厚生労働科学研究費補助金
エイズ対策研究事業

新作用機序の抗 HIV-1 薬剤の開発に関する研究

平成１８年度　総括研究報告書

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研究要旨　HIV-1 アクセサリー蛋白 Nef が ERK の活性を変化させることにより M-CSF の受容体経路を阻害すること、ゴルジ体における M-CSF 受容体の成熟を障害するという新しい事実を見出した。また、Nef 蛋白を標的とした抗 HIV-1 薬のスクリーニングを行い、いくつかの候補物質を得た。免疫不全マウスにヒト臍帯血を移植することによりヒトのＴ細胞が構築し、HIV-1 感染可能な可能な系を確立した。

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A. 研究目的

エイズの原因ウィルスであるヒト免疫不全ウィルス(HIV-1)は、年々その感染者が増加している。近年 HAART 療法のような有効な薬剤治療法が開発され、HIV-1 感染者の治療状況は大幅に改善された。しかし、現在の治療法では HIV-1 の完全な排除は不可能であり、薬剤の長期服用による副作用や薬剤耐性の出現など多くの問題が生じている。そのため、新たな作用機序を持つ薬剤開発は厚生労働行政上急務である。

HIV-1 アクセサリー蛋白 Nef はエイズ発症の病原性因子として良く知られている。近年、Nef と宿主細胞内チロシンリン酸化酵素 Hck との会合が発症に大きな役割を果たす事が明らかにされた。Hck は単球・マクロファージ特異的酵素である事から、Nef が Hck との会合を通して単球・マクロファージに機能変化をもたらす事が発症の引き金と予想されるが、その分子機序は不明である。この分子機序を解明し、その作用点を選択的に標的とする薬剤を見出すことにより新たな作用機序を持つ抗 HIV-1 薬を開発し、薬剤耐性ウィルスの克服に貢献する事が本研究の大きな目的である。先ず、Nef により単球・マクロファージ内のシグナル伝達機構がどのように撹乱されるかを明らかにすると共に Nef を標的とした新規抗 HIV-1 薬の *in vitro* スクリーニング系を確立する。特に、Hck はサイトカインシグナルの下流に於いても重要な働きを果たす分子である事から、Nef-Hck 会合のサイトカイン受容体経路に及ぼす影響に焦点を当て研究を行う。同時に、ヒト造血系・免疫系を構築したマウスを作成して、HIV-1 を感染させることにより、エイズのモデルマウスを作製し、エイズの病態解明と新たな治療法の開発の基盤とすることを目的としている。特に、本マウスを用いた抗 HIV-1 薬の *in vivo* 評価系の確立は、スクリーニングで得られた化合物等の評価のみならず、今後の様々な機序の抗 HIV-1 薬開発に極めて有用な系になると考えられる。

B. 研究方法

HIV-1 Nef 蛋白がサイトカインシグナルに及ぼす影響を、特に、単球・マクロファージの増殖・分化・活性化を司る最も主要なサイトカインである M-CSF, GM-CSF, IL-4 に着目して解析を行った。その為に、これらサイトカインに依存性に増殖する 2 種のヒト白血病細胞株（TF-1-fms は M-CSF に、TF-1 は GM-CSF と IL-4 に依存）を用いた。これら細胞株にエストロゲン受容体と融合させた Nef 蛋白質を発現させて安定株を樹立した。合成エストロゲン 4-HT で

Nef 活性化を誘導する事で Nef のサイトカイン系に及ぼす影響を細胞増殖能の変化で解析した。尚、Nef の活性化は CD4 の発現低下が誘導される事で確認した。また、これら *in vitro* 培養系を用いて様々な化合物をスクリーニングした。具体的には、Nef 活性化により抑制される M-CSF/IL-4 依存性細胞増殖および増強される GM-CSF 依存性細胞増殖に影響するかを MTT 法により検討した。

また、293T 細胞に M-CSFR, Hck, Nef を遺伝子導入して、これらの遺伝子の相互作用を Western Blot 法と蛍光免疫染色法により解析した。

ヒト造血系・免疫系を構築したマウスを作製する為に、NOD/Scid/Jak-3 欠損マウスへヒト臍帯血由来造血幹細胞移植を行った。NOD/Scid/Jak-3 欠損マウスは、NOD/Scid マウスに Jak-3 欠損マウス（理化学研究所 RCAI 斉藤隆博士から供与）をバッククロスして作成した。純化したヒト臍帯血中 CD34 陽性細胞を 2.5 Gy 放射線照射したマウスへ移植し、経時的に末梢血や種々のマウス臓器中におけるヒト血液細胞の出現を Flow Cytometry 法等により解析した。また、ヒト末梢血及び臍帯血単核球を放射線照射した NOD/Scid/Jak-3 欠損マウスに移植し、ヒトの免疫細胞の構築が可能かどうかを検討した。更に作成されたマウスに HIV-1 実験室株(JRFL)の感染を試みた。

（倫理面への配慮）

ヒト由来試料（末梢血・臍帯血等）を用いた研究は、熊本大学大学院医学薬学研究部等倫理委員会の承認を受け、規則に従い実施している。また、免疫不全マウスの作成及び移植実験等の動物実験は、熊本大学本荘地区動物実験委員会の承認を得た上で「熊本大学動物実験指針」に従い実施している。

1) 研究対象者に対する人権擁護上の配慮

研究に用いる臍帯血・末梢血は、他の研究目的には使用しない。臍帯血・末梢血は匿名処理を行うため、個人情報が流出することはない。また、同意書に署名後も試料採取・使用までの期間に同意を撤回することを可能としている。

2) 研究方法による研究対象者に対する利益・不利益

本研究により、直接提供者が医学上の利益・不利益を得ることはない。

3) 危険性の排除

臍帯血は臍帯を切り離した後で、臍帯・胎盤に残った血液を採取するため、新生児と母体への影響はない。また、臍帯血の採取は母子共に安全な分娩のみに限るとし、臍帯血採取によって分娩時の危険性が増す可能性を排除している。

末梢血は、医師が問診した上で健康に問題ないと判断した場合に限り、医師が採血している。採血に伴う身体への危険性はありうるが、これは通常の診療行為を越えるものではない。一回の採取量は 10-100 ml であり、採血量は、本人の了解のもとに決定している。

4) インフォームドコンセントに係わる状況

臍帯血採取に関しては、協力医療機関の医療スタッフ（医師）が本研究の趣旨を説明し、臍帯血提供の同意を得られた方のみ同意書に署名していただいている。この際、説明を行った医療スタッフ名を明記し、同意書は協力医療機関において厳重に保管している。

