tor and drugs elevating extracellular adenosine on erythropoietic recovery following 5-fluorouracil-induced haematotoxicity in mice. Eur J Haematol 2000;65:310–6.

[20] Zong Y, Zhou S, Fatima S, Sorrentino BP. Expression of mouse Abcg2 mRNA during hematopoiesis is regulated by alternative use of multiple leader exons and promoters. J Biol Chem 2006;281:29625–32.

[21] Jonker JW, Buitelaar M, Wagenaar E, van der Valk MA, Scheffer GL, Scheper RJ, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci USA 2002;99:15649–54.

[22] Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem 2004;279:24218–25.

[23] Yin T, Li L. The stem cell niches in bone. J Clin Invest 2006;116:1195–201.

[24] Suda T, Arai F, Hirao A. Hematopoietic stem cells and their niche. Trends Immunol 2005;26:426–33.

[25] Schranzhofer M, Schifrer M, Cabrera JA, Kopp S, Chiba P, Beug H, et al. Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. Blood 2006;107:4159–67.

[26] Nakajima O, Takahashi S, Harigae H, Furuyama K, Hayashi N, Sassa S, et al. Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload. EMBO J 1999;18:6282–9.

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Heat shock suppresses human NK cell cytotoxicity via regulation of perforin

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Abstract

Human natural killer (NK) cell, which is an important lymphocyte for immune surveillance, is highly sensitive to heat, but the nature of its response to and its mechanistic regulation by heat remain unclear. Here we determined the effect of *in vitro* heat shock and *in vivo* hyperthermia on human NK cell cytotoxicity. Human peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers were subjected to heat shock *in vitro* (42°C, 1 h). PBMC from cancer patients receiving intentional hyperthermia (42°C, 1 h) for cancer therapy were also obtained. NK cytolytic activity was determined in these samples. NK cell cytotoxicity was down-regulated by heat shock *in vitro* at 5 h, but at 24 h after heat shock, the NK cytotoxicity was comparable to that with its respective control. Furthermore, we observed that the mRNA and protein expression levels of perforin, which is the cytolytic granule of NK cells, were regulated by heat shock in a similar manner as NK cytotoxicity at 5 h and at 24 h after heat shock. Heat regulation involved the perforin protein in $CD56^{dim}$ but not in $CD56^{bright}$ NK cell subset. Heat shock neither induced cell death nor altered the expression of some NK activating receptors and adhesion molecules. Moreover, whole-body hyperthermia at 42°C for 1 h of cancer patients also suppressed the cytotoxicity of NK cells but recovered to basal level 1 week after hyperthermia. Heat shock *in vitro* and *in vivo* temporarily represses the cytotoxicity of human NK cells.

Keywords: *Heat shock, human NK cells, NK cell cytotoxicity, $CD56^{dim}$ NK subset, perforin*

Introduction

Hyperthermia is used in conjunction with other established cancer therapy for the treatment of malignant disease [1]. With increased accordance on the close connection between cancer and immune system, many studies have been focused on the understanding of hyperthermia-induced immune responses (reviewed in [2]). In this context, substantial attention has been given to natural killer (NK) cell, a large lymphocyte that functions as important innate immune defense against viral-infected and tumor cells. Human NK cells can be divided into two subsets based on the cell-surface density of CD56: the $CD56^{dim}$ and $CD56^{bright}$ NK subset. These two subsets exhibit distinct phenotypic and functional properties. $CD56^{dim}$ cells, which are the majority of human NK cells (~90%), contain more cytolytic granules, such as perforin, and are

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naturally more cytotoxic than $CD56^{bright}$ NK cells but produce negligible amount of cytokines [3, 4]. On the other hand, $CD56^{bright}$ NK cell is an immunoregulatory subset that functions as major producer of NK-derived cytokines [3].

Although NK cells have been demonstrated to be highly sensitive to heat [5, 6], and that thermal stress *in vitro* reduces NK cell lytic function [7, 8], the actual mechanism underlying heat-regulation of NK cells remain ambiguous. We have previously demonstrated that the murine NK cell cytotoxicity was suppressed after heat shock through the down-regulation of perforin [9]. However, considering that human and mouse NK cells are different from each other in terms of their functional subsets [10], we considered it relevant and necessary for clinical applications to investigate the effect of heat shock on human NK cells. Here we demonstrate that heat shock continuously suppressed cytotoxicity of human NK cells until 24 h by down-regulating perforin mRNA and protein expression. Heat shock regulation of perforin protein selectively occurs in $CD56^{dim}$ NK cell population. The temporary suppression of NK cell activity was also observed in tumor patients receiving hyperthermia. Our findings clarify to a certain extent one of the possible mechanisms of hyperthermia regulation of human NK cell function.

Materials and methods

Cell lines

The erythroleukaemia cell line, K562, was obtained from RIKEN Cell Bank (Tsukuba, Japan). EGFP-transfected K562 (EGFP-K562) cells were established as described previously [11]. The cells were cultured in RPMI1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Biowest, France), 50 IU/ml penicillin and 50 μg/ml streptomycin (GIBCO). Cells were maintained at 37°C in 5% CO_2.

Isolation and culture of peripheral blood mononuclear cells and NK cells

Human peripheral blood samples were collected from adult donors after informed consent was obtained. Investigations were carried out in accordance to ethical standards authorized by the ethics committee of the Kumamoto University Graduate School of Medical Sciences or the ethics committee of Luke Hospital (Nakano, Tokyo). Peripheral blood mononuclear cells (PBMC) were isolated using lymphocyte separation medium (LSM, MP Biomedicals, OH). NK cells were isolated from freshly prepared PBMC by CD56 MicroBeads using magnetic cell-sorting technique (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The binding of CD56 monoclonal antibody has been shown not to affect NK cell cytotoxicity [4].

Hyperthermia

In vitro hyperthermia was performed by immersing the cells in 42°C water bath for 1 h. Cells were immediately cooled down in an ice bath before the medium was changed, and were re-incubated at 37°C. Control cells were left in the incubator at 37°C.

For whole-body hyperthermia, patients were anaesthetized during the procedure, which utilized a far-infrared radiation heat device (RHD2002 and RHS7500; Enthermics Medical Systems Inc., WI) [12]. The temperature was maintained at 42–43°C locally in the hyperthermic chamber and 41.5–42°C systemically (rectal temperature) for 1 h as described previously [13]. Peripheral blood samples were collected before, and at 3 h and 1 week after treatment from eight patients receiving intentional hyperthermia therapy for malignant tumors.

Flow cytometry

For cell surface expression analysis, 1×10^6 cells were stained with the following monoclonal antibodies: flourescein isothiocyanate (FITC)-conjugated anti-CD2 CD94, CD58 (BD PharMingen, San Diego, CA), PE-conjugated anti-NKG2D, NKp46 (Beckman Coulter, Fullerton, CA), CD3 (Caltag, Burlingame, CA), APC-conjugated anti-CD56 (Beckman Coulter), PECy7-conjugated anti-CD3 (eBioscience, San Diego, CA). Cells were incubated on ice for 30 min, washed twice and resuspended in staining medium. For staining of intracellular perforin, cells were fixed with 1% paraformaldehyde for 10 min at room temperature after staining of cell surface molecules, permeabilized with 0.1% saponin for 10 min at room temperature and stained with anti-perforin-FITC (eBioscience) for 30 min on ice. Cells were washed twice with permeabilization buffer and once with staining medium. Data analysis was performed using FACSCalibur flow cytometer and Cell Quest Software (Becton Dickinson).

Cytolytic assay

Using freshly prepared PBMCs isolated from healthy donors, the NK activity in PBMC against EGFP-K562 cells was measured by flow cytometry with propidium iodide (PI) as described before [11] with some modifications. Specifically, non-heat shock control cells were kept at 37°C while heat shocked samples were placed in water bath maintained at 42°C for 60 min. Heat shock-treated cells were then transferred to 37°C incubator.

