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ORIGINAL PAPER

Copy number variations of *CCL3L1* and long-term prognosis of HIV-1 infection in asymptomatic HIV-infected Japanese with hemophilia

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Abstract We set up a cohort of HIV-infected, asymptomatic Japanese patients with hemophilia for follow-up study in 1995. All subjects who had been infected with HIV-1 for more than 10 years met the criteria for long-term nonprogressors (LTNPs) at the time of entry; however, some of them later developed lymphopenia and required antiretroviral treatment during five more years of observation. In this study, we investigated the impacts of the *CCL3L1* dose on the long-term prognosis in the subjects with chronic HIV-1 infection. We collected genomic DNA from 95 long-term survivors including 48 nonprogressors and 47 subjects receiving antiretroviral treatment. The distributions of *CCL3L1* copy number significantly differed between the 95 HIV-1-infected subjects with hemophilia and 205 controls. Average copy number of *CCL3L1* in the HIV-1-infected subjects was significantly lower than in control (5.00±0.22 vs 3.35±0.24, $p<0.001$). Moreover, the subjects possessing two or less copies of *CCL3L1* had significantly higher risk of acquiring HIV-1. However, *CCL3L1* copy number variations had no significant effect on the disease progression among the LTNP subjects who had been afflicted with chronic HIV-1 infection for more than 15 years, when compared between nonprogressors and patients under treatment (3.68±0.37 vs 3.02±0.29, ns). Furthermore, variations in the *CCL3L1* copy number had little effect on the levels of HIV-1 load among them. We conclude that variation in the *CCL3L1* copy number is apparently not a factor that determines the prognosis of chronic HIV-1 infection, even though it is linked to HIV-1 susceptibility.

Keywords HIV-1 infection · HIV-1 susceptibility · Long-term nonprogressors · *CCL3L1*

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Introduction

Worldwide, more than 50 million people have become infected with HIV-1 in the past 25 years, and a third of them have died (UNAIDS 2006). However, the clinical course of HIV-1 infection varies considerably from person to person. Some patients rapidly progress to acquired immunodeficiency syndrome (AIDS), while others spontaneously achieve viral control and relative immunologic stability. Much attention has been given to the small proportion of people, called "long-term nonprogressors" (LTNPs; Pantaleo et al. 1995; Hogan and Hammer 2001; Muñoz et al. 1995), who maintain sufficient CD4+T cell numbers not to develop symptoms for many years after infection. Understanding the mechanisms underlying the delayed disease progression has been one of the major areas of investigation for AIDS researchers over the last decade.

Several factors are linked to the delayed progression of HIV-1/AIDS. The main correlate with delayed progression is low viral burden (Henrard et al. 1995; Mellors et al. 1997), which probably reflects an effective immunologic response by HIV-1-specific CD4+T cells in the early course of infection. Thus, host genetic factors that determine the immune response to HIV-1 could account for the delayed disease progression. Some insights into the genetic determinants of HIV-1/AIDS susceptibility have come from studies of LTNPs. One of them involves genetic variations in chemokine receptor 5 (*CCR5*). Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of CCR5, decrease the susceptibility to HIV-1 infection (Dragic et al. 1996; McDermott et al. 1998; Ioannidis et al. 2001).

Recently, copy number variations in CC chemokine ligand 3-like 1 (*CCL3L1*) have also been reported to be linked to the susceptibility to HIV-1 infection (Gonzalez et al. 2005). CCL3L1, also known as macrophage-inflammatory protein 1α (MIP-1αP) or LD78β, is a natural ligand for the HIV-1 coreceptor CCR5 (Menten et al. 2002). *CCL3L1* exhibits copy number variations on chromosome 17q11.2 (Hirashima et al. 1992; Townson et al. 2002), and the possession of a lower copy number of *CCL3L1* is associated with markedly enhanced HIV/AIDS susceptibility. However, Shao et al. (2007) could not replicate the association in adolescents, and it remains controversial.

In an effort to understand the nature of protective immunity to HIV-1 infection, considerable attention has been given to the small proportion of people, LTNPs. Although called "LTNPs", many of these patients do eventually progress to disease, and it is important to identify the genetic factors linked to the prognosis and outcome under long-term chronic HIV-1 infection. In 1995, we set up a cohort of HIV-infected, asymptomatic patients with hemophilia for a follow-up study (Munkanta et al. 2005). All subjects who had been infected with HIV-1 for more than 10 years met the criteria for LTNPs at the time of entry; however, some of them later developed lymphopenia and required antiretroviral treatment during a further 5 years of observation. This cohort was deemed to be suitable to evaluate the genetic factors to determine the prognosis under chronic HIV-1 infection. In this study, we investigated the impact of *CCL3L1* dose on the long-term prognosis of chronic HIV-1 infection.

Materials and methods

Subjects

The protocol for the present study was approved by the Ethics Review Board of the Medical Research Institute of Tokyo Medical and Dental University as well as all of the hospitals in which the samples were taken. Upon the set-up of the cohort of HIV-1-infected Japanese patients with hemophilia in 1995, all patients had been infected for longer than 10 years but were nevertheless asymptomatic without any antiviral measures. Blood samples were collected from 95 well-characterized patients who were selected from the cohort after obtaining written informed consent. At the time of sample collection, 48 of them were still asymptomatic and maintained their CD4+T cells at a level of no less than 200/mm^3, but the rest of the 47 subjects were under antiretroviral treatment (HAART) because of a reduction of the CD4 count to beneath this level (less than 200/mm^3). Quantification of viral RNA in the plasma was evaluated by the Roche Amplicor version 1.5 assay (Roche Diagnostics, NJ). Controls were obtained from a random sampling of healthy volunteers (n=205). DNA was prepared from the blood sample by the use of the QuickGene DNA whole blood kit S (FUJIFILM, Japan).

Estimation of the copy number of *CCL3L1* in the genome

The copy number of *CCL3L1* in the genome was estimated according to the method of Gonzalez et al. (2005), with a few modifications. Briefly, real-time polymerase chain reaction (PCR) was performed by using a Bio-Rad iCycler iQ Real-Time PCR Detection Systems (Bio-Rad) detecting the emitted 6-carboxyfluorescein (FAM) fluorescence from the probe for *CCL3L1* and the probe for the β-*globin* gene (*HBB*) during amplification. The *CCL3L1* primer sequences were as follow: sense primer 5′-TGCCTATCTCCGTCTAGAGAGCTT-3′; antisense primer 5′-AGAAGGAGGCAGCAGGACACT-3′; probe 5′-FAM-TGACTCCAGGCAAGGG-MGB. The *HBB* primer sequences were: sense primer 5′-GGCAACCCTAAGGTGAAGGC-3′; antisense 5-GGTGAGCCAGGCCATCACTA-3′; probe 5′-FAM-CATGGCAAGAAAGTGCTCGGTGCCT-TAMRA (Applied Biosystems).