末梢血採取に関しては、熊本大学エイズ学研究センターのスタッフ（医師）が直接本研究の趣旨を説明し、末梢血提供の同意を得られた方のみ同意書に署名していただいている。この際、説明を行った医師名を明記し、同意書はエイズ学研究センターにおいて厳重に保管している。

5) 実験動物に対する動物愛護上の配慮

動物実験は、「熊本大学動物実験指針」を遵守し、極力動物の苦痛軽減に配慮して行っている。動物実験における実験処置に対する倫理基準では、カテゴリーB（動物に対してほとんど不快感を与えないと思われる実験）レベルの実験であり、解析時には「動物の処分方法に関する指針」を遵守して、頸椎脱臼により安楽死させている。

C. 研究結果

本研究は、以下の３カ年計画で行った。①平成１６年度：HIV-1 Nef 蛋白による単球・マクロファージの機能攪乱の機序解明とヒトの免疫系を構築したマウスの確立、②平成１７年

度：Nef 特異的な機能阻害作用を有する低分子化合物のスクリーニングとエイズマウスモデルの確立、③平成１８年度：Nef 蛋白の機能阻害物質の同定とモデルマウスを用いた in vivo スクリーニングと毒性判定。

平成１６年度は、HIV-1 Nef 蛋白が非受容体型チロシンキナーゼ Hck と会合することにより M-CSF 受容体のシグナル伝達を阻害することを見いだした。この結果を基に Nef 蛋白の機能阻害物質のスクリーニング系を開発した。また、様々な免疫不全マウスにヒト臍帯血・末梢血由来の単核球や造血幹細胞を移植することにより、ヒトの免疫系を構築したマウスを作製した。平成１７年度は、Nef 蛋白の機能阻害物質のスクリーニングを行い、いくつかの候補化合物を得た。また、免疫不全マウスにヒト NK 細胞と T 細胞が特異的に生着する系を開発した。

平成１８年度は、①Nef 蛋白の単球・マクロファージ機能阻害機構の更なる解析②Nef 蛋白による M-CSF 受容体成熟障害機構の解析、③Nef 蛋白の機能阻害物質のスクリーニングの継続、④ヒトの免疫系を構築したマウスの作製と HIV-1 感染実験、を行い、以下のような結果を得た。

1) HIV-1 Nef 蛋白による単球・マクロファージの機能攪乱機序の解明

Nef 活性化により M-CSF 依存性細胞増殖と IL-4 依存性細胞増殖が抑制された。一方、GM-CSF 依存性増殖はむしろ増強したことから Nef は、サイトカイン選択的に攪乱を誘導することが示唆された。更に、SH3 領域特異的阻害剤がこれらの Nef の作用を解除する傾向を示した。平成１７年度に Nef による M-CSF 受容体経路の阻害は、Nef が Hck を活性化し Hck と M-CSF 受容体の非生理的な会合をもたらす為であることを明らかにしたが(Blood, 2005)、更に Nef が ERK 活性化を介した M-CSF 受容体シグナル伝達経路も攪乱するために、単球・マクロファージ系の増殖と分化を修飾することを見出した（J Cell Physiol, 印刷中)。

2) Nef 蛋白による M-CSF 受容体蛋白成熟障害機構の解析

293T 細胞に M-CSFR, Nef, Hck を遺伝子導入することにより、Nef 蛋白の存在下では、M-CSF 受容体が未成熟なままでゴルジ体に留まることを証明した。このため、細胞表面の M-CSF 受容体量が減少することも、HIV-1 感染によるマクロファージの機能障害の一因となることが示唆された（論文投稿中)。

3) HIV-1 Nef 蛋白を標的とした新たな抗 HIV-1 薬のスクリーニング

Nef-Hck の会合を阻害する新規作用機序の抗 HIV-1 薬のスクリーニングを行った。本法では、TF-1-*fms*-Nef-ER 細胞に候補となる薬剤を加えて培養し、4-HT を添加して Nef の活性化を誘導した際に、TF-1-*fms*-Nef-ER 細胞の増殖を阻害するか否かで判定する。本法により、①熊本大学薬学部より供与された生薬を含む様々な化合物、②既にチロシンキナーゼ阻害作用等があると報告されている既知の化合物、③低分子化合物ランダムライブラリー（２万種、国立感染症研究所、駒野淳博士、武部豊博士より供与)、のスクリーニングを行った。その結果、熊本大学薬学部より供与された植物由来の物質、既知のチロシンキナーゼ阻害作用のある物質に Nef の機能阻害作用があることを見出した。また、低分子化合物ライブラリーは、1.6 万種までスクリーニングが終了し、３種類の候補物質を得た。更に、GST 結合 Nef 蛋白を用いた二次スクリーニング系により、このうちの一部の物質は、Nef 蛋白と Hck の結合を阻害することが判明している。

4) ヒトの免疫系を構築したマウスとエイズモデルマウスの作製

臍帯血単核球を移植した NOD/Scid/Jak-3 欠損マウスでは、移植後２－６週に渡りヒト T 細胞の構築が認められた。一部に CD4 が発現した活性化 CD8 陽性 T 細胞が認められた。HIV-1 実験室株 JRFL を感染させたところ、感染２週後にマウス末梢血において ELISA 法により p24 が同定されたことから、マウス体内で HIV-1 の感染・増殖が起きていることが示唆された。また、本マウスにおいて、活性化 CD8 陽性 T 細胞の一部に CD4 が発現し、HIV-1 が感染することを見出した（投稿準備中)。

一方、臍帯血由来 CD34 陽性ヒト造血幹細胞を移植した NOD/Scid/Jak-3 欠損マウスでは、移植後２０週以上に渡りヒト由来の細胞による造血系の構築が確認された。マウス骨髄と脾臓において、ヒト由来のＢ細胞と CD14 陽性の単球系細胞の安定的な生着が確認された。更に、NOD/Scid/Jak-3 欠損マウスにおいては、移植後

８週目からＴ細胞と樹状細胞の出現も確認され、その割合は週令が進むにつれて増加した。また、本マウスにおいてHIV-1の感染成立が確認された。

D. 考察

Nef 蛋白が存在することにより、M-CSF 受容体シグナル伝達経路においてERKの活性化を修飾することにより、単球・マクロファージ系の増殖・分化に影響することを示した。また、Nef 蛋白は、ゴルジ体におけるM-CSF受容体の成熟を障害するという新たな知見を得た。これらの結果は、Nef がサイトカイン系を多段階で制御する事で、単球・マクロファージに機能的な偏りを生じさせ、その事がエイズ発症に繋がる一因である可能性を示唆する。