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After 5 h or 24 h, cells were used for cytolytic assay. For the assay, mixtures of effector-target cells at effector-to-target (E:T) ratios of 160 : 1 and 40 : 1 were incubated at 37°C for 4 h. Total of 50 µl of 2 µg/ml PI was added with 15 min incubation. GFP and PI were measured by flow cytometry. Cytolytic activity was calculated as described previously [11], that is: % Lysis = A/(A + B) × 100 − C (%), where A is the percentage of PI^+EGFP^+ cells; B is the percentage of PI^-GFP^+ cells at each E/T ratio; C is the percentage of spontaneous PI^+ cells without effector cells (A/(A + B) × 100 (%) at E/T ratio = 0).

Cell death assay

Detection of apoptotic cells was performed using flow cytometric analysis of Annexin V (Annexin V-PE; BD Biosciences) stained cells according to the manufacturer's instructions. Briefly, cells were first stained with anti-CD3-PC7 (Caltag) and anti-CD56-APC (Beckman Coulter) for 30 min on ice. Cells were washed with cold PBS and resuspended in Annexin V binding buffer (0.01 M Hepes, 0.14 M NaCl, 2.5 mM $CaCl_2$). Annexin V-PE and Viaprobe (7-AAD; BD Biosciences) were added into 1×10^5 cell suspension and incubated for 15 min at room temperature. Fluorescence intensity of Annexin V and Viaprobe was analyzed in $CD3^-CD56^+$ cells.

Real time quantitative-polymerase chain reaction (Q-PCR)

NK cells were purified from PBMC using magnetic beads (Miltenyi Biotec, Auburn, CA). This positive selection with anti-CD56 microbeads has no experimental problems at least for the real time Q-PCR experiment, because it is well-known that CD56 does not contribute cellular function in human NK cells [14]. Total RNA was isolated from NK cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using iScript cDNA Synthesis kit (Bio-Rad Laboratories) and real-time quantitative PCR was performed using iQ SYBER GreenSupermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Real time PCR was carried out at 95°C for 3 min, then 40 cycles of denaturation (95°C for 10 s) and annealing/extension (60°C, 1 min). The sequences of perforin primers were described previously [15] and GAPDH (internal control) primers were as follows: GAPDH (up) 5′-CGGGAAGCTTGTGATCAATGG3′ and GAPDH (down) 5′-GGCAGTGATGGCATG GACTG-3′.

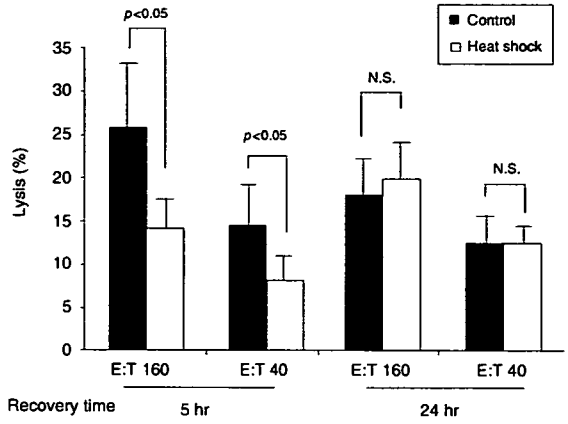

Figure 1. Heat shock temporarily suppresses cytotoxicity of human NK cells *in vitro*. PBMC obtained from healthy donors were heat shock treated at 42°C for 1 h or maintained at 37°C (control). Five or twenty-four hours after heat shock, the heat-shocked and control PBMC were each co-cultured with EGFP-transduced K562 cells at 37°C for 4 h followed by 15 min incubation with PI to assess for the cytolytic activity of PBMC. Effector : Target (E:T) ratios were 160 : 1 and 40 : 1. After the incubation, the percentages of PI^+ cells (dead cells) within $EGFP^+$ cells were analyzed with flow cytometry, and cytotoxicity was calculated as described in the Section, 'Methods'. Percentage means of specific lysis ± S.E. from three donors are displayed. *P* was assessed by paired *t*-test. N.S. = not significant.

Statistical analysis

The different groups were tested for statistical significance using paired two-tailed Student's *t* test. Value of $p < 0.05$ was considered to be statistically significant.

Results

Heat shock continuously suppresses cytotoxicity of human NK cells in vitro *until 24 h*

Heat-treated (1 h, 42°C) and control (37°C) PBMC from three healthy donors were recovered at indicated times and the cytotoxicity against a highly susceptible NK target, the K562 cell line, was determined by flow cytometric analysis. We observed that at 5 h after heat shock, NK cytotoxicity was dramatically reduced in heat-treated human primary PBMC compared with nonheat shock control. However at 24 h, NK cytotoxicity in heat-treated PBMC was comparable to the control group (Figure 1). These results suggest that heat shock suppresses NK cytotoxicity and that this suppression is temporary and reversible.

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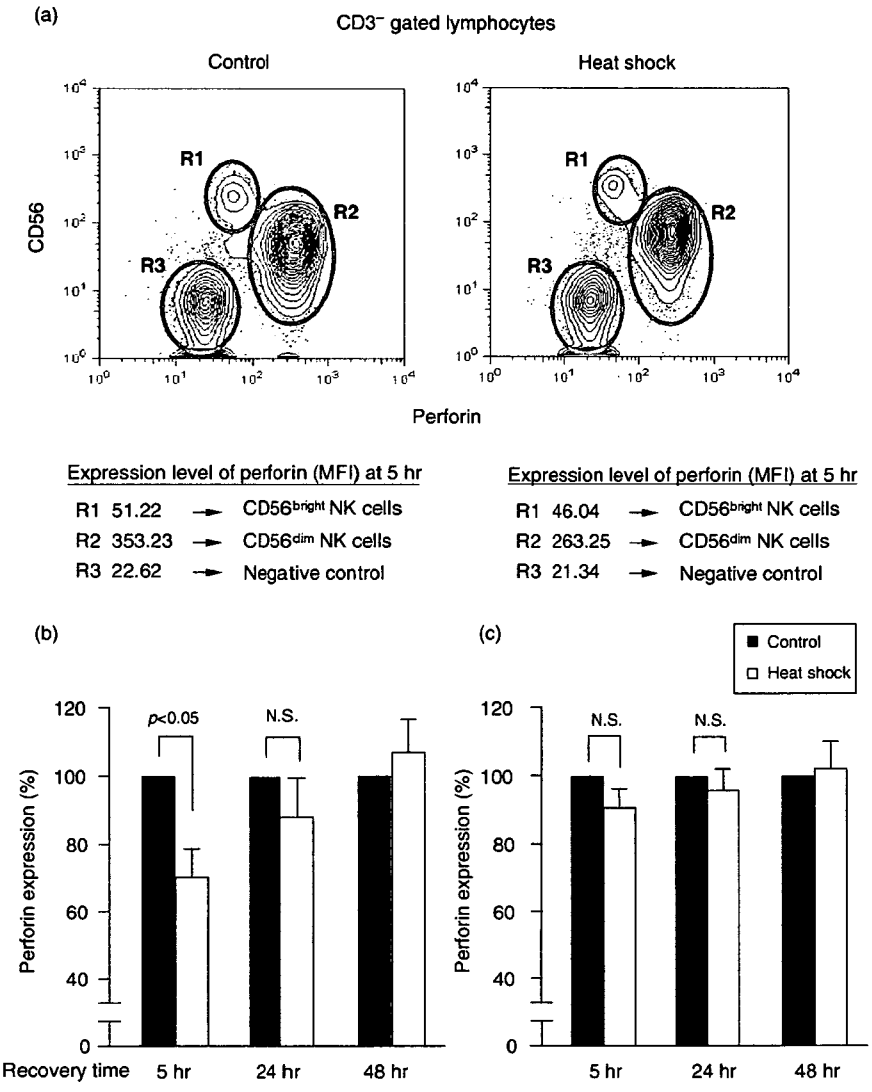

Figure 2. Heat shock down-regulates perforin expression on $CD56^{dim}$ NK cells. (a) PBMC were maintained at 37°C or heat shock treated at 42°C for 1 h and were recovered at 5, 24, and 48 h. Cells were stained with anti-CD56-APC and anti-CD3-PE. Then cells were fixed, permeabilized, and stained with anti-perforin-FITC. Lymphocytes were gated for $CD3^-$ population and perforin expression in different lymphocyte subpopulations was determined by flow cytometry. $CD3^-CD56^-$ populations (B cells) were used as negative controls. Non-specific binding was not observed with isotype control mAbs (data not shown). Percentage means of perforin expression ± S.E. ($n = 3$) in heat-treated and control of (b) $CD56^{dim}$ NK cells and (c) $CD56^{bright}$ NK cells at the indicated times are presented. P was assessed by paired t-test. N.S. = not significant. MFI = Mean fluorescence intensity.

