Nef induces apoptosis by activating JNK signaling pathway and inhibits NF-κB-dependent immune responses in *Drosophila*

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Summary

The human immunodeficiency virus type 1 (HIV-1) *nef* gene encodes a 27-kDa protein that plays a crucial role during AIDS pathogenesis, but its exact functional mechanism has not been fully elucidated and remains controversial. The present study illuminated the in vivo functions of Nef using *Drosophila*, in which genetic analyses can be conveniently conducted. Using *Drosophila* transgenic lines for wild-type Nef, we demonstrated that Nef is not involved in the regulation of cell proliferation but rather specifically induces caspase-dependent apoptosis in wings in a cell-autonomous manner. Interestingly, myristoylation-defective Nef completely failed to induce the apoptotic wing phenotypes, consistent with previous reports demonstrating a crucial role for membrane localization of Nef in vivo. Further genetic and immunohistochemical studies revealed that Nef-dependent JNK activation is responsible for apoptosis. Furthermore, we found that ectopic expression of Nef inhibits *Drosophila* innate immune responses including Relish NF-κB activation with subsequent induction of an antimicrobial peptide, diptericin. The in vivo functions of Nef in *Drosophila* are highly consistent with those found in mammals and so we propose that Nef regulates evolutionarily highly conserved signaling molecules of the JNK and NF-κB signaling pathways at the plasma membrane, and consequently modulates apoptosis and immune responses in HIV target cells.

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Key words: Apoptosis, *Drosophila*, Nef, JNK, NF-κB

Introduction

The HIV-1 protein Nef is a myristoylated protein produced during early stages of infection (Kim et al., 1989). Significantly, several HIV-1 strains containing mutations in *nef* have been isolated from a few long-term non-progressing AIDS patients (Kirchhoff et al., 1995), implicating a central role of Nef in AIDS pathogenesis. Because apoptosis is one of the main causes of T-cell depletion in AIDS (Groux et al., 1992; Meyaard et al., 1992) and because Nef can induce apoptosis in lymphoid cells (for a review, see Roshal et al., 2001), it has been suggested that Nef might facilitate the progression of HIV infection to AIDS by positively regulating apoptosis in T cells. This notion was further supported by previous reports that transgenic expression of Nef in $CD4^+$ T cells in mouse causes AIDS-like immune symptoms, including dramatic $CD4^+$ T-cell depletion (Hanna et al., 1998), and that ectopic expression of CD8-Nef hybrid protein in Jurkat T cells leads to apoptosis (Baur et al., 1994). In addition, rhesus macaques infected with the simian immunodeficiency virus (SIV) containing deletions in its *nef* gene do not exhibit AIDS-like symptoms owing to a dramatic reduction of apoptosis in lymphoid cells compared with the wild-type virus-infected control (Xu et al., 1997).

Although the downstream molecules mediating Nef-dependent apoptosis have not been clearly identified, it has been revealed that Nef interacts functionally with various signaling pathways involved in the control of apoptosis, including the c-Jun N-terminal kinase (JNK) (Fackler et al., 1999; Varin et al., 2003), the phosphoinositide 3-kinase (PI3K) (Wolf et al., 2001; Blagoveshchenskaya et al., 2002), the ERK/mitogen-activated-protein kinase (ERK-MAPK) (Schrager et al., 2002) and the nuclear factor-κB (NF-κB) (Niederman et al., 1992; Mahlknecht et al., 2000; Varin et al., 2003) pathways. In mammalian cells, the JNK pathway is activated by Nef through an interaction between Nef and several upstream signaling proteins of JNK, such as Vav, PAK, Cdc42 and Rac1 (Fackler et al., 1999), and is mainly involved in the activation of viral replication by regulating the activity of the transcription factor activator-protein 1 (AP-1) (Varin et al., 2003). Interestingly, the JNK pathway is well documented to regulate apoptosis in other systems (for a review, see Leppa and Bohmann, 1999) and to control the process of negative selection of thymocytes by regulating apoptosis in vivo (Rincon et al., 1998).

In addition to the JNK pathway, the PI3K pathway and the ERK-MAPK pathway are also involved in the regulation of

apoptosis by inducing antiapoptotic signals. Intriguingly, Nef interacts directly with PI3K, specifically with its p85 regulatory subunit, which leads to the activation of PI3K and downregulation of major histocompatibility complex I through ARF6 activation (Blagoveshchenskaya et al., 2002). Consequently, these PI3K-mediated Nef functions enable HIV-1 to escape immune surveillance, and increase viral replication in the host cell (Wolf et al., 2001; Blagoveshchenskaya et al., 2002). In addition, the ERK-MAPK pathway is activated by Nef in a T-cell-receptor (TCR) dependent manner, which augments HIV-1 replication (Schrager et al., 2002).

