厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書

サル指向性 HIV-1 感染による宿主免疫応答に関する解析

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研究要旨

エイズ動物モデルは、HIV-1 感染症・エイズ発症機序の解明および抗エイズ治療薬・ワクチンの開発のために必要である。しかし現時点では、ヒトと同じように HIV-1 が持続感染しエイズ発症に至る動物モデルは存在せず、サル免疫不全ウイルス（SIV）感染マカクサルモデルが最適のエイズ動物モデルと考えられている。本研究課題は、HIV-1 感染サルモデルの開発を目的とするが、私はその HIV-1 感染による宿主免疫反応の解析を担当することとした。平成 19 年度は、カニクイサルへの第 1 世代サル馴化型 HIV-1 感染実験における末梢血リンパ球数の変化およびウイルス特異的 T リンパ球反応の解析を行った。その結果、感染急性期の末梢血 CD4 陽性 T リンパ球数、CD8 陽性 T リンパ球数とも大きな変化はなく、このウイルス感染のリンパ球系に与える影響は病原性 SIV ほどは強くはないと考えられた。急性期において、HIV-1 特異的 CD8 陽性 T リンパ球反応は認められ、これが HIV-1 持続感染成立阻止に加担している可能性が考えられたが、HIV-1 特異的 CD4 陽性 T リンパ球反応は認められず、この点についてはこの HIV-1 がある程度の病原性を発揮している可能性が考えられた。これらの結果は、今後の第 2 世代あるいは第 3 世代のサル馴化型 HIV-1 感染実験の際の比較基準として重要である。

A. 研究目的

エイズ動物モデルは、HIV-1 感染症・エイズ発症機序の解明および抗エイズ治療薬・ワクチンの開発において必要である。しかし現時点では、ヒトと同じようにHIV-1 が持続感染しエイズ発症に至る動物モデルは存在せず、サル免疫不全ウイルス（SIV）感染マカクサルモデルが最適のエイズ動物モデルと考えられている。

ヒト HIV-1 感染とサル SIV 感染による慢性持続感染におけるいくつかの重要なポイントについて両者間で共通点が認められるわけではあるが、ウイルス Nef 蛋白の機能など、両者間でいくつかの異なる点があることも知られている。そこで、ヒトとサルの宿主間の違いはともかく、HIV-1 と SIV というウイルス間の違いの問題をできるだけ解消すべく、HIV-1 感染サルモデルの開発を目的とする本研究課題が計画された。私はその HIV-1 感染による宿主免疫反応の解析を担当することとした。

これまでのサルエイズモデルの解析により、病原性 SIV 感染により、急性期に比較的大きな宿主リンパ球数の変化が認められることが知られている。また、ウイルス特異的 CD8 陽性細胞傷害性 T リンパ球（CTL）反応が誘導され、急性期体内ウイルス量のピークからセットポイント期にかけての減少に中心的役割を担っていることも知られている。そこで、平成 19 年度は、カニクイサルでの第 1 世代サル馴化型 HIV-1 感染実験にお

ける末梢血リンパ球数の変化およびウイルス特異的Tリンパ球反応の解析を行うこととした。

B. 研究方法

主任研究者が行ったカニクイサル2頭への第1世代HIV-1チャレンジ実験において、チャレンジ直前、チャレンジ後1週目、2週目、3週目、4週目に採血された血液を用い、末梢血リンパ球数、CD4陽性Tリンパ球数、CD8陽性Tリンパ球数の測定を行った。

この2頭のHIV-1特異的Tリンパ球反応を測定するために、チャレンジ実験前に、まず各々のサル由来のBリンパ芽球株（BLCL）の樹立を行った。このBLCLに、第1世代HIV-1分子クローンDNAをトランスフェクションし、この細胞とチャレンジ後4週目の末梢血単核球（PBMC）との共培養を行った後、Tリンパ球中に特異的に誘導されるインターフェロンγ（IFN-γ）を細胞内染色により測定した。

（倫理面への配慮）

全ての動物実験は、倫理面も含めて、東京大学医科学研究所、国立感染症研究所および医薬基盤研究所の動物実験委員会の審査をうけ、その承認を得てから医薬基盤研究所霊長類医科学研究センターにて開始した。用いた組換え生物等については、第二種使用等拡散防止措置確認申請承認（大臣確認）済みである。

C. 研究結果

第1世代HIV-1チャレンジを行ったカニクイサル2頭の感染急性期の末梢血CD4陽性Tリンパ球数およびCD8陽性Tリンパ球数の変化を図1に示す。CD4陽性Tリンパ球数およびCD8陽性Tリンパ球数ともに大きな変化は認められなかった。

これら2頭の4週目のHIV-1特異的Tリンパ球反応の解析結果を図2に示す。上段はmock BLCLとの共培養による非刺激実験（対照実験）、下段はHIV-1導入BLCLとの共培養によるHIV-1特異的刺激実験後の細胞内染色結果を示すFACSのdot plotである。いずれもCD3陽性リンパ球（Tリンパ球）分画で、各々につき、左パネル（X軸：CD4、Y軸：IFN-γ）はCD4陽性Tリンパ球反応を、右パネル（X軸：CD8、Y軸：IFN-γ）はCD8陽性Tリンパ球反応を示している。非刺激実験では、CD4陽性Tリンパ球反応、CD8陽性Tリンパ球反応ともに認められなかったが、HIV-1特異的刺激実験により、CD8陽性Tリンパ球反応（IFN-γ陽性CD8陽性細胞）が認められ、HIV-1特異的CD8陽性Tリンパ球反応が誘導されていることが確認できた。一方、HIV-1特異的CD4陽性Tリンパ球反応は認められなかった。

D. 考察

感染急性期の末梢血CD4陽性Tリンパ球数、CD8陽性Tリンパ球数とも大きな変化はなく、このウイルス感染のリンパ球系に与える影響は病原性SIVほどは強くはないと考えられた。

HIV-1特異的Tリンパ球反応の解析では、急性期においてHIV-1特異的CD8陽性Tリンパ球反応（CTL反応）が認められたことから、検出可能レベルの適応免疫を誘導する程度にはウイルス増殖が起こっていると考えられた。HIV-1特異的CTL反応は、ウイルス複製に対して抑制力を有するとされる重要な反応であり、エイズ動物モデルにおける重要な評価対象である。CTL反応は、HIV-1持続感染成立阻止に加担している可能性が高いことから、今後の第2世代あるいは第3世代のサル馴化型HIV-1感染実験の際におけるこの反応の影響が注目される。

一方、HIV-1特異的CD4陽性Tリンパ球反応が認められなかったことから、感染の標的となるCD4陽性Tリンパ球がある程度の損傷をうけている可能性が考えられた。これらの結果は、今後の第2世代あるいは第3世代のサル馴化型HIV-1感染実験の際の比較基準として重要である。

E. 結論

カニクイサルへの第１世代サル馴化型HIV-1感染実験における末梢血リンパ球数の変化およびHIV-1特異的Ｔリンパ球反応の解析を行った。感染急性期の末梢血Ｔリンパ球数の大きな変化はなく、このウイルス感染のリンパ球系に与える影響は病原性 SIV ほどは強くはないと考えられたが、HIV-1特異的CD4陽性Ｔリンパ球反応は認められず、この点についてはこのHIV-1がある程度の病原性を発揮している可能性が考えられた。一方、感染急性期の HIV-1 特異的 CD8 陽性Ｔリンパ球反応は認められ、これがHIV-1持続感染成立阻止に加担している可能性が考えられた。これらの結果は、今後の第２世代あるいは第３世代のサル馴化型HIV-1感染実験の際の比較基準として重要である。

G. 研究発表

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(4) Takeuchi H, Matano T. Host factors involved in resistance to retroviral infection. Microbiol Immunol., in press.