The cycle number at which the fluorescence reached a fixed threshold, termed the cycle threshold (CT), was determined. Eight serial 1:2 dilutions (100–0.78 ng/reaction) of genomic DNA having four copies of *CCL3L1* were used to generate standard curves of the CT value for *HBB* and for the *CCL3L1* gene. For each test sample, at least duplicate wells were set up for *CCL3L1* and *HBB*, the CT as determined, and converted into template quantity using the standard curves. The copy number is the ratio of the template quantity for *CCL3L1* to the template quantity for *HBB* multiplied by four.

Southern blotting

CCL3L1 copy numbers for the standard DNA sample and several additional DNA samples were evaluated by standard Southern blotting and a hybridization method (Hirashima et al. 1992) using AlkPhos Direct Labelling and Detection System (GE Healthcare, UK).

Statistical analysis

All statistical analyses in this study were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software. CA).

Results

Copy number variations of CCL3L1 and HIV-1/AIDS susceptibility in HIV-1-infected Japanese with hemophilia

We evaluated the copy number of *CCL3L1* in 95 asymptomatic HIV-1-infected Japanese with hemophilia and 205 controls. All 95 asymptomatic subjects were classified into LTNPs at the entry. The copy number was estimated by the quantitative ratio of *CCL3L1* to *HBB* in the template genomic DNA. The quantities of *CCL3L1* and *HBB* in the template genomic DNA were evaluated by the quantitative TaqMan PCR method (Fig. 1).

The distributions of the copy number of *CCL3L1* were different between the HIV-1-infected subjects with hemophilia and controls (Fig. 2). The subjects with a low copy number were more common among the HIV-1-infected subjects with hemophilia than in the control individuals. The average copy number of *CCL3L1* in the HIV-1-infected subjects with hemophilia was significantly lower than in the control (5.00±0.22 vs 3.35±0.24, $p<0.001$ by unpaired t test). The average copy number in the control was almost identical to that reported by Gonzalez et al. (2005). Moreover, the subjects possessing two or less copies of *CCL3L1*, who had a markedly enhanced risk of acquiring HIV-1 (Table 1), were more common among the HIV-1-infected subjects with hemophilia (Table 2).

Copy number variations of CCL3L1 and long-term prognosis of HIV-1 infection

Ninety five HIV-1-infected subjects with hemophilia were divided into two groups based on the clinical courses during further 5 years of follow-up; one with 48 subjects who maintained their CD4+T cell counts at a certain level (no less than 200/mm^3) without antiretroviral treatment (nonprogressor) and the other with 47 subjects who required antiretroviral treatment due to the depletion of CD4+T cells beneath this level (slow progressor). According to the AIDS surveillance case definitions (CDC 1992), individuals in the first group were categorized as A1 or A2 and the second group as A3. When compared, there was no significant difference in the distribution of the copy number of *CCL3L1* (Fig. 2). The average copy number of *CCL3L1* in nonprogressors was 3.68±0.37 and 3.02±0.29 in the patients undergoing antiretroviral treatment.

Copy number variations of CCL3L1 and HIV-1 load

The data for HIV-1 load after 7 to 8 years of observation period were available from 74 of the 95 subjects. There was no correlation between the *CCL3L1* copy number variations and HIV-1 load (data not shown). Moreover, the copy number of *CCL3L1* had little effect on the distribution of HIV-1 load in nonprogressors or slow progressors (Fig. 3). The levels of HIV-1 load from 28 subjects, 7 nonprogressors and 21 slow progressors, were under the detectable

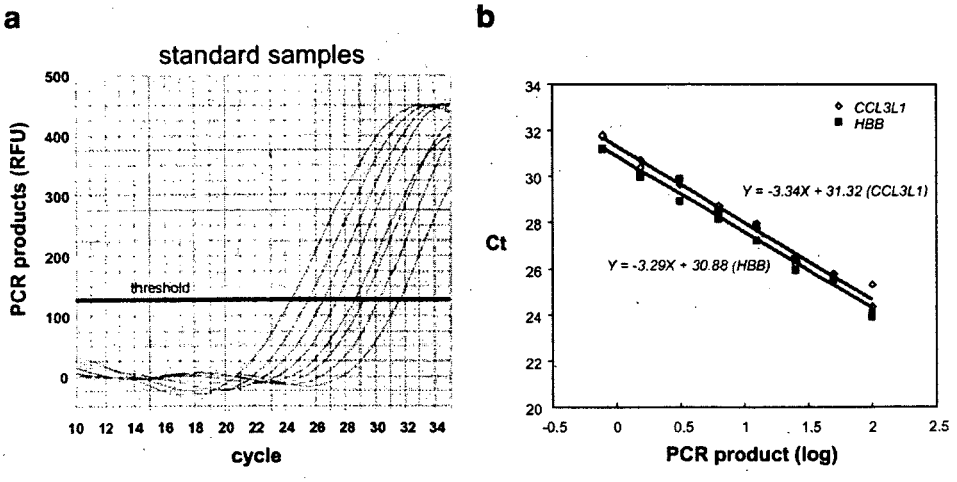

Fig. 1 **a** The amplification curves obtained using eight (1:2) serial dilutions of standard human genomic DNA with four copies of *CCL3L1* per diploid genome ranging from 100 to 0.56 ng. **b** The standard curves with the slope and the Pearson correlation coefficient (R^2) for CCL3L1 (*open diamonds*) and HBB(*closed squares*)

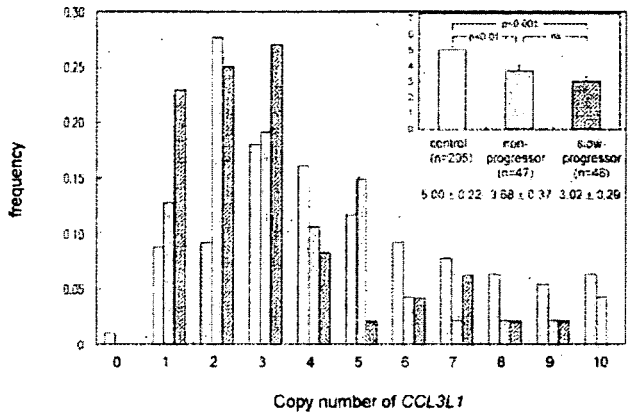

Fig. 2 The distribution of the copy number of *CCL3L1* in the HIV-1-infected asymptomatic subjects with no treatment (n=48), the HIV-1-infected asymptomatic subjects with antiretroviral treatment (n=47), and controls (n=205)

level; however, the rate of them in nonprogressors [0.133 (2/15) in CCL1L1≤2 vs 0.217(5/23) in CCL3L1>2, ns] and slow progressors [0.556(10/18) in CCL1L1≤2 vs 0.217 (11/18) in CCL3L1>2, ns] was not significantly different by the copy number of *CCL3L1*.