また、新規作用機序の抗HIV-1薬剤スクリーニングのための、極めて簡便で且つ多検体をスクリーング出来る *in vitro* 培養系を用いて様々な物質のスクリーニングを行った。本系は細胞を用いたバイオアッセイであり、細胞毒性のある物質はスクリーニングにより除外されるため、薬剤として不適格な物質は予め除かれるというメリットがある。現在までに、数種類の候補物質が得られており、その一部では、実際にNefとHckの結合を阻害していることを確認している。これらの物質は、その分子機構にフィットした抗HIV-1薬の開発に役立つものと考えられる。

また、様々な免疫不全マウスにヒト血液細胞を移植することにより、HIV-1が感染するモデルマウス系を確立することができた。そして、これらのマウスを用いて記憶CD4陽性T細胞や活性化CD8陽性T細胞の一部にHIV-1が感染することを示した。これらのマウスは、上記研究で得られた研究成果を *in vivo* の系で確認するのみでなく、今後薬剤やワクチン等の治療法の開発やHIV-1の病態解明に非常に有用なツールとなりうると考えられる。

E. 結論

HIV-1 Nef 蛋白が単球・マクロファージ系の機能を阻害する機序として、①ERK の活性化を変化させる、②ゴルジ体における M-CSF 受容体の成熟を障害するという新たな２つの機序を見出した。また、化合物のスクリーニングにより Nef 蛋白を分子標的とした薬剤開発のリードとなりうる候補物質を得た。

我々が見出した Nef によるサイトカインシグナル伝達の撹乱作用は、新しい知見であり、薬剤開発に最も適した分子標的のひとつであると考えられた。今後、より詳細な作用機構の解明を進めると同時に、薬剤のスクリーニングを継続する。一方、ヒトの免疫系を構築したマウス系を確立し、HIV-1 の感染を確認できた。これらの *in vitro*、 *in vivo* の系を有機的に駆使することにより、Nef をターゲットとした新規薬剤の開発とその評価系の確立を目指す。

F. 健康危機情報

該当なし

G. 研究発表

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H. 知的所有権の出願・取得状況（予定を含む）

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抗 HIV-1 薬剤のスクリーニング系及びスクリーニング方法

研究成果の刊行に関する一覧表

書籍

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雑誌

発表者名	論文タイトル	発表誌名	巻号	ページ	出版年
Ohsugi T, Kumadaka T, Okada S, and Urano T	HTLV-1 Tax promotes oncogenesis not only in immature T cells but also mature T cells.	***Nature Medicine***		印刷中	2007
Suzu S, Hiyoshi M, Yoshidomi Y, Harada H, Takeya M, Kimura F, Motoyoshi K, and Okada S	M-CSF-mediated macrophage differentiation but not proliferation is correlated with increased and prolonged ERK activation.	***J Cell Physiol***		印刷中	2007

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M-CSF-Mediated Macrophage Differentiation but not Proliferation Is Correlated with Increased and Prolonged ERK Activation

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M-CSF is a cytokine essential for both the proliferation and differentiation of monocytes/macrophages. In this study, we established a new M-CSF-mediated differentiation-inducing system, and examined how the level and duration of the activation of ERK preceded M-CSF-mediated differentiation. TF-1-fms human leukemia cells rapidly proliferated in response to M-CSF. However, in the presence of a phorbol ester, TPA, TF-1-fms cells definitely switched their responsiveness to M-CSF from proliferation to differentiation, as evidenced by a more drastic morphological change and the appearance of cells with a higher level of phagocytic activity. In TF-1-fms cells expressing HIV-1 Nef protein in a conditionally active-manner, both M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation were inhibited by the activation of Nef. The Nef-active cells showed perturbed patterns of ERK activation. Under the proliferation-inducing conditions (TPA-free), parental or Nef-inactive cells showed modest ERK activation following M-CSF stimulation, whereas Nef-active cells showed an earlier and transient ERK activation, despite a decrease in their proliferation rate. Under the differentiation-inducing conditions, parental or Nef-inactive cells showed increased and prolonged ERK activation following M-CSF stimulation, whereas Nef-active cells showed transient ERK activation. These results supported the idea that the increased and prolonged ERK activation led to M-CSF-mediated macrophage differentiation but not to proliferation.

J. Cell. Physiol. 9999: 1–7, 2007.

Macrophage colony-stimulating factor (M-CSF) is a cytokine that supports both proliferation and differentiation of the cells of monocytic lineage (Roth and Stanley, 1992). The biological effects of M-CSF are mediated by a receptor tyrosine kinase, Fms (Sherr et al., 1985). The binding of M-CSF leads to the autophosphorylation of tyrosine residues in the cytoplasmic domain of Fms and subsequent interactions of the phosphorylated residues with other proteins, resulting in the initiation of multiple pathways (Bourette and Rohrschneider, 2000). Thus, to investigate which pathway leads to the proliferation or differentiation, mutant Fms proteins in which the tyrosine residues are substituted with phenylalanine have been generated and expressed in various cell types, such as NIH3T3 fibroblasts (Roussel et al., 1990), Rat-2 fibroblasts (van der Geer and Hunter, 1993), FDC-P1 myeloid progenitor cells (Bourette et al., 1995) and M1 myeloid cells (Marks et al., 1999). However, because the pathway that is predominantly utilized and whether cells proliferate or differentiate in response to M-CSF depend on the cell type, the exact differences in the signaling events between these distinct cellular responses to M-CSF are still unclear. Culture systems in which the same cells distinctly respond to different stimuli (proliferation versus differentiation) are useful for clarifying this issue. For example, rat neuronal PC12 cells proliferate in response to EGF, whereas stimulation with NGF causes neuronal differentiation (Marshall, 1995). Of importance, studies with this culture system have revealed that the increased and prolonged activation of extracellular signal-regulated kinase (ERK) is critical for the neuronal differentiation of PC12 cells, but not for proliferation (Marshall, 1995). It has been also shown that the increased and prolonged activation of ERK is critical for the differentiation of megakaryocytes (Melemed et al., 1997; Racke et al., 1997) and muscle cells (Gredinger et al., 1998). Furthermore, the functional maturation of macrophages induced by lipopolysaccharide seemed to require increased and prolonged ERK activation (Valledor et al., 2000). However, the role of ERK activation in M-CSF-mediated macrophage differentiation is not fully understood.

