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新作用機序の抗 HIV-1 薬剤の開発に関する研究

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厚生労働科学省研究費補助金（エイズ対策研究事業）
総合研究報告書

新作用機序の抗HIV-1薬剤開発に関する研究

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研究要旨　HIV-1 アクセサリー蛋白 Nef がマクロファージのチロシンキナーゼ Hck と会合することにより M-CSF の受容体経路を阻害する事を見いだした。そして、Nef を標的とする新たな抗 HIV-1 薬のスクリーニング系を開発し、数種類の候補化合物を得た。また、Nef が ERK の活性を変化させることにより M-CSF の受容体シグナルを修飾すること、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。一方、新規高度免疫不全マウスを樹立し、本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。現在、感染者は全世界で４千万人を超え、近年インドや中国では著しく HIV-1 感染者が増加している。本邦においても HIV-1 感染者は１万人を突破し、その数は確実に増加している。近年、HAART 療法のような有効な薬剤治療法が開発され、HIV-1 感染者の生命予後は大幅に改善された。しかし、現在の HIV-1 の生活環阻害に基づいた治療法では 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きている。そのため、作用機序の異なる新たな抗 HIV-1 薬の開発は厚生労働行政上急務である。

HIV-1 は、いくつかのアクセサリー蛋白を有しており、これらのアクセサリー蛋白が、エイズの病態に深く関わっていることが知られている。特に、Nef 蛋白は、HIV-1 の複製能を高めるなどエイズの発症を助長する重要な病原因子である。そのため、Nef 蛋白を分子標的とした化合物は、新たな作用機序を持つ抗 HIV-1 薬として潜伏感染や薬剤耐性ウィルスへの効果が期待される。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ことが期待できる。

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、これらのサイトカイン依存性に増殖する 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4-HT投与によりCD4の発現低下が誘導される事で確認した。4-HTによるNefの活性化の影響をMTT法による細胞増殖の変化、様々な抗リン酸化抗体を用いたWestern Blot法により解析を行った。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。

Nef 活性化誘導型細胞株（TF-1-fms-Nef）がNef の活性化により増殖抑制をきたすことを指標に、新たなバイオアッセイ系を樹立した。本系では、添加薬剤によりNefの活性化が阻害されると、TF-1-fms-Nefの増殖抑制が解除されることを指標にしたバイオアッセイである。TF-1-fms-Nefを96穴プレートに撒き、4-HTと低分子物質を添加し、MTT 法により細胞増殖を計測し、4-HT単時投与と比べて細胞増殖が回復している化合物を候補薬剤とした。本法は、細胞株を用いたバイオアッセイであるため細胞毒性のある物質はスクリーニングにより最初から除外されるという長所がある。Nef蛋白は、その Proline-rich 領域と非受容体型チロシンキナーゼHckのSH3領域を介して結合する事が既に明らかになっている。そこで、GST 蛋白質と融合させたNef蛋白を用いたPull-down法による二次スクリーニング法を樹立し、スクリーニングで得られた候補物質がNefとHckの結合を阻害するか否かを解析した。

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１０世代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様々な免疫不全マウス（NOD/Scid マウス, NOD/Scid/β2micro 欠損マウス、NOD/Scid/Jak-3欠損マウス等）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, NL43等)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、規則に従い実施した。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」に従い実施した。

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た。

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、医師が採血した。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、毎回本人の了解のもとに決定した。

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た。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た。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た。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た。

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。

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 活性化の単球・マクロファージ系への影響を解析するために、4-HT 添加により Nef の活性化が誘導される細胞株（TF-1-fms-Nef-ER, TF-1-Nef-ER）を樹立した。結果 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。その分子機序として、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(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）。また、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。

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 A、既知のチロシンキナーゼ阻害作用のある物質 B 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、物質 B は、Nef 蛋白と Hck の結合を阻害することが判明した。物質 B は、HIV-1 Nef 蛋白を標的とした薬剤開発のリードとして期待される。

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

Jak-3欠損マウスとNOD/Scidマウスを１０世代交配して、NOD/Scid/Jak-3欠損マウスを樹立した。本マウスは、すべてのリンパ球（Tリンパ球、Bリンパ球、NK細胞、NKT細胞）が欠損し、補体活性、マクロファージ機能、樹状細胞機能が低下している重度の免疫不全マウスである。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。

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。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。本マウスにおいては、ヒトのナイーブT細胞から記憶T細胞までが同時に出現するが、HIV-1は、主にCD62L陰性CD45RA陰性の記憶CD4陽性T細胞に感染することが判明した。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）。

NOD/Scidマウス腹腔内にヒト腎癌細胞株GHINK-1と臍帯血単核球を同時に移植することにより、ヒトNK細胞が特異的に増殖する系を開発した（Eur J Immunol, 2005）。この系では、T細胞も同時に存在することからHIV-1とNK細胞の相互作用を解析するのに有用であると考えられる。

D. 考察

Nef-Hckの会合がHck-M-CSF受容体の恒常的会合という非生理的な変化をもたらす事で、M-CSF受容体の活性化を障害し、結果としてNefがM-CSFの生物活性を抑制する事を明らかにした(Blood, 2005)。また、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・分化を修飾することを示した（J Cell Physiol, in press）。更に、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。一方、単球・マクロファージに作用する主要なサイトカインであるGM-CSF受容体系にはNefは促進的に働き、IL-4受容体系には抑制的に働くことを示した。これらの結果は、Nefがサイトカイン系を異常制御する事で、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。今後、その機能的偏りを規定する分子群の同定がエイズ発症の新たな機構を明らかにすると予想される。また、Nef-Hck会合がM-CSF系のみを阻害するという特異性は、本研究計画で目指す選択性高い新規作用機序の抗HIV-1薬開発が可能とするものである。

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。本スクリーニング系により得られる候補物質は、その分子機構にフィットしたものであり、Nefを標的とした新作用機序の抗HIV-1薬の開発のリードとなることが期待される。

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、①短期間（４週間程度）ヒトT細胞が生着し、HIV-1が感染するマウス、②ヒトNK細胞が特異的に生着するマウス。③長期に渡りT細胞を含むヒト造血・免疫細胞が生着するマウス、を作製することができた。そしてこれらのマウス系でHIV-1の感染を確認することができた。本マウスは、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。NOD/Scid/Jak-3欠損マウスには、ヒトの造血・免疫系細胞のみならず様々

なヒト細胞・癌細胞が生着可能であり、癌研究・感染症研究など、厚生労働行政上重要な疾患研究への応用が期待できる。

HIV-1 Nef 蛋白を標的とした薬剤は、これまでに開発された薬剤とまったく作用機序が異なる抗 HIV-1 薬として、既存の薬剤との併用療法で効果が期待される。また、その作用機序から潜伏感染や薬剤耐性ウィルスに対する効果も期待できることから、その開発が急がれる。

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、①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、②ERK の活性化を修飾する、③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３つの知見得た。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、薬剤開発を推し進めることが必要である。一方、新規免疫不全マウスを樹立し、そのマウスを用いて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* の評価系は、今後の様々な新規薬剤の開発研究において重要な役割を果たすことが期待される。

F. 健康危機情報

該当なし

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発明者：岡田誠治、鈴 伸也

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

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HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and inhibits M-CSF bioactivities