Discussion

Much attention has been paid to the small proportion of people, LTNPs, who maintain normal CD4+T cell numbers and remain symptom free for many years after infection. Many of these patients do eventually progress to disease. In our cohort, about half of the subjects who met the LTNP criteria at the time of entry also developed lymphopenia and required antiretroviral treatment during a further 5 years of observation. It is urgent to identify the genetic factors which determine the prognostic outcome of the patients afflicted with chronic HIV-1 infection, especially because of the recent dramatic increase in the number of such patients thanks to the remarkable progress of antiretroviral treatment. In this study, we investigated the impact of *CCL3L1* dose on this long-term prognosis.

Gonzalez et al. (2005) have reported the copy number variations of *CCL3L1* to be linked to the susceptibility to HIV-1 infection. Possession of a low *CCL3L1* copy number was found to be a determinant of enhanced HIV-1 susceptibility among the study population. Our study replicated these results in the subjects in our study with hemophilia. The subjects with a low *CCL3L1* copy number were more common among the HIV-1-infected subjects with hemophilia, and the subjects possessing two or fewer copies of *CCL3L1* had a higher risk of acquiring HIV-1. Independent studies have shown that an increased copy number of *CCL3L1* results in the enhanced secretion of CCL3L1 by activated leukocytes (Gonzalez et al. 2005;

Table 1 Copy number of *CCL3L1* and risk of acquiring HIV-1 when compared between asymptomatic HIV-infected Japanese (n=95) with hemophilia and controls (n=205)

CCL3L1 copy number	Odds ratio	95% CI	Chi-square test with Yates correction	p-value
1	2.26	1.11–4.62	4.39	0.036*
2	3.50	1.81–6.74	13.74	0.0002**
3	1.37	0.76–2.48	0.77	0.379
4	0.55	0.25–1.19	1.85	0.174
5	0.69	0.30–1.61	0.43	0.511
6	0.43	0.14–1.30	1.69	0.194
7	0.52	0.17–1.60	0.83	0.362
8	0.32	0.07–1.44	1.64	0.200
9	0.38	0.08–1.75	0.97	0.324
10	0.32	0.07–1.44	1.64	0.200

*Significant association, p>0.05
**Significant association, p>0.001

Table 2 Copy number of *CCL3L1* among LTNPs (n=95) and controls (n=205)

	Control	LTNPs	
Mean of CCL3L1 copy number	5.00±0.22	3.35±0.25	p<0.001[a]
The rate of the subjects with CCL3L1≤2	0.190 (39/205)	0.389 (37/95)	p=0.0004 (χ^2=12.589)[b]

[a] Evaluated by unpaired t test
[b] Evaluated by chi-square test with Yates correction

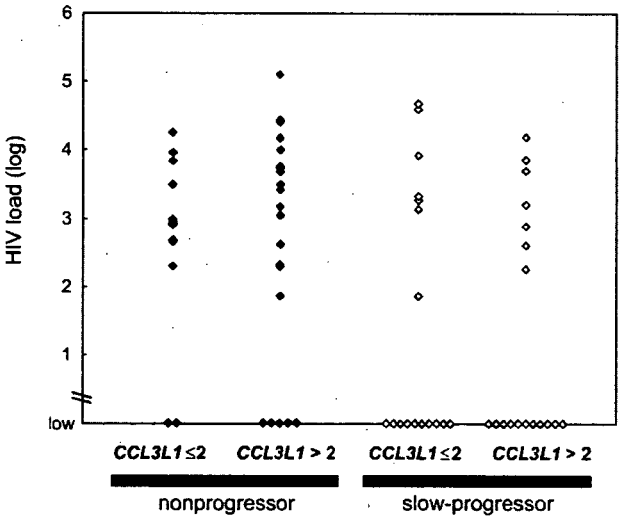

Fig. 3 Copy number of *CCL3L1* and HIV-1 load. *Low* means the plasma viral RNA levels under the detectable level by the Roche Amplicor version 1.5 assay (Roche Diagnostics, NJ)

Townson et al. 2002), so that altering the genetic expression of *CCL3L1* through copy number variations might contribute to the susceptibility to HIV-1 infection. It is reasonable that the reduced expression of *CCL3L1* might be associated with enhanced HIV-1 susceptibility because CCL3L1, which is the most potent known ligand for HIV-1 coreceptor CCR5, is a dominant HIV-1-suppressive chemokine (Menten et al. 2002).

In addition to influencing HIV-1 acquisition, Gonzalez et al. (2005) have reported the association of these variations with prognosis in HIV-1-infected patients. A lower *CCL3L1* dose was associated with an increased risk of progressing rapidly to AIDS or death. *CCL3L1* copy number was also associated with the viral set point and rate of change in CD4+T cell counts in a dose-dependent manner, which are well-established predictors of clinical outcome (Henrard et al. 1995; Mellors et al. 1997). Blocking of the interaction between HIV-1 glycoprotein 120 and CCR5 and/or the immune reaction against HIV through CCL3L1 itself might be related to the *CCL3L1* dose-dependent protective mechanism. However, we could not identify significant differences in the distribution of the *CCL3L1* copy number variations between the two groups with chronic HIV-1 infection, the one which maintained their CD4+T cells with no antiretroviral treatment and the other which received antiretroviral treatment due to the reduction of CD4+T cells. *CCL3L1* copy number variations had no significant effects on disease progression among subjects with chronic HIV-1 infection of more than 15 years. Furthermore, *CCL3L1* copy number variations had little effect on the levels of HIV-1 load in asymptomatic HIV-1-infected subjects with hemophilia. A possible explanation of this inconsistency is that the copy number of *CCL3L1* was associated with an effective immunologic response in the acute phase of HIV-1 infection but not chronic phase. Some patients with asymptomatic chronic HIV-1 infection nevertheless exhibit high rates of HIV-1 replication and destruction of CD4+T cells daily (Wei et al. 1995; Ho et al. 1995). Cell death and replacement are in near balance during the chronic phase of the illness in these patients. Thus, the maintenance of a steady state of cell counts and the viral load seems to be tightly linked to the long-term prognosis under chronic HIV-1 infection. It is unlikely that the *CCL3L1* dose would play a crucial role in maintaining this balance of HIV-1 replication with the destruction of CD4+T cells during the chronic phase. Further analyses are required to clarify the specific functional role of *CCL3L1* on the chronic phase of HIV-1 infection.

We conclude that the copy number variation of *CCL3L1* appears not to be a determining factor in the prognosis of chronic HIV-1 infection, even though it is linked to HIV-1 infection susceptibility. Our studies also suggest that the genetic factors which do determine the prognosis of HIV-1/AIDS may be different between the acute and chronic phases.