In this study, we first attempted to establish a new M-CSF-mediated differentiation-inducing system, using human leukemia TF-1-fms cells which essentially showed a proliferative response to M-CSF (Suzu et al., 1997). We assessed whether 12-*O*-tetradecanoylphorbol 13-acetate (TPA) triggered their differentiation and M-CSF accelerated the process. TPA has been characterized by its ability to induce the differentiation of several leukemia cell lines (Kitamura et al., 1989; Racke et al.,

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1997; He et al., 1999). We next attempted to clarify whether the increased and prolonged activation of ERK preceded M-CSF-mediated differentiation, by utilizing TF-1-fms cells expressing a conditionally active HIV-1 Nef protein (Suzu et al., 2005). Previously, we showed that Nef activation inhibited the M-CSF-mediated proliferation of TF-1-fms cells but enhanced the activation of ERK following M-CSF treatment (Suzu et al., 2005). Nef is a major determinant of the pathogenicity of HIV-1 (Fackler and Baur, 2002; Peterlin and Trono, 2003; Qiao et al., 2006), and has been shown to bind to and activate Hck, a Src kinase (Saksela et al., 1995; Moarefi et al., 1997). The activation of Hck was one possible molecular mechanism by which Nef caused the inhibition of M-CSF-mediated cell proliferation (Suzu et al., 2005) and the enhancement of ERK activation (Schrager et al., 2002; He et al., 2004). Based on these findings, we carefully examined how Nef affected the M-CSF/TPA-mediated differentiation of TF-1-fms cells and the level/duration of ERK activation in the differentiation-inducing conditions.

Materials and Methods

Cell culture and reagents

TF-1 cells (Kitamura et al., 1989) were routinely cultured with RPMI 1640 medium (Sigma, St. Louis, MO) — 10% fetal calf serum (FCS) in the presence of recombinant human granulocyte/macrophage-CSF (GM-CSF) (2 ng/ml; PeproTech, London, UK). TF-1-fms cells (Suzu et al., 1997) were maintained with RPMI 1640 — 10% FCS in the presence of M-CSF (100 ng/ml; a gift from Morinaga Milk Industry, Kanagawa, Japan) and G418 (200 μg/ml; Life Technologies, Grand Island, NY). TF-1-fms cells expressing the HIV-1 Nef-murine estrogen receptor hormone-binding domain (Nef-ER) fusion protein (TF-1-fms-Nef-ER) (Suzu et al., 2005) were maintained with RPMI 1640 — 10% FCS containing M-CSF, G418, and puromycin (1.5 μg/ml; Sigma). In this system, Nef was basally inactive but its function was inducibly activated by the estrogen analogue, 4-hydroxytamoxifen (4-HT; Sigma) (Suzu et al., 2005). In this study, we also established TF-1 cells expressing the Nef-ER fusion protein with the plasmid pEBB-Nef-ER-IRES-puro (Walk et al., 2005). TF-1-Nef-ER cells were maintained with RPMI 1640 — 10% FCS containing GM-CSF and puromycin. To activate Nef, 4-HT was added to the culture at a final concentration of 1 μM. TPA (Sigma) was added to the culture at a final concentration of 100 ng/ml. PD98059 (ERK kinase inhibitor) and PP2 (Src kinase inhibitor) were purchased from Sigma. U0126 was purchased from Calbiochem (San Diego, CA).

Cell count and viability analysis

The viable cell counts were obtained by enumerating the cells that excluded trypan blue dye on a hemocytometer. The adherent cells were harvested by trypsinization. The viability of cells was also examined with the propidium iodide (PI) exclusion method (Okada et al., 1998). Cells were suspended in phosphate-buffered saline (PBS) containing 0.1% NaN_3, 3% FCS and 2 μg/ml PI. The uptake of PI in each cell was analyzed with a FACSCalibur using Cell Quest Software (Becton Dickinson, Mountain View, CA).

Analyses of expression of Fms and CD204, and phagocytic activity

The cell surface expression of Fms was analyzed by flow cytometry using Flag-tagged M-CSF (Suzu et al., 2005). In brief, cells were incubated with the Flag-tagged M-CSF followed by biotin-labeled anti-Flag M2 antibody (Sigma) and phycoerythrin (PE)-labeled streptavidin (PharMingen, San Jose, CA). The analyses were performed with a FACSCalibur. The cell surface expression of CD204 was determined using anti-CD204-FITC (clone E-5) (Tomokiyo et al., 2002). The phagocytic activity was determined by measuring the uptake of fluorescent microspheres (Fluoresbrite Carboxylate Microspheres, 0.7 μm in diameter, Polysciences, Warrington, PA). Cells cultured on a 6-well tissue culture plate were incubated with the fluorescent microspheres for 5 h and washed with PBS. The cells showing phagocytized particles were analyzed by flow cytometry.

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed essentially as described previously (Suzu et al., 2000, 2005). Cells were depleted of M-CSF for 14 h in RPMI 1640 — 10% FCS with or without TPA, and then stimulated with M-CSF for the indicated periods. In selected experiments, 4-HT was added to the culture at the initiation of M-CSF deprivation/TPA pre-treatment. Then, the cells were solubilized with the Nonidet P-40 lysis buffer. The immunoprecipitation was performed with anti-phosphotyrosine mouse IgG conjugated to agarose (PY99; Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies (purchased from Santa Cruz) used for Western blotting were as follows: anti-Fms (C-20), anti-phosphotyrosine (PY99), anti-ERK (K-23), and anti-phosphorylated ERK (E-4). The rabbit antiserum to Nef was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH[Q1]). The detection was performed using the ECL system (Amersham, Buckinghamshire, UK). The relative intensity of bands on scanned gel images was quantified by using the NIH Image software.

Results

Proliferative- and differentiative properties of TF-1-fms cells

TF-1-fms cells were derived from TF-1 cells by introducing the wild-type *c-fms* gene into the parental cells (Fig. 1A). The proliferation of TF-1 and TF-1-fms was entirely dependent on GM-CSF and M-CSF, respectively (Fig. 1B) (Kitamura et al., 1989; Suzu et al., 1997). TF-1-fms cells lost their responsiveness to GM-CSF, due to a loss of the expression of the GM-CSF receptor α chain (data not shown). In the presence of M-CSF, TF-1-fms cells neither adhered to dishes (Fig. 1C) nor showed a differentiated morphology (Fig. 1D, top part). On the other hand, when cultured with media containing TPA alone, the cells tended to adhere to dishes and show a flattened morphology (Figs. 1C and D, middle part). Of note, M-CSF accelerated the differentiation-like process. Most TF-1-fms cells cultured in the presence of both M-CSF and TPA adhered to dishes and showed a mature macrophage-like morphology (Figs. 1C and D, bottom part), which closely resembled the primary macrophages obtained by culturing human peripheral blood monocytes with M-CSF (Hashimoto et al., 1999). In parallel with the morphological change, these cells showed more of an increase in granularity than cells cultured with TPA alone (Fig. 1E). More importantly, these cells showed higher phagocytic activity: the percentage of cells phagocytizing the microbeads and their mean fluorescence intensity (MFI) were higher in the culture containing M-CSF and TPA than in the culture containing TPA alone (Fig. 1F, left part). In addition, these cells showed a higher level of CD204 (class A macrophage scavenger receptor) (Fig. 1F, right part). These results indicated that the cells obtained by culturing TF-1-fms cells with both M-CSF and TPA were functionally mature macrophages.
As mentioned above and shown in the upper panel of Figure 2A, the culture of TF-1-fms cells with TPA resulted in the appearance of adherent cells and the addition of M-CSF further increased the number of adherent cells. However, there was no significant difference in total number of viable cells between the two treatments (TPA alone versus M-CSF + TPA) (Fig. 2A, lower part). Moreover, there was no significant difference in the percentage of PI-positive dead cells between the two treatments (Fig. 2B), excluding the possibility that the acceleration of TF-1-fms cell differentiation by M-CSF and TPA reflected a survival-enhancing or anti-apoptotic function of M-CSF. It was therefore likely that TF-1-fms cells definitely switched their responsiveness to M-CSF from proliferation to macrophage differentiation in the presence of TPA.