Shinya Suzu, Hideki Harada, Takahiro Matsumoto, and Seiji Okada

HIV-1 Nef protein is a major determinant of the pathogenicity of the virus. It has been shown that Nef activates Hck, a member of Src family kinase, in monocytes/macrophages and that the interaction is critical for AIDS-like disease progression in a mouse model. However, it was unclear how the molecular interaction in monocytes/macrophages leads to disease progression. Here, we show for the first time that Nef interferes with the macrophage colony-stimulating factor (M-CSF)/M-CSF receptor signal pathway. In this study, we introduced a conditionally active Nef into myeloid leukemia TF-1–*fms* cells and analyzed their responsiveness to M-CSF. We found that Nef-activated Hck constitutively associated with the M-CSF receptor complex. The formation of the molecular complex should occur under physiologic conditions, that is, on M-CSF stimulation. Because of aberrant molecular association, the tyrosine-phosphorylation/activation of the receptor in response to M-CSF was markedly diminished in Nef-active cells. Consequently, Nef activation caused the inhibition of M-CSF–mediated proliferation of TF-1–*fms* cells and macrophage differentiation of the cells induced by M-CSF and 12-*O*-tetradecanoylphorbol 13-acetate. These results indicate that HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and thereby inhibits its M-CSF functions in monocytes/macrophages. (Blood. 2005;105:3230-3237)

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Introduction

Nef is an accessory protein of HIV-1, a causative virus for AIDS. A number of reports, including studies of HIV-1–infected patients and of animal models, have demonstrated that the Nef protein is a major determinant of the pathogenicity of HIV-1.[1-4] Transgenic mice expressing the complete coding sequences of HIV-1 under the regulatory sequences of human CD4 gene developed severe AIDS-like abnormalities: loss of $CD4^+$ T cells, thymus atrophy, failure to thrive, diarrhea, wasting, premature death, interstitial pneumonitis, and tubulo-interstitial nephritis.[4] Using this mouse model and the introduction of mutation into selected HIV-1 gene(s), Hanna et al[4] clearly demonstrated that Nef harbored a major disease determinant. Therefore, much attention has been given to Nef to explain its contribution to HIV-1 pathogenesis and to investigate it as a target for antiviral drug development.

$CD4^+$ T cells and monocytes/macrophages are the principal target cells for HIV-1, and the functions of Nef in $CD4^+$ T cells are generally accepted as accounting for many aspects of viral pathogenesis (reviewed in Fackler and Baur[5] and Peterlin and Trono[6]). For example, Nef has been shown to cause the down-regulation of cell surface molecules such as cell surface receptor CD4[7] and major histocompatibility complex (MHC) class I in $CD4^+$ T cells.[8] The down-regulation of MHC class I is considered to diminish the recognition of HIV-1–infected cells by cytotoxic T cells. In contrast, the contribution of monocytes/macrophages to viral pathogenesis is less well understood. Several lines of evidence support the idea that monocytes/macrophages and $CD4^+$ T cells are important for the development and progression of AIDS. Recent studies have demonstrated that Nef induces the production of CC chemokines (macrophage inflammatory proteins-1α and -1β) and soluble forms of CD23 and intracellular adhesion molecule-1 by macrophages.[9,10] These Nef-induced factors from macrophages might stimulate the chemotaxis and activation of resting $CD4^+$ T cells, thereby promoting the permissiveness of $CD4^+$ T cells to HIV-1 infection.[9,10] This Nef function is likely to be mediated by the activation of NF-κB transcription factor.[10]

Another important feature of Nef is the binding at high affinity to myeloid lineage–specific Src family kinase Hck to activate its kinase activity.[11,12] The proline-rich (PxxP) motif in Nef binds to the Src homology 3 (SH3) domain of Hck.[11] Interestingly, Hck was found to bind preferentially and with higher affinity to Nef than other Src kinases.[11] The pathologic relevance of the molecular interaction in vivo was revealed by studies of HIV-1 transgenic mice. The mutation in the SH3-binding motif of Nef abolished the development of AIDS-like disease in the HIV-1 transgenic mice.[13] Moreover, the breeding of transgenic mice expressing the complete coding sequences of HIV-1 on a $hck^{-/-}$ background resulted in the delay of disease development.[13] These studies suggest that the modulation of macrophage functions by Nef is important for disease development because the molecular interaction between Nef and Hck occurs in cells of macrophage lineage but not in $CD4^+$ T cells. However, it is unclear how the molecular interaction in monocytes/macrophages contributes to HIV-1 pathogenesis. To answer the question, it is necessary to identify intracellular signaling pathways that would be perturbed by the Nef-Hck interaction.

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Macrophage colony-stimulating factor (M-CSF) is a primary cytokine for monocytes/macrophages and regulates their development and various effector functions (reviewed in Roth and Stanley[14]). The importance of M-CSF in macrophage development in vivo has been clearly proven using a naturally occurring *osteopetrotic* (*op/op*) mouse.[15] The M-CSF biologic effects are mediated by a unique receptor encoded by the proto-oncogene *c-fms*, a receptor tyrosine kinase (reviewed in Bourette and Rohrschneider[16]). The binding of M-CSF induces the dimerization of the receptor and the activation of its kinase activity.[16] This leads to the autophosphorylation of specific tyrosine residues in the cytoplasmic domain and the subsequent interactions of the phosphorylated residues with other proteins, each initiating signaling along specific pathways.[16] The Src family is one of the well-documented partners for the activated M-CSF receptor. M-CSF stimulation results in the activation of Src kinases, including Hck; simultaneously, these kinases associate with the ligand-activated M-CSF receptor.[17-21] We and other investigators[17-21] have demonstrated that the activation of Src kinases is required for biologic effects of M-CSF such as mitogenic signal and differentiation-inducing signal. This finding prompted us to examine whether the molecular interaction between HIV-1 Nef and Hck would affect M-CSF receptor signaling and thereby modulate M-CSF bioactivities. In this paper, we demonstrate for the first time that Nef interferes with M-CSF-induced activation of its receptor, the earliest signaling event induced by the ligand, through Hck activation and thereby inhibits M-CSF functions on monocytes/macrophages.

Materials and methods

Cells and culture conditions

TF-1-*fms* cells were established by introducing the human M-CSF receptor (*c-fms*) gene into a human myeloid leukemia cell line, TF-1, as described previously.[22] Original TF-1 cells were growth factor–dependent cells,[23] and the proliferation of TF-1-*fms* cells was dependent on M-CSF.[22] In this study, we established a TF-1-*fms* clone expressing a fusion protein composed of Nef and murine estrogen receptor hormone–binding domain (Nef-ER) by introducing pEBB-Nef-ER-IRES-puro plasmid[24] using Effectene reagent (Qiagen, Valencia, CA). The *Nef* gene was derived from NL4-3 strain of HIV-1,[24] and its product has been shown to activate the kinase activity of Hck.[11,12] The transfection was performed according to the manufacturer's recommendations. Transfected cells were selected in medium containing 1.5 μg/mL puromycin (Sigma, St Louis, MO), followed by limiting dilution to establish stable cell lines. The stable TF-1-*fms* clones were analyzed for Nef-ER expression by Western blotting using anti-Nef rabbit antiserum.[25] We also established a TF-1-*fms* clone expressing enhanced green fluorescent protein (EGFP). This was achieved with a retrovirus vector (MSCV2.2/IRES-EGFP),[26] as described previously.[27] In brief, the amphotropic packaging PT67 cells (Clontech, Palo Alto, CA) were transfected with MSCV2.2/IRES-EGFP vector using LipofectAMINE2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Then, TF-1-*fms* cells were cocultured with the transfected PT67 cells in the presence of 8 μg/mL Polybrene (Sigma). The stable TF-1-*fms* clones were obtained by limiting dilution and were analyzed for EGFP expression by flow cytometry. Parental TF-1-*fms* cells and EGFP-expressing cells were maintained with RPMI 1640 (Sigma)–10% fetal calf serum (FCS) in the presence of recombinant human M-CSF (100 ng/mL)[22] and 200 μg/mL G418 (Life Technologies, Grand Island, NY). Nef-ER-expressing cells were maintained with RPMI 1640–10% FCS in the presence of M-CSF, G418, and puromycin. To activate the Nef-ER protein, an estrogen analog, 4-hydroxytamoxifen (4-HT; Sigma), was added to the cultures.[24] Unless otherwise stated, 4-HT was used at a final concentration of 1 μM. To induce the differentiation of TF-1-*fms*-Nef-ER cells into macrophages, cells were cultured with M-CSF and 100 ng/mL 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Sigma).

