/Tim4/$\alpha\beta$ cells were incubated at 37°C for 120 min with pHrodo-labeled apoptotic thymocytes in the absence or presence of 0.1 μg/ml mouse MFG-E8. The cell mixture was subjected to flow cytometry for GFP and pHrodo. The pHrodo staining profile of the GFP-positive population is shown. The experiment was performed three times, and representative data are shown. (B) Microscopic observation. BaF/GFP/Rac/$\alpha\beta$ or BaF/GFP/Rac/Tim4/$\alpha\beta$ cells were incubated as described above with pHrodo-labeled apoptotic thymocytes in the presence of 0.1 μg/ml mouse MFG-E8. The cells were transferred to a Lab-Tek chamber and observed by fluorescence microscopy. (C) Enhanced engulfment of apoptotic cells by Tim4 and MFG-E8. BaF/GFP/Rac (−), BaF/GFP/Rac/$\alpha\beta$ ($\alpha\beta$), BaF/GFP/Rac/Tim4 (Tim4), or BaF/GFP/Rac/Tim4/$\alpha\beta$ (Tim/$\alpha\beta$) cells were incubated at 37°C for 120 min with pHrodo-labeled apoptotic thymocytes in the absence (−) or in the presence (+) of 0.1 μg/ml mouse MFG-E8. The cell mixture was transferred to a Lab-Tek chamber and observed by fluorescence microscopy. The phagocytosis index was determined for at least 3,500 Ba/F3 cells in 10 fields and is plotted. (D) No effect of integrin or MFG-E8 on the binding of apoptotic cells to Ba/F3 cells. BaF/GFP, BaF/GFP/Tim4, or BaF/GFP/$\alpha\beta$ cells were incubated at 37°C with CellTracker Orange-labeled apoptotic thymocytes in the absence or presence of 0.1 μg/ml mouse MFG-E8 for the indicated periods of time. The percentage of GFP-positive Ba/F3 cells that bound apoptotic cells was determined by flow cytometry. The experiments were done in triplicate, and the average values are plotted with SDs (bars). (E) Tim4-dependent tethering and uptake of apoptotic cells by mouse resident peritoneal macrophages. (Left) Peritoneal cells from the wild-type and Tim4$^{-/-}$ mice were incubated with pHrodo-labeled apoptotic thymocytes. The cells were stained with APC-conjugated anti-mouse Mac1 and subjected to flow cytometry for APC and pHrodo. The engulfment was determined as the percentage of pHrodo-positive cells in the Mac1-positive population. The experiment was carried out in triplicate, and the average values were plotted with SDs (bars). (Right) Peritoneal cells from the wild-type and Tim4$^{-/-}$ mice were incubated at room temperature with CellTracker-labeled apoptotic thymocytes for the indicated periods of time. After staining with APC-labeled anti-Mac1, the percentage of Mac1-positive cells that bound apoptotic cells was determined by flow cytometry. The experiments were done in triplicate, and the average values are plotted with SDs (bars). (F) Requirement of Ca^{2+} for the uptake but not binding of apoptotic cells to peritoneal macrophages. (Left) Peritoneal cells from the wild-type mice were incubated at 37°C for 60 min with pHrodo-labeled apoptotic thymocytes in PBS containing 2% FCS in the absence or presence of 0.4 mM Ca^{2+}. The cells were stained with APC-labeled anti-Mac and analyzed by flow cytometry as described above. The experiments were done in triplicate, and the average values are plotted with SDs (bars). (Right) Peritoneal cells from the wild-type mice were incubated at room temperature in PBS containing 2% FCS in the absence or presence of 0.4 mM Ca^{2+} with CellTracker-labeled apoptotic thymocytes for the indicated periods of time and analyzed by flow cytometry as described above. The experiments were done in triplicate, and the average values are plotted with SDs (bars).