Requirement of ERK activation for M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation of TF-1-fms cells

We next examined whether the activation of ERK was required for M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation of TF-1-fms cells, using pharmacological

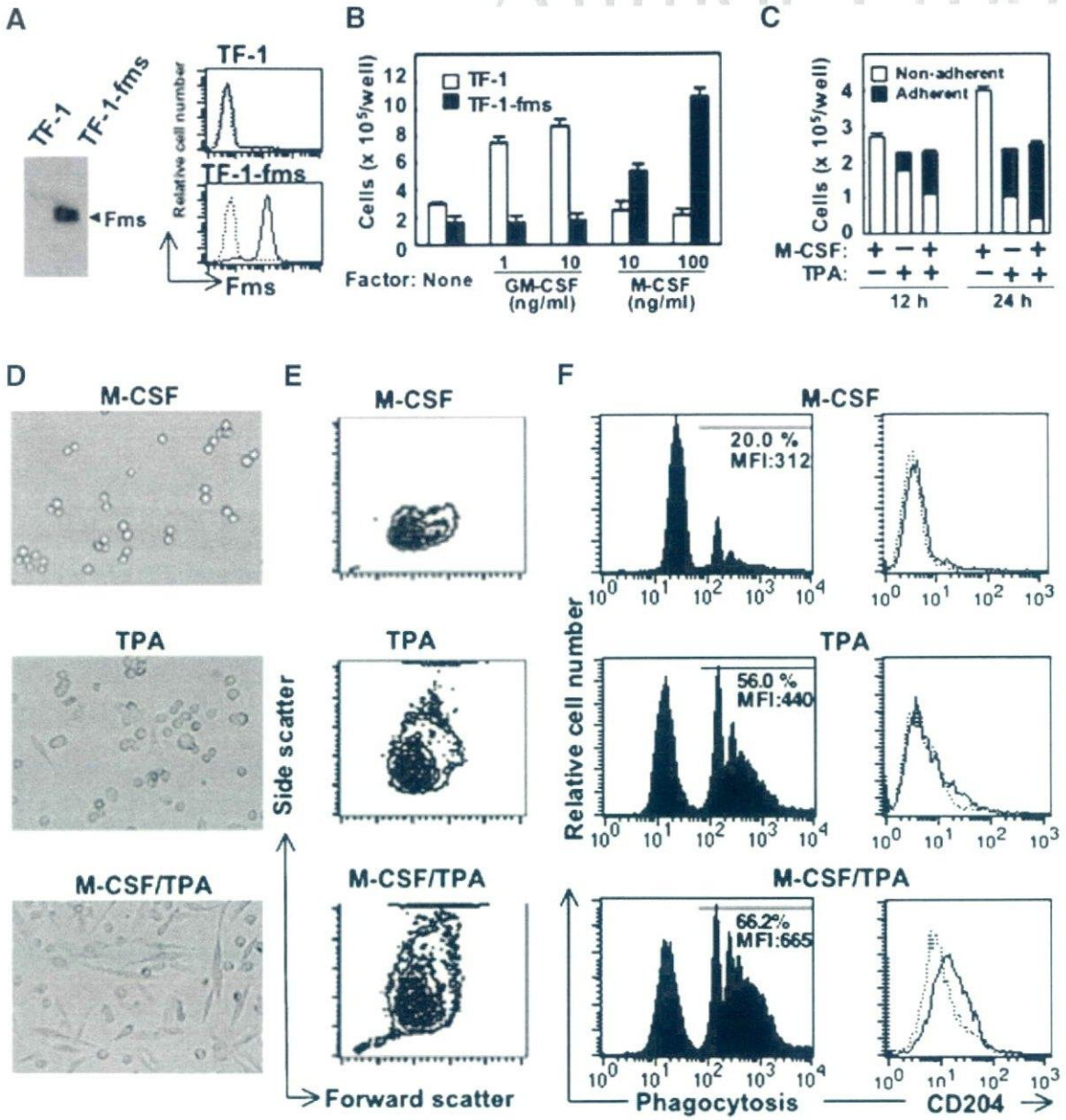

Fig. 1. The expression of Fms in and proliferative and differentiative properties of TF-1-fms cells. A: The total cell lysates from TF-1 or TF-1-fms cells were analyzed for the expression of Fms by Western blotting. Alternatively, the level of cell surface Fms expression was analyzed by flow cytometry with Flag-tagged M-CSF (solid lines). The profiles of cells incubated with a Flag-tagged protein, which is unrelated to M-CSF, are also shown as a control (broken lines). B: TF-1 or TF-1-fms cells were seeded into 6-well culture plates at a density of 5×10^4 cells/ml in the absence or presence of the indicated concentrations of cytokines. TF-1 and TF-1-fms cells were cultured for 3 and 2 days, respectively. After the cultures, viable cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. C: TF-1-fms cells were seeded at a density of 1×10^5 cells/ml, and cultured in the presence of M-CSF, TPA, or both. After culturing for 12 or 24 h, cells adhering to the dishes and non-adherent cells were enumerated. D–F: TF-1-fms cells were seeded at a density of 1×10^5 cells/ml, and cultured for 2 days in the presence of M-CSF (M-CSF), TPA (TPA), or both (M-CSF/TPA). D: The morphology of cells after culturing is shown. The cells cultured with M-CSF alone were photographed after a 5-fold dilution with media. E: The results of flow cytometric analyses of cells for forward and side scatters are shown. An equal number of cells were analyzed and the results are presented as counter plots. F: The phagocytic activity of cells was determined by the procedures described in the Materials and Methods (left panels). The percentage and mean fluorescence intensity (MFI) of cells in the region indicated by solid lines are shown. Alternatively, the cells were analyzed for the expression of CD204 by flow cytometry (right parts).