Cell count and flow cytometry

Viable cell counts were obtained by enumerating cells that excluded trypan blue dye. Adherent cells were harvested by trypsinization.

To detect apoptotic cells, the flow cytometric analysis of propidium iodide (PI)–stained nuclei was performed as described previously.[28] In brief, cells were washed with phosphate-buffered saline (PBS) and then incubated in hypotonic lysing buffer (0.1% sodium citrate, 0.3% Nonidet P-40, and 50 μg/mL PI) at 4°C for 30 minutes. Alternatively, cells were stained with annexin V conjugated to phycoerythrin (PE) (PharMingen, San Jose, CA) and vital dye 7-aminoactinomycin D (7-AAD; PharMingen). Cultured cells were washed with PBS, resuspended in binding buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 140 mM NaCl, and 2.5 mM $CaCl_2$), and incubated with annexin V-PE and 7-AAD (1.25 μg/mL) at room temperature for 15 minutes. Flow cytometry analyses were performed with FACSCalibur using Cell Quest Software (Becton Dickinson, Mountain View, CA).

In this study, we analyzed the expression level of M-CSF receptor on TF-1-*fms*-Nef-ER cells by flow cytometry using Flag-tagged M-CSF protein. The Flag (DYKDDDDK) sequence was introduced into the C-terminus of mature M-CSF polypeptides[22] by polymerase chain reaction (PCR) amplification with an upstream primer (5'-TGCCGGGACCCAGCTGCCCGTATGA-3') and a downstream primer (5'-CTCACTTGTCGTCATCGTCTTTGTAGTCTCGGCTAGAGCA-3'). Codons specifying the Flag sequence are underscored. The PCR product was subcloned into an expression vector, pEF-BOS.[29] The resultant plasmid was transfected into COS7 cells using LipofectAMINE2000 reagent (Invitrogen),[30] and the culture supernatant was used as a source of Flag-tagged M-CSF. For analysis, cells were incubated with the Flag-tagged M-CSF, followed by biotin-labeled anti–Flag M2 antibody (10 μg/mL; Sigma) and PE-labeled streptavidin (5 μg/mL; PharMingen).[31]

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed essentially as described previously.[17] Cells were growth-factor depleted for 14 hours in RPMI 1640–10% FCS and then stimulated with M-CSF (100 ng/mL) for the indicated periods. For the activation of Nef, 4-HT (1 μM) was added to the culture at the initiation of M-CSF deprivation. Then cells were solubilized with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris HCl, 150 mM NaCl) containing protease inhibitors (1 mM EDTA [ethylenediaminetetraacetic acid], 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). Cell lysates were centrifuged, and the resultant supernatants were subjected to Western blotting or immunoprecipitation. Antibodies (purchased from Santa Cruz Biotechnology, Santa Cruz, CA) used for Western blotting were as follows: anti–M-CSF receptor rabbit immunoglobulin G (IgG) (C-20), antiphosphotyrosine mouse IgG (PY99), anti-ERK rabbit IgG (K-23), antiphosphorylated ERK mouse IgG (E-4), and anti-Hck rabbit IgG (N-30). Anti-Hck mouse IgG (clone 18; Transduction Laboratories, Lexington, KY) was also used. Antibodies used for immunoprecipitation were as follows: anti–M-CSF receptor rat IgG (12-2D6; Zymed, South San Francisco, CA), anti-Hck rabbit IgG (Santa Cruz Biotechnology), and antiphosphotyrosine mouse IgG (PY99; Santa Cruz Biotechnology). Immune complexes were precipitated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Cleared cell lysates and immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and proteins were transferred to a nylon membrane (Hybond-P; Amersham, Buckinghamshire, United Kingdom). Detection was performed using the Enhanced Chemiluminescence Western Blotting Detection System (Amersham) with horseradish peroxidase–coupled anti-immunoglobulin (Amersham).

Expression of M-CSF receptor, Nef, and Hck in 293T cells

Expression plasmid for human M-CSF receptor was prepared as described previously.[22] The plasmids for Nef and human Hck were provided by E. T. Sawai[32] and Y. Murakami,[33] respectively. The *Nef* gene was derived from the SF2 strain of HIV-1,[32] and its product has been shown to activate the kinase activity of Hck.[34] The human embryonic kidney cell line 293T was obtained from the American Type Culture Collection (Manassas, VA) and was maintained with Dulbecco modified Eagle medium (DMEM; Sigma)–10% FCS. Cells were grown on a 6-well tissue culture plate and were transfected with plasmids for M-CSF receptor (0.4 μg), Nef (0.8 μg), and Hck (0.4 μg), using LipofectAMINE2000 reagent (Invitrogen). After transfection, the cells were cultured in DMEM–10% FCS for 36 hours and then cultured in DMEM–0.1% FCS for an additional 12 hours. In a selected experiment, cells were stimulated with M-CSF for 2 minutes. Cells were solubilized with Nonidet P-40 lysis buffer, and the cleared cell lysates were subjected to immunoprecipitation with anti–M-CSF receptor rat IgG (Zymed) or anti–Hck rabbit IgG (Santa Cruz Biotechnology). Immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine mouse IgG (Santa Cruz Biotechnology), anti–M-CSF receptor rabbit IgG (Santa Cruz Biotechnology), or anti–Hck mouse IgG (Transduction Laboratories).

Results

Inhibition of M-CSF–mediated cell proliferation and differentiation by Nef

We initially attempted to determine whether Nef would affect the biologic effects of M-CSF. To achieve this, we used the human myeloid leukemia cell line TF-1–*fms*, the proliferation of which was dependent on M-CSF.[22] We established the TF-1–*fms* clone expressing Nef-murine estrogen receptor hormone–binding domain fusion protein (TF-1–*fms*-Nef-ER) (Figure 1A). The Nef-ER fusion protein could be seen as a 60-kDa band on Western blot (Figure 1B). In this system, Nef was basally inactive, but its function could be inducibly activated by the estrogen analog 4-HT.[24] As shown in Figure 1C, we found that the proliferation of TF-1–*fms*-Nef-ER cells mediated by M-CSF was markedly inhibited on the addition of 4-HT (ie, the activation of Nef). The inhibitory effect was dependent on the concentrations of 4-HT added to the cultures and was specific because 4-HT never affected the proliferation rate of the parental TF-1–*fms* cells (Figure 1C). Furthermore, we found that Nef activation was associated with the appearance of apoptotic subdiploid cells in the culture of TF-1–*fms*-Nef-ER cells (Figure 1D). In parallel, Nef activation increased the percentage of annexin V–positive but 7-AAD–negative cells in the culture of TF-1–*fms*-Nef-ER cells (Figure 1E). These findings indicated that Nef induced apoptotic cell death in TF-1–*fms*-Nef-ER cells.