Heat shock regulates perforin expression selectively in $CD56^{dim}$ NK cells

Because perforin is the key mediator of NK-mediated cell killing [16, 17], we investigated using PBMC from three healthy donors, the effect of heat shock on perforin expression in the two subsets of NK cells, $CD56^{dim}$ and $CD56^{bright}$ NK cells, at various recovery times. Heat-treated and control NK cells were tested for the expression of intracellular perforin by flow cytometry. Interestingly, heat shock predominantly affected perforin expression in $CD56^{dim}$ NK cells, which is a highly cytotoxic subset (Figure 2(a)). Perforin expression in $CD56^{dim}$ NK cells treated with heat shock was significantly reduced compared with control at 5 h after heat shock. At 24 h, the expression level of perforin in heat shock-treated cells was slightly but not significantly lower than that of the control cells (Figure 2(b)) and at 48 h after heat shock, a complete

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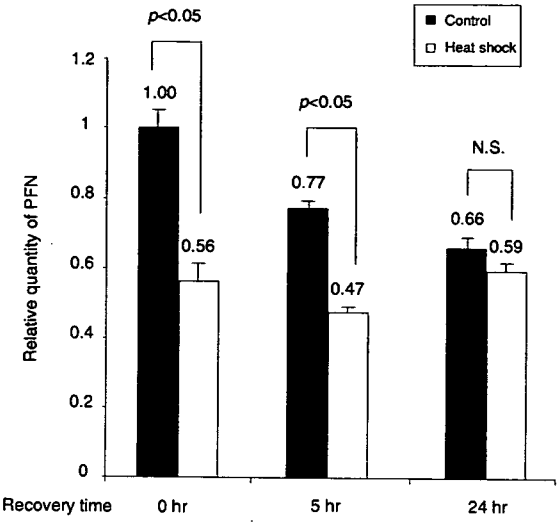

Figure 3. Heat shock regulates perforin mRNA expression in NK cells. Primary NK cells were heat-shock treated or kept at 37°C (control). Total RNA was recovered from control and treated cells at 0, 5, and 24 h. RNA extracts were subjected to real time Q-PCR using perforin and GAPDH primers. Quantity of perforin mRNA normalized to GAPDH is shown as mean ± S.E. ($n = 3$).

recovery of perforin expression was observed in the treated cells. On the other hand, there was no significant difference in the perforin expression of $CD56^{bright}$ NK cells between control and heat shock-treated cells at various time points (Figure 2(c)). Therefore, heat shock seemed to modulate perforin protein in $CD56^{dim}$ but not in $CD56^{bright}$ NK cell subset.

Heat shock regulates perforin mRNA expression in NK cells

We determined whether the down-regulation of perforin by heat shock was at the transcriptional level. As shown in Figure 3, the relative quantity of perforin mRNA in heat-treated purified NK cells was significantly suppressed immediately (0 h) and at 5 h after heat shock compared with their respective controls, indicating that heat shock affects the transcription of perforin in NK cells. When the recovery time was extended to 24 h, the quantity of perforin mRNA in NK cells subjected to heat shock was comparable to that of the control group (Figure 3), consistent with the findings on perforin protein expression and cytolytic activity.

Heat shock does not induce cell death and down-regulation of surface molecules

We next asked whether the profound decrease of NK cell activity and suppression of perforin could be due to cell death. We performed cell death analysis by flow cytometry with Annexin V and 7-AAD staining on untreated and heat-treated PBMC from four donors. At 5 h after heat shock, we did not observe a significant difference in the percentage of apoptotic NK cells between the control and heat-treated groups (Figure 4(a)). Moreover, both groups were undergoing cell death at spontaneous level. These data suggest that heat shock at 42°C for 1 h did not induce cell death in NK cells.

The balance of signal transmitted from cell surface activating and inhibitory receptors could determine NK cell activity [8]. Thus, we investigated the potential relationship between heat shock and expression of NK cell receptors and adhesion molecules. Heat-treated and control PBMC from three donors were stained with anti-CD56, anti-CD3, and the indicated antibodies, and were analyzed by flow cytometry. However, we observed that at 5 h after heat shock, expression levels of CD94, activating receptors (NKG2D, NKp46) and adhesion molecules (CD58, CD2) in heat-treated cells did not significantly differ from those of the control group (Figure 4(b)), suggesting that heat shock did not significantly affect the expression of these cell surface receptors in NK cells.

Whole-body hyperthermia temporarily suppresses cytotoxicity of NK cells

The cytotoxicity of NK cells before and after whole-body hyperthermia was evaluated in blood samples from eight cancer patients undergoing hyperthermia therapy at 41.5–42°C for 1 h. There was a significant decrease of NK cell cytotoxicity after 3 h treatment but it reverted to basal level after 1 week (Figure 5). These results reflected those of *in vitro* heat shock (Figure 1) and support our observations that NK cell activity is temporarily down-regulated by heat shock.

Discussion

In this article, we have shown that at 5 h after heat shock treatment, the cytotoxicity of human NK cells was suppressed but at 24 h after heat shock, the NK cytolytic activity of heat shock-treated cells was similar to that of its respective control. Moreover, we observed a similar temporary suppression of NK cell activity in tumor patients receiving hyperthermia. The inhibition of human NK cell-mediated cytotoxicity by hyperthermia has also been reported by other researchers [7, 18–21], however, these studies did not clearly establish the mechanism underlying the regulation of NK cell cytotoxicity by heat. In our previous work, we showed that cytotoxicity of NK cells from whole-body heat-shocked mice was suppressed via down-regulation of perforin expression [9]. However, mouse NK biology differs