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H. 知的財産権の出願・登録状況

無し。

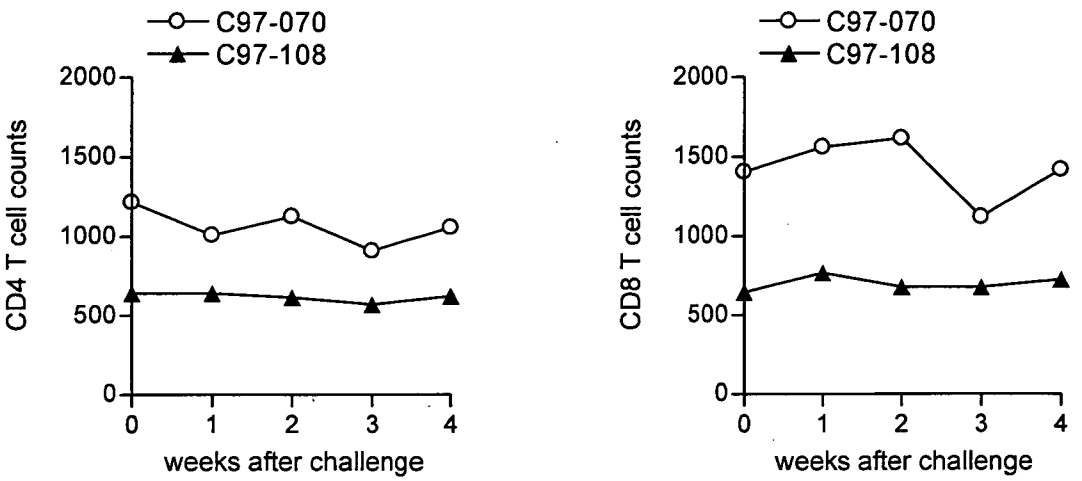

図1
HIV-1チャレンジ後の末梢血CD4陽性Tリンパ球数（左図）およびCD8陽性Tリンパ球数（左図）の変化

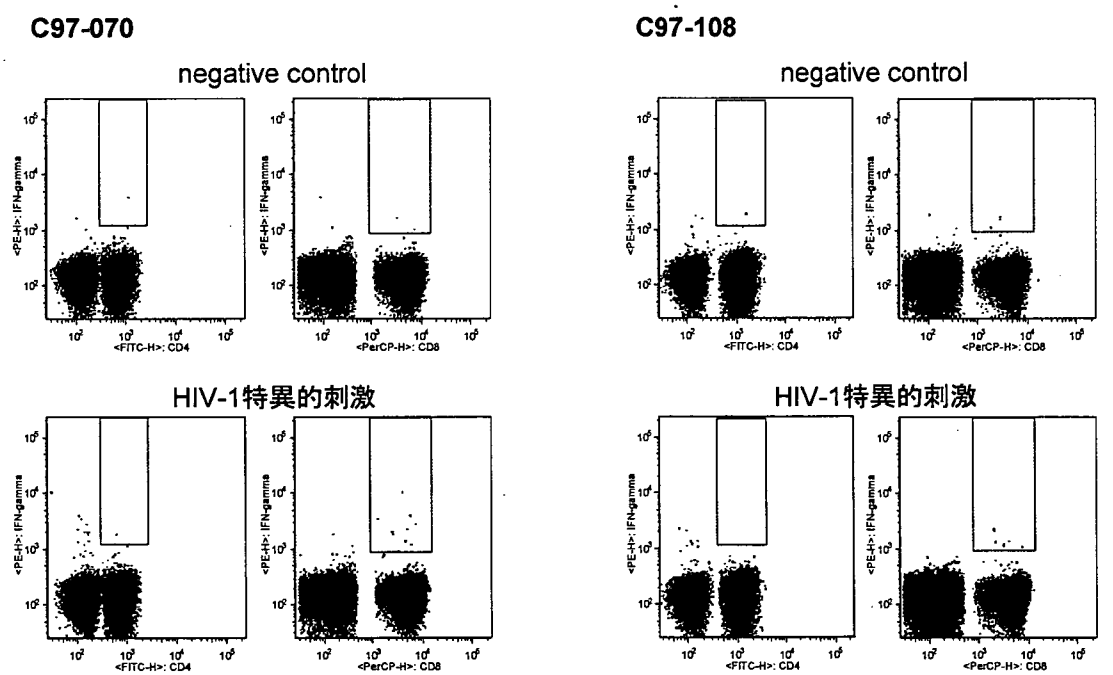

図2　HIV-1特異的Tリンパ球反応
チャレンジ後4週目の末梢血CD4（左パネル）およびCD8（右パネル）Tリンパ球における
HIV-1特異的IFN-γ誘導を示すdot plot

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
明里宏文	医学実験用霊長類を用いた病原体感染実験施設の管理運営におけるコンプライアンスとバイオセーフティ	獣医畜産新報JVM	60	641-645	2007
Hiyoshi, M., Suzu, S., Yoshidomi, Y., Hassan, R., Harada, H., Sakashita, N., Akari, H., Motoyoshi, K., Okada, S.	Interaction between Hckand HIV-1 Nef negatively regulates cell surfaceexpression of M-CSF receptor.	Blood	111	243-250	2007
Piroozmand, A., Yamamoto, Y., Khamsri, B., Fujita, M., Uchiyama, T., and Adachi, A.	Generation and characterization of APOBEC3G-positive 293T cells for HIV-1 Vif study.	J. Med. Invest.	54	154-158	2007
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足立昭夫、鎌田和弥、八町和樹、山下知輝、内山恒夫、野間口雅子	HIV-1の病原性とアクセサリー遺伝子.	蛋白質核酸酵素	52	1261-1267	2007
Kawada, M., Tsukamoto, T., Yamamoto, H., Takeda, A., Igarashi, H., Watkins, D.I., and Matano, T.	Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after non-sterile protection by acytotoxic T lymphocyte-based vaccine.	J. Virol.	81	5202-5211	2007
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Tsukamoto, T., Dohki, S., Ueno, T., Kawada, M., Takeda, A., Yasunami, M., Naruse, T., Kimura, A., Takiguchi, M., and Matano, T.	Determination of a major histocompatibility complex class I restricting simian immunodeficiency virus Gag241-249 epitope.	AIDS		in press	

Takeuchi, H., and Matano, T.	Host factors involved in resistance to retroviral infection.	Microbiol. Immunol.		in press	

Ⅳ．研究成果の刊行物・別刷

医学実験用霊長類を用いた病原体感染実験施設の管理運営におけるコンプライアンスとバイオセーフティ

明里宏文

要　約

近年の新興再興感染症の拡大は我が国のみならず世界的にも危急の問題となっており，この克服に向けた基盤的研究が精力的に国内外で進められている。特にその原因微生物による疾患の病態解明や予防治療法開発において，医学実験用霊長類を用いた動物モデル研究は不可欠となっている。本項では，このような研究を推進する上で必要な病原体感染実験施設におけるコンプライアンスについて，主にバイオセーフティの観点から概説したい。

序　論

感染症研究における霊長類を用いた動物モデルの意義

20 世紀初頭まで人類全体の脅威であった「伝染病」は，医学研究の進歩，特にワクチン開発および抗生物質の発見によりその多くが致死的疾病から免れることができるようになり，もはや脅威ではなくなったかに見えた。しかし，こうした予想に反して，現在では新たな感染症の脅威 - 新興再興感染症に人類は直面している。すなわち交通手段の高速化・グローバル化，一方では輸血や注射等非衛生的条件下における様々な医療行為等は，エンデミックな病原微生物の世界的規模での拡散を可能とした。AIDS はこの代表例としてあげられる。当初 AIDS はアフリカ一地方の風土病であったものが 1970 ～ 1980 年代に欧米に拡散し，現在もアジア・アフリカ諸国において感染者が増大の一途を辿っている。近年では，C 型肝炎，プリオン病（牛の海綿状脳症，人では変異型クロイツフェルト・ヤコブ病），SARS（重症呼吸器症候群），鳥インフルエンザなど枚挙にいとまがない。

このような新興再興感染症による脅威に立ち向かい制圧していくにあたっては，その原因微生物による疾患の病態やその発症機序を分子レベルで解明し，その研究成果を基に新規治療薬や有効なワクチンを開発していく事が期待される。このような基礎・応用研究を推進し，臨床現場に反映させるためには，動物モデルを用いた実験感染系の確立およびこれを用いた候補薬等の安全性・有効性評価に関する前臨床試験（いわゆるトランスレーショナルリサーチ）が不可欠である。

こうした動物モデル研究では通常マウスやラットなどの小動物が第一選択となる。ところが新興再興感染症における研究の場合，実際には病原体がこうした小動物に感染・発症しない場合が多く見られる。他方，人にもっとも近縁な動物であるサル類をモデル動物として用いることにより，原因病原体に感染し人で生じるような病態を再現できる場合が多く認められる。このため新興再興感染症研究における霊長類モデルの重要性は近年非常に高まっているのが実情である。こうした社会的要請から，独立行政法人医薬基盤研究所・霊長類医科学研究センターでは，実験用サル類を用いた各種感染症の動物モデル研究を実施している。これまでに，AIDS 研究を始めとしてプリオン病，結核，ウイルス肝炎，マラリア，デング熱など様々な感染症に関する重要な知見が得られている。

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当センター着任後，本来の研究テーマであるウイルス感染症モデル開発およびその基盤的研究を進める傍ら，霊長類感染症実験施設の管理運営という二足の草鞋を穿くことになりました。当初は様々な困難に直面しましたが，多くの方々からのサポートのおかげで，ようやくある程度の基礎ができつつあるところです。

実験用霊長類を用いる感染症モデル研究実施におけるコンプライアンス

感染症モデル研究（もちろんそれ以外の医科学研究も含めて）において実験用霊長類を用いるに当たっては，その特殊性から様々なコンプライアンスが求められ，さらに多くのリスクを伴うことから，その研究実施に当たってはサル類以外の選択肢がない，すなわちサル類を用いること以外での代替法がない場合で，かつその実施によりリスクを上回る重要性や成果が期待される場合に限られなければならない。関連する主なコンプライアンスを以下に示す。

■サル取扱いに関するコンプライアンス

①動物愛護管理法：特にサル類を医科学実験に用いる際には，動物福祉面や倫理上の観点から充分な配慮と厳密な規制が必要

②ワシントン条約（CITES）：サルおよびサル材料の輸出入制限

■遺伝子組換え微生物の取扱に関するコンプライアンス

①カルタヘナ議定書：生物多様性に関する国際条約に基づき制定

②遺伝子組換え生物等の使用規制法：遺伝子組換え微生物等の取扱い

■病原体に関するコンプライアンス

①改正感染症法：バイオテロ対策

②バイオセーフティ管理：病原体取扱における安全管理

■薬物，化学物質の取扱に関するコンプライアンス

①獣医師法，薬事法：動物医薬品の安全管理

②麻薬取締法：特に昨年度からの塩酸ケタミン麻薬指定

ここでは本題である医学実験用霊長類におけるバイオーセーフティ管理について解説する。それ以外のコンプライアンスについては他項の総説をご参照願いたい。

実験用霊長類を用いる感染症モデル研究実施におけるバイオハザードの可能性

感染症モデル動物としてサル類を用いるに当たっては，その人との近縁性からバイオハザード（生物学的災害）によるリスクを充分に認識しておくことが肝要である。

1）病原体取扱者（病原体研究者，動物作業従事者，獣医師，施設管理者を含む）のバイオハザード

①研究目的のためサル類に感染させた病原微生物に，誤って人が暴露することにより感染・発症する可能性が考えられる。本来，人に病原性を有する微生物でありバイオハザードのリスクは高いが，予め各実験ごとに事故時の対応法，治療法などのマニュアルを作成しておくことでリスク低減を図ることが可能である（後述）。