We next attempted to determine whether Nef also could inhibit another biologic activity of M-CSF. The phorbol ester TPA is well known to induce macrophage differentiation of various myeloid leukemia cell lines.[23] Recently, we found that, in the presence of TPA, M-CSF markedly stimulates the differentiation of TF-1–*fms* cells into mature macrophages but that it does not stimulate the proliferation of the cells (S. Suzu et al, unpublished results, February 2004). This was based on several observations. First, the combination of TPA and M-CSF caused more drastic morphologic changes in TF-1–*fms* cells than did treatment with TPA alone. Second, culture in the presence of TPA and M-CSF contained more adherent cells than in the presence of TPA alone. Third, the phagocytic activity of cells treated with TPA and M-CSF was significantly higher than that of cells treated with TPA alone. Using

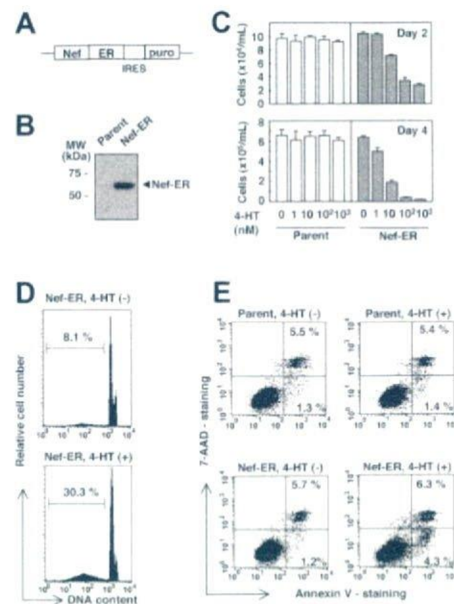

Figure 1. TF-1– *fms* cells expressing Nef-ER show impaired proliferation and apoptotic cell death on Nef activation. (A) Schematic diagram of the Nef-ER-IRES-puro construct. ER indicates estrogen receptor hormone–binding domain; IRES, internal ribosomal entry sequence; puro, puromycin resistance gene. (B) Total cell lysates from the parental TF-1–*fms* cells (parent) or the TF-1–*fms* clone stably expressing Nef-ER (Nef-ER) were analyzed for the expression of Nef-ER by Western blotting with –Nef rabbit antiserum. (C) Parental TF-1–*fms* (□) or TF-1–*fms*-Nef-ER cells (▥) were seeded at a density of 1×10^4 cells/mL in the presence of M-CSF (100 ng/mL) and increasing concentrations of 4-HT. Cells were cultured for 2 days (top row) or 4 days (bottom row), and viable cells were enumerated. Error bars from triplicate assays are shown. Results are representative of 3 independent experiments. Error bars indicate standard deviation (SD). (D) TF-1–*fms*-Nef-ER cells were cultured with M-CSF in the absence (top) or the presence of 1 μM 4-HT (bottom) for 48 hours. Apoptotic subdiploid cells was detected by flow cytometry. The percentages of subdiploid cells are shown. (E) Parental TF-1–*fms* (top row) or TF-1–*fms*-Nef-ER cells (bottom row) were cultured with M-CSF in the absence (left column) or the presence of 1 μM 4-HT (right column) for 24 hours. Cells were analyzed for the presence of apoptotic cells by staining with annexin V–PE, and 7-AAD (lower right quadrant). The percentages in cells of the upper right corners and lower right corners are shown.

this culture system, we examined the effect of Nef on the macrophage differentiation–inducing activity of M-CSF. As shown, the morphologic changes in TF-1–*fms*-Nef-ER cells induced by TPA and M-CSF were markedly inhibited on the activation of Nef (Figure 2A). Furthermore, the number of cells adhering to the dishes in the 4-HT–containing culture was lower than that in the 4-HT–free culture (Figure 2B). The inhibitory effect was dependent on the concentration of 4-HT added to the cultures (Figure 2C).

Inhibitory effect of Nef on M-CSF bioactivity is not mediated by soluble factors or down-regulation of M-CSF receptor

The inhibitory effects of Nef on M-CSF–induced cell proliferation (Figure 1) and macrophage differentiation (Figure 2) suggested that Nef affected signaling pathways transduced by M-CSF. However, because Nef has been shown to induce the expression of a number of soluble factors (chemokines and cytokines) in monocytes/macrophages,[9,10,35-37] the inhibitory effects of Nef observed in our culture systems might be mediated through the secretion of inhibitory molecule(s) by Nef rather than through a direct effect on the M-CSF receptor signaling pathways. To address this issue, we conducted a coculture experiment with parental TF-1–*fms* cells and TF-1–*fms*-Nef-ER cells. Parental cells were engineered to express EGFP protein, allowing us to distinguish parental cells from Nef-ER cells in the cocultures through flow cytometric analysis. As

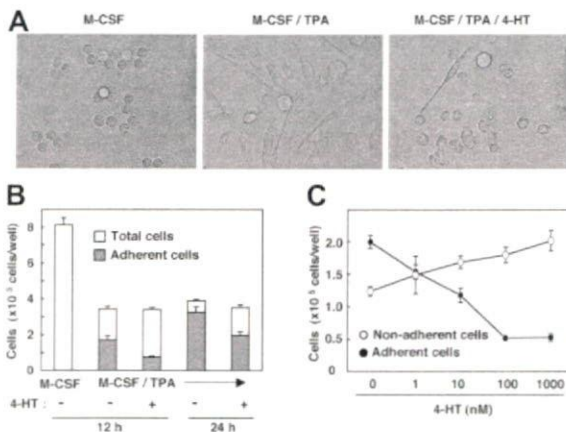

Figure 2. Nef activation causes inhibition in macrophage differentiation. (A) Morphologies of TF-1–*fms*-Nef-ER cells cultured for 24 hours with M-CSF (100 ng/mL), M-CSF/TPA (100 ng/mL), or M-CSF/TPA/4-HT (1 μM), as indicated in each case. (B) TF-1–*fms*-Nef-ER cells were seeded into 6-well culture plates at a density of 2×10^5 cells/well. Cells were cultured in the presence of M-CSF, M-CSF/TPA, or M-CSF/TPA/4-HT for 12 hours or 24 hours. After culture, total cells in the wells (□) and cells that adhered to the dishes (▨) were enumerated. (C) TF-1–*fms*-Nef-ER cells were seeded as in panel B. Cells were cultured for 12 hours in the presence of M-CSF, TPA, and increasing concentrations of 4-HT. Cells adhering to the dishes (●) or remaining in suspension (○) were enumerated. (B-C) Error bars from triplicate assays are shown and represent SD. Results are representative of 3 independent experiments.

shown in Figure 3A, the relative cell number of Nef-ER cells was comparable to that of the parental cells in the absence of 4-HT (left panel) but was markedly low in the presence of 4-HT (right panel). In fact, the absolute cell number of Nef-ER cells in 4-HT–containing cocultures was reduced to approximately 10% when compared with that in 4-HT–free cocultures (Figure 3B). However, and of importance, the absolute cell numbers of the parental cells remained unchanged between 4-HT–containing cocultures and 4-HT–free cocultures (Figure 3B). These results suggested that soluble factors from Nef-active cells, if any, were not involved in the proliferation inhibitory effect of Nef observed in our culture system.