inhibitors. As shown, PP2 (the inhibitor specific for Src kinases), PD98059 (the inhibitor specific for ERK kinase, MEK), and U0126 (another MEK inhibitor) significantly reduced the rate of M-CSF-mediated proliferation (Fig. 3A). The reduced proliferation rate correlated well with the increase in the percentage of PI-positive dead cells (Fig. 3A). Thus, we could not exclude the possibility that the inhibitory effect of these inhibitors on M-CSF-mediated proliferation reflected their cytotoxicity. Of importance, however, these inhibitors, in particular U0126, significantly inhibited the differentiation of

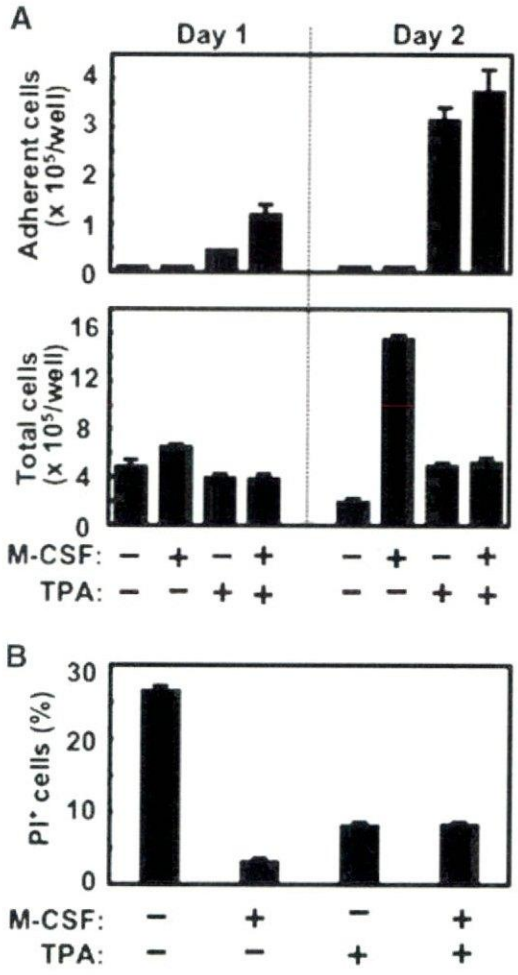

Fig. 2. A comparison of cell viability/death between the culture with TPA alone and that with M-CSF plus TPA. A and B: TF-1-fms cells were seeded into 6-well culture plates at a density of 1×10^5 cells/ml. Then, the cells were cultured in the absence of additives, or the presence of M-CSF, TPA, or both. A: After culturing for 1 or 2 days, cells adhering to the dishes (upper part) and all viable cells (lower part) were enumerated. B: After culturing for 2 days, the percentage of PI-positive dead cells in the wells was determined by flow cytometry. Error bars from triplicate assays are shown. These results are representative of two independent experiments.

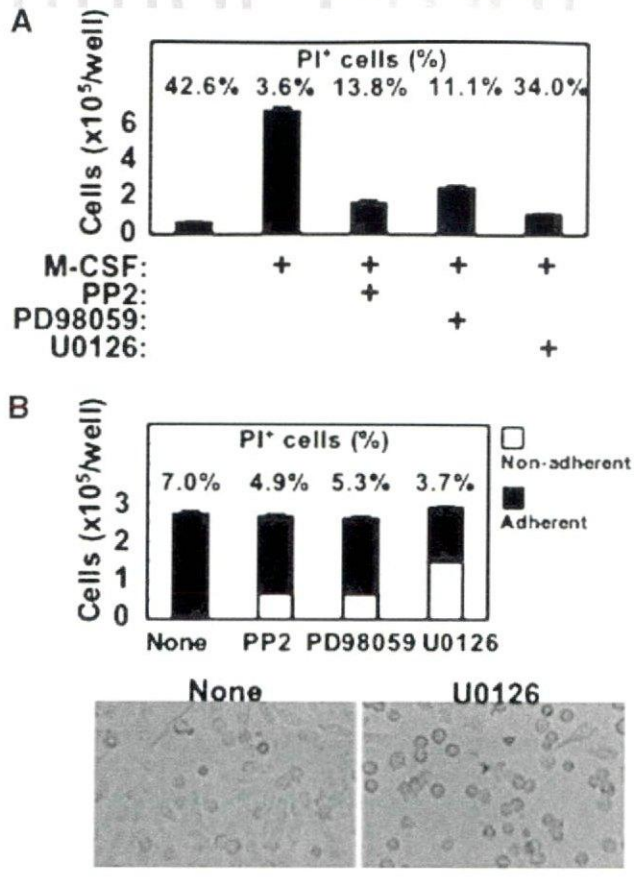

Fig. 3. Effects of pharmacological inhibitors on the proliferation and differentiation of TF-1-fms cells. A: TF-1-fms cells were seeded into 6-well culture plates at a density of 5×10^4 cells/ml in the absence or presence of the indicated inhibitors. M-CSF was added at a final concentration of 100 ng/ml. Both PP2 and U0126 were added at a final concentration of 10 μM. PD98059 was used at a final concentration of 50 μM. After culturing for 2 days, viable cells were enumerated. Error bars from triplicate assays are shown. Simultaneously, the percentage of PI-positive dead cells was determined by flow cytometry. These results are representative of two independent experiments. B: TF-1-fms cells were suspended at 1×10^5 cells/ml in medium containing M-CSF and TPA, and then cultured for 1 day in the absence or presence of the indicated inhibitors. The cells adhering to the dishes and non-adherent cells were enumerated. Error bars from triplicate assays are shown. Simultaneously, the percentage of PI-positive dead cells was determined by flow cytometry. These results are representative of two independent experiments. The morphology of cells after culturing is shown.

TF-1-fms cells induced by M-CSF and TPA without increasing the percentage of PI-positive dead cells (Fig. 3B). These results indicated that the differentiation of TF-1-fms cells was dependent on the activation of ERK.

The level and duration of ERK activation in parental TF-1-fms cells

We next examined whether the increased and/or prolonged activation of ERK preceded the differentiation. We pretreated TF-1-fms cells with TPA or left them untreated, stimulated then with M-CSF or left them un-stimulated, and analyzed the activation of ERK by using an antibody specific for phosphorylated ERK (Fig. 4). Consistent with an earlier report (He et al., 1999), the treatment of TF-1-fms cells with TPA led to the ERK activation (Figs. 4A and B). Following M-CSF stimulation, a number of molecules were shown to be rapidly tyrosine-phosphorylated in both TPA-pretreated cells and untreated cells (Fig. 4A). The most prominent band at 150–160 kD, the tyrosine-phosphorylation level of which was reduced in the pretreated cells, seemed to be Fms (Suzu et al., 2005). The reduction of Fms phosphorylation in the pretreated cells was likely to be due to the reduced cell surface expression of Fms, because the pretreatment caused an increase in the intracellular form of Fms (gp130) and concomitant decrease in the cell surface form of Fms (gp150) (Fig. 4A), as seen in TPA-treated p388D1 macrophages (Wilhelmsen and van der Geer, 2004). Despite the reduced Fms phosphorylation, the level of ERK activation following M-CSF stimulation in TPA-pretreated cells was higher than that in untreated cells (Figs. 4A and B). Although the higher level of ERK activation following M-CSF stimulation seemed to be due to the high baseline level (Fig. 4A, the bar graph), the extent of the activation apparently correlated with the differentiation of TF-1-fms cells.