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HLA-DPβ chain may confer the susceptibility to hepatitis C virus-associated hypertrophic cardiomyopathy

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Summary

Hypertrophic cardiomyopathy (HCM) is a heart muscle disease characterized by hypertrophy and diastolic dysfunction of cardiac ventricles. It is suggested that one possible aetiology of HCM is the hepatitis C virus (HCV) infection, but molecular mechanisms underlying development of HCV-associated HCM (HCV-HCM) remains unknown. Because the human leucocyte antigen (HLA) molecule is involved in the control of progression/suppression of viral infection, extensive *HLA* allelic diversity may modulate the post-infectious course of HCV and pathogenesis of HCV-HCM. Here we undertook a case-control study with 38 patients with HCV-HCM and 132 unrelated healthy controls to reveal the potential impact of polymorphisms in seven classical and two non-classical *HLA* genes on the pathogenesis of HCV-HCM. It was found that *DPB1*0401* and *DPB1*0901* were significantly associated with increased risk to HCV-HCM in dominant model ($P < 0.028$, OR = 3.94, 95% confidence interval (CI) = 1.19, 13.02) and in recessive model ($P < 0.007$, OR = 9.85, 95% CI = 1.83, 53.04), respectively. The disparity in the gene–dose effect by two susceptible DPB1 alleles may be attributable to the difference between the susceptible (36 A and 55 A) and resistant (8L, 9F, 11G, 57E and 76M) residue-combination consisting of DPβ anchor pocket for antigenic peptide-binding. These results implied that the HLA-DP molecules with specificity pocket appropriate for HCV antigen(s) might confer the progressive process of HCM among the HCV-infected individuals.

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Introduction

Primary cardiomyopathy is a heart muscle disease that often leads to severe heart failure and sudden death. There are two different types of cardiomyopathy; one is hypertrophic cardiomyopathy (HCM) characterized by cardiac hypertrophy and diastolic ventricular dysfunction, and the other is dilated cardiomyopathy (DCM) characterized by chamber dilation and contractile dysfunction (Seidman & Seidman, 2001). It is well known that more than half of patients with HCM and about 20–30% of patients with DCM have apparent family history of the disease (familial cardiomyopathy) mainly consistent with the autosomal dominant genetic trait, and mutations in the genes for sarcomere or Z-disc components could be found in the patients with familial cardiomyopathy (Seidman & Seidman, 2001; Richard *et al.*, 2006). On the other hand, there are many patients who have no family history of the disease (30–50% of HCM patients and 70–80% of DCM patients), that is, patients with sporadic cardiomyopathy. Molecular mechanisms underlying the pathogenesis of sporadic HCM or sporadic DCM have not been fully understood, because gene mutations are usually not found in the patients with sporadic cardiomyopathy albeit that a part of sporadic HCM patients and sporadic DCM patients carried antibodies to hepatitis C virus (HCV). These findings have suggested a possible involvement of HCV in the pathogenesis of sporadic HCM and/or sporadic DCM (Matsumori *et al.*, 1995, 1996, 2006; Teragaki *et al.*, 2003; Matsumori, 2005).

HCV causes not only liver disease but also a broad clinical spectrum of extrahepatic manifestation (Mayo, 2003). Immunohistological studies demonstrated the presence of HCV in extrahepatic organs including heart (Takeda *et al.*, 1999), and it was reported that the prevalence of patients with anti-HCV antibodies (seropositive patients) was about 9.5% and 6.7% in Japanese HCM and DCM, respectively (Matsumori *et al.*, 2002). On the other hand, transgenic mice with high expression of HCV-core in the heart exhibited morphological and functional changes similar in part to both HCM and DCM without apparent inflammatory cell infiltration (Omura *et al.*, 2005), demonstrating the role of HCV infection in the development of cardiomyopathy. However, not all of the HCV infected-patients exhibited abnormal findings in the electrocardiogram and echocardiography such as arrhythmia and

ventricular hypertrophy (Matsumori *et al.*, 1998), suggesting the involvement of host genetic factors in the pathogenesis of HCV-associated HCM (HCV-HCM) and/or HCV-associated DCM (HCV-DCM). Molecular mechanisms of predisposition to HCV-HCM and/or HCV-DCM are largely unknown, by which HCV leads not to liver diseases but to two different types of cardiomyopathy. However, it is plausible that the predisposing factors include the variations or diversity in both the virus and host genome.

Human major histocompatibility complex (MHC), also called human leucocyte antigen (HLA) complex, on chromosome 6p21.3 plays a crucial role in immune response against foreign as well as self-antigens so that it may control the susceptibility and/or resistance to infectious diseases (McCluskey & Peh, 1999). The striking hallmark of HLA is highly duplicated and polymorphic genes than any other region of the human genome (Marsh *et al.*, 2002; Horton *et al.*, 2004). In principal, foreign antigens including invading pathogens provoke immune response through their recognition by T-cell receptors in peptide form loaded on the HLA molecules (Rudolph *et al.*, 2006). The antigen-presenting capacity of a given HLA molecule depends on the structure of peptide-binding motif and the amino acid preferences of the specificity pockets (Rammensee *et al.*, 1995). For example, HCV entry into host cell may be achieved regardless of *HLA* genotype of hosts but, in the post-infectious stage, course of the viral infection can be influenced by *HLA* allele-specific manner. Specifically, previous reports suggested that *DRB1*1302* carriers remained asymptomatic (Kuzushita *et al.*, 1996), whereas *DRB1*0405* carriers tended to develop chronic liver disease (Seki *et al.*, 1992).

Considering such pivotal role of HLA system in the course of HCV infection, *HLA* genes are hypothesized to control the genetic predisposition to HCV-HCM and/or HCV-DCM. In accordance with the hypothesis, we have previously carried out microsatellite analyses to delineate the susceptible loci to HCV-HCM and/or HCV-DCM within the MHC region. While the susceptible locus to HCV-DCM was successfully mapped within the 180 kb interval encompassing *TNF* to *MICA* located in the HLA class III–HLA class I boundary, none of the microsatellite markers showed significant associations with HCV-HCM, suggesting that the contribution of HLA-linked genes to the disease susceptibility was completely different between HCV-HCM and HCV-DCM (Shichi *et al.*, 2005). However, focusing on the microsatellite association data for HCV-HCM might overlook subtle disease-influencing variations in the MHC region. In the present study, we investigated nine *HLA* genes to assess whether particular alleles of HLA system would exert advantageous or deleterious effects on the HCV-HCM pathogenesis.