②サル類はその人との類似性から，サル・人共に病原性を有するいわゆる人獣共通感染症（ズーノーシス）の原因微生物に感染している可能性が考えられる。特にズーノーシスとして留意すべき病原体としてBウイルスが挙げられる。Bウイルスについては，以下のウェブサイトに詳述されている。

http://wwwsoc.nii.ac.jp/jsvs/05_byouki/infect/07-B-virus.html

http://idsc.nih.go.jp/idwr/kansen/k00-g45/k00_41/k00_41.html

概　説：Bウイルスはαヘルペスウイルスに分類され，アジア産マカクに広く感染している。ただしこれまでに人への病原性が報告されているものはアカゲザル由来ウイルスのみである。通常は神経節に潜伏感染し，実験操作や輸送によるストレスや免疫抑制により再活性化し唾液や排泄物中にウイルスを排出する。これが人への感染源となる。サルには口腔内潰瘍などの病原性を示す程度だが，人への感染は風邪様症状の後，時として神経症状を呈し死の転帰を辿る。これまでに40例ほどの死亡例が報告されている。神経症状発症までの抗ヘルペス薬服用が有効である。

③研究に供したサル個体が臨床的に健康であり，かつ既知の病原微生物に感染していない場合であっても，サル類自身には特段の病原性を発揮せずに自然感染している微生物が，種を越えて人に感染した際に強い病原性を呈する可能性は否定できない。また実験的処置による免疫抑制状態において，サル類に自然感染している非病原性微生物が活性化すると考えられることから，実験内容により予防的措置が必要である。またサル輸送にともなうストレスでも同様の可能性があることから，導入時の検疫措置は不可欠である。当センターでは，施設内サルの安全確保のため，海外からの輸入あるいは他の国内施設からの導入サル全頭に対して検疫を実施している。検疫は，それら対象動物がどのような病原体に感染しているか不明であるが故に，少なくともBSL2病原体に対応が可能な動物施設で行うことが

必要であろう。

2）サルにおけるバイオハザード

①研究目的のためサル類に感染させた病原微生物が他のサル個体に施設内感染する可能性は常に考慮しなければならない。特にエアロゾル若しくは空気伝播しうる病原微生物（例：結核菌，麻疹ウイルス）では，こうした水平感染を予防するための措置が必要となる。

②サル類が自然感染している微生物が，他の個体に感染した際に病原性を呈する場合。例えばマカク属サルに感染が見られるサル水痘ウイルス（SVV）やタイプDサルレトロウイルス（SRV-D）は感染性，病原性共に強く，時としてサルコロニーに甚大な被害を与えることが知られている。またリスザルを自然宿主としているヘルペスウイルスサイミリ（HVS）はリスザル自体には病原性を有さないが，マーモセットやタマリンといった他の新世界ザルへの感染により重篤なリンパ性白血病を呈することから厳重な注意が必要である。

③人自身が感染している病原体がサル類に伝播するといったケースについても想定が必要である。結核や麻疹，風疹，インフルエンザなどに病原体取扱者が罹患している場合，動物施設内への入室を禁止，制限しなければならない。

バイオセーフティ管理のための基本とその概要

サル類を用いた病原性微生物の感染実験を行うための実験施設を安全に管理運営するに当たっては，ひとたびバイオハザード事故が起こればその被害は計り知れないものになる恐れがあることを念慮し，日常よりバイオハザードを防止するための安全対策が充分なされなければならない。バイオセーフティ対策としては，米国CDC/NIHが推奨するバイオセーフティレベルに応じた動物実験室基準を参考にすることが一般的である（表1）。基本的には，ハードウエアとソフトウエアにより安全確保する。

1）ハードウエア概要

第一にハードウエア，すなわち実験施設および病原体の安全操作に必要な設備による病原体の拡散を物理的に封じ込めることがバイオセーフティの前提であり最も重要である。これらは建築構造体および空調設備，安全キャビネット，サル用アイソレータ，オートクレーブ等機器類の正確な動作に依存するため，定期的メンテナンスによる保守管理はもちろんのこと，老朽化を前提とした計画的な整備計画が事故防止に不可欠である。またBSL3病原体を取扱う実験室では自家発電装置を設け，停電時にも設備機器機能が維持されるように備えておくことが必要である。これは災害発生時における病原体取扱者の安全確保に特に重要である。実際，2005年米国南部を襲ったハリケーン（カトリーナ）によりツーレン国立霊長類研究センターも甚大な被害を被ったが，充分な燃料備蓄を保有した自家発電装置により実験用サル類への被害を食い止めることができている。

2）ソフトウエア概要

前述のハードウエアを充分に機能させ，安全な病原体取扱を行うためには，バイオセーフティのための様々なルールが病原体取扱者によって遵守されなければならない。また必要に応じて病原体取扱者間による情報交換・問題提起，改善の場を設けることが望ましい。

（1）病原体取扱に関する各種規則の制定

現在我が国では，病原体取扱に関する法規制はなく（今年度改正された感染症法はバイオテロ対策が目的であり，病原体のバイオセーフティ管理といった面は必ずしも考慮されていない），そのため各研究機関等ごとに自主的な規制を設けている。多くの場合は国立感染症研究所の病原体等安全管理規程を基に，各研究機関や施設の状況を加味して策定されている。特に施設管理責任者や各病原体取扱におけるバイオセーフティ管理者を置くなどの安全管理態勢を確立し，責任の所在を明確化することが重要である。また具体的な病原体取扱方法や実験室への入退室，防護装備の着脱滅菌法，機器操作手順および緊急時の対応法などについてはそれぞれ安全操作マニュアルや危機対応マニュアルの作成が必要である。なおバイオハザードのリスクが高いABSL3施設では，外部委員を含む安全監視委員会を設け施設運営管理に関する定期的な審査・査察を実施することにより，バイオセーフティの判断に客観性を持たせることが望ましい。ちなみに当センターのABSL3施設では，外部委員を含む安全監視委員会による年1回の定期的査察を実施している。

（2）バイオセーフティに関する教育（再教育）の実施

病原体取扱者は，上記の各種規則のみならず，取扱う病

表1　微生物実験室のバイオセーフティ（CDC/NIH 監修，4th edition，1999 年発行）

動物実験施設のバイオセーフティレベル 2（animal biosafety level 2：ABSL-2）基準

・動物用施設の管理者は，すべての操作および動物飼育舎への立入りの方針，手順およびプロトコルを確立しなければならない。
・職員用の適切な医療監視プログラムを設けなければならない。
・安全操作マニュアルを作成し，導入しなければならない。
・バイオハザード標示を扉および他の適切な場所に標示し，使用している感染性病原体を明示すること。
・施設は，掃除および維持管理が容易に行えるように設計しなければならない。
・扉は内側に開き，自動的に閉まる構造でなければならない。
・暖房，換気および照明は十分でなければならない。
・機械換気を行う場合，空気流は内側に向かっていなければならない。排気は屋外に排出し，建物のどの部分にも再循環しないこと。
・許可された者以外の立入りを制限しなければならない。
・実験用途以外の動物を入れないこと。
・節足動物およびげっ歯類防除対策を設けること。
・窓がある場合は，頑丈で耐破損性であり，開閉可能ならば節足動物を防ぐ網戸をはめなければならない。
・作業面の使用後は，効果的な消毒剤で汚染除去しなければならない。
・エアゾールの発生を伴う可能性のある作業には，HEPA フィルターを通して排気する安全キャビネット（クラス I または II），または隔離飼育ケージを設けなければならない。
・現場または近くにオートクレーブを用意しなければならない。
・動物用床敷きの材料は，エアゾールおよび粉塵の発生を最小限に抑えるような方法で取り除かなければならない。
・すべての廃材および床敷きは，廃棄する前に汚染除去しなければならない。
・鋭利な機器の使用は，可能な限り禁止すること。鋭利物は必ず，穴の開かない蓋付きの容器に回収し，感染性のものとして扱うこと。
・オートクレーブまたは焼却する材料は，閉めた容器で安全に輸送しなければならない。
・動物ケージは，使用後汚染除去しなければならない。
・動物の死体は，焼却しなければならない。
・施設内では防護衣および装置を着用し，退出の際に脱がなければならない。適切な手袋を用意し，着用すること。
・手洗い設備を設けなければならない。職員は，動物用施設を退出する前に手を洗わなければならない。
・すべての外傷は，どんなに軽微なものでも報告し記録しなければならない。
・施設内では飲食および化粧を禁じなければならない。
・すべての作業者は，適切な教育訓練を受けなければならない。

動物実験施設のバイオセーフティレベル 3（animal biosafety level 3：ABSL-3）基準

・ABSL-2 のすべての要件を満たさなければならない。
・立入りを厳密に管理しなければならない。
・扉を 2 つ備える部屋によって控え室を形成し，施設を他の実験室および動物舎から区画しなければならない。
・更衣室に手洗い設備およびシャワーを設けなければならない。
・全室に確実に連続した空気を流すため，機械換気を行わなければならない。排気は再循環させず，大気に放出する前に HEPA フィルターを通さなければならない。換気システムは，不測の逆流が生じず，動物舎のどの部分も昇圧しないように設計しなければならない。
・動物舎内の生物危害が収容されている場所に好都合の位置に，オートクレーブを用意しなければならない。感染性廃棄物は，施設の他の区域に移動される前にオートクレーブすること。
・BSL3 病原体に感染させた動物は，アイソレーター内のケージ，またはケージ後部に換気の排気装置を配置した部屋で飼育しなければならない。
・床敷きは，可能な限り粉塵がないようにすること。
・施設内では，実験室用防護衣を着用しなければならない。この防護衣は実験室外に退出する前に汚染除去しなければならない。
・窓は閉めて密閉し，耐破損性でなければならない。
・必要に応じて，職員に予防接種を行うこと。