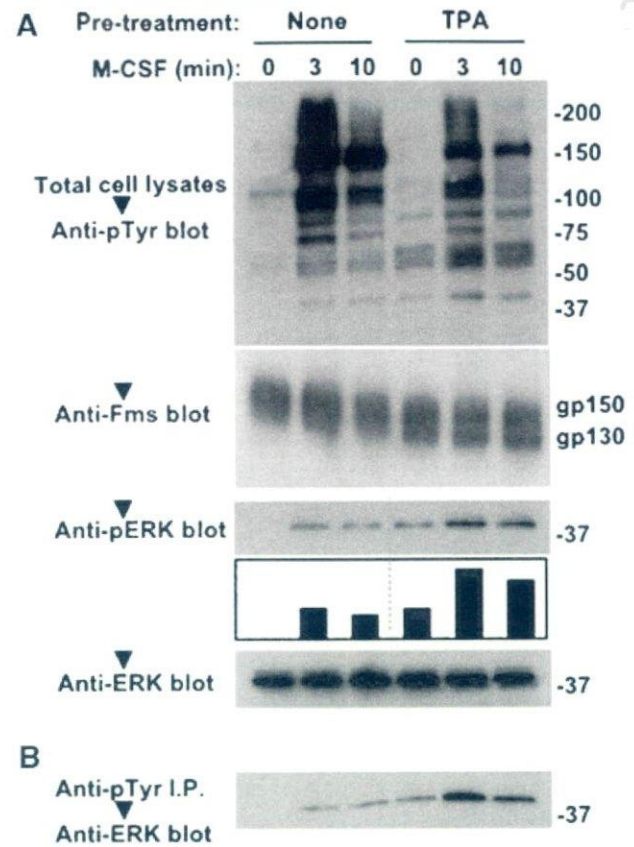

Fig. 4. The level of phosphorylation of ERK in TF-1-fms cells after pre-treatment with TPA and re-stimulation with M-CSF. TF-1-fms cells were deprived of M-CSF with or without TPA for 14 h and then re-stimulated with M-CSF for the indicated periods. A: Total cell lysates were analyzed with Western blotting using an antibody specific for phosphotyrosine (pTyr), Fms, phosphorylated ERK (pERK), or total ERK. The bottom blot was to show that comparable amounts of proteins were loaded in the upper blots. The relative intensities of bands on scanned gel images were quantified using the NIH Image software and the level of phosphorylated ERK normalized to the total amount of ERK is shown in the bar graph. B: The immunoprecipitates with anti-pTyr antibody were analyzed using anti-ERK antibody.

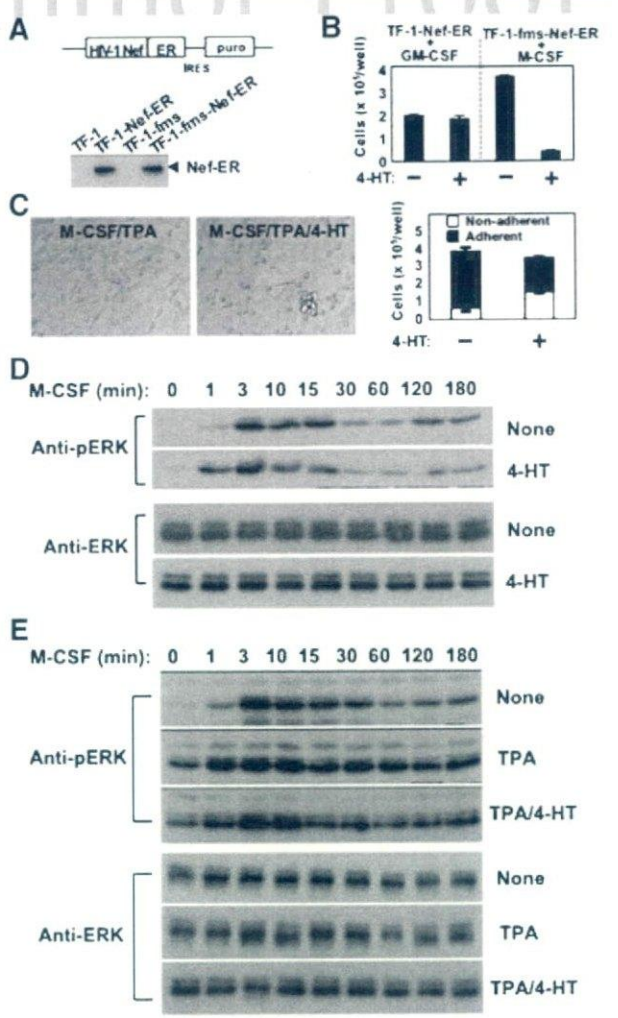

Fig. 5. Establishment of cells expressing the conditionally active Nef, proliferative/differentiative properties, and ERK activation. A: Schematic diagram of the Nef-ER-IRES-puro construct. ER, estrogen receptor hormone-binding domain; IRES, internal ribosomal entry sequence; puro, puromycin resistance gene. Total cell lysates from parental cells (TF-1 and TF-1-fms) or cells stably expressing Nef-ER (TF-1-Nef-ER and TF-1-fms-Nef-ER) were analyzed for the expression of Nef-ER by Western blotting with anti-Nef antibody. B: TF-1-Nef-ER cells were seeded at a density of 2×10^4 cells/ml and cultured in the presence of GM-CSF alone or GM-CSF plus 4-HT for 4 days. Similarly, TF-1-fms-Nef-ER cells were seeded at a density of 1×10^4 cells/ml and cultured in the presence of M-CSF alone or M-CSF plus 4-HT for 4 days. After the cultures, viable cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. C: TF-1-fms-Nef-ER cells were suspended in medium containing M-CSF and TPA, and then cultured for 2 days in the absence (M-CSF/TPA) or the presence of 4-HT (M-CSF/TPA/4-HT). The cells adhering to the dishes and non-adherent cells were enumerated. Error bars from triplicate assays are shown. These results are representative of two independent experiments. D: TF-1-fms-Nef-ER cells were deprived of M-CSF for 14 h and then re-stimulated with M-CSF for the indicated periods. 4-HT was added to the culture at the beginning of the period of deprivation. Total cell lysates were prepared and analyzed by Western blotting using the antibody specific for phosphorylated ERK (upper part) or anti-total ERK antibody (lower part). E: As in D, TF-1-fms-Nef-ER cells were deprived of M-CSF for 14 h and then re-stimulated with M-CSF for the indicated periods. TPA or 4-HT was added to the culture at the beginning of the period of deprivation, as indicated. Total cell lysates were analyzed by Western blotting using the antibody specific for phosphorylated ERK (upper part) or anti-total ERK antibody (lower part). The result is representative of two independent experiments.