Materials and methods

Subjects

Thirty-eight patients with HCM and 21 patients with DCM, who all were positive for anti-HCV antibody, and 132 healthy individuals selected at random were the subjects. None of the patients had a family history of HCM or DCM. Diagnosis of HCV- HCM and HCV- DCM was described previously (Matsumori *et al.*, 1998). All subjects participating in this study were of Japanese ethnicity. Informed consent was acquired from each subject and the study protocol was approved by the Ethics Reviewing Committees of Tokai University School of Medicine, Kyoto University School of Medicine, and Medical Research Institute, Tokyo Medical and Dental University.

Genotyping analysis of classical and non-classical HLA genes

Blood DNA was extracted by the guanidine hydrochloride method from each subject. A total of nine *HLA* genes (three class I and six class II *HLA* genes) were analysed in this study. High-resolution typing for three *HLA* class I genes (*HLA-A*, *-B* and *-Cw*) and two *HLA* class II genes (*HLA-DPB1* and *-DRB1*) was carried out by the sequence-based typing method using available kit according to manufacturer's instructions (Forensic Analytical, Hayward, CA, USA) and/or according to standard protocols of the 13th International Histocompatibility Complex Working Group (http://www.ihwg.org/components/sbtover.htm). These *HLA* alleles were assigned by using the MATCHMAKER allele identification program (Applied Biosystems, Foster City, CA, USA). The remaining four *HLA* class II genes (*HLA-DQA1*, *-DQB1*, *-DMA* and *-DMB*) were genotyped by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis that was designed to distinguish all known alleles found in the Japanese population (Inoko & Ota, 1993; Kuwata *et al.*, 1996; Naruse *et al.*, 1996). When only one allele was deduced from the RFLP pattern, the subject was considered to be homozygous for that allele.

Statistical analysis

The PYPOP version 0.6.0 program (http://allele5.biol.berkeley.edu/pypop/) was used to analyse the deviations of genotypic distribution from Hardy–Weinberg equilibrium in multiple *HLA* alleles (Lancaster *et al.*, 2003). Distribution of *HLA* alleles and amino acid polymorphisms among subjects was assessed by Fisher's exact test along with odds ratios (OR) and 95% confidence intervals using R software version 2.4.1 (http://www.r-project.org/) (Ihaka & Gentleman, 1996). The Bonferroni's inequality method was applied for multiple comparisons. A level of P and corrected P (Pc) value of less than 0.05 was accepted as statistical significance.

Results

HLA polymorphism in HCV-HCM

Nine *HLA* genes were investigated for polymorphisms in association with the development of HCV-HCM. Genotypic distribution of three *HLA* class I genes (*HLA-A*, *-B*

Table 1. Frequencies of *DPB1* alleles in the patients with HCV-associated cardiomyopathy and controls

DPB1 Allele	HCV-HCM ($2n = 76$)	HCV-DCM ($2n = 42$)	Control ($2n = 264$)
**0201*	0.145	0.143	0.205
**0202*	0.026	0.024	0.015
**0301*	0.053	0.095	0.057
**0401*	0.079[a]	0.024	0.023
**0402*	0.105	0.095	0.170
**0501*	0.368	0.381	0.413
**0601*	0.013	—	0.004
**0901*	0.184[a]	0.119	0.095
**1301*	0.013	0.048	0.004
**1401*	—	0.048	0.008
**1701*	0.013	—	—
**1901*	—	0.024	0.004
**4101*	—	—	0.004

DPB1 allele frequencies were listed. Dash (–) indicates no observation of the corresponding allele in the subject. Both DPB1*0401 and DPB1*0901 were significantly associated with HCV-HCM ([a] $P < 0.05$, see Table 2 for detail), whereas none of *DPB1* allele demonstrated significant association with HCV-DCM.
HCV, hepatitis C virus; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy.

and -*Cw*) and six *HLA* class II genes (*HLA-DPB1*, *-DQB1*, *-DQA1*, *-DRB1*, *-DMA* and *-DMB*) was not departed from Hardy–Weinberg equilibrium in both patients and unrelated healthy controls. Of the nine *HLA* loci studied, eight *HLA* gene loci other than *DPB1* showed no significant association with HCV-HCM (data not shown), consistent in part with our previous microsatellite mapping study (Shichi *et al.*, 2005). On the other hand, two *HLA-DPB1* alleles, *DPB1*0401* and **0901*, showed significantly higher frequencies in the patients with HCV-HCM than healthy controls (Table 1). In contrast, no *DPB1* alleles showed significant association with HCV-DCM, suggesting that the association with the *DPB1* alleles was specific to HCV-HCM and not to HCV infection itself (Table 1). Further investigation of the association between DPB1 and HCV-HCM revealed that the two predisposing *HLA-DPB1* alleles conferred susceptibility to HCV-HCM in different manner, i.e. *DPB1*0401* showed significant risk in a dominant model ($P < 0.028$, OR = 3.94, 95% CI = 1.19, 13.02), whereas *DPB1*0901* conferred risk in a recessive model ($P < 0.007$, OR = 9.85, 95% CI = 1.83, 53.04) (Table 2).

Because there were weak but significant linkage disequilibrium (LD) between *DPB1* alleles and alleles of *DRB1* or *DQB1* locus in the Japanese population (Saito *et al.*, 2000), we examined the association between *DPB1*0401* and alleles of DR/DQ loci in both patients and controls. It was found that *DRB1*1302* and *DQB1*0604* were in LD with *DPB1*0401* in both patients and controls, and that the frequencies of *DRB1*1302* and *DQB1*0604* were slightly increased in the patients, but not reached to statistical significance (data not shown). In addition, to assess the involvement of DQ/DR loci in association with DP locus for the susceptibility to HCV-HCM, we investigated the possible effect of DQ/DR loci on the DP locus by the two-locus-analysis method (Svejgaard & Ryder, 1994). The results, however, showed no additive or synergistic effect of any DR/DQ alleles on the susceptibility conferred by *DPB1*0401* (data not shown). These observations suggested that *DPB1*0401* per se or *DPB1*0401*-linked polymorphisms conferred the susceptibility to HCV-HCM.

Table 2. Association of hepatitis C virushypertrophic cardiomyopathy risk in individuals with the *HLA-DPB1* alleles

		Cases ($n = 38$)	Controls ($n = 132$)	OR (95% CI)	*P*	*Pc*
*DPB1*0401*						
Genotype[a]	Homozygotes (Hz)	0	0			
	Heterozygotes (Ht)	6	6			
	Non-carrier (NC)	32	126			
Genotype comparison						
Dominant model	(Hz + Ht vs. NC)			3.94 (1.19–13.02)	0.028	0.364
Recessive model	(Hz vs. Ht + NC)			3.44 (0.07–176.32)	1.000	13.000
Allele	Positive	6	6	3.69 (1.15–11.78)	0.030	0.390
	Negative	70	258			
*DPB1*0901*						
Genotype[a]	Homozygotes (Hz)	5	2			
	Heterozygotes (Ht)	4	21			
	Non-carrier (NC)	29	109			
Genotype comparison						
Dominant model	(Hz + Ht vs. NC)			1.47 (0.61–3.52)	0.258	3.351
Recessive model	(Hz vs. Ht + NC)			9.85 (1.83–53.04)	0.007	0.085
Allele	Positive	14	25	2.16 (1.06–4.40)	0.029	0.377
	Negative	62	239			

[a] Hz, homozygous carrier with the targeted polymorphisms; Ht, heterozygous; NC, non-carrier.