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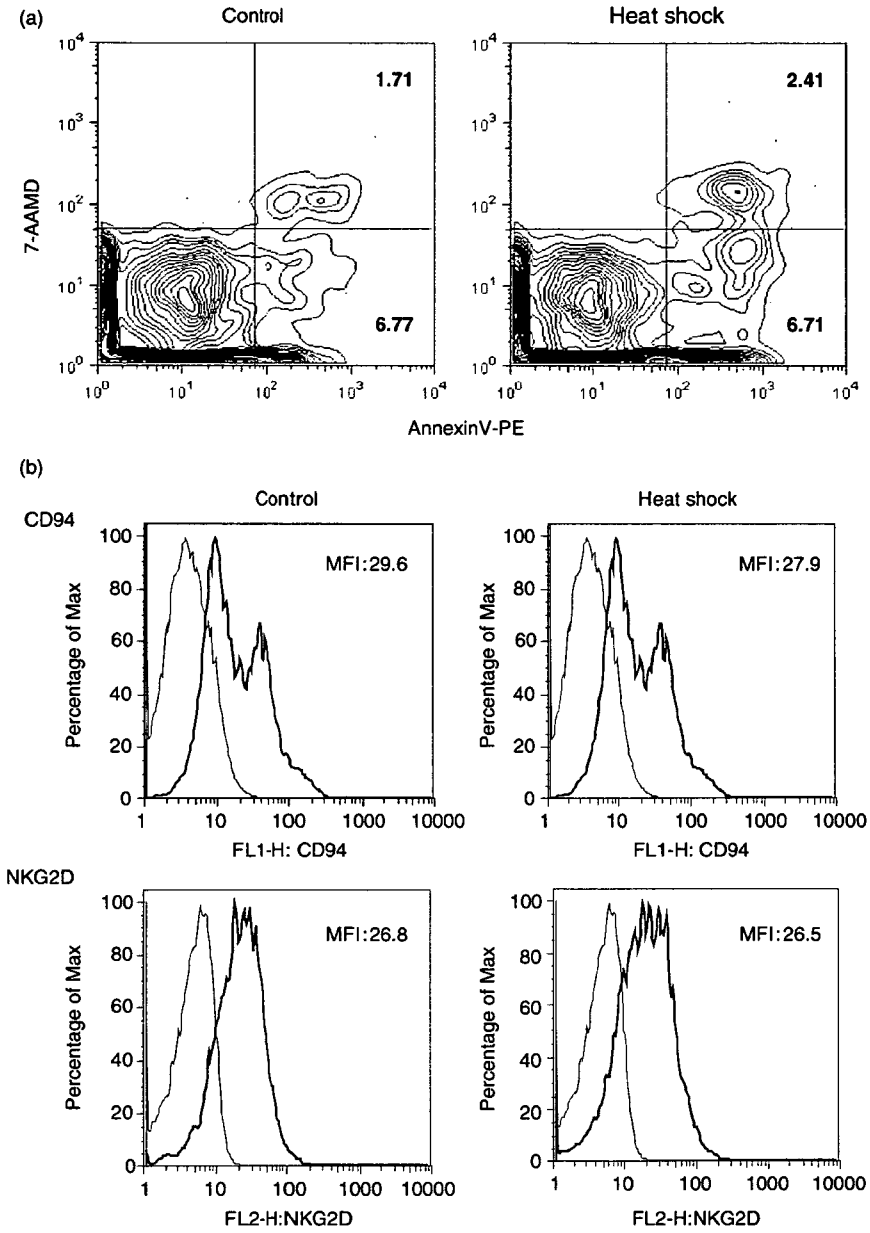

Figure 4. Heat shock does not induce cell death and down-regulation of surface molecules. (a) PBMC were recovered at 5 h after heat shock, stained with anti-CD56-APC and anti-CD3-PECy7, Annexin V-PE (for apoptotic cells) and 7-AAMD (for dead cells). NK ($CD3^{-}CD56^{+}$) cell population was analyzed by the fluorescence intensities of Annexin V and 7-AAMD. Numbers at the right side of each panel indicate percentages of each fraction. Representative data from four donors are shown. (b) Heat-treated NK cells in (a) were analyzed for the expression of indicated surface molecules with FITC-or PE-conjugated mAbs. Thick lines indicate the cell surface receptor expression with or without heat shock and thin lines indicate the isotype-matched controls. Representative data was obtained from three donors.

in certain aspects to human NK biology, notably, the lack of murine homolog of CD56 subsets [22]. Hence, we have undertaken the investigation of the effect of heat shock on human NK cells. In the present article, we show that the suppression of human NK cytotoxicity by heat shock involved the perforin protein expression in $CD56^{dim}$ NK cell subset (Figure 2), a novel finding that could not be noted in mouse model [9]. But although we have shown that heat shock predominantly affected the expression of perforin in $CD56^{dim}$ NK cell subset, the actual mechanism of how heat shock

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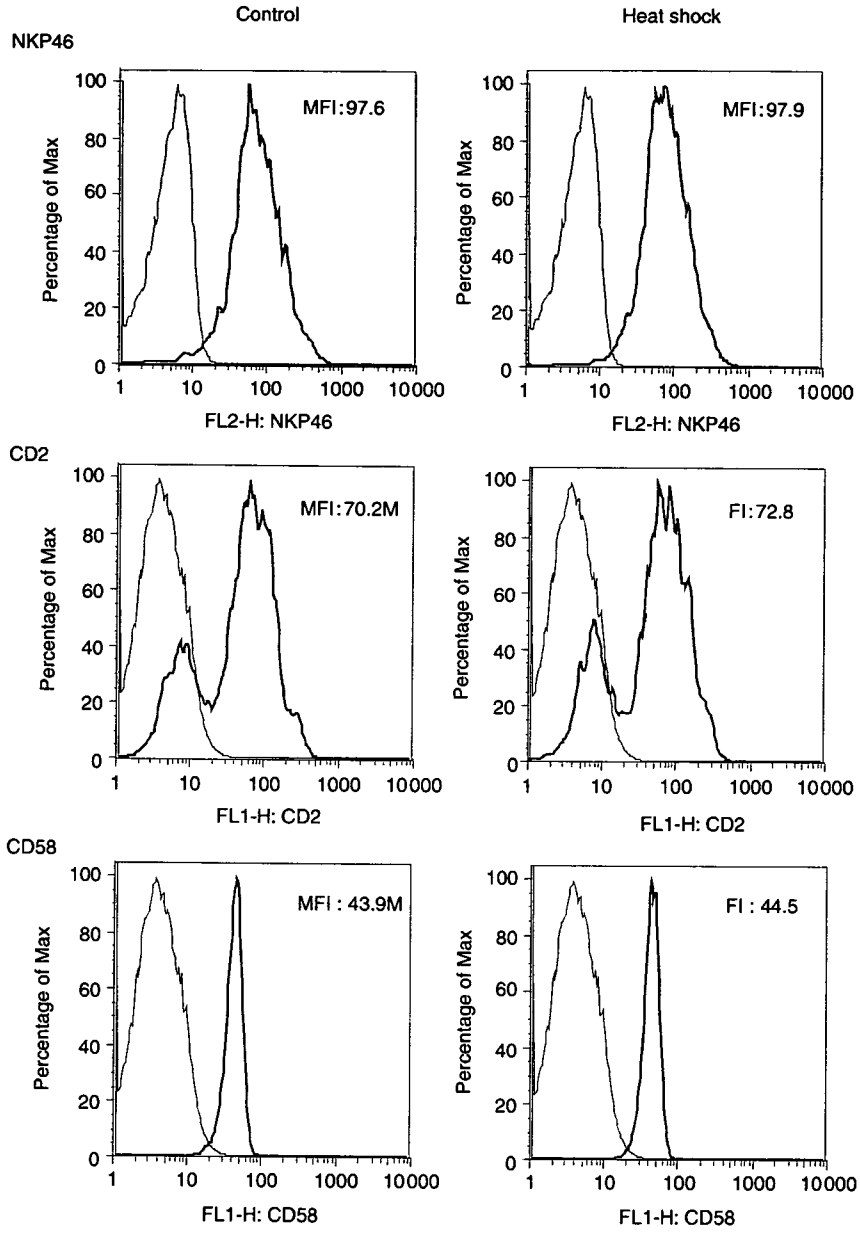

Figure 4. Continued.

preferentially regulates perforin in $CD56^{dim}$ NK cells remains to be elucidated. Further studies into the selective regulation by heat on subsets of human NK cells may provide insight into the distinct functional properties of these human NK subsets in heat-regulated immune response.

We observed that the cytolytic activity was fully recovered in heat shock-treated cells after 24 h but the level of perforin protein at this time point in treated cells was slightly lower than that of nonheat shock control (Figure 2(b)). However, the difference in perforin expression between control and heat shock-treated cells was not significant and the level of perforin in the treated cells at 24 h might be enough for the NK cells to be fully functional. Nonetheless, we cannot totally rule out the possibility of a perforin-independent-pathway [23].