原体ごとの性状（BSL分類，感染様式，病原性など）や滅菌方法などの基本知識を把握していることが不可欠である。またサル類を用いる動物実験を実施する場合には，サル類ごとの特性に基づく取扱い方法にも習熟していなければならない。このため，定期的なバイオセーフティ講習会を実施し病原体取扱者の受講（必要に応じてペーパーテストや実習を行う）を義務づけるようにする。なお新規病原体を用いようとする研究者は，施設管理者や動物技術者等への病原体に関する情報（不活化方法，リスク評価，事故時の対応法など）を公開し，充分な安全対策を図るようにする。

（3）特別定期健康診断の実施

前述の実験用霊長類を用いる感染症モデル研究実施におけるバイオハザードの可能性を考慮し，病原体取扱者に対して年1～2回程度の特別定期健康診断受診を義務化する。特に結核予防のため，胸部X線撮像による診断が不可欠である。取り扱う病原体により，医師による診察や抗体価測定検査を実施し，また必要に応じてワクチンの接種を勧告し，職員等も職務上必要と考えられる場合はワクチンの接種を要求できるようにする。さらに，健康診断の結果に基づき，健康管理上必要と認められる事項については病原体取扱者ごとに記録を作成し保存することが大切である。

なお病原体取扱に際しては，万が一の病原体への暴露事故が生じた場合にその病原体への感染有無を評価する目的で血清の採取・保存を実施する。血清の採取・保存は予め承諾を得た上で行い，その使用に当たっては，個人（退職者を含む）の健康障害の発生またはそのおそれのある場合に限り，バイオセーフティ委員会の承認の下で行うこととする。なお，これら健康診断，血清保存に関する病原体取扱者の個人情報については，充分かつ適切な保護がなされなければならない。

（4）運営会議の実施

病原体取扱者（特に施設管理者，飼育管理技術者，獣医師など施設運営実務者）間における情報交換・問題提起，改善の場として運営会議を定期的に設けることが望ましい。特にサル類を用いる病原体感染実験では，既に述べたようにバイオセーフティ以外にも様々なコンプライアンスが求められることから，実務者個人の当事者意識の向上が安全かつ健全な施設運営のために非常に重要である。

終わりに

本項では，バイオセーフティの観点から，医学実験用霊長類を用いた病原体感染実験施設の管理運営におけるコンプライアンスについて当センターにおける例を挙げながら概説した。特にバイオセーフティ管理は自主規制であることからそのルール徹底が難しく，また特殊施設であるがための維持管理経費も莫大なものとなる。しかしながら狂牛病やSARSにおける経緯からわかるように，経済立国である我が国にとって新興再興感染症の予防治療対策は必須のものであり，そのトランスレーショナルリサーチにおいて医学実験用霊長類を用いた病原体感染実験施設は今後さらにその重要性を増すものと思われる。

稿を終えるに当たり，当施設の管理運営にご協力いただいた疾患制御研究室および社団法人予防衛生協会の職員各位に深謝したい。

Interaction between Hck and HIV-1 Nef negatively regulates cell surface expression of M-CSF receptor

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Nef is a multifunctional pathogenetic protein of HIV-1, the interaction of which with Hck, a Src tyrosine kinase highly expressed in macrophages, has been shown to be responsible for the development of AIDS. However, how the Nef-Hck interaction leads to the functional aberration of macrophages is poorly understood. We recently showed that Nef markedly inhibited the activity of macrophage colony-stimulating factor (M-CSF), a primary cytokine for macrophages. Here, we show that the inhibitory effect of Nef is due to the Hck-dependent down-regulation of the cell surface expression of M-CSF receptor Fms. In the presence of Hck, Nef induced the accumulation of an immature under–N-glycosylated Fms at the Golgi, thereby down-regulating Fms. The activation of Hck by the direct interaction with Nef was indispensable for the down-regulation. Unexpectedly, the accumulation of the active Hck at the Golgi where Nef prelocalized was likely to be another critical determinant of the function of Nef, because the expression of the constitutive-active forms of Hck alone did not fully down-regulate Fms. These results suggest that Nef perturbs the intracellular maturation and the trafficking of nascent Fms, through a unique mechanism that required both the activation of Hck and the aberrant spatial regulation of the active Hck. (Blood. 2008;111:243-250)

Introduction

HIV-1 infections lead to the development of AIDS by causing progressive degeneration of the immune system.[1-3] The main cellular targets of HIV-1 are CD4$^+$ T cells and macrophages, and the depletion of CD4$^+$ T cells caused by an infection is suggested to account for many aspects of the pathogenesis of HIV-1.[1-3] Meanwhile, a number of studies have revealed the functional aberration of HIV-1–infected macrophages.[4,5] Infected macrophages showed an altered profile of the production of cytokine/chemokines[4] or migratory capacity,[5] which might contribute to the uncontrolled homeostasis of the immune system. Indeed, functional analyses of HIV-1 Nef protein have revealed that macrophages as well as CD4$^+$ T cells play an important role in the development of AIDS.

Nef is a 25- to 30-kDa protein with no enzymatic activity encoded by the HIV-1 genome.[6,7] Studies of HIV-1–infected patients have clearly demonstrated Nef to be a critical determinant of the development of AIDS: HIV-1 strains without an intact Nef gene were frequently isolated from nonprogressive long-term survivors.[8,9] Subsequent study of HIV-1 transgenic mice confirmed the pathogenetic activity of Nef: targeted expression of the entire coding sequence of HIV-1 in CD4$^+$ T cells and macrophages caused a severe AIDS-like disease in mice, which was completely abolished by the disruption of the Nef gene.[10] Importantly, only an amino acid substitution in the proline-rich (PxxP) motifs of Nef was sufficient to protect mice from the development of AIDS-like disease.[11] A number of studies have revealed that Nef interacts with a subset of cellular Src family tyrosine kinases, via the PxxP motifs.[12-15] The Nef PxxP motifs had an affinity for the Src homology (SH3) domain of Hck, Lyn, and possibly c-Src, but not of Fgr, Fyn, Lck, and Yes.[12-15] In particular, the interaction between the Nef PxxP motifs and the Hck SH3 domain was likely to be important, because the interaction caused the activation of Hck.[13-15] Indeed, a study with HIV-1 transgenic mice clearly demonstrated the importance of the Nef-Hck interaction for the development of AIDS: the appearance of the AIDS-like disease was significantly delayed when the HIV-1 transgenic mice expressing an intact Nef gene were crossed with an *hck*$^{-/-}$ background.[11] Given that Hck is expressed in macrophages but not in CD4$^+$ T cells,[16] the finding indicates that the Nef-Hck interaction in macrophages is at least in part responsible for the development of AIDS. However, little is known of the molecular mechanisms by which the Nef-Hck interaction contributes to the functional aberration of macrophages and the development of AIDS. The fact that Src kinases including Hck have both positive and negative roles in cell signaling pathways[16-19] makes it difficult to predict the functional consequences of the Nef-Hck interaction.

A well-characterized function of Nef is the down-regulation of the cell surface expression of CD4[6,7,20] or major histocompatibility complex class I (MHC I).[6,7,21-23] Nef accelerates the endocytosis of CD4,[20] the receptor for HIV-1,[1-3] which allows an efficient viral release from the host cells.[6,7] Nef reduces the level of the surface expression of MHC I through multiple mechanisms,[21-23] which diminishes the recognition of the infected cells by cytotoxic T cells.[6,7] However, these hallmark functions of Nef may not fully account for the functional significance of the Nef-Hck interaction,

Submitted April 17, 2007; accepted September 18, 2007. Prepublished online as *Blood* First Edition paper, September 24, 2007; DOI 10.1182/blood-2007-04-086017.

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because the down-regulation of CD4 or MHC I occurs even in the absence of Hck (ie, in $CD4^+$ T cells).[20-23] Meanwhile, we and others have recently identified the functions of Nef that are dependent on Hck.[24-26] Drakesmith et al demonstrated that Nef down-regulated the surface expression of HFE, an iron homeostasis regulator expressed on macrophages, which was abolished by a dominant-negative Hck.[24] Briggs et al demonstrated that Nef mimicked the cell growth–promoting activity of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that supports the proliferation and differentiation of monocyte/macrophages,[27] possibly through a mechanism that required Hck and the Stat3 transcription factor.[25] Nef might contribute to the survival of macrophages by mimicking GM-CSF receptor pathways, allowing long-term viral replication.[25] In contrast to the latter finding, we demonstrated that Nef inhibited the growth of human myeloid leukemia TF-1-fms cells mediated by macrophage colony-stimulating factor (M-CSF),[26] another cytokine essential for the proliferation and differentiation of monocytes/macrophages.[28] The growth inhibition of the cells correlated well with the impaired activation of the M-CSF receptor Fms,[26] which is a tyrosine kinase encoded by the proto-oncogene *c-fms*.[28] Impaired activation of Fms was also observed in human embryonic 293 cells coexpressing Nef and Hck, but not in cells expressing Nef alone or Hck alone.[26] Thus, these data indicated that Nef inhibited the activation of Fms through a mechanism that required Hck.