Another well-known function of Nef is the down-regulation of cell surface molecules such as CD4 and MHC class I.[7,8] Therefore, we next examined whether Nef activation caused the down-regulation of the receptor for M-CSF in TF-1–*fms*-Nef-ER cells. As shown by the culture in Figure 4A, on which Western blotting analysis was performed, we did not find any change in the expression level of M-CSF receptor, even when TF-1–*fms*-Nef-ER

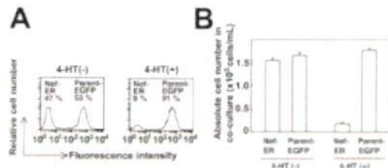

Figure 3. Secretion of soluble factors is not involved in the impaired proliferation of TF-1–*fms*-Nef-ER cells induced by Nef activation. Parental TF-1–*fms* cells, but not TF-1–*fms*-Nef-ER cells, were engineered to express EGFP protein by the retroviral infection system. The cell density of the TF-1–*fms*-EGFP (parent-EGFP) and TF-1–*fms*-Nef-ER (Nef-ER) cells was adjusted to 1×10^4 cells/mL, and the cells were cultured after an equal volume of the cell suspensions was combined. These cultures were incubated for 3 days with M-CSF in the presence or the absence of 4-HT. (A) After the cocultures, cells were subjected to flow cytometric analysis to determine the relative cell numbers. (B) Absolute cell numbers of the parental cells and Nef-ER cells were calculated from the relative cell numbers (A) and the total cell numbers in the cocultures. Error bars from triplicate assays are shown and represent SD. These results are representative of 3 independent experiments.

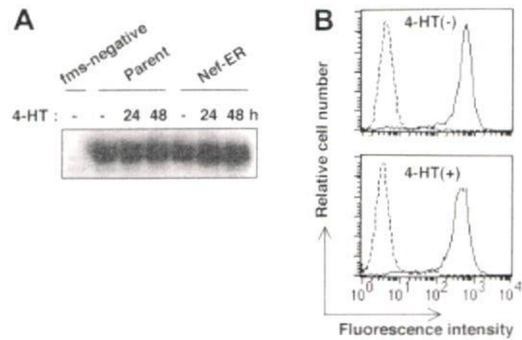

Figure 4. Down-regulation of M-CSF receptor is not involved in the impaired proliferation of TF-1–*fms*-Nef-ER cells induced by Nef activation. (A) Parental TF-1–*fms* and TF-1–*fms*-Nef-ER cells were cultured with 4-HT for the indicated periods, and the levels of M-CSF receptor proteins were analyzed by Western blotting. Total cell lysates from TF-1 cells, but not from TF-1–*fms* cells, in which the M-CSF receptor gene had not been introduced, were included in the analysis as a negative control (*fms*-negative). (B) TF-1–*fms*-Nef-ER cells were left untreated (top) or were treated with 4-HT for 24 hours (bottom), and the level of cell surface M-CSF receptor expression was analyzed by flow cytometry with Flag-tagged M-CSF (solid lines). Profiles of cells incubated with a Flag-tagged protein,[30] unrelated to M-CSF, are also shown as a control (broken lines).

cells were cultured for up to 2 days in the presence of 4-HT. Flow cytometric analysis with the Flag-tagged M-CSF also revealed that the level of cell surface expression of M-CSF receptor in TF-1–*fms*-Nef-ER cells treated for 24 hours with 4-HT was comparable with that in Nef-inactive cells (Figure 4B). Taken together with the result of the coculture experiment (Figure 3), these results strongly suggested that Nef affected intrinsic M-CSF receptor signaling pathways and thereby inhibited M-CSF activities.

Mechanism by which Nef inhibits M-CSF receptor signaling

Because M-CSF receptor is a receptor tyrosine kinase,[14,16] we initially compared the M-CSF–induced protein tyrosine phosphorylation in Nef-active TF-1–*fms*-Nef-ER cells with that in Nef-inactive cells. On M-CSF stimulation, a number of signaling molecules, such as proteins, with molecular weights of 150 kDa to 160 kDa, 100 kDa, and 60 kDa were shown to be rapidly tyrosine phosphorylated in Nef-inactive cells (Figure 5A, open arrowheads). However, the tyrosine phosphorylation of these proteins in response to M-CSF stimulation was markedly diminished in Nef-active cells (Figure 5A). Yet, the blot also showed that the effect of Nef was not an overall reduction in tyrosine phosphorylation. The proteins, seen as a closely spaced doublet at approximately 55 kDa, were phosphorylated only on M-CSF stimulation in Nef-inactive cells but were phosphorylated before stimulation in Nef-active cells (Figure 5A, asterisks). Moreover, the tyrosine phosphorylation of protein, seen at 40 to 45 kDa, was elevated in Nef-active cells (Figure 5A, filled arrowhead). Judging by its molecular weight, the latter protein (40-45 kDa, filled arrowhead) seemed to be extracellular signal-related protein kinase (ERK).[17] This was confirmed by further analyses in which total cell lysates were immunoprecipitated with antiphosphotyrosine antibody and then were probed with anti-ERK antibody or in which total cell lysates were directly probed with phosphorylated ERK-specific antibody (Figure 5B). Using a similar approach, we identified the protein seen at 150 to 160 kDa, the tyrosine phosphorylation of which was induced by M-CSF stimulation but was diminished in Nef-active cells (Figure 5A, top open arrowhead) as M-CSF receptor (Figure 5C, top and middle panels). The blot shown in the middle panel also revealed that the 60-kDa protein (Figure 5A, bottom open arrowhead) was a component of M-CSF receptor

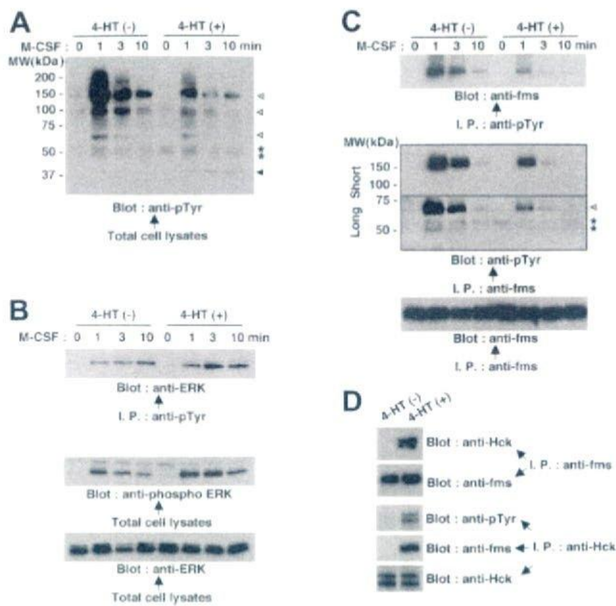

Figure 5. Nef activation causes perturbations in M-CSF receptor signaling in TF-1–*fms*-Nef-ER cells. (A-C) TF-1–*fms*-Nef-ER cells were deprived of M-CSF for 14 hours and restimulated with M-CSF for the indicated periods. For the activation of Nef, 4-HT was added to the culture at the initiation of M-CSF deprivation. Total cell lysates or immunoprecipitates were analyzed by Western blotting. (A) Total cell lysates from untreated or 4-HT–treated cells were analyzed with antiphosphotyrosine (pTyr) antibody. See "Mechanism by which Nef inhibits M-CSF receptor signaling" for explanations of the symbols in detail. (B) Immunoprecipitates with anti–pTyr antibody were analyzed with anti–ERK antibody (top). Alternatively, total cell lysates were analyzed with antibody specific for phosphorylated ERK (middle). The blot shown in the bottom panel, in which the total cell lysates were analyzed with –ERK antibody, verified that comparable amounts of proteins were loaded in the top panel. (C) Immunoprecipitates with anti–pTyr antibody were analyzed with anti–M-CSF receptor (*c-fms*) antibody (top). Alternatively, the immunoprecipitates with anti–M-CSF receptor antibody were analyzed with anti–pTyr antibody (middle). The blot was exposed to autoradiography film for 10 seconds (short) or 1 minute (long). See "Mechanism by which Nef inhibits M-CSF receptor signaling" for explanations of the symbols in detail. The blot shown in the bottom panel, in which the immunoprecipitates were analyzed with anti–M-CSF receptor antibody, is a loading control experiment for the upper panel. (D) TF-1–*fms*-Nef-ER cells were cultured with or without 4-HT for 14 hours under M-CSF–free conditions. Total cell lysates were prepared and subjected to immunoprecipitation with anti–M-CSF receptor (*c-fms*) antibody or anti–Hck antibody. Anti–M-CSF receptor immunoprecipitates were analyzed with anti–Hck antibody or anti–M-CSF receptor antibody. Anti–Hck immunoprecipitates were analyzed with anti–pTyr antibody, anti–M-CSF receptor antibody, or anti–Hck antibody.