The initial suppression of NK cytotoxicity by heat shock was not attributed to the reduction of cell count caused by cell death or to an altered expression of the major NK cell receptors and adhesion

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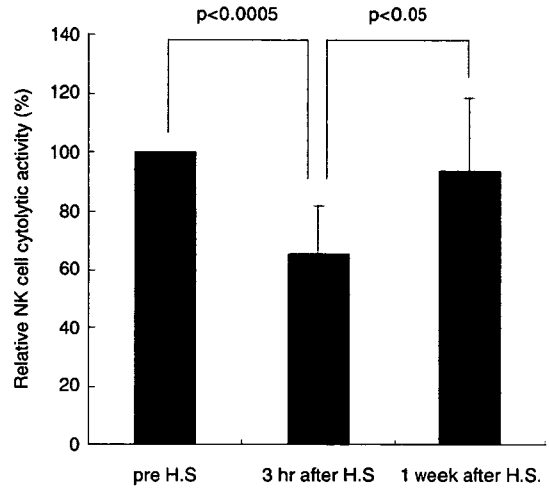

Figure 5. Whole-body hyperthermia temporarily suppresses cytotoxicity of NK cells. PBMC from donor patients were recovered before, and at 3 h and 1 week after hyperthermia. Mixtures of effector-target cells were incubated at 37°C for 4 h followed by 15 min incubation with PI. Cytotoxicity was determined by flow cytometry as described above. Percentage means of specific lysis are displayed ($n=8$). P was assessed by paired t-test.

molecules (Figure 4). Surface density of natural cytotoxicity receptors (NCRs) on NK cells has been reported to correlate with the magnitude of NK cytotoxicity against target cells [24]. However, the heat regulation of NK cytotoxicity might not involve these surface receptors since there were no alterations by heat on these molecules. As shown by Yang et al., even though NK cells were completely inactivated for target cell lysis at a high-thermal condition of 44°C, they maintained their recognition and binding functions [20].

Finally, the purpose of this study was to accumulate knowledge about the effects of heat treatment on NK cells. We anticipate that further investigations will yield a deeper understanding on the response of the immune system to hyperthermia, which could further improve current hyperthermia modalities.

Acknowledgments

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References

1. Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, Felix R, Schlag PM. Hyperthermia in combined treatment of cancer. Lancet Oncol. 2002;3:487–497.
2. Baronzio G, Gramaglia A, Fiorentini G. Hyperthermia and immunity. A brief overview. *In vivo* (Athens, Greece) 2006;20:689–695.
3. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633–640.
4. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986;136:4480–4486.
5. Azocar J, Yunis EJ, Essex M. Sensitivity of human natural killer cells to hyperthermia. Lancet 1982;1:16–17.
6. Onsrud M. Effect of hyperthermia on human natural killer cells. Recent Results Cancer Res. 1988;109:50–56.
7. Fuggetta MP, Alvino E, Tricarico M, D'Atri S, Pepponi R, Prete SP, Bonmassar E. *In vitro* effect of hyperthermia on natural cell-mediated cytotoxicity. Anticancer Res. 2000;20:1667–1672.
8. Yang H, Lauzon W, Lemaire I. Effects of hyperthermia on natural killer cells: Inhibition of lytic function and microtubule organization. Int. J. Hyperthermia 1992;8:87–97.
9. Koga T, Harada H, Shi TS, Okada S, Suico MA, Shuto T, Kai H. Hyperthermia suppresses the cytotoxicity of NK cells via down-regulation of perforin/granzyme B expression. Biochem Biophys Res Commun 2005;337:1319–1323.
10. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol 2006;176:1517–1524.
11. Harada H, Suzu S, Ito T, Okada S. Selective expansion and engraftment of human $CD16^+$ NK cells in NOD/SCID mice. Eur J Immunol 2005;35:3599–3609.
12. Robins HI, Dennis WH, Neville AJ, Shecterle LM, Martin PA, Grossman J, Davis TE, Neville SR, Gillis WK, Rusy BF. A nontoxic system for 41.8°C whole-body hyperthermia: Results of a Phase I study using a radiant heat device. Cancer Res 1985;45:3937–3944.
13. Sawaji Y, Sato T, Takeuchi A, Hirata M, Ito A. Anti-angiogenic action of hyperthermia by suppressing gene expression and production of tumour-derived vascular endothelial growth factor *in vivo* and *in vitro*. Br J Cancer 2002;86:1597–1603.
14. Lanier LL, Chang C, Azuma M, Ruitenberg JJ, Hemperly JJ, Phillips JH. Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). J Immunol 1991;146:4421–4426.
15. Shacklett BL, Cox CA, Quigley MF, Kreis C, Stollman NH, Jacobson MA, Andersson J, Sandberg JK, Nixon DF. Abundant expression of granzyme A, but not perforin, in granules of $CD8^+$ T cells in GALT: Implications for immune control of HIV-1 infection. J Immunol 2004;173:641–648.
16. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SE, Yagita H, Takeda K, van Dommelen SL, Degli-Esposti MA, Hayakawa Y. Activation of NK cell cytotoxicity. Mol Immunol 2005;42:501–510.
17. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer 2002;2:850–861.
18. Yoshioka A, Miyachi Y, Imamura S. Immunological effects of in vitro hyperthermia. J Clin Lab Immunol 1989;29:95–97.

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19. Kalland T, Dahlquist I. Effects of in vitro hyperthermia on human natural killer cells. Cancer Res 1983;43:1842–1846.
20. Yang HX, Mitchel RE. Hyperthermic inactivation, recovery and induced thermotolerance of human natural killer cell lytic function. Int J Hyperthermia 1991;7:35–49.
21. Shen RN, Lu L, Young P, Shidnia H, Hornback NB, Broxmeyer HE. Influence of elevated temperature on natural killer cell activity, lymphokine-activated killer cell activity and lectin-dependent cytotoxicity of human umbilical cord blood and adult blood cells. Int J Radiat Oncol Biol Phys 1994;29:821–826.
22. Hayakawa Y, Huntington ND, Nutt SL, Smyth MJ. Functional subsets of mouse natural killer cells. Immunological Rev 2006;214:47–55.
23. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nature Rev 2002;2:735–747.
24. Sivori S, Parolini S, Marcenaro E, Castriconi R, Pende D, Millo R, Moretta A. Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. J Neuroimmunol 2000;107:220–225.

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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)

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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,

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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

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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.

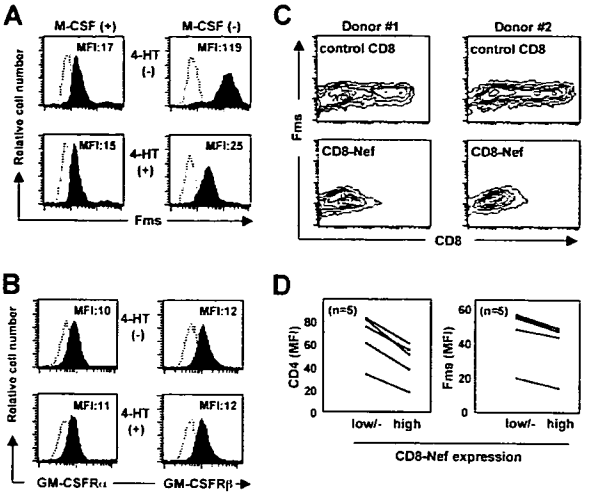

Figure 1. Nef inhibits surface expression of Fms. (A) In the left histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–containing media in the absence (upper) or presence (lower) of 0.1 mM 4-HT for 24 hours. In the right histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–free media 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.

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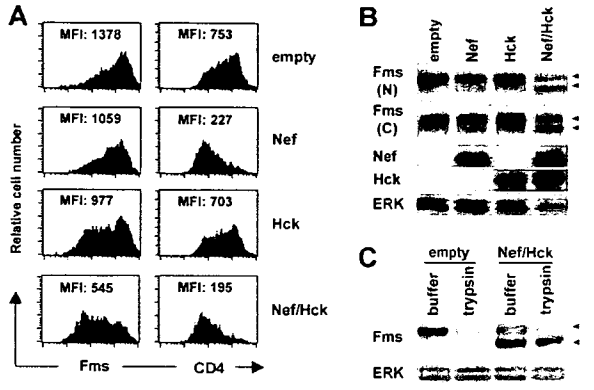

Figure 2. Nef reduces surface expression of Fms in 293 cells and increases intracellular gp130Fms, in the presence of Hck. (A) In the left panels, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef (CD8-Nef) or Hck (IRES-EGFP), and then stained 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.