Table 3. Association with polymorphic residues of DPβ chain in hepatitis C virus-associated hypertrophic cardiomyopathy

Position	Amino acid residue	Cases ($n = 38$)		Controls ($n = 132$)		OR (95% CI)	P	Pc
		Positive	Negative	Positive	Negative			
DPβ 8	L	32	6	129	3	0.12 (0.03–0.52)	0.004	0.008
DPβ 9	F	32	6	129	3	0.12 (0.03–0.52)	0.004	0.012
DPβ 11	G	32	6	129	3	0.12 (0.03–0.52)	0.004	0.008
DPβ 36	A	7	31	7	125	4.03 (1.32–12.35)	0.017	0.034
DPβ 55	A	7	31	7	125	4.03 (1.32–12.35)	0.017	0.051
DPβ 57	E	32	6	129	3	0.12 (0.03–0.52)	0.004	0.008
DPβ 76	M	32	6	129	3	0.12 (0.03–0.52)	0.004	0.012

Table 4. Amino acid sequence comparison of DPβ chain carrying susceptible or protective residues

Alleles	OR	Polymorphic positions															
		8 (6)	9 (9)	11 (6)	35 (9)	36 (9)	55 (9)	56 (9)	57 —	65 —	69 (4)	76 (4)	84 (1)	85 (1)	86 (1)	87 (1)	91 (1)
*DPB1*0201*	0.66	L	F	G	F	V	D	E	E	I	E	M	G	G	P	M	R
*DPB1*0202*	1.76	—	—	—	L	—	E	A	—	—	—	—	—	—	—	—	—
*DPB1*0301*	0.92	V	Y	L	—	—	—	—	D	L	K	V	D	E	A	V	—
*DPB1*0401*	3.69	—	—	—	—	A	A	A	—	—	K	—	—	—	—	—	—
*DPB1*0402*	0.57	—	—	—	—	—	—	—	—	—	K	—	—	—	—	—	—
*DPB1*0501*	0.83	—	—	—	L	—	E	A	—	—	K	—	D	E	A	V	—
*DPB1*0601*	3.51	V	Y	L	—	—	—	—	D	L	—	—	D	E	A	V	F
*DPB1*0901*	2.16	V	H	L	—	—	—	—	D	—	—	V	D	E	A	V	—
*DPB1*1301*	3.51	V	Y	L	Y	A	A	A	—	—	—	I	D	E	A	V	—
*DPB1*1401*	0.69	V	H	L	—	—	—	—	D	L	K	V	D	E	A	V	—
*DPB1*1701*	10.51	V	H	L	—	—	—	—	D	—	—	M	D	E	A	V	—
*DPB1*1901*	1.15	—	—	—	—	—	E	A	—	—	—	I	D	E	A	V	—
*DPB1*4101*	1.15	—	—	—	—	—	—	—	—	F	—	—	—	—	—	—	—

The polymorphic amino acid sequences are aligned with *DPB1*0201* as consensus, which is shown with the conventional one-letter code. Each odds ratio (OR) for *DPB1* alleles was shown. Dashes indicate sequence identity to the consensus at that position. Numbers in parentheses refer to the specificity pockets of DP molecule.

Identification of polymorphic amino acid residue of DPβ chain involved in HCV-HCM

To further assess the contribution of *HLA-DPB1* genes in the susceptibility to HCV-HCM, we attempted to identify which of the polymorphic amino acid residues within the DPβ chain showed primary association. Of 32 polymorphic positions in the DPβ chain from 115 known four-digit *DPB1* alleles provided by the IMGT/HLA database (www.ebi.ac.uk/imgt/hla, release 2.16.0, 12 January 2007), 16 positions were polymorphic in the studied subjects and they were used as analytical data. The polymorphic residues located in P9 pockets (at positions 36 A and 55 A) showed positive associations with HCV-HCM ($P < 0.017$, OR = 4.03, 95% CI = 1.32, 12.35) (Table 3). In contrast, five polymorphic residues showed significant negative associations as follows: 76M in P4 pocket; 8L and 11G in P6 pocket; 9F in P9 pocket and 57E adjacent to P9 pocket ($P < 0.004$, OR = 0.12, 95% CI = 0.03, 0.52) (Table 3). Quite interestingly, all the residues showing significant positive or negative associations composed of *DPB1*0401*. None of characteristic components of *DPB1*0901* allele demonstrated significant associations (data not shown).

HCV-HCM-associated amino acid motif shared within the HLA-DPB1 patchwork structure

Since DPβ chain has a patchwork structure with constant and polymorphic segment (Naruse *et al.*, 1995), we aligned amino acid sequence alignment of DPβ chain to refine a core structure for the progression and/or protection of HCV-HCM (Table 4). Both residues of DPβ chain, 36 A and 55 A, are the characteristic compositions of *DPB1*0401* allele associated with HCV-HCM. On the other hand, representative of *HLA-DPB1* alleles carrying the protective residues (8L, 9F, 11G, 57E and 76M) against HCV-HCM development share the majority of Japanese population, such as *DPB1*0201* (allele frequency: 0.205), *DPB1*0402* (0.170) and *DPB1*0501* (0.413) (Table 4). Quite interestingly, the susceptible allele, *DPB1*0401*, also included not only two susceptible residues (36 A and

55 A) but also resistant residues (8L, 9F, 11G, 57E, 76M). In contrast, the other susceptible allele, *DPB1*0901*, had neither the two susceptible nor the five resistance residues in the polymorphic positions.