complex, and its association with the complex was markedly diminished in Nef-active cells (Figure 5C, open arrowhead). These results indicated that Nef activation caused inhibition in the tyrosine phosphorylation/activation of M-CSF receptor and the molecular association with the activated receptor, which was the earliest signaling event induced by M-CSF.

We next examined whether the activation of Hck by Nef was involved in the inhibitory effect of Nef on M-CSF receptor signaling. We focused attention on the proteins seen as a closely spaced doublet at approximately 55 kDa. The pattern in tyrosine phosphorylation was different between Nef-inactive and Nef-active cells—the proteins were tyrosine phosphorylated in response to M-CSF stimulation in Nef-inactive cells but were phosphorylated before stimulation in Nef-active cells (Figure 5A, asterisks). Moreover, and of interest, the proteins were recruited to the complex of M-CSF receptor only after M-CSF stimulation in Nef-inactive cells but pre-existed in the complex in Nef-active cells irrespective of M-CSF stimulation (Figure 5C, asterisks). We confirmed that the proteins were Hck. When total cell lysates were prepared from cells that were cultured without M-CSF, immunoprecipitated with anti–M-CSF receptor antibody, and analyzed for the presence of Hck protein in the M-CSF receptor complex, we found that Hck was present in the complex in Nef-active cells but not in Nef-inactive cells (Figure 5D). Analyses of anti–Hck immunoprecipitates further revealed the tyrosine phosphorylation of Hck and its association with M-CSF receptor complex in Nef-active cells (Figure 5D). Given that Src family kinases, including Hck, should associate with M-CSF receptor only if the receptor is activated by its ligand,[18-20] the constitutive association of Hck with M-CSF receptor complex that occurred in Nef-active TF-1–*fms*-Nef-ER cells was an aberrant interaction.

Finally, we performed transient expression experiments with 293T cells and confirmed that Nef induced the activation of Hck, the aberrant molecular interaction between Hck and M-CSF receptor, and the impaired M-CSF receptor activation. Tyrosine phosphorylation of Hck in 293T cells was enhanced by cotransfection with Nef (Figure 6A, top panel), possibly reflecting the finding that Nef stimulated the autophosphorylation of Hck.[12,33,34,38] The association between Hck and M-CSF receptor, in the absence of M-CSF stimulation, was stimulated by cotransfection with Nef (Figure 6A, middle panel; Figure 6B, upper panel). The tyrosine phosphorylation of M-CSF receptor in response to M-CSF was impaired in 293T cells coexpressing M-CSF receptor, Nef, and Hck (Figure 6C, upper panel). Such impairment was not observed in 293T cells coexpressing M-CSF receptor and Nef or M-CSF receptor and Hck (Figure 6C, upper panel). In summary, our data strongly suggested that HIV-1 Nef interfered with the activation of M-CSF receptor in response to its ligand by means of the activation of Hck and the subsequent induction of an aberrant molecular association between Hck and M-CSF receptor complex, thereby inhibiting M-CSF bioactivities.

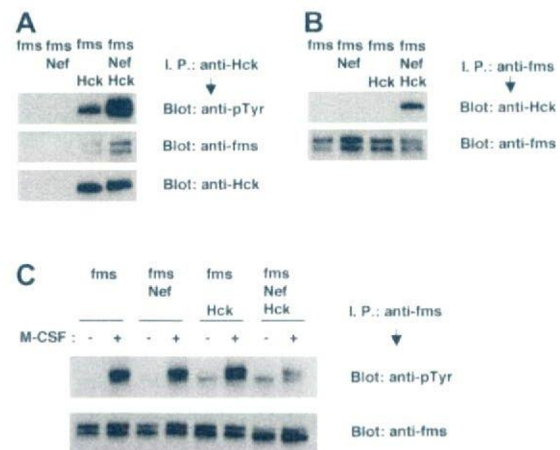

Figure 6. Nef causes tyrosine phosphorylation of Hck, constitutive association of Hck with M-CSF receptor complex, and inhibition in tyrosine phosphorylation of M-CSF receptor in 293T cells. (A-C) 293T cells were transfected with M-CSF receptor (*c-fms*) expression plasmid, alone or in combination with Nef or Hck expression plasmids. Total cell lysates were prepared and subjected to immunoprecipitation/Western blotting analyses. (C) Transfected 293T cells were treated in the absence or presence of M-CSF for 2 minutes before lysis. (A) Immunoprecipitates with anti–Hck antibody were analyzed with antiphosphotyrosine (pTyr) antibody (top) or anti–M-CSF receptor antibody (middle). The blot shown in the bottom panel, in which the immunoprecipitates were analyzed with anti–Hck antibody, is a loading control experiment for upper panels. (B) Immunoprecipitates with anti–M-CSF receptor antibody were analyzed with anti–Hck antibody (top). The blot shown in the bottom panel, in which the immunoprecipitates were analyzed with anti–M-CSF receptor antibody, is a loading control experiment for the top panel. (C) Immunoprecipitates with anti–M-CSF receptor antibody were analyzed with anti–pTyr antibody (top). The blot shown in the bottom panel, in which the immunoprecipitates were analyzed with anti–M-CSF receptor antibody, is a loading control experiment for the top panel.

Discussion

The present study identified for the first time a dysregulated signaling event in monocytes/macrophages induced by the interaction between HIV-1 Nef and the cellular kinase Hck. We have demonstrated that Nef activates Hck and recruits the activated Hck to the M-CSF receptor complex. By means of the induction of the unphysiologic signaling event, Nef interferes with M-CSF receptor signaling pathways, thereby inhibiting M-CSF bioactivities and possibly causing dysregulated functions of HIV-1–infected monocytes/macrophages.

Since Hanna et al[13] reported the importance of the interaction between Nef and Hck for the development of AIDS-like disease through studies with HIV-1 transgenic mice and Hck knockout mice, efforts have been made to identify the signaling molecules for which activation is affected by Nef-Hck interaction in monocytes/macrophages. Signal transducer and activator of transcription (STAT) family molecules were considered possible candidates for the Nef-Hck complex. A recent study reported that Nef induced the activation of STAT3 in a myeloid cell line.[34] However, it was not established whether the Nef-Hck interaction directly led to STAT3 activation.[34] Similarly, another recent study reported that Nef activated STAT-1α and -1β in human macrophages derived from peripheral blood monocytes, but the activation occurred through the secretion of unidentified soluble factors.[36] Thus, the STAT family molecules may not be direct targets for the Nef-Hck complex. The secretion of soluble factors, such as cytokines and chemokines, from monocytes/macrophages is a well-established function of Nef,[9,10,35-37] and macrophage inflammatory proteins 1α and 1β are well-known examples, as reported by Swingler et al.[9] Interestingly, the possibility that the Nef-Hck interaction did not participate in the secretion of these chemokines was raised by the authors.[9] After all, in spite of its importance in disease development, the role of the Nef-Hck interaction in the modulation of monocyte/macrophage signaling remains to be determined.