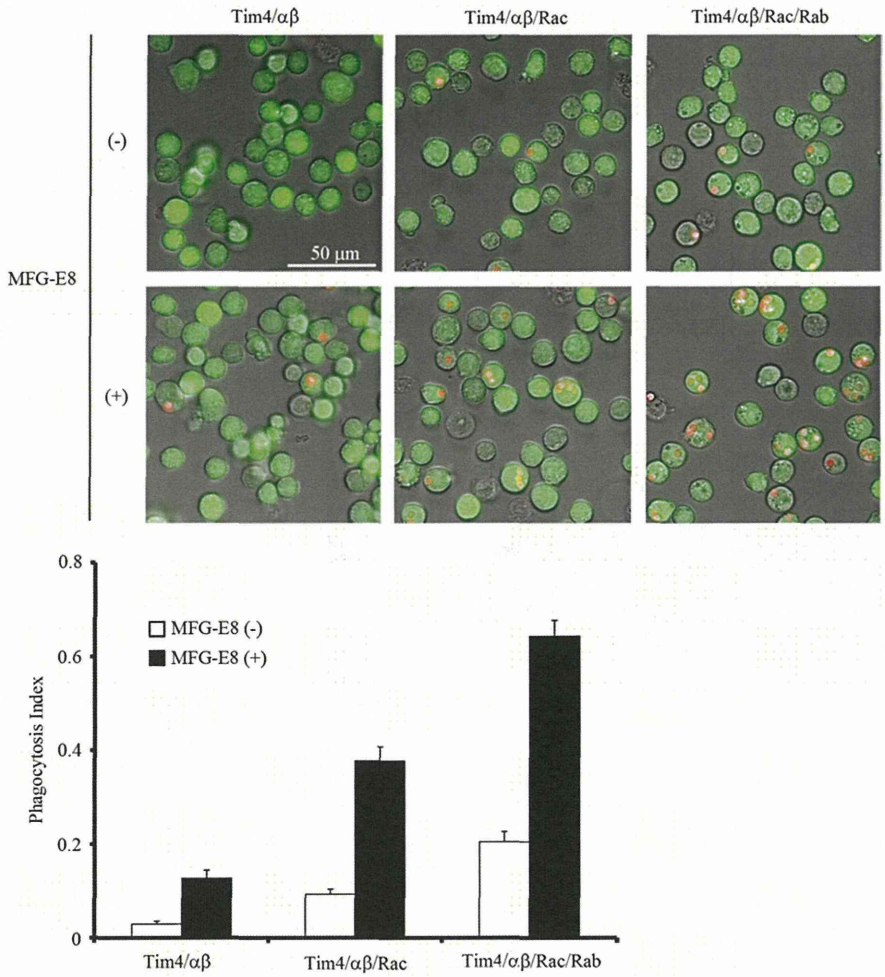

FIG 5 Effect of Rac1 and Rab5 on the engulfment of apoptotic cells by Ba/F3 cells. BaF/GFP/Tim4/$\alpha\beta$ cells (Tim4/$\alpha\beta$) were transformed with Rac1 (Tim4/$\alpha\beta$/Rac) or Rac1 and Rab5 (Tim4/$\alpha\beta$/Rac/Rab) and incubated at 37°C for 120 min with pHrodo-labeled apoptotic thymocytes in the absence (open bars) or presence (filled bars) of 0.1 μg/ml mouse MFG-E8. Ba/F3 cells were sorted by FACS, transferred to a Lab-Tek chamber, and observed by fluorescence microscopy. (Bottom) The phagocytosis index for more than 4,500 Ba/F3 cells in 10 to 12 fields is plotted.

Tim4/$\alpha\beta$ cells strongly enhanced the MFG-E8-dependent engulfment of apoptotic cells, increasing the phagocytic index from 0.1 to 0.4. The addition of Rab5 expression further increased the index to 0.65. That is, less than 10% of the BaF/GFP/Tim4/$\alpha\beta$ cells engulfed apoptotic thymocytes in the presence of MFG-E8. In contrast, about half the BaF/GFP/Tim4/$\alpha\beta$ cells expressing Rac1 and Rab5 engulfed dead cells, and some of them contained several dead cells. These results support the idea that the engulfment of apoptotic cells in the Ba/F3 suspension culture efficiently reproduces the process of apoptotic cell engulfment by macrophages.

DISCUSSION

The reconstitution of apoptotic cell engulfment in a system that uses defined molecules is essential for understanding the detailed molecular mechanisms involved. We and others have used fibroblasts and epithelial cell lines such as NIH 3T3, LR73, and 293T cells as host cells to reconstitute engulfment and have identified several molecules (MFG-E8/integrins, Tim4, stabillin 2, BAI1, etc.) that bind to PS (6, 15, 20, 23, 24). When they are expressed in fibroblasts, they enhance the engulfment of dead cells, suggesting that each molecule is independently involved in this process. In particular, Tim4 alone endows NIH 3T3/LR73 cells with the ability to efficiently engulf apoptotic cells, yet its cytoplasmic region is not required for the engulfment of apoptotic cells, suggesting that Tim4 works at the tethering step and LR73 cells express one or more additional molecules that comediate the engulfment of apoptotic cells with Tim4 (22).