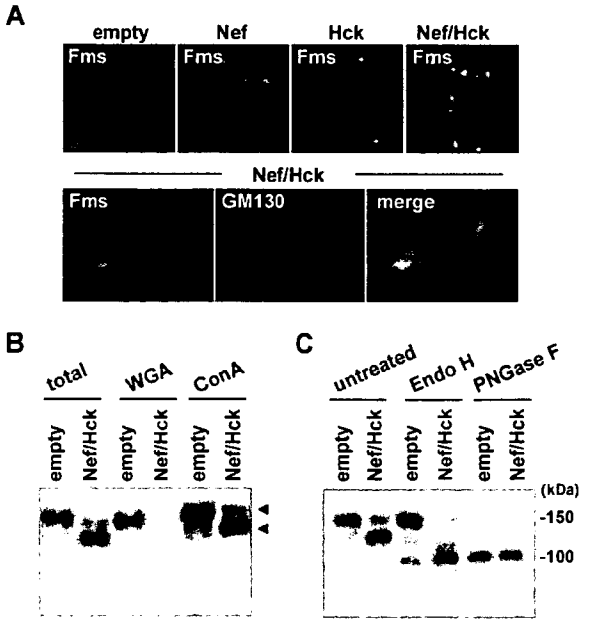

Figure 3. gp130Fms appearing in 293 cells coexpressing Nef/Hck is Golgi-localized underglycosylated Fms. (A) 293 cells stably expressing Fms were transfected with the control CD8 plasmid (empty) or CD8-Nef plasmid (Nef). Similarly, 293 cells stably coexpressing Fms and Hck were transfected with the control 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

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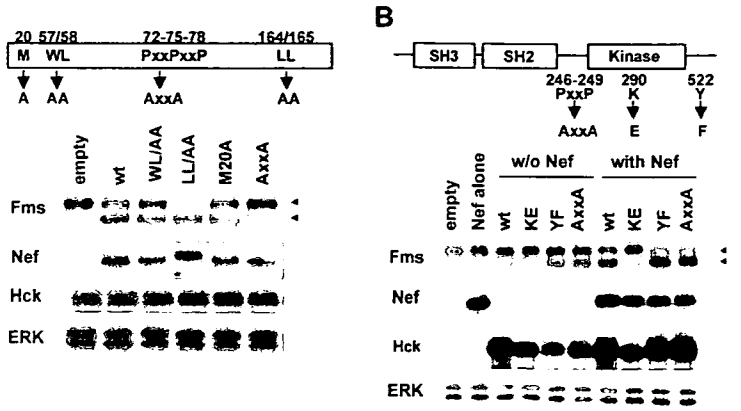

Figure 4. Activation of Hck by Nef is essential but not sufficient for accumulation of gp130Fms. (A) The Nef mutants used (M20A, WL/AA, AxxA, and LL/AA) are schematically shown. All the constructs are CD8-Nef chimeras. 293 cells stably expressing Fms were cotransfected with wild-type Hck and the plasmid 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.

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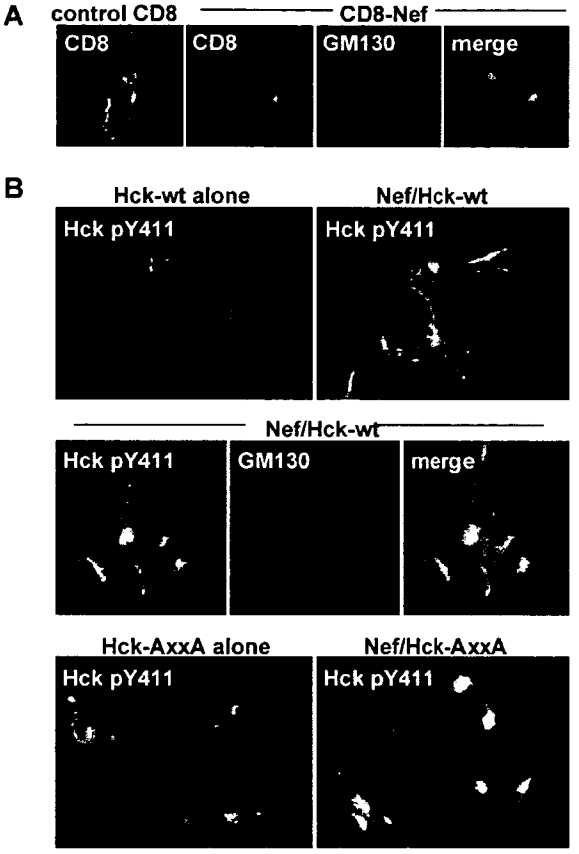

Figure 5. Nef induces Golgi localization of active Hck. (A) Parental 293 cells were transfected with the control CD8 plasmid or CD8-Nef plasmid, and then stained with anti-CD8 antibody (green), anti-GM130 antibody (red), or DAPI (blue). (B) In the top panels, parental 293 cells were transfected with 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

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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.

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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.

M.H. and S.S. contributed equally to this study.

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References

1. Pantaleo G, Fauci AS. Immunopathogenesis of HIV infection. Annu Rev Microbiol. 1996;50:825-854.
2. Stevenson M. HIV-1 pathogenesis. Nat Med. 2003;9:853-860.
3. Letvin NL, Walker BD. Immunopathogenesis and immunotherapy in AIDS virus infections. Nat Med. 2003;9:861-866.
4. Kedzierska K, Crowe SM. Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother. 2001;12:133-150.
5. Maslin CLV, Kedzierska K, Webster NL, Muller WA, Crowe SM. Transendothelial migration of monocytes: underlying molecular mechanisms and consequences of HIV-1 infection. Curr HIV Res. 2005;3:303-317.
6. Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. Immunity. 2002;16:493-497.
7. Peterlin BM, Trono D. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol. 2003;3:97-107.
8. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med. 1995;332:228-232.
9. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science. 1995;270:988-991.
10. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell. 1998;95:163-175.
11. Hanna Z, Weng X, Kay DG, Poudrier J, Lowell C, Jolicoeur P. The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. J Virol. 2001;75:9378-9392.
12. Saksela K, Cheng G, Baltimore D. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. 1995;14:484-491.
13. Moarefi I, LaFevre-Bernt M, Sicheri F, et al. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature. 1997;385:650-653.
14. Lerner EC, Smithgall TE. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat Struct Biol. 2002;9:365-369.
15. Briggs SD, Lerner EC, Smithgall TE. Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo. Biochemistry. 2000;39:489-495.
16. Lowell CA. Src-family kinases: rheostats of immune signaling. Mol Immunol. 2004;41:631-643.
17. Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. Oncogene. 2004;23:7957-7968.
18. Zhang H, Meng F, Chu CL, Takai T, Lowell CA. The Src family kinases Hck and Fgr negatively regulate neutrophil and dendritic cell chemokine signaling via PIR-B. Immunity. 2005;22:235-246.
19. Mermel CH, McLemore ML, Liu F, et al. Src family kinases are important negative regulators of G-CSF-dependent granulopoiesis. Blood. 2006;108:2562-2568.
20. Garcia JV, Miller AD. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature. 1991;350:508-511.
21. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med. 1996;2:338-342.
22. Greenberg ME, Iafrate AJ, Skowronski J. The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. EMBO J. 1998;17:2777-2789.
23. Williams M, Roeth JF, Kasper MR, Filzen TM, Collins KL. Human immunodeficiency virus type 1 Nef domains required for disruption of major histocompatibility complex class I trafficking are also necessary for coprecipitation of Nef with HLA-A2. J Virol. 2005;79:632-636.
24. Drakesmith H, Chen N, Ledermann H, Screaton G, Townsend A, Xu XN. HIV-1 Nef down-regulates the hemochromatosis protein HFE, manipulating cellular iron homeostasis. Proc Natl Acad Sci U S A. 2005;102:11017-11022.
25. Briggs SD, Scholtz B, Jacque J-M, Swingler S, Stevenson M, Smithgall TE. HIV-1 Nef promotes survival of myeloid cells by a Stat3-dependent pathway. J Biol Chem. 2001;276:25605-25611.
26. Suzu S, Harada H, Matsumoto T, Okada S. HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and inhibits M-CSF bioactivities. Blood. 2005;105:3230-3237.
27. Gasson J. Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood. 1991;77:1131-1145.
28. Roth P, Stanley ER. The biology of CSF-1 and its receptor. Curr Top Microbiol Immunol. 1992;181:141-167.
29. Hashimoto S, Suzuki T, Dong HY, Yamazaki N, Matsushima K. Serial analysis of gene expression in human monocytes and macrophages. Blood. 1999;94:837-844.
30. Kitamura T, Tange T, Terasawa T, et al. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. J Cell Physiol. 1989;140:323-334.