Our present study strongly suggests that M-CSF receptor is a target for the Nef-Hck complex. We demonstrated that Nef caused the inhibition of the activation of M-CSF receptor in response to M-CSF and the molecular association with the activated receptor, which were the earliest signaling events for M-CSF receptor pathways (Figures 5A,C, 6C). As an inevitable consequence, Nef caused the inhibition of biologic activities of M-CSF, such as cell proliferation (Figure 1) and macrophage differentiation (Figure 2) of TF-1-*fms* cells. We observed a similar effect of Nef in primary myeloid cells. When cord blood CD34$^+$ cells were retrovirally transduced with the *Nef* gene and then cultured with M-CSF, we found a marked decrease in the proportion of Nef-positive cells during the culture with M-CSF (data not shown). Although further analyses are necessary, the finding raises the possibility that Nef does inhibit M-CSF receptor signaling in primary myeloid cells. The proliferation inhibitory effect of Nef was not mediated by the secretion of inhibitory molecules (Figure 3) or by the down-regulation of M-CSF receptor (Figure 4), further supporting the conclusion that Nef affected intrinsic M-CSF receptor signaling. The most likely mechanism by which Nef interferes with M-CSF receptor signaling is the unphysiologic behavior of Hck. The Hck proteins seem to be constitutively phosphorylated in Nef-active TF-1-*fms*-Nef-ER cells (Figure 5A, C). The induction of tyrosine phosphorylation of Hck by Nef was confirmed by transient expression experiments in 293T cells (Figure 6A). More important, Hck constitutively formed the complex with M-CSF receptor in active Nef-expressing cells (Figures 5D, 6A,B). In contrast, Hck associates with M-CSF receptor only if the receptor is activated by its ligand (Figure 5C,D).[18-20] It is reasonable to expect that the aberrant constitutive association of Nef-activated Hck to M-CSF receptor complex would inhibit the conformational change of the receptor that would be induced by its ligand and would be required for initiating signaling cascades. Therefore, we concluded that Nef interfered with M-CSF receptor signaling through the activation of Hck and its recruitment to M-CSF receptor complex.

Given that Nef activates the kinase activity of Hck[11,12] and that the activation of Src kinases, including Hck, is required for biologic effects of M-CSF such as mitogenic signal and differentiation-inducing signal,[17-21] one may speculate that Nef mimics the effect of M-CSF on monocytes/macrophages. However, this is not the case. Nef caused the inhibition of biologic activities of M-CSF such as cell proliferation (Figure 1) and macrophage differentiation (Figure 2). In addition, the activation of Nef did not cause M-CSF–independent proliferation of TF-1-*fms*-Nef-ER cells (data not shown). The reason Nef-activated Hck does not mimic M-CSF functions might be explained by the difference in the mode of Hck activation. The activity of Src family kinases, including Hck, is suppressed by 2 intramolecular interactions, SH2-tail and SH3-linker.[39,40] Nef has been shown to activate Hck by binding to its SH3 domain, thus preventing the SH3–linker interaction.[12,38] In contrast, the ligand-activated M-CSF receptor has been shown to associate with the SH2 domain but not with the SH3 domain of Src kinases.[19] It is reasonable to expect that the different modes of Hck activation might generate distinct output signals. That Nef-activated Hck formed an unphysiologic molecular association with M-CSF receptor complex supported this idea.

Nef activation did not necessarily cause the overall reduction of M-CSF receptor pathways. Tyrosine phosphorylation/activation of ERK in response to M-CSF was enhanced by Nef activation (Figure 5B). This finding raises the possibility that Nef-activated Hck inhibits the process of "full activation" of M-CSF receptor. The activation process of M-CSF receptor is considered to involve the ligand-induced dimerization and subsequent autophosphorylation of specific tyrosine residues in their cytoplasmic region.[16] Phosphorylated tyrosine residues then serve as docking sites for SH2 domain–containing molecules, each initiating signaling cascades along specific pathways.[16] Future studies in which we determine whether Nef-activated Hck directly binds to M-CSF receptor and identify site(s) within the cytoplasmic region of the receptor to which Nef-activated Hck binds will clarify the mechanism for the selective inhibition of Nef on the M-CSF receptor pathways.

Our finding that Nef inhibits the process of macrophage differentiation mediated by M-CSF (Figure 2) explains the mechanism for dysfunction, such as defective phagocytosis of opportunistic pathogens, of monocytes/macrophages in HIV-1–infected persons. It is well documented that the phagocytosis of pathogens such as *Mycobacterium avium* complex, *Pneumocystis carinii*, *Toxoplasma gondii*, and *Candida albicans* by peripheral blood monocytes, tissue macrophages, and monocyte-derived macrophages is impaired by in vitro and in vivo HIV-1 infection (reviewed in Kedzierska et al[41]). M-CSF is an important cytokine for macrophage differentiation[15] and has been shown to stimulate various effector functions of macrophages.[14] In particular, a number of in vitro and in vivo studies have shown that M-CSF activates antimicrobial activities of macrophages.[42-45] Moreover, the clinical efficacy of M-CSF has been proven in patients with invasive fungal infections.[46,47]

We showed that the percentage of apoptotic cells in M-CSF–containing culture of Nef-active TF-1–*fms*-Nef-ER cells was significantly higher than that of Nef-inactive cells (Figure 1D, E). In contrast, the degree of apoptotic cell death induced by M-CSF withdrawal in Nef-active TF-1–*fms*-Nef-ER cells was similar to that in Nef-inactive cells (data not shown). These results indicate that Nef does not actively induce apoptotic cell death in the cells but inhibits the signaling from M-CSF. On the other hand, a recent study has revealed that Nef does not inhibit, but that it mimics signaling initiated by granulocyte/macrophage-CSF (GM-CSF),[34] another cytokine that stimulates the proliferation and differentiation of monocytes/macrophages. That HIV-1 Nef selectively inhibits the M-CSF/M-CSF receptor system may address how monocytes/macrophages contribute to the development and progression of AIDS in vivo. Monocytes/macrophages are considered to be involved in the disease progression process by their secretion of soluble factors;[9,10] the profile of gene expression of M-CSF–derived macrophages differs from that of GM-CSF–derived macrophages.[48] It is possible that the selective inhibition of Nef on cytokine signaling results in the modification of macrophage functions, such as cytokine/chemokine production, in HIV-1–infected persons. This may create an immunologic environment favorable for HIV-1 virus production. Alternatively, this may lead to abnormal tissue distribution of cells of the lineage, thereby facilitating the transfer of HIV-1 virus to bystander $CD4^+$ T cells. Our findings provide a novel clue to understanding how monocytes/macrophages contribute to the development and progression of AIDS and to clarifying the role of the Nef-Hck interaction in the disease process.