Lymphocytes that grow in suspension usually do not engulf apoptotic cells. On the other hand, immature dendritic cells established from the mouse spleen grow in suspension and can take up mycobacteria (21). We therefore thought that cells of a lymphocyte cell line could be a good host for reconstituting apoptotic cell engulfment, when coupled with the pHrodo-labeled apoptotic cell engulfment assay. Here, we found that, unlike Tim4 in NIH 3T3 cells, the expression of Tim4 in Ba/F3 cells was not sufficient for apoptotic cell engulfment, although apoptotic cells bound to the Tim4-expressing cells. Integrin $\alpha_v\beta_3$ was also required for the efficient engulfment of apoptotic cells. However, the failure of apoptotic cells to bind to Ba/F3 cells expressing integrin $\alpha_v\beta_3$, but not Tim4, even in the presence of MFG-E8, indi-

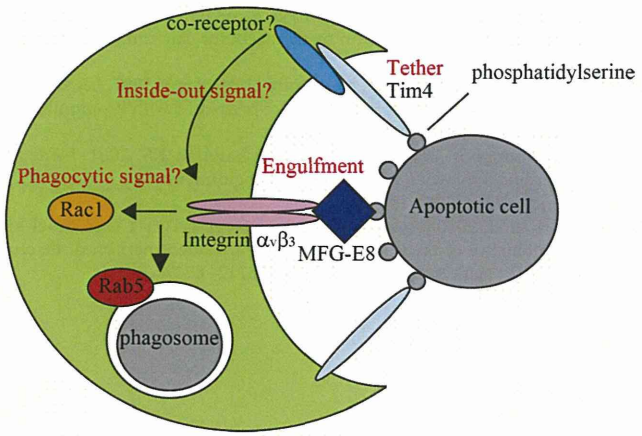

FIG 6 The tether-and-uptake model for the engulfment of apoptotic cells. In the tethering step, Tim4 catches apoptotic cells by recognizing phosphatidylserine. A putative coreceptor that associates with Tim4 then activates the integrin $\alpha_v\beta_3$ complex to initiate uptake, in which MFG-E8 binds to phosphatidylserine on apoptotic cells and to the activated integrin $\alpha_v\beta_3$ complex on the phagocytes. The signal from the integrin $\alpha_v\beta_3$ complex then activates the macrophages' internalization of apoptotic cells into lysosomes. This final step is mediated by Rac1, Rab5, and other, as-yet-unidentified molecules.

cated that the role of integrin $\alpha_v\beta_3$/MFG-E8 is different from that of Tim4. Our experiments indicated that apoptotic cells are recruited by Tim4 and then passed to the integrin $\alpha_v\beta_3$/MFG-E8 complex for uptake (Fig. 6). This mechanism agrees with the tether-tickle model for the engulfment of apoptotic cells, proposed by Hoffmann et al. (8). A similar two-step engulfment, Tim4-dependent tethering and Ca^{2+}-dependent uptake, was demonstrated by mouse resident peritoneal macrophages, indicating that the engulfment system reconstituted with the Ba/F3 cell line well represents the engulfment that naturally takes place with macrophages.

Integrins are activated by an inside-out signaling pathway or can be artificially activated with manganese (9, 17). Accordingly, when the Ba/F3 cells expressing integrin $\alpha_v\beta_3$ were treated with 0.5 mM manganese, they engulfed apoptotic cells in the presence of MFG-E8 (S. Toda, R. Hanayama, and S. Nagata, unpublished observation), suggesting that integrin $\alpha_v\beta_3$ is activated in Ba/F3/Tim4/integrin $\alpha_v\beta_3$ cells by inside-out signaling during the engulfment of apoptotic cells. Since Tim4 does not directly associate with the integrin complex (Toda et al., unpublished), it is possible that there is another unidentified factor that links Tim4 with the integrin $\alpha_v\beta_3$/MFG-E8 system (Fig. 6).

The tingible-body macrophages in the spleen express MFG-E8, and MFG-E8$^{-/-}$ tingible-body macrophages do not engulf apoptotic cells (7). The strong association of apoptotic cells with these macrophages supports the idea that MFG-E8 functions at the uptake step, but not at the tethering step. Resident peritoneal macrophages express Tim4, but not MFG-E8, whereas thioglycolate-elicited peritoneal macrophages express MFG-E8, but not Tim4 (15). Yet, these resident and thioglycolate-elicited peritoneal macrophages efficiently engulf apoptotic cells, suggesting that some factor other than MFG-E8 is working in the uptake step in the resident macrophages and some factor besides Tim4 is working in the tethering step in the thioglycolate-elicited macrophages. Recently, the engulfment of apoptotic cells by macrophages has been recognized to be as complicated as neurotransmission or lymphocyte activation (5, 12). A synapse-like structure consisting of many molecules called "engulfment synapse" has been proposed. The engulfment system established here, using Ba/F3 cells, will help in classifying these molecules into the tethering and uptake steps and also in identifying new factors involved in these processes.

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