NF-κB is a transcription factor composed of homo- and heterodimers of Rel-family proteins, and is known to be one of the main mediators of HIV-1 long-terminal-repeat (LTR) dependent transcription (Varin et al., 2003). In addition to its role in HIV-1 viral transcription, NF-κB activity is required for the proper functioning of immune systems in the host cell (Ghosh et al., 1998), which suggests a possibility that disruption of NF-κB activity by Nef might contribute to the lowered immune responses observed in AIDS patients. Interestingly, Nef regulates NF-κB activity either positively or negatively, depending on the experimental conditions in mammalian cells (Niederman et al., 1992; Mahlknecht et al., 2000).

To identify the signaling molecules responsible for the in vivo functions of Nef in AIDS pathogenesis, we used a transgenic approach in *Drosophila*. Unlike research carried out with mammalian cells, *Drosophila* allows us to investigate the physiological effects of Nef through well-defined phenotypic evaluations and genetic approaches using tissue-specific and inducible expression systems (Fischer et al., 1988; Battaglia et al., 2001; Leulier et al., 2003; Lee et al., 2003). Notably, *Drosophila* transgenic lines encoding the HIV-1 proteins Tat and Vpu have been previously generated and successfully used to demonstrate the functional interaction between Tat and tubulin (Battaglia et al., 2001), and the in vivo role of Vpu in inhibition of NF-κB-dependent immune responses (Leulier et al., 2003). In the present study, we have generated *Drosophila* transgenic lines for Nef and found that Nef expression induces JNK-dependent apoptosis and also inhibits *Drosophila* innate immune responses mediated by the Relish NF-κB pathway.

Materials and Methods

Drosophila strains

The upstream activation sequence (UAS) fly lines *basket* (*bsk*; *Drosophila JNK*) and *hemipterous* (*hep*; *Drosophila MKK7*) were gifts from M. Mlodzik (EMBL-Heidelberg, Germany). The hep^{1} (a loss-of-function mutant for *hep*) fly line was kindly provided by S. Noselli (CNRS, Nice, France). The fly lines bsk^{1} (a loss-of-function mutant for *bsk*), hep^{r75} (a loss-of-function mutant for *hep*), heat-shock flipase (hsFLP), *hsp70*-GAL4, *ap*-GAL4, *en*-GAL4, ay-GAL4s and UAS-JNK^{DN} (a dominant negative form of *bsk*) were obtained from the Bloomington Stock Center (Bloomington, IN). The *puckered*-lacZ [*puckered* (*puc*) encodes a *Drosophila* JNK-specific phosphatase] reporter fly line and UAS-hep^{CA} (a constitutively active form of *hep*) line were kindly provided by T. Adachi-Yamada (Kobe University, Kobe, Japan). The MS1096-GAL4 driver line was a gift from M. Freeman (MRC Laboratory of Molecular Biology, Cambridge, UK). EP(X)1516, a *Drosophila TRAF2* expression line, was obtained from the Szeged *Drosophila melanogaster* P Insertion Mutant Stock Center, Szeged, Hungary.

Transgenic *nef* fly lines

Wild-type *nef* (GenBank accession number AF011469) and a myristoylation-site mutant of *nef* (G2A; a mis-sense point mutant that converts glycine-2 to alanine) were each subcloned into a pUAST vector. Standard procedures for microinjection of these plasmids into eggs were followed (Ashburner, 1989) using a microinjector (model IM300, Narishige, Japan) and an Axiovert 25 micromanipulator (Carl Zeiss, Germany).

Reverse transcription PCR

For reverse transcription PCR (RT-PCR), total RNA was isolated from wild-type and *nef*-expressing third-instar larvae using the easy-Blue™ system (Intron, Korea) and reverse-transcribed into cDNA. PCR was performed with *nef*-specific primers (5′-ATGGGTGGCAAGTGGTC-3′ forward and 5′-TCAGCAGTCTTTGTAGTAG-3′ reverse) to amplify the full-length *nef*.

Histochemical analysis

Drosophila wing imaginal discs were dissected and fixed in 4% paraformaldehyde in PBS. They were washed in PBST (PBS plus 0.1% Triton X-100) and then blocked in PBST with 3% bovine serum albumin. The samples were co-stained overnight at 4°C with mouse anti-β-galactosidase antibody (JIE7, 1:100 dilution; DSHB, IA) with either rabbit antibody against active Drice (a *Drosophila* homolog of mammalian caspases) [1:200 dilution, a gift from B. A. Hay (California Institute of Technology, Pasadena, CA)] or anti-phosphospecific JNK rabbit antibody (1:100 dilution; Promega, WI). Rabbit anti-Relish antibody [1:200 dilution; a gift from S. Stoven (Umea University, Umea, Sweden)] and anti-phosphospecific histone-H3 rabbit antibody (1:200 dilution; Upstate Biotechnology, VA) were also used appropriately. To detect these primary antibodies, the samples were further incubated for 4 hours at room temperature with a Cy5-labeled anti-mouse secondary antibody (1:100 dilution; Jackson ImmunoResearch, PA) and an FITC-labeled anti-rabbit secondary antibody (1:200 dilution; Sigma, MO) for co-staining. The FITC-labeled anti-rabbit secondary antibody alone was used to detect either rabbit anti-Relish or rabbit anti-phosphospecific histone-H3 antibody. The immunostained samples were visualized with an LSM510 laser confocal microscope (Carl Zeiss, Germany). In order to visualize the nuclear structure, the fat-body cells from third-instar larvae were stained with BOBO-3 iodide (1:10,000 dilution; Molecular Probes, OR) for 20 minutes. For acridine orange (Sigma, MO) staining, third-instar larval wing discs were dissected in PBS and incubated for 5 minutes in 1.6 μM acridine orange solution in PBS.