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31. Suzu S, Kimura F, Ota J, et al. Biologic activity of proteoglycan macrophage colony-stimulating factor. J Immunol. 1997;159:1860-1867.
32. Walk SF, Alexander M, Maier B, Hammarskjold M-L, Rekosh DM, Ravichandran KS. Design and use of an inducibly activated immunodeficiency virus type 1 Nef to study immune modulation. J Virol. 2001;75:834-843.
33. Shugars DC, Smith MS, Glueck DH, Nantermet PV, Seillier-Moiseiwitsch F, Swanstrom R. Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo. J Virol. 1993;67: 4639-4650.
34. Sawai ET, Baur A, Struble H, Peterlin BM, Levy JA, Cheng-Mayer C. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc Natl Acad Sci U S A. 1994;91:1539-1543.
35. Murakami Y, Fukazawa H, Kobatake T, et al. A mammalian two-hybrid screening system for inhibitors of interaction between HIV Nef and the cellular tyrosine kinase Hck. Antiviral Res. 2002; 55:161-168.
36. Tahara-Hanaoka S, Ushijima Y, Tarui H, et al. Differential level of co-down-modulation of CD4 and CXCR4 promed by HIV-1 gp120 in response to phorbol ester, PMA, among HIV-1 isolates. Microbiol Immunol. 2000;44:489-498.
37. Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J Virol. 2000;74:2907-2912.
38. Suzu S, Tanaka-Douzono M, Nomaguchi K, et al. $p56^{dok-2}$ as a cytokine-inducible inhibitor of cell proliferation and signal transduction. EMBO J. 2000;19:5114-5122.
39. Spivak JL, Avedissian LS, Pierce JH, Williams D, Hankins WD, Jensen RA. Isolation of the full-length murine erythropoietin receptor using a baculovirus expression system. Blood. 1996;87: 926-937.
40. Schmidt-Arras DE, Bohmer A, Markova B, Choudhary C, Serve H, Bohmer F. Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases. Mol Cell Biol. 2005;25:3690-3703.
41. Xiang Z, Kreisel F, Cain J, Colson A, Tomasson MH. Neoplasia driven by mutant c-KIT by intracellular, not plasma membrane, receptor signaling. Mol Cell Biol. 2007;27:267-282.
42. Suzu S, Hiyoshi M, Yoshidomi Y, et al. M-CSF-mediated macrophage differentiation but not proliferation is correlated with increased and prolonged ERK activation. J Cell Physiol. 2007;212: 519-525.
43. Lee PS, Wang Y, Dominguez MG, et al. The Cbl protooncogene stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J. 1999;18: 3616-3628.
44. Puthenveedu MA, Bachert C, Puri S, Lanni F, Linstedt AD. GM130 and GRASP65-dependent lateral cisternal fusions allows uniform Golgi-enzyme distribution. Nat Cell Biol. 2006;8:238-248.
45. Xu Y, Wong SH, Tang BL, Subramaniam VN, Zhang T, Hong W. A 29-kilodalton Golgi soluble N-ethylmaleimide-sensitive factor attachment protein receptor (Vti1-rp2) implicated in protein trafficking in the secretory pathway. J Biol Chem. 1998;273:21783-21789.
46. Helenius A, Aebi M. Intracellular functions of N-linked glycans. Science. 2001;291:2364-2369.
47. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. 1985;41:665-676.
48. Roussel MF, Downing JR, Rettenmier CW, Sherr CJ. A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. Cell. 1988;55:979-988.
49. Woolford J, McAuliffe A, Rohrschneider LR. Activation of the feline c-fms proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. Cell. 1988;55:865-977.
50. Sherr CJ. Colony-stimulating factor-1 receptor. Blood. 1990;75:1-12.
51. Courtneidge SA, Dhand R, Pilat D, Twamley GM, Waterfield MD, Roussel MF. Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. EMBO J. 1993; 12:943-950.
52. Alonso G, Koegl M, Mazurenko N, Courtneidge SA. Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors. J Biol Chem. 1995;270:9840-9848.
53. Marks DC, Csar XF, Wilson NJ, et al. Expression of a Y559F mutant CSF-1 receptor in M1 myeloid cells: a role for Src kinases in CSF-1 receptor-mediated differentiation. Mol Cell Biol Res Commun. 1999;1:144-152.
54. Rohde CM, Schrum J, Lee AWM. A juxtamembrane tyrosine in the colony-stimulating factor-1 receptor regulates ligand-induced Src association, receptor kinase function, and down-regulation. J Biol Chem. 2004;279:43448-43461.
55. Carreno S, Gouze M, Schaak S, Emorine LJ, Maridonneau-Parini I. Lack of palmitoylation redirects $p59^{Hck}$ from the plasma membrane to $p61^{Hck}$-positive lysosomes. J Biol Chem. 2000; 275:36223-36229.
56. Chiu VK, Bivona T, Hach A, et al. Ras signaling on the endoplasmic reticulum and the Golgi. Nat Cell Biol. 2002;4:343-350.
57. Kasahara K, Nakayama Y, Ikeda K, et al. Trafficking of Lyn through the Golgi caveolin involves the charged residues on αE and αI helices in the kinase domain. J Cell Biol. 2004;165:641-652.
58. Bard F, Mazelin L, Pechoux-Longin C, Malhorta V, Jurdic P. Src regulates Golgi structure and KDEL receptor-dependent retrograde transport to the endoplasmic reticulum. J Biol Chem. 2003;47: 46601-46606.
59. Komuro I, Yokota Y, Yasuda S, Iwamoto A, Kagawa KS. Regulation of Hck and C/EBPβ represent a heterogeneous susceptibility of monocyte-derived macrophages to M-tropic HIV-1 infection. J Exp Med. 2003;198:443-453.