Discussion

Clinical outcome of the HCV infection is known to vary substantially among the individuals despite the same viral aetiology. The classical *HLA* alleles, especially *HLA-DRB1* alleles, provide one with the major genetic contribution to HCV-related pathogenesis (Kuzushita *et al.*, 1998). Our hypothesis for this diversity is that specific *HLA* polymorphism(s) may influence the clinical outcome in HCV-associated cardiac disease as seen for the liver disease. Observations in the present study suggest that the *HLA-DPB1* gene plays an important role in the development of HCV-HCM, because we demonstrated that polymorphic residues within the P4, P6, and P9 pockets of DPβ chain were associated with the predisposition to HCV-HCM. In clear contrast, such association was not observed for HCV-DCM, suggesting that different HLA-linked genes were contributed to the pathogenesis of HCV-related cardiomyopathy. Although we could not exclude a possibility that the susceptibility to HCV-HCM was controlled by HLA-DPB1-linked genes, this was unlikely since we found that two different DPB1 alleles were associated with HCV-HCM as different genetic traits; *DPB1*0401* appeared to be a dominant trait while *DPB1*0901* was a recessive trait. Given that none of microsatellite markers within the MHC region showed significant association with the susceptibility to HCV-HCM (Shichi *et al.*, 2005), the finding in this study suggested that the microsatellite markers used in the previous mapping could not capture the susceptible SNP or residues within the patchwork pattern of DPβ chain. Even direct screening at the nucleotide level, both disease-predisposing alleles, *DPB1*0401* and *DPB1*0901*, showed marginal association. This may be due to the small number of patients, but the limitation of detectable resolution might also be due to the structures of *HLA* allelic polymorphisms. In particular, *DPB1* allelic variations are expected to result from the shuffling of sequence motifs through inter-allelic gene conversion (Zangenberg *et al.*, 1995).

Considering the role of HLA in the natural history of the HCV infection, the observations in this study lead to a speculation that the DP polymorphisms controlled the viral load and clearance/persistence of HCV. As for the viral load, majority of the patients with HCV-HCM showed relatively low HCV RNA titre (Matsumori *et al.*, 1999; Teragaki *et al.*, 2003) as compared to the patients with HCV hepatitis (Strader *et al.*, 2004). These data implied that HCV-HCM per se was associated with the lower viral load and hence the involvement of DP molecule in the pathogenesis of sporadic HCM. On the other hand, because we had no data on the HCV titre in the analysed patients, we could not investigate whether the DP polymorphisms influence the clearance or persistence of HCV.

HLA-DP resembles to the other classical HLA class II molecules, DR and DQ, in terms of structure. However, the roles of HLA-DP molecule in the immunological processes and disease susceptibility have not been well established because of the limited description of peptide interactions (Dong *et al.*, 1995; Hori *et al.*, 1996; Castelli *et al.*, 2002) and function of polymorphic positions (Berretta *et al.*, 2003; Diaz *et al.*, 2003). From the aspect of immune system, several reports, however, suggested the involvement of DPβ chain polymorphism in the susceptibility to diseases (Taylor *et al.*, 2002; de Graaff *et al.*, 2004). It is not very common to find that DP locus was primarily associated with infectious or autoimmune diseases. However, there are several reports demonstrating that *HLA-DPB1* was the primary locus to control the immune response to foreign antigens (Hori *et al.*, 1996; Sakaguchi *et al.*, 2002) or disease susceptibility (Fontenot *et al.*, 2000; Takahashi *et al.*, 2006). Molecular mechanisms of the susceptibility to HCV-HCM might at least in part involve the immunological function of the HLA-DP molecule.

The classical HLA class II molecules have five main specificity pockets (numbered P1, P4, P6, P7, and P9) that accommodate the side chains of antigenic peptide (Stern *et al.*, 1994; Jardetzky *et al.*, 1996). The diversity of DPβ chains carrying the susceptible and/or resistant residues to HCV-HCM was concentrated at the P9 pocket, suggesting that the P9 pocket is a core element for determining the course to HCM after the HCV infection. For instance, two HLA-DP4 subtypes observed in the Japanese population, susceptible *DPB1*0401* and non-susceptible *DPB1*0402*, differ only in three amino acids at positions 35, 36 and 56 composed of the P9 pocket, although these two alleles were reported to exhibit similar binding motifs (Castelli *et al.*, 2002; Busson *et al.*, 2006). Interestingly, *DPB1*0401* contains both susceptibility-associated residues (36 A and 55 A) and resistance-associated residues (8L, 9F, 11G, 57E, and 76M) facing into the peptide-binding groove. An explanation for this phenomenon is that the susceptibility would be dominant over the resistance. Alternatively, because all the polymorphic residues composing peptide-binding groove should participate in the binding of predictive disease-related antigenic peptide, even subtle changes in space, shape, and charge at a specificity pocket could influence the differential antigenic peptide loading and T-cell receptor accessibility to DP molecule. Therefore, the presence of two susceptible substitutions, 36 A and 55 A, within the P9 pocket of *DPB1*0401* might be responsible for the susceptibility conferred by DPβ chain in HCV-HCM. Conversely, the absence of protective residues (8L, 9F, 11G, 57E, and 76M) at the P4, P6 and P9 pockets as found in *DPB1*0901* may lead to less susceptible effect on HCV-HCM risk than the predominant *DPB1*0401* allele, thereby requiring double gene dose to exert sufficient effect on the susceptibility to HCV-HCM. Thus, both the susceptible alleles and their copy number (heterozygous or homozygous) probably confer risk to HCM-HCM by presenting distinct peptide with accessibility to the specificity pocket. Future studies are required to identify the possible disease-related antigenic peptides.

Our study, albeit preliminary, provides new and suggestive information on the immunological involvement of *DPB1* gene in the HCV-HCM development. Specifically, the polymorphic amino acids residues by which the DPβ chain adopt specificity pocket appear to influence on disease susceptibility at the allelic manner level. The existence of different risk alleles among HCV-related diseases including chronic liver disease, asymptomatic carrier and HCV-DCM suggests that each clinical outcome may arise from distinct pathogenic conditions on the basis of differential HLA-meditated immune responses. Involvement of proinflammatory cytokines such as interleukin-6 and tumour necrosis factor-alpha (TNF-α) which lead to hypertrophy of cardiomyocytes was also speculated in the pathogenesis of HCV-HCM (Matsumori *et al.*, 1994; Zen *et al.*, 2005). Several studies have demonstrated that TNF-α have an functional impact on cellular hypertrophy (Yokoyama *et al.*, 1997; Higuchi *et al.*, 2002) and on various cardiac ion channels (El-Ani & Zimlichman, 2003; Kawada *et al.*, 2006). Hence, immunological studies on function of disease-predisposing alleles are apparently needed to understand the HCV-related pathogenesis.

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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)

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

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.