puc-lacZ reporter assay

For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining, wing imaginal discs were fixed in 4% formaldehyde in PBS for 10 minutes and then incubated in standard X-Gal staining solution [4.9 mM X-Gal, 3.1 mM $K_4Fe(CN)_6$, 3.1 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.3% Triton X-100] for 4 hours at 37°C before observation.

BrdU labeling experiment

Wing imaginal discs were dissected from third-instar larvae in Ringer's solution and incubated in the presence of 100 μg ml^{-1} 5-bromo-2′-deoxyuridine (BrdU; Roche, Germany) in Shields and Sang M3 insect medium for 1 hour at room temperature. The samples were fixed in Carnoy's fixative (ethanol:acetic-acid:chloroform=6:3:1) for 30 minutes at 25°C and sequentially treated with 70% ethanol in PBST, 50% ethanol in PBST, and 30% ethanol in PBST for 3 minutes each. Next, the samples were incubated in 2 N HCl for 1 hour and the incorporated BrdU was visualized using mouse anti-BrdU antibody

(G3G4, 1:200 dilution; DSHB, IA) and a TRITC-labeled anti-mouse secondary antibody (1:200 dilution; Jackson ImmunoResearch, PA). The samples were observed using a confocal laser microscope (Carl Zeiss, Germany).

Clonal expression of *nef*

In a hsFLP/ay-GAL4 system, heat-shock-induced expression of flipase derived from hsFLP renders consistent expression of GAL4 by flipping out the inhibitory region inserted within the GAL4 promoter of an ay-GAL4 driver (Ito et al., 1997). To generate *nef*-expressing clones, the second-instar larvae containing both hsFLP/ay-GAL4 and UAS-*nef* were subjected to heat shock for 1 hour, then incubated at 25°C for 48 hours before immunohistochemical analysis.

Bacterial infection

Bacterial challenges to induce immune responses were performed by pricking third-instar larvae with a thin needle that had been dipped into a concentrated bacterial culture of *Escherichia coli*. The larvae were pretreated with a heat shock for 30 minutes at 37°C followed by incubation for 90 minutes at 25°C to induce *nef* expression. The infected larvae were incubated at 25°C for another 3 hours in a Petri dish containing the standard *Drosophila* medium before conducting subsequent experiments.

Northern-blot analysis

Total RNA, extracted by the easy-Blue system (Intron, Korea), was separated by electrophoresis on denaturing formaldehyde-agarose gels in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, pH 5.2, 1 mM EDTA), transferred onto a nylon membrane and successively hybridized with nick-translated ^{32}P-labeled cDNA probes of full-length *nef*, full-length *puc* or full-length *Diptericin* (*Dpt*) (Lee et al., 2001). Hybridized probes were visualized by autoradiography.

TUNEL assay for wing imaginal discs

Third-instar larval wing discs were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. The samples were then washed with PBS and permeabilized by incubation in a solution containing 0.1% sodium citrate and 0.1% Triton X-100 at 65°C for 30 minutes. After extensive washing, the samples were submerged in a terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) reaction solution (Roche, Germany) and incubated in a 37°C chamber for 3 hours. After rinsing three times with PBS, the wing discs were observed using a confocal laser microscope (Carl Zeiss, Germany).