The proliferative/differentiative properties and the level/duration of ERK activation in TF-1-fms cells expressing conditionally active HIV-1 Nef

As mentioned above, we previously established TF-1-fms cells expressing the conditionally active HIV-1 Nef protein (the Nef-ER fusion protein) and showed that the activation of Nef by 4-HT caused the inhibition of the M-CSF-mediated proliferation of TF-1-fms cells (Figs. 5A and B; Suzu et al., 2005). By using the newly established TF-1 cells expressing the Nef-ER (Fig. 5A), we excluded the possibility that the inhibitory effect of Nef was due to non-specific cytotoxic activity of the viral protein: the activation of Nef did not affect GM-CSF-mediated proliferation of TF-1 cells (Fig. 5B). In this study, we found that Nef activation also caused the inhibition of the M-CSF/TPA-mediated differentiation of TF-1-fms cells (Fig. 5C). Consistent with its inhibitory effect on both M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation, Nef perturbed the patterns of ERK activation following the stimulation of TF-1-fms-Nef-ER cells with M-CSF (Figs. 5D and E). The untreated control cells

showed a biphasic ERK activation following M-CSF treatment. The early phase occurred between 1 and 3 min whereas the late phase started after 120 min (Fig. 5D). In contrast, the Nef-active cells whose proliferation rate was low showed an earlier but transient ERK activation following M-CSF stimulation (Fig. 5D). On the other hand, TPA-pretreated cells showed an increased and prolonged activation following M-CSF treatment when compared to the untreated control cells (Fig. 5E). Of importance, however, the Nef-active TPA-pretreated cells showed a transient ERK activation (Fig. 5E). These results suggested that the increased and prolonged activation of ERK correlated well with M-CSF/TPA-mediated differentiation, but not with M-CSF-mediated proliferation, of TF-1-fms cells.

Discussion

We established a new macrophage differentiation-inducing system that was dependent on M-CSF activity. TF-1-fms cells definitely switched their responsiveness to M-CSF from proliferation to differentiation in the presence of TPA (Fig. 1). Although the treatment with TPA alone triggered the macrophage differentiation of the cells, the presence of M-CSF enhanced the process: (1) the combination of both M-CSF and TPA caused more drastic morphological changes (Figs. 1C and D); (2) the culture in the presence of both M-CSF and TPA contained more adherent cells than that in the presence of TPA alone (Fig. 2A, upper part); (3) the phagocytic activity and the expression of CD204 were significantly higher in cells treated with M-CSF and TPA than those treated with TPA alone (Fig. 1E). The macrophage differentiation by M-CSF and TPA did not reflect the survival-enhancing/anti-apoptotic function of M-CSF (Fig. 2A, lower part and B).

The experiments with the pharmacological inhibitor U0126 showed that M-CSF/TPA-mediated differentiation of TF-1-fms was dependent on ERK activation (Fig. 3B). That the Src inhibitor PP2 was also a potent inhibitor of the responses (Fig. 3B) might reflect the finding that the activation of ERK by M-CSF was in part dependent on the activity of Src kinases (Cheng et al., 1999; McMahon et al., 2001). However, the involvement of the increased and/or prolonged ERK activation in M-CSF-mediated differentiation is somewhat controversial. Unlike murine M1 cells expressing wild-type Fms, the cells expressing Y559F mutant Fms showed an impaired differentiative response to M-CSF and a reduced level of ERK activation (McMahon et al., 2001). The enforced expression of a scaffolding protein, Gab2, in wild-type Fms-expressing FDC-P1 cells resulted in an acceleration of the differentiation process and increased ERK activation (Liu et al., 2001). In contrast, the enforced expression of an adapter protein, Mona, in wild-type Fms-expressing FDC-P1 cells resulted in increased and prolonged ERK activation, but not an acceleration of M-CSF-mediated differentiation (Bourgin et al., 2000). Moreover, 32D myeloid cells expressing the Y559F mutant Fms showed a "hyper-proliferative" response to M-CSF and prolonged ERK activation (Rohde et al., 2004). Our studies with parental and Nef-expressing TF-1-fms cells (Figs. 4 and 5) supported the former idea that increased and prolonged ERK activation led to M-CSF-mediated macrophage differentiation, but not to cell proliferation. The TPA-pretreated parental TF-1-fms cells (differentiative) showed increased and prolonged ERK activation following M-CSF stimulation (Fig. 4). In contrast, the Nef-active TPA-pretreated TF-1-fms (un-differentiative) showed transient ERK activation (Figs. 5C and E). The pharmacological agent GF109203X, a potent inhibitor of the expression of MAPK phosphatase-1 (Valledor et al., 1999), caused TF-1-fms cells to differentiate in the presence of M-CSF and the cells pretreated with GF109203X showed a sustained ERK activation following M-CSF treatment (data not shown), further supporting the idea.

The molecular mechanisms whereby Nef inhibited the M-CSF-mediated proliferation and M-CSF/TPA-mediated differentiation of TF-1-fms cells remained to be elucidated. In TF-1-fms cells, Nef activation induced the activation of Hck and its constitutive association with Fms (Suzu et al., 2005). The unphysiological behavior of Hck might explain the inhibitory effect of Nef on the responsiveness to M-CSF. Yet, under the proliferation-inducing conditions (TPA-free), the activation of Nef resulted in an earlier ERK activation (Fig. 5D). This might be explained by the finding that Nef induced ERK activation in $CD4^+$ T cells (Schrager et al., 2002) and podocytes (He et al., 2004) in a Src-dependent manner. Further experiments are required to understand the molecular mechanisms whereby Nef rapidly terminated ERK activation in the presence of TPA (Fig. 5E). In summary, we showed that M-CSF-mediated macrophage differentiation, but not proliferation was correlated with increased and prolonged ERK activation, by using a newly established macrophage-inducing system. The culture system with Nef-expressing TF-1-fms cells provides a useful tool for determining the temporal regulatory mechanism of ERK activation and its contribution to M-CSF-mediated proliferation/differentiation.

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