The functions of macrophages are distinctly regulated by M-CSF and GM-CSF,[27,28] as evidenced by the marked difference in the morphology of macrophages derived from these cytokines.[29] Moreover, these macrophages showed different profiles of the production of chemokines/cytokines.[29] Thus, it is possible that Nef affects the functions of macrophages by differently modulating the activities of M-CSF and GM-CSF, contributing to the uncontrolled immune system. However, little is known of the molecular mechanisms by which Nef differently modulates the activities of these cytokines, through the common target Hck. In this study, we therefore attempted to clarify how the Nef-Hck interaction caused the impaired activation of Fms.

Methods

Hematopoietic cell lines and culture conditions

Human myeloid leukemia TF-1 cells[30] were maintained with RPMI1640 medium supplemented with 10% FCS and 2 ng/mL recombinant human GM-CSF (rhGM-CSF; PeproTech, Rocky Hill, NJ). TF-1-fms cells,[31] which were obtained by introducing the plasmid pCEF-c-fms encoding the human *c-fms* gene into the TF-1 cells, were maintained with RPMI1640–10% FCS in the presence of 100 ng/mL rhM-CSF (a gift from Morinaga Milk Industry, Kanagawa, Japan) and 200 μg/mL G418 (Calbiochem, Darmstadt, Germany). TF-1-fms-Nef-ER cells[26] were obtained by introducing pEBB-Nef-ER-IRES-puro[32] into TF-1-fms cells, and maintained in the presence of rhM-CSF, G418, and 1.5 μg/mL puromycin (Sigma, St Louis, MO). The plasmid encoded the Nef-ER fusion protein composed of Nef (derived from the NL4–3 strain of HIV-1) and the hormone-binding domain of the murine estrogen receptor (ER).[32] In this system, Nef was basally inactive but it was induced to function by the estrogen analog, 4-hydroxytamoxifen (4-HT; Sigma).[32] We also established TF-1 cells expressing the Nef-ER fusion protein (TF-1-Nef-ER) by using the same plasmid. The transfection was performed with Lipofectin reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. Transfected cells were selected in media containing rhGM-CSF and puromycin, followed by limiting dilution to isolate stable clones. The expression of Nef-ER in these clones was determined by Western blotting[26] with anti-Nef rabbit antiserum obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD).[33] The cell growth was determined by colorimetric assay with MTT reagent (Sigma), and the absorbance of each culture was measured at 595 nm with a microplate reader (Thermo Electron, Vantaa, Finland). The expression of Fms on TF-1-fms-Nef-ER cells and that of GM-CSF receptors on TF-1-Nef-ER cells was analyzed on a FACSCalibur using Cell Quest Software (Becton Dickinson, Mountain View, CA).[26] Anti-Fms rat monoclonal IgG (clone 12-2D6; Zymed, South San Francisco, CA) was labeled with FITC using Fluorescein Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan). FITC-labeled anti–GM-CSF receptor α chain (clone 4H1) and PE-labeled anti–GM-CSF β chain (clone 1C1) were purchased from eBioscience (San Diego, CA).

Macrophages and nucleofection

Human peripheral blood samples were collected from adults donors after informed consent was obtained in accordance with the Declaration of Helsinki and based on a protocol approved by the Institutional Review Board of the Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. Monocytes were enriched from peripheral blood mononuclear cells by adherence to dishes for 1 hour. Macrophages were prepared by culturing the monocytes with RPMI1640 medium supplemented with 15% FCS and 100 ng/mL rhM-CSF for 5 to 7 days. The nucleofection with the Human Macrophage Nucleofector Kit and the Nucleofector II device (Amaxa, Cologne, Germany) was performed according to the manufacturer's recommendations. In brief, 5×10^5 macrophages were nucleofected with 5 μg plasmid and then cultured with Macrophage-SFM medium (Gibco, Grand Island, NY) supplemented with 15% FCS and 10 ng/mL rhGM-CSF for 8 to 12 hours. The nucleofected macrophages were cultured with GM-CSF, because M-CSF caused the down-regulation of Fms (Figure S2B,C). To identify the Nef-expressing macrophages, we used the pRc/CMV-CD8-Nef plasmid[34] encoding Nef (derived from the SF2 strain of HIV-1) fused to the extracellular/transmembrane regions of CD8. As a control, we used the plasmid encoding only those regions of CD8 (pRc/CMV-CD8).[34] The nucleofected macrophages were detached from the culture dishes using the enzyme-free cell dissociation buffer (Gibco), and then subjected to flow cytometric analysis on a FACSCalibur. Labeled antibodies used were PE-labeled anti-Fms (clone 3-4A4; Santa Cruz Biotechnology, Santa Cruz, CA), APC-labeled anti-CD8 (clone DK25; Dako, Glostrup, Denmark), and PE-labeled anti-CD4 (clone S3.5; Caltag, Burlingame, CA).

293 cell lines, transfection, and plasmids

Human embryonic kidney 293 cells (Invitrogen) were maintained with DME medium supplemented with 10% FCS. We also used 293 cells stably expressing Fms, both Fms and Hck, or CD4. 293-Fms cells were established by transfecting pCEF-c-fms[31] followed by the enrichment of Fms^{high} cells with a JSAN cell sorter (Bay bioscience, Kobe, Japan). 293-Fms/Hck cells were established by further transfecting a human Hck expression plasmid into the 293-Fms cells. For this purpose, Hck cDNA[35] cloned in the vector pIRES2-EGFP (Clontech, Mountain View, CA) was subcloned into pIRES-bleo3 (Clontech). An Hck^{high} clone was isolated from the transfected cells by Western blotting. 293-CD4 cells were established by transfecting pEneoMOS-CD4[36] followed by the enrichment of $CD4^{high}$ cells by the sorting. These cells were maintained with media containing 200 μg/mL G418 or 200 μg/mL phleomycin D1 (Invitrogen), or both. Transient transfection experiments with these 293 cell lines were performed essentially as described previously.[26] In brief, cells grown on a 12-well tissue culture plate were transfected with a total of 1.6 μg plasmid using LipofectAMINE2000 reagent (Invitrogen).

The transient expression of Fms was achieved with pCEF-c-fms. The transient expression of Hck was mostly achieved with Hck cDNA cloned in pcDNA3.1 (Invitrogen), except for the flow cytometric analysis in which Hck cDNA cloned in pIRES2-EGFP was used (Figure 2A). Based on an earlier report,[14] we also prepared constitutive-active (YF and AxxA) and kinase-dead (KE) forms of Hck by using QuikChange II Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA). The transient expression of Nef was achieved mostly with pRc/CMV-CD8-Nef,[34] the Nef of which was

derived from the SF2 strain of HIV-1. In a selected experiment (Figure 4A), we used Nef of the NL4-3 strain, as the mutants used in the analysis were derived from the strain. WL/AA, LL/AA, and AxxA mutants were provided by A. Adachi (University of Tokushima, Tokushima, Japan) and subcloned into the vector pRc/CMV-CD8. The M20A mutant[37] was also subcloned into this vector.

Western blotting, flow cytometry, and immunofluorescence with 293 cells

The preparation of total cell lysate and Western blotting were performed essentially as described.[26,38] In a selected experiment (Figure 2C), a monolayer of transfected 293 cells was treated with trypsin or control PBS buffer for 3 minutes at room temperature immediately prior to the lysis. Total cell lysate was also subjected to a lectin pull-down assay,[39] using wheat germ agglutinin (WGA)–agarose and concanavalin A (Con A)–agarose (both from Wako, Osaka, Japan). Alternatively, total cell lysate was treated with either endo-β-*N*-acetylglucosaminidase H (Endo-H) or peptide-*N*-glycosidase F (PNGase F) (both from Roche, Mannheim, Germany), according to the manufacturer's recommendations. Primary antibodies used were as follows: anti–N-terminal portion of Fms (H-300; Santa Cruz Biotechnology), anti–C-terminal portion of Fms (C-20; Santa Cruz Biotechnology), anti-Nef rabbit antiserum,[33] anti-Hck (clone 18; Transduction Laboratories, Lexington, KY), and anti-ERK (K-23; Santa Cruz Biotechnology).

The transfected cells were detached from the culture dishes and subjected to a flow cytometric analysis with anti–Fms-PE, anti–CD4-PE, or anti–CD8-APC as above. For immunostaining, cells were directly fixed in 2% paraformaldehyde, permeabilized with ethanol, and stained with primary antibodies for 12 hours followed by labeled secondary antibodies.[40,41] The primary antibodies used were as follows: anti-Fms rat IgG (clone 3-4A4-E4; Abcam, Cambridge, MA), anti-GM130 mouse IgG (Transduction Laboratories), anti-CD8 rabbit IgG (H-160; Santa Cruz Biotechnology), and rabbit IgG specific for Hck phosphorylated at Tyr411 (Santa Cruz Biotechnology). The labeled secondary antibodies used were as follows: anti–rat IgG-AlexaFluo488, anti–mouse IgG-AlexaFluo568, and anti–rabbit IgG-AlexaFluo488 (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (Molecular Probes). The fluorescent signals were visualized with a BZ-8000 fluorescence microscope (Keyence, Osaka, Japan) equipped with Plan-Fluor ELWD 20×/0.45 objective lenses (Nikon, Tokyo, Japan). Image processing was performed using BZ-Analyzer (Keyence) and Adobe Photoshop software (Adobe Systems, San Jose, CA).