Results

Drosophila lines transgenic for *nef*

To understand the in vivo functions of the HIV-1 Nef protein, we have generated transgenic fly lines for *nef* (GenBank accession number AF011469). Ectopic expression of *nef* was induced in a tissue-specific manner using wing-specific GAL4 drivers (MS1096-GAL4 and *ap*-GAL4, which drive *GAL4* expression in the whole region and the dorsal region of *Drosophila* wing, respectively). The whole-wing size was reduced when *nef* was expressed by the MS1096-GAL4 driver (compare Fig. 1B with its control, 1A,G). Moreover, the overall size of larval-wing imaginal discs was also reduced by *nef* expression (Fig. 1G). This Nef-induced phenotype became more severe when the gene dosage of *nef* was doubled (Fig. 1C,G). Consistently, a convex wing phenotype was observed

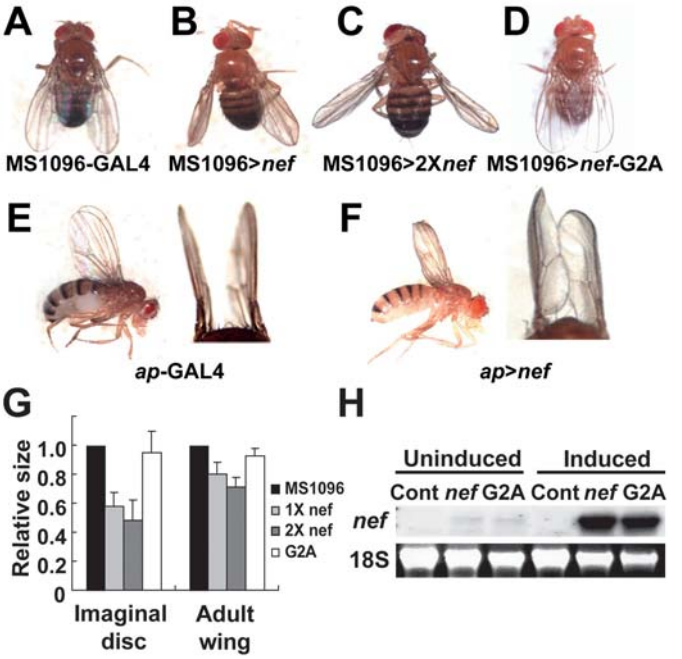

Fig. 1. Characterization of *Drosophila* wing phenotypes induced by *nef* expression. (A) MS1096/Y. (B) MS1096/Y;; UAS-*nef*/+. (C) MS1096/Y;; UAS-*nef*/UAS-*nef*. (D) MS1096/Y;; UAS-*nef*-G2A/+. (E) *ap*-GAL4/bc. (F) *ap*-GAL4/+; UAS-*nef*/+. (G) Comparison between tissue sizes of *nef*-expressing wings and control wings. Wing imaginal discs were obtained from third-instar larvae. The standard deviation is obtained by examining 20 samples of the same genotype. Fly genotypes: MS1096 (MS1096/X). 1×*nef* (MS1096/X;; UAS-*nef*/+). 2×*nef* (MS1096/X;; UAS-*nef*/UAS-*nef*). G2A (MS1096/X; UAS-*nef*-G2A/+). (H) Determination of expression and induction levels of *nef* transcript using northern-blot analysis. To induce expression of wild-type and G2A *nef*, third-instar larvae were subjected to 2 hours of heat shock at 37°C. 18S rRNA was used as a control. Fly genotypes were as follows. Cont: Control, *hsp70*-GAL4/+. *nef*: *hsp70*-GAL4/+; UAS-*nef*/+. G2A: *hsp70*-GAL4/UAS-*nef*-G2A.

when *nef* was expressed by the *ap*-GAL4 driver (compare Fig. 1F with its control, 1E), supposedly caused by unproportionally reduced surface area of the dorsal layer compared with the ventral layer of the two-cell-layered wing blade.

Interestingly, however, expression of *nef*-G2A, encoding a myristoylation-site point mutant of Nef, under the same conditions did not induce any detectable phenotypes (Fig. 1D,G). Because the expression levels of *nef* and *nef*-G2A were similar (Fig. 1H, see Fig. S1A in supplementary material), these data strongly imply that proper localization of Nef to the plasma membrane is crucial for its in vivo activity, consistent with previous reports demonstrating the importance of the membrane localization of Nef for carrying out its proper functions in mammalian cells (Guy et al., 1990; Baur et al., 1994).

Caspase-dependent apoptosis induced by Nef

In multicellular organisms, size reduction of tissues can be caused either by increased cell death or by inhibited cell proliferation. Therefore, in order to determine the cause of the

Nef-induced wing phenotype, we examined whether *nef* expression can induce apoptosis in the wing by carrying out vital dye (acridine orange) staining (Fig. 2A-C) and immunostaining (Fig. 2D-F) with an antibody against active Drice (a *Drosophila* homolog of mammalian caspases). Expression of *nef* dramatically increased apoptotic cell death and caspase activation in the wing imaginal disc (Fig. 2B,E) compared with the control (Fig. 2A,D), whereas expression of *nef*-G2A did not induce either apoptosis or caspase activation (Fig. 2C,F).

Next, to determine whether Nef is also capable of affecting cell proliferation, we conducted BrdU incorporation experiments and immunostaining with a phosphorylated-histone-H3-specific antibody that labels mitotic cells in M phase (Brodsky et al., 2000). As shown in Fig. 2G-L, however, expression of neither wild-type nor G2A mutant *nef* in the wing imaginal disc affected cell proliferation.