Acknowledgments

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Selective expansion and engraftment of human CD16^{+} NK cells in NOD/SCID mice

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NK cells are large granular lymphocytes that represent a critical component of the innate immunity. Investigations of human NK cell function are largely based on *in vitro* assays because of the lack of suitable animal models. Here we have established conditions leading to the development of human NK cells in NOD/SCID (severe combined immunodeficiency) mice receiving grafts of cord blood mononuclear cells (CBMC), and GFP-transduced HFWT inducing NK cells (GHINK-1), which have been shown to support the selective expansion of NK cells from human PBMC and CBMC *in vitro*. Significant numbers of CD56^{dim}CD16^{+} cytotoxic and CD56^{-}CD16^{+} immature NK cells appeared in peripheral blood (PB), peritoneal cavity, spleen, bone marrow and liver of the mice. The newly generated NK cells did not express activation markers such as CD25, CD69 and NKp44, the expression of which was augmented by IL-2 *in vitro*. The NOD/SCID mice engrafted with human NK cells exhibited antitumor activity against K562 erythroleukemia *in vitro* and *in vivo*. Thus, we succeeded in developing a CD56^{dim}CD16^{+} cytotoxic NK cell populations in NOD/SCID mice closely resembling the main NK fraction in human PB and CD56^{-}CD16^{+} immature NK cells. Our model provides not only information about the development and dynamics of physiological human NK cells but also an important pre-clinical system for immunotherapeutic strategies.

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Introduction

NK cells are defined functionally by their ability to lyse target cells without deliberate prior sensitization and without restriction by MHC antigens [1–4]. Early studies identified two distinct subsets of human NK cells based on the density of surface CD56 (NCAM, neural cell adhesion molecule) expression and CD16 (FcγRIII) expression. Approximately 90% of human peripheral blood (PB) NK cells are CD56^{dim}CD16^{+}, whereas a minority (approximately 10%) are CD56^{bright}CD16^{-}. The former exhibit high natural cytotoxicity, while the latter produce large amounts of cytokines. CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells differ in their proliferative response to IL-2, intrinsic cytotoxic capacity, NK cell receptor (NKR) repertoire, and adhesion molecule expression [5]. As NK cells represent the most efficient cytolytic effector cells, clinical trials attempting to harness the antitumor effect of NK cells, either through *in vivo* or *in vitro* activation, have been done [6, 7]. To date, the general impression has been that of this form of treatment is still inefficient. However, recent advances in the study of receptors activating and inhibiting NK cells[8–12] will help us to control NK cell activity and to

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Abbreviations: **CB:** cord blood · **CBMC:** cord blood mononuclear cells · **GHINK-1:** GFP-transduced HFWT inducing NK cells · **PB:** peripheral blood · **PC5:** PE-Cy5 · **SCID:** severe combined immunodeficiency

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develop new clinical treatments for malignancy and infectious diseases.

Most human NK cell studies aimed at clinical applications have used *in vitro* assays, but these surrogate assays have been shown not to correctly reflect the physiological activities of NK cells. Recent technical advances using immunodeficient mice have enabled investigators to evaluate the function of human hematopoietic cells in the mice [13–18]. The NOD/SCID (severe combined immunodeficiency)mouse strain was reported to have a variety of immunological abnormalities, and as a result, has been used as a recipient for the xenotransplantation of human hematopoietic cells [13]. This mouse strain has shown to have an appropriate microenvironment for the homing and retention of human NK cells [19]. Recently, Kalberer *et al.* [20] reported the engraftment of human NK cells by administration of IL-15 and Flt-3 ligand into $CD34^+$ cell-transplanted NOD/SCID mice. In this mouse model, the administration of these growth factors generated $CD56^+$ mature NK cells, *i.e.,* the minor population of PB NK cells, and the $CD34^-CD7^+$ NK precursor. However, $CD56^{dim}CD16^+$ NK cells, the major NK population in human PB and cord blood (CB), could not be generated in their system [20]. In addition, although IL-2 and IL-15 are known to support the differentiation and expansion of NK cells from progenitors both *in vitro* [21–24] and *in vivo* [20, 25], these cytokines also activate NK cells [26–28]. Thus, it is important to establish a mouse model with human steady state NK cells for clinical applications.

We have previously reported that a human Wilms' tumor cell line, HFWT, selectively stimulated proliferation of human NK cells *in vitro* via direct cell-to-cell interaction [29]. The NK cells generated showed mainly the $CD56^+CD16^+$ phenotype, which differs from the cytokine-generated $CD56^{bright}CD16^-$ NK cells *in vitro*. In this study, we attempted to generate human NK cells in NOD/SCID mice by intraperitoneal co-transplantation of a HFWT-derived cell line, GHINK-1 (GFP-transduced HFWT inducing NK cells) [30], and CB mononuclear cells (CBMC). NK cells generated in NOD/SCID mice are $CD56^+CD16^+$ resembling human PB and CB NK cells, and show cytotoxic activity against K562 erythroleukemia cells *in vitro* and reduce the growth of K562 *in vivo*. Thus, NOD/SCID mice with selective engraftment of human NK cells provide a useful pre-clinical *in vivo* tool for immunotherapy as well as the investigation of NK cell development.

Results

GHINK-1 cells induce proliferation of human NK cells in NOD/SCID mice

The NOD/SCID xenotransplantation system was chosen to investigate the function of human NK cells *in vivo*. As the Wilms' tumor cell line HFWT selectively stimulates the proliferation of human NK cells *in vitro* [29], we attempted to transplant the human CBMC with an EGFP-expressing subline of HFWT, GHINK-1.

GHINK-1 cells were co-transplanted with CBMC intraperitoneally into NOD/SCID mice. After 12 days, PB was obtained and analyzed by flow cytometry to verify the engraftment of human cells. The percentage of human $CD45^+$ cells was about fivefold higher among PB leukocytes of the co-transplanted mice than those of the mice transplanted with CBMC alone (Fig. 1A, upper panel). Further analysis revealed that the $CD45^+$ cell population mainly consisted of $CD3^-CD56^+$ and $CD3^-CD56^-$ cells in the co-transplanted mice. By contrast, the CBMC-transplanted mice mainly had $CD3^+CD56^-$ T lymphocytes (Fig. 1A, under panels). The percentage of typical mature NK ($CD3^-CD56^+$) cells was approximately thirtyfold higher in the co-transplanted mice than CBMC-transplanted mice [$36.5 \pm 6.5\%$ ($n=8$) and $1.2 \pm 6.5\%$ ($n=6$), respectively]. Some $CD3^+CD56^-$ T cells were generated in the co-transplanted mice but fewer than in the CBMC-transplanted mice (Fig. 1B). The generated human $CD45^+$ cells were mainly positive for CD16 and dim/negative for CD56 (Fig. 1C). These populations, $CD56^{dim}CD16^+$ and $CD56^-CD16^+$, were also detected in CBMC. Although CD16 (FcγRIII) is known to be expressed on human NK cells, neutrophils and macrophages [31], we could not detect human neutrophils and macrophages in the mouse PB by Wright Giemsa staining (data not shown). These results revealed that the $CD56^-CD16^+$ cells detected in the mice were most likely immature NK cells that have been shown to exist in CBMC [32–34]. Both $CD56^+$ $CD16^+$ and $CD56^-CD16^+$ NK cells were detected in the peritoneal cavity, spleen, bone marrow and liver as well as in PB at 1 wk post transplantation (Fig. 2A). Peak of the NK cell numbers in each organ was at 2–3 wks, and the NK cells were detectable until 6 wks (Fig. 2B). These results suggested that human mature and immature NK cells were selectively generated and engrafted in NOD/SCID mice by co-transplantation of CBMC and GHINK-1 cells.

GHINK-1 cells support the selective proliferation of human NK cells in NOD/SCID mice

To analyze the kinetics of NK cell development in the NOD/SCID mice, we used the cell division-sensitive

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