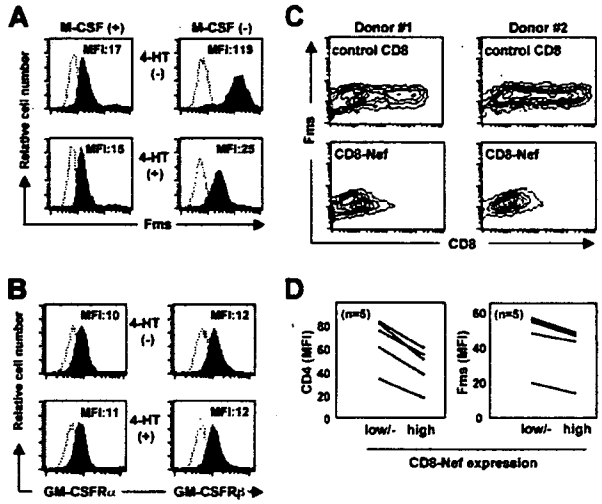

Figure 1. Nef inhibits surface expression of Fms. (A) In the left histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–containing media in the absence (upper) or presence (lower) of 0.1 mM 4-HT for 24 hours. In the right histograms, TF-1-fms-Nef-ER cells were precultured with M-CSF–free media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

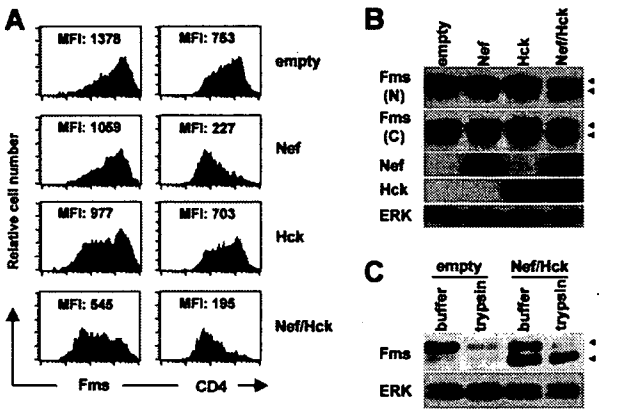

Figure 2. Nef reduces surface expression of Fms in 293 cells and increases intracellular gp130Fms, in the presence of Hck. (A) In the left panels, parental 293 cells were transfected with the Fms plasmid, alone or in combination with the plasmid for Nef (CD8-Nef) or Hck (IRES-EGFP), and then stained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

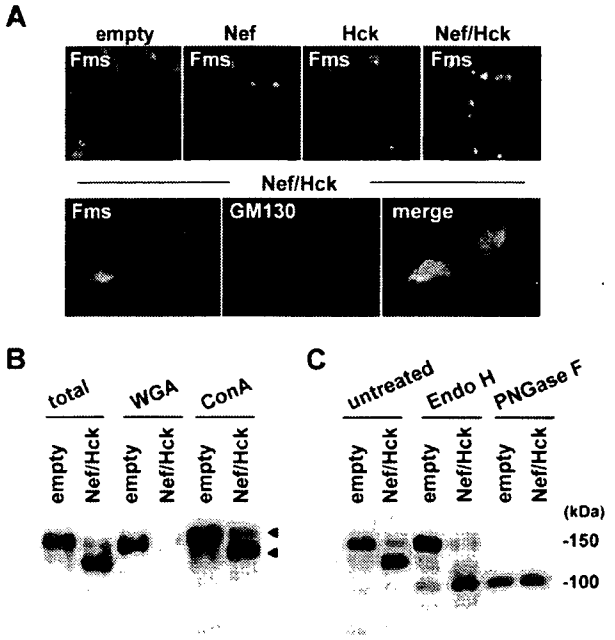

Figure 3. gp130Fms appearing in 293 cells coexpressing Nef/Hck is Golgi-localized underglycosylated Fms. (A) 293 cells stably expressing Fms were transfected with the control CD8 plasmid (empty) or CD8-Nef plasmid (Nef). Similarly, 293 cells stably coexpressing Fms and Hck were transfected with the control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

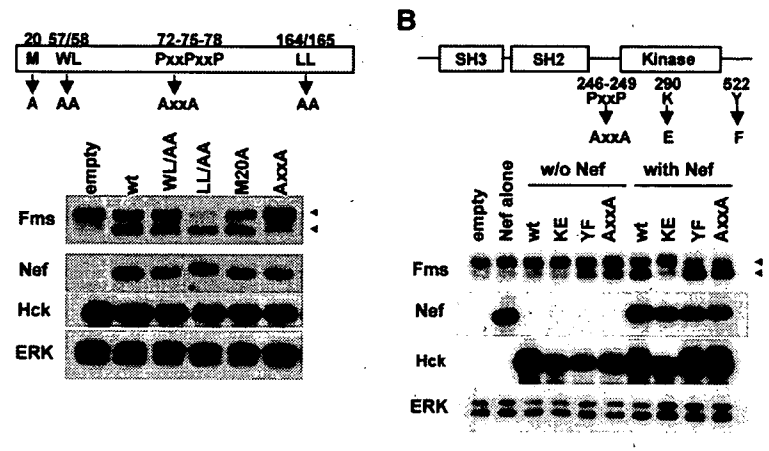

Figure 4. Activation of Hck by Nef is essential but not sufficient for accumulation of gp130Fms. (A) The Nef mutants used (M20A, WL/AA, AxxA, and LL/AA) are schematically shown. All the constructs are CD8-Nef chimeras. 293 cells stably expressing Fms were cotransfected with wild-type Hck and the plasmid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

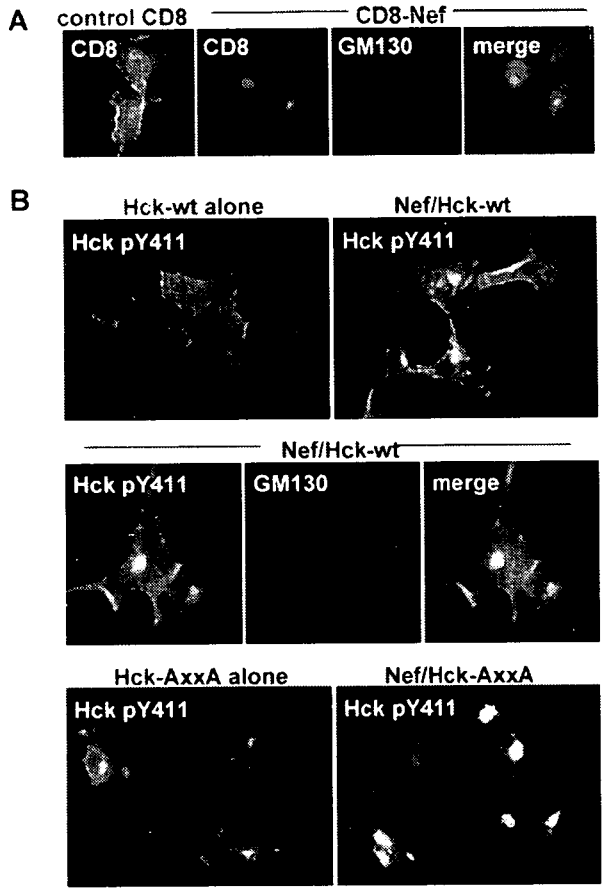

Figure 5. Nef induces Golgi localization of active Hck. (A) Parental 293 cells were transfected with the control CD8 plasmid or CD8-Nef plasmid, and then stained with anti-CD8 antibody (green), anti-GM130 antibody (red), or DAPI (blue). (B) In the top panels, parental 293 cells were transfected with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