Collectively, these results demonstrate that the reduced wing size observed in Nef transgenic flies is caused by Nef-induced caspase-dependent apoptosis.

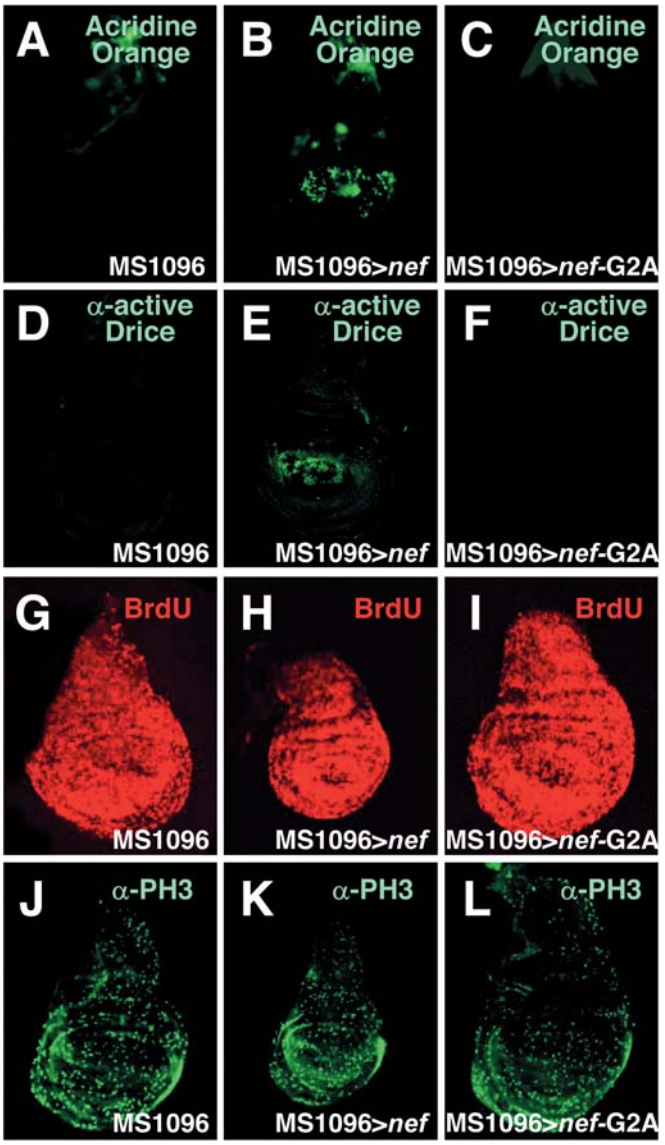

Fig. 2. Caspase-dependent apoptosis in wings expressing *nef*. (A-F) Nef-induced caspase-dependent apoptosis in wings. Wing imaginal discs were stained with acridine orange (A-C) or antibody against active Drice (D-F). (G-L) Nef did not affect cell proliferation in wings. Wing imaginal discs were examined by BrdU incorporation assay (G-I) or by immunostaining with antibody against phosphospecific histone H3 (J-L).

Activation of JNK signaling by Nef

To identify cellular signaling pathways responsible for the Nef-dependent apoptosis observed in the fly system, we investigated in vivo (genetic) interactions between Nef and various signaling molecules including rl/ERK, JNK, p38 and Akt as representative components for the MAPK and PI3K signaling pathways. Interestingly, whereas other signaling molecules did not show any notable interactions with Nef (data not shown), we found that JNK (basket, *Drosophila* JNK) interacted strongly with Nef.

Simultaneous expression of either *bsk* (encoding *Drosophila* JNK) (Fig. 3C) or *hep* (encoding *Drosophila* MKK7, a direct upstream kinase of *Drosophila* JNK) (Fig. 3D) with *nef* resulted in a more-severe wing phenotype than the phenotype induced by *nef* expression alone (Fig. 1B), whereas expression of either *bsk* (Fig. 3A) or *hep* (Fig. 3B) alone by the MS1096-GAL4 driver did not induce any detectable phenotypes. These data suggest the possibility that the JNK pathway is responsible for the apoptotic wing phenotypes induced by *nef* expression. Indeed, this was confirmed by the subsequent observation that, when *nef* was expressed in either a heterozygous bsk^1 (Fig. 3F) or a hemizygous hep^1 genetic background (Fig. 3H) (bsk^1 and hep^1 are the loss-of-function mutants for *bsk* and *hep*, respectively), the wing phenotypes induced by *nef* expression (Fig. 1B, Fig. 3G) were strongly suppressed. Consistently, apoptosis induced by *nef* expression in the wing imaginal disc (Fig. 3J) was also strongly suppressed in heterozygous bsk^1 genetic background (Fig. 3K).