Results

Nef selectively inhibits M-CSF–dependent growth and down-regulates Fms

In this study, we initially attempted to confirm the stimulatory effect of Nef on GM-CSF reported by another group,[25] using the same system in which we found the inhibitory effect on M-CSF.[26] We previously established human myeloid TF-1-fms cells expressing a conditionally active Nef-ER fusion protein.[26,32] Although TF-1-fms was an M-CSF–dependent clone derived from GM-CSF–dependent TF-1 cells,[30,31] TF-1-fms cells lost their growth response to GM-CSF due to long-term maintenance with M-CSF.[42] Thus, we also established TF-1 clones expressing the Nef-ER fusion proteins, the level of which was comparable with that in the pre-established TF-1-fms-Nef-ER clone (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The inducible activation of Nef by the estrogen analog 4-HT was verified by the down-regulation of CD4 expression (data not shown). As shown (Figure S1A,B) and consistent with the results of the other group,[25] the activation of Nef did not inhibit but enhanced the GM-CSF–dependent growth of TF-1-Nef-ER cells, albeit slightly. However, the activation of Nef markedly inhibited the M-CSF–dependent growth of TF-1-fms-Nef-ER cells (Figure S1A,C). These results confirmed that Nef did not actively induce the death of these cells but selectively inhibited the activity of M-CSF.

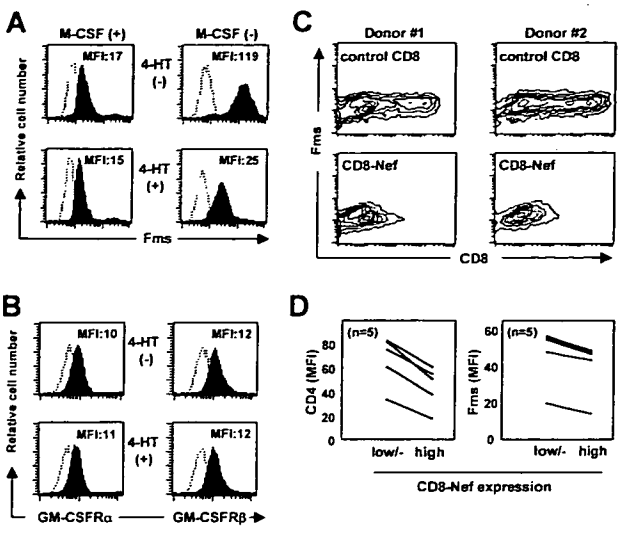

Figure 1. Nef inhibits surface expression of Fms. (A) In the left histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–containing media in the absence (upper) or presence (lower) of 0.1 mM 4-HT for 24 hours. In the right histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–free media in the absence (top) or presence (bottom) of 0.1 μM 4-HT for 12 hours. The expression of Fms on these cells was analyzed by flow cytometry with PE-labeled anti-Fms antibody. The mean fluorescence intensity (MFI) of Fms expression is indicated. (B) TF-1-Nef ER cells were precultured with GM-CSF–free media in the absence (top) or presence (bottom) of 0.1 μM 4-HT for 12 hours. The surface expression of GM-CSF receptors was analyzed with FITC-labeled anti-α chain (left) and PE-labeled anti-β chain (right) antibodies. The MFI of GM-CSF receptor expression is indicated. (C) Macrophages were nucleofected with the control CD8 plasmid or CD8-Nef plasmid and then costained with APC-labeled anti-CD8 and PE-labeled anti-Fms. Results with macrophages obtained from 2 different donors are shown as contour plots. (D) As in panel C, the nucleofected macrophages were costained with APC-labeled anti-CD8 and PE-labeled anti-Fms, or with APC-labeled anti-CD8 and PE-labeled anti-CD4. The MFI of the expression of Fms or CD4 in the populations of $CD8^{low/-}$, $CD8^{high}$, $CD8\text{-}Nef^{low/-}$, or $CD8\text{-}Nef^{high}$ was analyzed. The results with macrophages obtained from 5 different donors are summarized.

Next, we carefully examined whether Nef down-regulated the surface expression of Fms, as a possible mechanism for the selective inhibitory effect of Nef on the activity of M-CSF. In a previous study in which TF-1-fms-Nef-ER cells cultured under M-CSF–containing conditions were used, we failed to observe an obvious down-regulation of Fms expression by Nef.[26] However, the effect of Nef might have been underestimated under such conditions, because M-CSF itself down-regulated the expression by inducing the internalization/degradation of Fms.[43] Indeed, the addition of M-CSF caused the down-regulation of Fms in both TF-1-fms-Nef-ER cells (Figure S2A) and primary macrophages (Figure S2B) in a dose-dependent manner and an obvious effect of Nef on the surface level of Fms was not detected under such conditions (Figure 1A left panels). However, under the M-CSF–free Fms-high conditions, a significant reduction in the surface expression of Fms was observed in the Nef-active TF-1-fms-Nef-ER cells (Figure 1A right panels). The surface expression of CD29 (integrin β1), CD33, and CD54 (ICAM-1) was unaffected by the same treatment (data not shown). Furthermore, such down-regulation was not observed with the α chain and β chain of GM-CSF receptors (Figure 1B). Thus, the inhibitory effect of Nef on the activity of M-CSF but not of GM-CSF was likely to be due to the selective down-regulation of Fms expression.

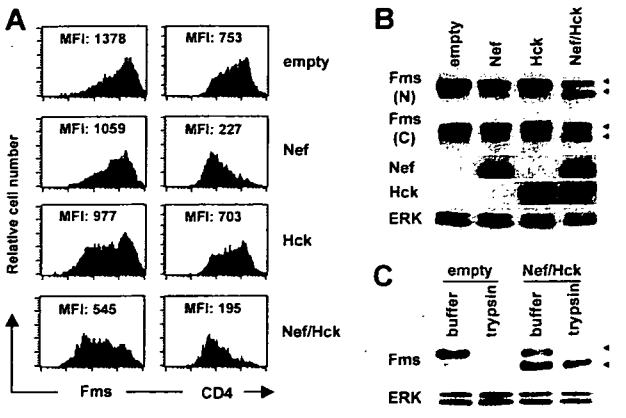

Figure 2. Nef reduces surface expression of Fms in 293 cells and increases intracellular gp130Fms, in the presence of Hck. (A) In the left panels, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef (CD8-Nef) or Hck (IRES-EGFP), and then stained with PE-labeled anti-Fms. In the right panels, 293 cells stably expressing CD4 were transfected with the indicated plasmids and stained with PE-labeled anti-CD4. These cells were costained with APC-labeled anti-CD8, and the data for cells positive for both CD8 and EGFP are shown. The MFI of the expression of Fms or CD4 is indicated. (B) As in panel A, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef or Hck. Total cell lysate was prepared and subjected to Western blotting with antibodies against the N-terminal portion of Fms (N), the C-terminal portion of Fms (C), Hck, Nef, or ERK. (C) 293 cells stably expressing Fms were cotransfected with Nef and Hck (Nef/Hck), or transfected with empty vectors (empty), and then treated with trypsin or control buffer. Total cell lysate was prepared and subjected to Western blotting with antibodies against the C-terminal portion of Fms or ERK. (B,C) The ERK blot is a loading control. The ◀ indicate the position of gp150Fms or gp130Fms.

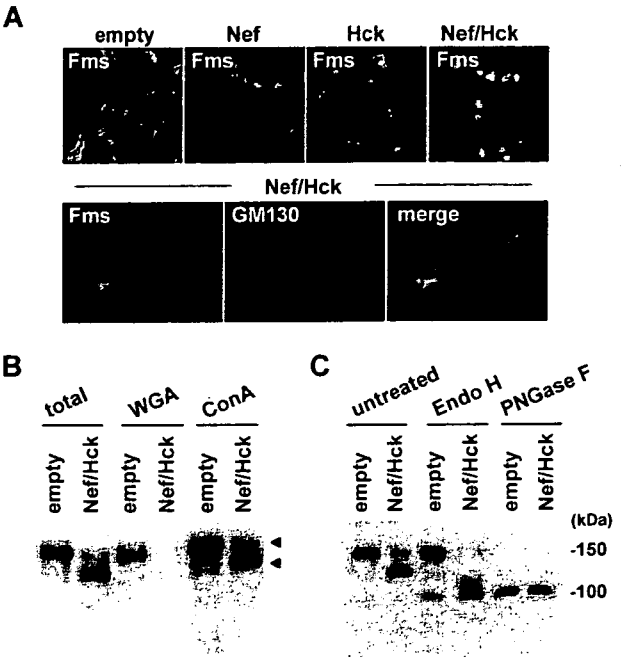

Figure 3. gp130Fms appearing in 293 cells coexpressing Nef/Hck is Golgi-localized underglycosylated Fms. (A) 293 cells stably expressing Fms were transfected with the control CD8 plasmid (empty) or CD8-Nef plasmid (Nef). Similarly, 293 cells stably coexpressing Fms and Hck were transfected with the control CD8 plasmid (Hck) or CD8-Nef plasmid (Nef/Hck). These cells were stained with anti-Fms antibody (top panels). In the bottom panels, 293 cells stably coexpressing Fms and Hck were transfected with CD8-Nef and costained with anti-Fms antibody (green), anti-GM130 antibody (red), and DAPI (blue). (B) 293 cells stably expressing Fms were cotransfected with Nef and Hck (Nef/Hck), or transfected with empty vectors (empty). The total cell lysate was subjected to Fms Western blotting directly (total) or after pull down with WGA-agarose or Con A–agarose. The arrowheads indicate the position of gp150Fms or gp130Fms. (C) Total cell lysate prepared as in panel B was subjected to Fms Western blotting directly (untreated) or after treatment with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F).