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Efficient induction of HIV-1 replication in latently infected cells through contact with CD4$^+$ T cells: Involvement of NF-κB activation

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Abstract

Reservoir cells latently infected with HIV-1 pose one of the major obstacles that hamper ultimate eradication of HIV-1 from infected patients. In this report, we showed that direct contact with MOLT-4 T cells induced HIV-1 replication in J_{22}-HL-60 latently infected cells without any additional stimulus. Neutralization experiments revealed that pro-inflammatory cytokines, whose production was increased following cell–cell contact, were unlikely to be primarily involved in the induced HIV-1 replication. Cell–cell contact, but not soluble components in the culture supernatant, caused a rapid phosphorylation and degradation of IκBα, which led to elevated NF-κB DNA binding activity in J_{22}-HL-60 cells. Furthermore, forced expression of a super-repressor form of IκBα or pretreatment with ritonavir efficiently blocked the activation of NF-κB and HIV-1 replication in J_{22}-HL-60 cells co-cultured with MOLT-4 T cells. Moreover, either resting or PHA stimulated primary CD4$^+$ T cells induced HIV-1 replication in J_{22}-HL-60 cells in a similar way with that of MOLT-4 cells. These results indicated that direct contact with CD4$^+$ T cells induced HIV-1 replication in latently infected cells and provide insight into the molecular mechanism of virus release from myeloid progenitor cells latently infected with HIV-1.
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Keywords: HIV-1; Reservoir; Cell–cell contact; Latency; Co-culture

Introduction

The use of highly active antiretroviral therapy (HAART) in HIV-1-infected individuals has dramatically improved the clinical outcome in a majority of infected patients (reviewed in Pomerantz and Horn, 2003). However, the presence of cellular reservoirs that contain latent provirus capable of producing infectious particles after cellular activation leads to a rebound of the viral load after interruption of HAART (Pierson et al., 2000). The persistence of these latently infected viral reservoirs, despite prolonged HAART treatments, represents a major obstacle to the eradication of HIV-1 in infected patients (Finzi et al., 1997; Wong et al., 1997). CD4$^+$ T cells are thought to be the major reservoirs of both actively replicating and latent HIV-1 throughout the course of the disease (Bukrinsky et al., 1991; Siliciano et al., 2003). In addition to CD4$^+$ T cells, cells of the monocytic lineage are infected by HIV-1 and accumulating evidence suggests that purified CD34 positive populations of myeloid progenitor cells are susceptible to HIV-1 infection as well (Louache et al., 1994). A greater understanding of the molecular mechanisms of viral latency and its reactivation is essential for eradicating HIV-1. Previous studies demonstrated that cell–cell contact was essential for

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induction of HIV-1 replication in latently infected cell lines (Devadas et al., 2004; Poli et al., 1994; Duh et al., 1989). However, the mechanism of HIV-1 replication induced by cell–cell contact in latently infected myeloid progenitor cells has remained unknown. In the present study, the J_{22}-HL-60 cell line, established from HL-60 promyelocytic/myeloblastic cell line after infection with monocyte-tropic HIV-1 JR-FL strain, was used as a standard model.

Induction of HIV-1 replication by various extracellular stimuli is often mediated by NF-κB, which is known to bind to the κB sites within the HIV-1 long terminal repeat (LTR) enhancer region (Fauci, 1996). NF-κB is a dimeric transcription factor composed of p50, p52, RelA, RelB or c-Rel. In most unstimulated cells, NF-κB is retained in the cytoplasm through binding to IκB proteins, which mask the nuclear localization signal of NF-κB. Cellular stimulation triggers phosphorylation of specific serine residues of IκB proteins followed by its ubiquitination and rapid proteasome-mediated degradation. Released NF-κB then translocates to the nucleus and enhances transcription of target genes by binding to specific consensus sequences in their promoter region. Protease inhibitors (PIs) such as ritonavir have been successfully used in the clinical treatment of human immunodeficiency virus 1 (HIV-1) infection, resulting in a marked decrease in HIV-1 viral load and subsequent increase in $CD4^+$ T-cell counts in patients (Collier et al., 1996a, 1996b). Other investigators showed that ritonavir suppressed proliferation of Kaposi's sarcoma and prostate cancer cells via inhibition of NF-κB activity; ritonavir treatment of the human dermal microvessel endothelial cells (HMEC) blocked TNF-α-induced NF-κB activation; PIs inhibited Toll-like receptor 2 (TLR2)- and TLR4-induced NF-κB activation (Pati et al., 2002; Equils et al., 2004). Ritonavir was also shown to inhibit the 20S proteasome function (Andre et al., 1998). In this study, we demonstrate a marked increase in virus release from J_{22}-HL-60 cells co-cultured with T cells, which is mediated by NF-κB and completely ablated by ritonavir.

Results

Co-culture induces HIV-1 replication in J_{22}-HL-60 cells

To investigate effects of cell–cell interaction on the induction of HIV-1 replication in chronically HIV-1-infected J_{22}-HL-60 cells, we co-cultured J_{22}-HL-60 cells with various B and T-cell lines such as L428, KM-H2, MOLT-4, Jurkat, MT-2 and MT-4 at a ratio of 1:1, and the viral p24 antigen released in culture supernatants was quantified at 24 and 48 h after co-cultivation. Although the JR-FL strain is originally infectious, HIV-1 released from J_{22}-HL-60 cells has proved not to be infectious (Qi et al., unpublished observation). Thus, p24 determined in this study is derived solely from J_{22}-HL-60 cells. As shown in Fig. 1, co-culture strongly enhanced HIV-1 p24 release from J_{22}-HL-60 cells. Notably, co-culture with T-cell lines induced p24 release from J_{22}-HL-60 cells more efficiently than that with B-cell lines. Since MOLT-4 cells are broadly used and also free of known viruses, we chose this cell line for further studies.

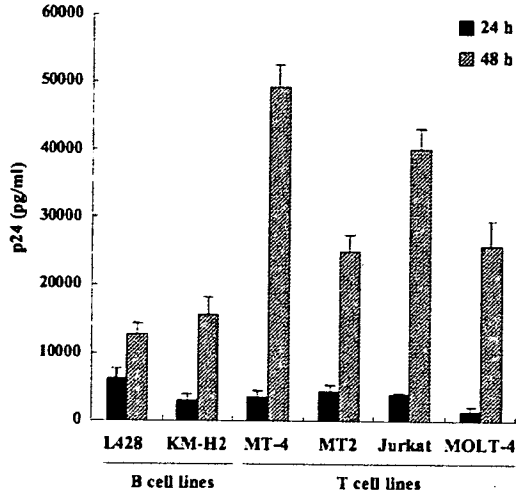

Fig. 1. HIV-1 replication in J_{22}-HL-60 cells induced by co-cultivation. J_{22}-HL-60 cells (2×10^5 cells/ml) were co-cultured with the equivalent number of the indicated cell lines. Culture supernatants were collected at 24 and 48 h after starting co-culture, and the HIV-1 p24 antigen in the supernatants was quantified with Lumipulse. Results shown are means±SD from three independent experiments done in duplicate.

Live cell contact is required for the co-culture-induced HIV-1 replication

We compared the kinetics of virus production induced by co-culture or TNF-α, one of the well-studied cytokines inducing HIV-1 replication in J_{22}-HL-60 cells. As shown in Fig. 2, significant increase in p24 release from J_{22}-HL-60 cells was first noticed at 36 h after co-cultivation, which continued throughout the experiment and finally exceeded the levels achieved by TNF-α stimulation. In contrast, TNF-α-induced p24 production was evident as early as 24 h after stimulation and reached a peak around 60 h (Fig. 2).

To determine whether cell–cell contact is essential for the induced HIV-1 expression in J_{22}-HL-60 cells, J_{22}-HL-60 and MOLT-4 cells were grown in separate trans-well compartments which allow exchange of soluble factors between the two cell lines, but not a physical contact between J_{22}-HL-60 and MOLT-4 cells. As shown in Fig. 3A, p24 production was much reduced when cells were cultured separately in trans-wells or when J_{22}-HL-60 cells were treated only with the supernatant of MOLT-4 cells. These results indicate that cell–cell contact is required for the maximal induction of HIV-1 replication in J_{22}-HL-60 cells after co-culture with MOLT-4 cells and that contribution of soluble factors in supernatants is quite limited.

We next examined whether live MOLT-4 cells are necessary for the induction of HIV-1 replication in J_{22}-HL-60 cells after co-culture with MOLT-4 cells. For this purpose, we used live, heat-inactivated, or 3% paraformaldehyde-fixed MOLT-4 cells for co-culture with J_{22}-HL-60 cells (Fig. 3B). Neither heat-inactivated nor paraformaldehyde-fixed MOLT-4 cells were able to induce HIV-1 replication in J_{22}-HL-60 cells, indicating that this induction requires the presence of live MOLT-4 cells. The results also suggest that the induction of HIV-1 replication is not simply a result of allo-reactive immunological response.