To support further the genetic interaction between Nef and the JNK pathway, we carried out a *puc*-lacZ reporter assay. The *puc* gene is a well-known downstream target of JNK in *Drosophila* (Tateno et al., 2000). Consistent with our previous data, we detected a strong induction of *puc* by Nef (Fig. 3M, see Fig. S1C,F in supplementary material), which was comparable to its expression induced by hep ectopic expression (Fig. 3O, see Fig. S1D in supplementary material). Moreover, the ectopic induction of *puc* by Nef expression was completely suppressed in a hemizygous hep^{r75} (a loss-of-function mutant for *hep*) genetic background (Fig. 3N, see Fig. S1G in supplementary material), demonstrating that the induction of *puc* expression by Nef was caused by Nef-dependent JNK activation. Interestingly, *puc* expression was induced only in the wing blade, whereas *nef* was expressed throughout the dorsal and posterior compartments of the wing discs by *ap*-GAL4 (Fig. 3M) and *en*-GAL4 (see Fig. S1F in supplementary material), respectively, suggesting that JNK activation in response to *nef* expression is not the general phenomenon common to all cell types.

From these results, we conclude that the JNK pathway mediates Nef signals to induce caspase-dependent apoptosis.

Induction of JNK activation and apoptotic activities by Nef in a cell-autonomous manner

To further support the correlation between the Nef-dependent

JNK activation and apoptosis and also to determine whether the Nef-induced apoptosis occurs in a cell-autonomous manner, we decided to conduct clonal analyses. Using the FRT-FLP system, clones expressing *nef* could be generated in the wild-type background of wing imaginal discs. In order to detect JNK activation and caspase-dependent apoptotic activities, the wing discs containing clones expressing *nef* were immunostained with anti-phosphospecific JNK antibody (Fig. 4A,D), which specifically labels activated JNK, and with antibody against active Drice (Fig. 4G,J), respectively. Notably, as shown in Fig. 4, the staining patterns of both antibodies (Fig. 4C,F,I,L) were correlated to the regions expressing *nef* (Fig. 4B,E,H,K), which were detected by using anti-β-galactosidase antibody (cytosolic β-galactosidase is a marker for GAL4 expression of the ay-GAL4 driver used in this experiment.). Interestingly, the active-JNK and active-Drice signals appeared in a scattered manner, a sign of apoptotic bodies, in *nef*-expressing clones. Furthermore, we also observed active Drice signals in the cytoplasm of the *nef*-expressing cells when we used a different ay-GAL4 driver that expresses its marker protein (β-galactosidase) in the nucleus (Fig. 4M-O). By contrast, we could not detect any signals for either JNK or caspase activation in the control cells expressing GAL4 driver alone (data not shown).

To determine whether JNK and caspase activation indeed correlate with apoptosis, the apoptotic cell death of the Nef-expressing cells was examined using TUNEL assay. As a result, TUNEL-positive apoptotic signals (red) were detected in most of the *nef*-expressing cells, marked by green-fluorescent-protein signals (green) (see Fig. S2 in supplementary material).

Collectively, these results clearly demonstrate that *nef* expression induces JNK activation and caspase-dependent apoptosis in a cell-autonomous manner.