The novel function of Nef was further confirmed by nucleofecting Nef into human primary macrophages (Figure 1C,D). The purity of the macrophage preparations was usually more than 95% and 85% when assessed by the expression of CD14 and Fms, respectively (Figure S3). We used the CD8-Nef plasmid encoding Nef fused to the extracellular/transmembrane regions of CD8[34] to identify Nef-positive macrophages. The nucleofection of the control CD8 plasmid encoding only those regions of CD8 did not affect the expression of Fms (Figure 1C "control CD8" panels). In contrast, in the CD8-Nef–nucleofected macrophages, the Fms^{high} population was reduced as the expression of CD8-Nef increased (Figure 1C "CD8-Nef" panels). Such down-regulation of Fms as well as CD4 in the CD8-Nef^{high} population was reproducibly observed with macrophages derived from different donors (Figure 1D). The supernatant obtained from macrophages nucleofected with the CD8-Nef plasmid did not affect the level of Fms in TF-1-fms cells (data not shown), suggesting that production of M-CSF, if any occurred, was not involved in the Nef-induced down-regulation of Fms in macrophages.

Down-regulation of Fms by Nef is Hck-dependent and due to inhibition of intracellular maturation/trafficking of Fms

As both TF-1-fms cells[26] and macrophages[16] endogenously expressed Hck, it was possible that Hck was involved in the down-regulation of Fms caused by Nef. To examine this possibility and clarify the molecular mechanisms by which Nef down-regulated Fms, we next performed a transfection experiment using human 293 cells. As shown (Figure 2A left panels), the cotransfection of Nef and Hck markedly reduced the surface expression of Fms, although the transfection of Nef alone or Hck alone was effective to a certain degree. This was in contrast with the finding that the transfection of Nef alone was almost sufficient to reduce the surface expression of CD4 (Figure 2A right panels). The reduced surface expression of Fms was confirmed by Western blotting. As shown (Figure 2B), the amount of Fms species with a molecular weight of 150 kDa (gp150Fms, upper arrowhead) in the cells coexpressing Nef/Hck was obviously less than that in cells expressing Nef alone or Hck alone. Indeed, gp150Fms was the cell surface form of Fms, because the treatment of the cell surface with trypsin resulted in the loss of gp150Fms (Figure 2C). The trypsin-resistant gp150Fms might represent an intracellular pool of mature Fms that would be rapidly inserted into the plasma membrane. Interestingly, in parallel with the decrease in the expression of gp150Fms, an increase in the expression of a lower molecular weight species (130 kDa, lower arrowheads) was observed in the cells coexpressing Nef/Hck (Figure 2C). The 130-kDa species was a Fms-related product, because the 2 antibodies against the different portions of Fms (the N-terminus and C-terminus) detected the species (herein referred to as gp130Fms). In contrast to gp150Fms, gp130Fms was an intracellular form of Fms, because it was unaffected by the trypsin treatment (Figure 2C). Thus, the down-regulation of Fms observed in TF-1-fms-Nef-ER cells and macrophages was reproducible in 293 cells cotransfected with Nef and Hck, and associated with the increase of the intracellular gp130Fms.

To further characterize the intracellular gp130Fms that appeared in the cells coexpressing Nef/Hck, we next performed immunofluorescence microscopy. As shown (Figure 3A top panels; Figure S4 top panels), the pattern of Fms staining in the coexpressing cells was quite different from that in cells expressing Nef alone

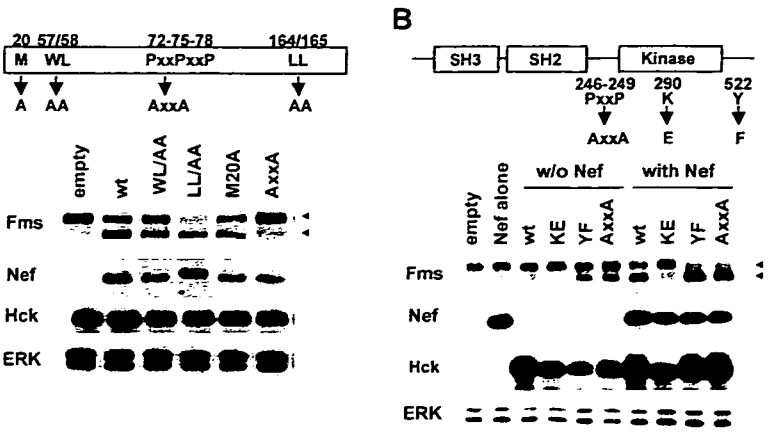

Figure 4. Activation of Hck by Nef is essential but not sufficient for accumulation of gp130Fms. (A) The Nef mutants used (M20A, WL/AA, AxxA, and LL/AA) are schematically shown. All the constructs are CD8-Nef chimeras. 293 cells stably expressing Fms were cotransfected with wild-type Hck and the plasmid indicated, and then analyzed for the expression of Fms, Nef, Hck, or ERK by Western blotting. (B) Schematic representations of Hck and the mutants used. KE is the kinase-dead form, whereas AxxA and YF are the constitutive-active forms.[14] 293 cells stably expressing Fms were transfected with empty vectors (empty), Nef plasmid (Nef), or the indicated Hck plasmid ("w/o Nef" lanes), or cotransfected with wild-type Nef and the indicated Hck plasmid ("with Nef" lanes). Then, the transfected cells were analyzed for the expression of Fms, Nef, Hck, or ERK by Western blotting. (A,B) The ERK blot is a loading control. The ◀ indicate the position of gp150Fms or gp130Fms.

or Hck alone. In a significant proportion of the cells coexpressing Nef/Hck, intense staining of Fms was detected in a perinuclear compartment (Figure 3A top Nef/Hck panel), which largely overlapped the signal for GM130, a marker for the Golgi apparatus[44] (Figure 3A bottom panels) and that for Vti1a, another Golgi marker[45] (Figure S4). Such intense staining of Fms in the perinuclear compartment was also detected in a few cells transfected with Nef alone or Hck alone (Figure 4A; Figure S4), which overlapped the signal for GM130 (Figure S4). Thus, it was highly likely that gp130Fms appeared in the coexpressing cells predominantly localized to the Golgi. As the N-glycosylation of many glycoproteins including Fms is known to be intimately linked with intracellular trafficking,[46-50] we then analyzed the state of the N-glycosylation of gp130Fms. For this purpose, we used 2 lectins, WGA and Con A, which recognize sialic acid and mannose, respectively.[39] As shown (Figure 3B), both gp150Fms and gp130Fms bound to Con A, whereas only gp150Fms bound to WGA, indicating that gp150Fms was modified with both mannose and sialic acid, but gp130Fms was not modified with sialic acid. Indeed, gp150Fms and gp130Fms showed similar electrophoretic mobility following the complete digestion of oligosaccharide groups by PNGase F, whereas only gp130Fms was sensitive to Endo-H, which selectively cleaves high-mannose type oligosaccharides (Figure 3C). These results suggested that the difference in their sizes was due to a difference in the N-glycosylation. Given that nascent Fms polypeptides are modified initially with mannose at the endoplasmic reticulum and terminally with sialic acid at the Golgi,[46-49] our results strongly suggested that the Nef/Hck-dependent accumulation of gp130Fms at the Golgi was due to the perturbation of intracellular N-glycosylation and/or trafficking of nascent Fms.

Down-regulation of Fms by Nef is dependent on activation of Hck and spatial regulation of active Hck

We next attempted to clarify the role of Hck in the down-regulation of Fms expression by Nef. Initially, we examined whether the direct interaction with Hck (Figure S5) was required for the function of Nef, using Nef mutants. As shown (Figure 4A), the AxxA mutant defective in the interaction with Hck[12] failed to down-regulate Fms, that is, the decrease of gp150Fms and the concomitant increase of gp130Fms. In contrast, the other 3 mutants still down-regulated Fms (Figure 4A). The WL/AA and LL/AA mutants, and the M20A mutant were shown to be defective in the down-regulation of CD4 and MHC I, respectively,[24,37] which was confirmed in our experimental system (data not shown). These results suggested that the down-regulation of Fms by Nef was mechanistically different from that of CD4 or MHC I, and dependent on the direct interaction with Hck. Thus, we next examined whether the activation of Hck by Nef was necessary and sufficient for the down-regulation of Fms, using Hck mutants. As shown (Figure 4B), Nef failed to down-regulate Fms when cotransfected with the kinase-dead KE mutant, but almost completely down-regulated Fms when cotransfected with the YF or AxxA mutant, both of which were the constitutive-active form. However, it should be noted that the transfection of these constitutive-active forms of Hck alone was not necessarily sufficient to achieve the full down-regulation of Fms (Figure 4B, see YF, AxxA, wt + Nef, YF + Nef, and AxxA + Nef lanes). These results clearly indicated that the activation of Hck was necessary but not sufficient for the Nef/Hck-induced down-regulation of Fms.

It has been shown that Nef distributes to the Golgi as well as the plasma membrane.[22,24] Indeed, intense signal of the CD8-Nef chimera was detected in the perinuclear compartment, which overlapped the signal for GM130 (Figure 5A). Thus, it was possible that the activation of Hck at the Golgi or the recruitment of the active Hck to the Golgi was another factor necessary for Nef to down-regulate Fms. To explore this possibility, we examined whether the active Hck in the Nef-expressing cells indeed localized to the Golgi and its existence at the Golgi correlated with the down-regulation of Fms. To detect the active Hck, we stained cells with the antibody specific for Hck phosphorylated at Tyr411, which was the major autophosphorylation site.[14] As shown (Figure 5B), an intense signal for the active Hck was indeed detected in the perinuclear compartment, in cells coexpressing Nef and wild-type Hck but not in cells expressing wild-type Hck alone (top panels), which largely overlapped the signal for GM130 (middle panels). Such colocalization of Nef and active Hck in the perinuclear compartment was also observed in macrophages nucleofected with the CD8-Nef plasmid (Figure S6). Moreover, the constitutive-active AxxA Hck tended to localize to the perinuclear compartment when expressed alone, and almost exclusively localized to the perinuclear compartment when coexpressed with Nef (Figure 5B bottom panels). Thus, the degree to which the active Hck accumulated at the Golgi correlated well with the observed down-regulation of Fms (Figure 4B). Taken together, these results suggest that the novel function of Nef (ie, the down-regulation of Fms expression by perturbing the maturation/trafficking of nascent Fms) is dependent on both the activation of Hck and the spatial regulation of the active Hck.