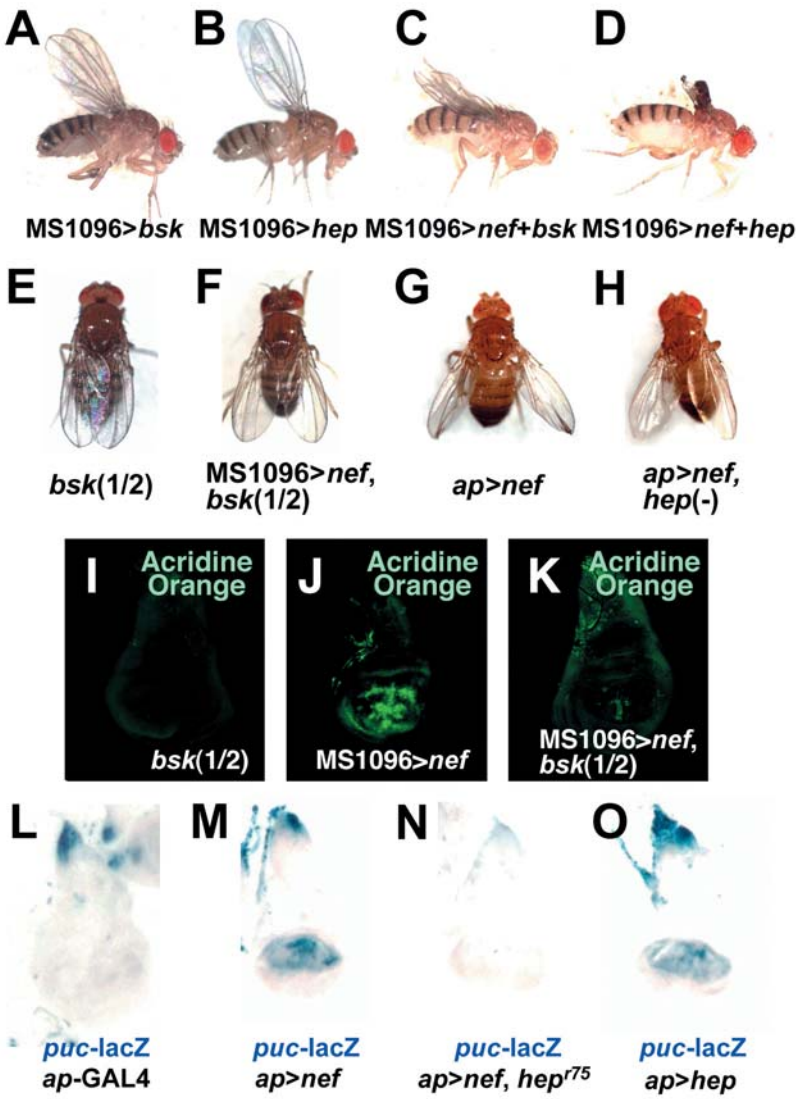

Fig. 3. JNK signaling pathway mediates Nef-induced apoptosis. (A-H) Functional interactions between Nef and the JNK pathway were determined by wing phenotypes. Fly genotypes were as follows. (A) MS1096/X; UAS-*bsk*/+. (B) MS1096/X; UAS-*hep*/+. (C) MS1096/X; UAS-*bsk*/+; UAS-*nef*/+. (D) MS1096/X; UAS-*hep*/+; UAS-*nef*/+. (E) bsk^{1}/+. (F) MS1096/Y; bsk^{1}/+; UAS-*nef*/+. (G) Basc/Y; *ap*-GAL4/+; UAS-*nef*/+. (H) hep^{1}/Y; *ap*-GAL4/+; UAS-*nef*/+. (I-K) Suppression of the Nef-induced apoptosis by reducing JNK gene dosage. Wing imaginal discs were stained with acridine orange. Fly genotypes were as follows. (I) bsk^{1}/+. (J) MS1096/X;; UAS-*nef*/+. (K) MS1096/Y; bsk^{1}/+; UAS-*nef*/+. (L-N) Enhanced *puc* expression caused by *nef* expression. Wing imaginal discs were stained with X-gal to determine β-galactosidase production. Fly genotypes were as follows. (L) *ap*-GAL4/+; *puc*-lacZ/+. (M) *ap*-GAL4/+; UAS-*nef*/*puc*-lacZ. (N) hep^{r75}/Y; *ap*-GAL4/+; UAS-*nef*/*puc*-lacZ. (O) *ap*-GAL4/UAS-*hep*; *puc*-lacZ/+.

Inhibition of *Drosophila* innate immune responses by Nef

Next, we decided to determine whether *nef* expression affects *Drosophila* immune responses. *Drosophila* innate immune responses against microbial infections include local melanization at the site of infection and systemic antimicrobial responses such as activation of NF-κB and concomitant induction of antimicrobial-peptide production (for a review, see Hoffmann and Reichhart, 2002).

First, we examined the melanization reaction after bacterial infection in third-instar larvae. Surprisingly, *nef* expression strongly inhibited the melanization reaction around the infected area in a highly reproducible manner (see Fig. S3A-C in supplementary material). To demonstrate further the regulation of immune responses by Nef, we next examined the transcriptional expression of *Dpt* using northern-blot analyses. *Dpt* encodes a representative *Drosophila* antimicrobial peptide and is transcriptionally induced mainly in fat-body cells by activated Relish NF-κB (an NF-κB in *Drosophila*) (Vidal et al., 2001; Lee et al., 2001). Interestingly, the induction of *Dpt* expression by bacterial infection was inhibited by *nef* expression (Fig. 5A,B), whereas expression of *nef*-G2A did not inhibit *Dpt* expression (Fig. 5A). In addition, the inhibition of *Dpt* expression by Nef occurred in a dose-dependent manner (Fig. 5B).

Because the induction of *Dpt* is fully dependent

on Relish NF-κB (Hedengren et al., 1999), we examined whether *nef* expression inhibits the activity of Relish NF-κB. We immunostained the fat-body cells dissected from *nef* transgenic and control larvae with anti-Relish antibody and BOBO-3 iodide, which stains the nucleus. We found that ectopic expression of *nef* inhibited the nuclear localization of Relish NF-κB induced by bacterial infections (Fig. 5C). These results demonstrate that Nef inhibits Relish NF-κB activation in *Drosophila*, further supporting the role of Nef in negatively regulating immune responses.