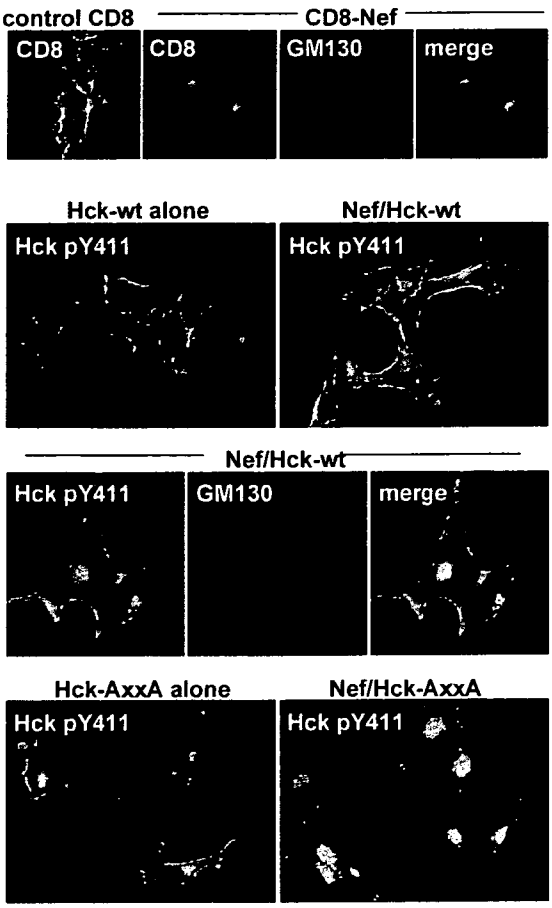

Figure 5. Nef induces Golgi localization of active Hck. (A) Parental 293 cells were transfected with the control CD8 plasmid or CD8-Nef plasmid, and then stained with anti-CD8 antibody (green), anti-GM130 antibody (red), or DAPI (blue). (B) In the top panels, parental 293 cells were transfected with wild-type Hck, or cotransfected with wild-type Hck and Nef, and then stained with the antibody specific for active Hck (ie, Hck phosphorylated at Tyr411). In the middle panels, parental 293 cells cotransfected with wild-type Hck and Nef were costained with anti-Hck pTyr411 antibody (green), anti-GM130 antibody (red), and DAPI (blue). In the bottom panels, parental 293 cells were transfected with the constitutive-active AxxA Hck (see Figure 4B), or cotransfected with the AxxA Hck and wild-type Nef, and then stained with anti-Hck pTyr411 antibody. See "Western blotting, flow cytometry, and immunofluorescence with 293 cells" for image acquisition information.

Discussion

In this study, we showed for the first time that Nef down-regulated the expression of Fms (Figures 1,2). The down-regulation was due to perturbation of the intracellular trafficking of nascent Fms (Figure 3), and likely to be a cause of the inhibitory effect of Nef on the activity of M-CSF because neither the activity of GM-CSF nor the cell surface expression of GM-CSF receptors was inhibited by Nef (Figure 1). Importantly, the present study strongly suggested that the down-regulation of Fms expression by Nef was due to a previously unreported mechanism that depended on both the activation of Hck and the aberrant spatial regulation of the active Hck (Figures 4,5).

The Nef-induced down-regulation of Fms was obviously mechanistically different from that of CD4 or MHC I in its dependence on Hck (Figures 2A,3A)[6,7,20-23] but appeared to resemble that of HFE. The Nef-induced down-regulation of HFE was abolished by either a mutation in the PxxP motifs of Nef or the overexpression of the dominant-negative Hck.[24] However, how Hck was involved in the Nef-induced down-regulation of HFE remains to be analyzed.[24] Interestingly, the YxxA motif in the cytoplasmic tail of HFE (^{342}YVLA) was shown to be required for Nef to down-regulate HFE.[24] The tyrosine-based YxxA motif was conserved in the kinase domain of Fms (^{873}YQMA, GenBank accession number P07333). However, when coexpressed with Hck, Nef also down-regulated a Fms mutant lacking the motif prepared by introducing the stop codon at ^{873}Y (data not shown). Thus, the mechanism for the Nef/Hck-induced down-regulation of Fms was likely to be somewhat different from that of HFE. Our earlier experiment revealed that gp130Fms was tyrosine phosphorylated in cells coexpressing Nef and Hck.[26] However, the ligand-independent tyrosine phosphorylation of Fms was not a direct cause of the down-regulation of Fms, because Nef also down-regulated a Fms mutant lacking the entire intracellular region when coexpressed with Hck (Figure S7).

The Nef/Hck-induced down-regulation of Fms was associated with the accumulation of the immature Fms at the Golgi (Figure 3). The experiment with Hck mutants clearly demonstrated that the activation of Hck was indispensable for the down-regulation of Fms (Figure 4B). The finding that Nef failed to down-regulate Fms when coexpressed with Lyn or Fgr (data not shown) further supported the conclusion, because Hck was the only Src kinase activated by Nef among Src kinases highly expressed in macrophages (ie, Hck, Lyn, and Fgr).[13-16] However, to our surprise, the activation of Hck was not the sole determinant of the down-regulation of Fms, because the expression of the constitutive-active Hck (YF or AxxA) alone was insufficient to fully achieve the down-regulation (Figure 4B). Our finding that the degree to which the active Hck accumulated at the Golgi correlated well with that of the down-regulation of Fms (Figures 4B,5B) strongly suggested that Nef down-regulated Fms through both the activation of Hck and the accumulation of the active Hck at the Golgi. The idea may answer why Hck, the downstream effector molecule important for the Fms signaling pathways,[38,50-53] is involved in the down-regulation of Fms by Nef.

A significant pool of Nef has been shown to localize to the Golgi.[22,24] Indeed, the CD8-Nef chimera used in this study localized to the Golgi as well as the plasma membrane (Figure 5A). This was not due to the fusion of the region of CD8 to the N-terminus of Nef, because the Nef-EGFP chimera, in which EGFP was fused to the C-terminus of Nef, also localized to the Golgi (data not shown). Thus, it was likely that the interaction with the Golgi-resident Nef or the recruitment of the active Hck led to the accumulation of the active Hck at the Golgi. However, it is unclear how this accumulation leads to a block of the intracellular trafficking of Fms in the same compartment. A plausible possibility might be direct interaction of the active Hck with Fms at the Golgi. Indeed, our earlier coimmunoprecipitation experiment revealed the formation of a molecular complex between Hck and Fms.[26] Meanwhile, it is known that the tyrosine located in the juxtamembrane domain of Fms (Y561 in human and Y559 in murine) serves as a binding site for Src kinases including Hck when the residue is autophosphorylated.[51-54] However, when coexpressed with Hck, Nef also down-regulated a Fms mutant in which the tyrosine residue was replaced with phenylalanine (data not shown). Thus, the active Hck at the Golgi may interact with Fms via unidentified site(s) or form complexes with Fms indirectly. Another possibility might be an alteration of the Golgi structure caused by the accumulation of the active Hck at the compartment. Recent studies revealed that Src kinases including Hck were present on the Golgi membrane as well as the plasma membrane.[55-57] The importance of the Golgi-localized Src kinases for the maintenance of the Golgi structure was clearly demonstrated by the finding that SYF

fibroblasts lacking the 3 ubiquitous Src kinases (Src, Yes, and Fyn) exhibited an aberrant morphology of the Golgi with collapsed stacks and bloated cisternae.[58] Interestingly, it was also demonstrated that the exogenous expression of the constitutive-active Src (E378G) in the SYF cells affected the distribution of some if not all Golgi-specific proteins.[58] Thus, it is possible that the accumulation of the active Hck affects the structure of the Golgi and thereby perturbs the trafficking of Fms.

A study with HIV-1 transgenic mice has clearly proved the importance of the interaction of Nef with Hck in macrophages for the development of AIDS.[11] Nevertheless, the functional consequences of the Nef/Hck interaction are not fully understood. The activation of Hck induced by the direct interaction with Nef is basically thought to cause the activation of macrophages, which may favor the replication of HIV-1. Indeed, Komuro et al demonstrated that the expression of Hck at a high level in macrophages correlated well with high titer replication of HIV-1.[59] Moreover, Briggs et al raised the possibility that the Nef-Hck interaction caused the activation of the Stat3 transcription factor, thereby mimicking the signaling pathway of the GM-CSF receptor.[25] However, the present study revealed that the Nef/Hck interaction also played a negative role: the molecular interaction caused the down-regulation of Fms and inhibition of the activity of M-CSF, which is likely to be due to the aberrant spatial regulation of the active Hck. The differential modulation of the activities of GM-CSF and M-CSF by Nef may alter the profile of production of cytokine/chemokines in HIV-1–infected macrophages, contributing to the development of AIDS. Future studies will clarify whether small compounds specifically targeting the Nef-Hck interaction prevent the progression of the disease. Moreover, a detailed mechanistic analysis of the unique function of Nef will help us to understand how Fms and Src kinases tightly regulate the signaling pathways and functions of macrophages.

Acknowledgments

We thank Y. Endo for secretarial assistance.

This work was supported in part by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan (S.O., S.S.).

Authorship

Contribution: M.H. and S.S. were responsible for the overall experimental work and design; Y.Y. and H.A., for DNA cloning; R.H., for Western blotting; H.H., for flow cytometry; N.S., for immunofluorescence; K.M. and S.O., for project planning and data analysis.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.

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