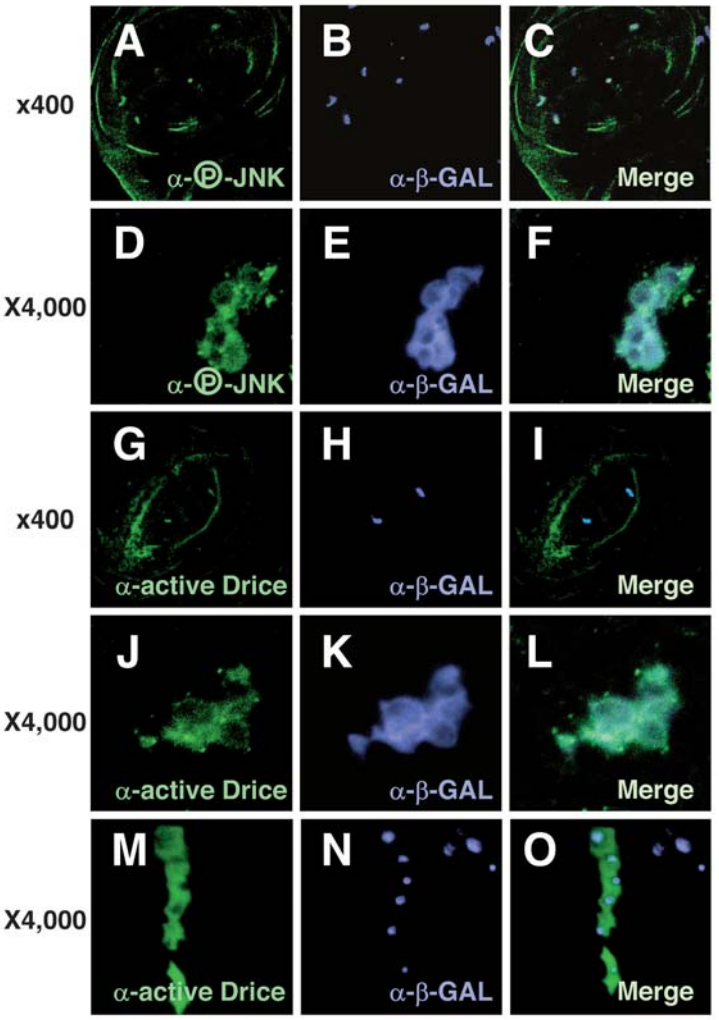

Fig. 4. JNK activation and apoptosis in the clones expressing *nef*. (A-F) Cells with active JNK and *nef*-expressing clones in wing imaginal discs were determined by immunostaining with anti-phosphospecific JNK antibody (green) (A,D) and anti-β-galactosidase antibody (purple) (B,E), respectively. Merged images are shown (C,F). In this experiment, Nef-expressing cells were marked by cytosolic β-galactosidase expression. (G-O) Cells with active Drice and *nef*-expressing clones in wing imaginal discs were determined by immunostaining with antibody against active Drice (green) (G,J,M) and anti-β-galactosidase antibody (purple) (H,K,N), respectively. Merged images are shown (I,L,O). In this experiment, *nef*-expressing cells were marked by either cytosolic β-galactosidase (H,K) or nuclear β-galactosidase (N). The wing disc images shown represent clones generated either in the columnar cell layer (A-L) or in the peripodial membrane cell layer (M-O) and were taken under a magnification of 400× (A-C,G-I) or 4000× (D-F,J-L,M-O).

Moreover, because *Drosophila* TRAF2 (DTRAF2), one of the *Drosophila* homologs of mammalian TRAFs, selectively triggers NF-κB-dependent induction of antimicrobial-peptide production without interacting with the JNK signaling pathway (Cha et al., 2003), we examined whether *nef* expression also inhibits DTRAF2-induced Relish NF-κB activation by measuring the *Dpt* expression level. Whereas ectopic expression of *DTRAF2* induced *Dpt* expression in a dose-dependent manner, simultaneous expression of *nef* with *DTRAF2* strongly suppressed the DTRAF2-induced *Dpt* expression (Fig. 5D). This result further supports our previous observation that Nef inhibits *Drosophila* innate immune responses, and suggests that the inhibition of Relish NF-κB activity by Nef is likely to occur downstream of TRAF in our system.

Finally, we examined whether the inhibition of *Drosophila* innate immune responses by Nef is also dependent on JNK activation. Interestingly, the inhibition of melanization by Nef was not significantly suppressed by reducing the gene dosage of *hep* or by simultaneous expression of a dominant negative form of JNK (JNK^{DN}) (see Fig. S3D in supplementary material). Moreover, Nef still strongly inhibited *Dpt* gene expression induced by bacterial infection (Fig. 5E) or *DTRAF2* expression (Fig. 5F) in the larvae with lowered JNK activities, caused by overproduction of JNK^{DN} and/or deletion of its upstream kinase (homozygous hep^{1} mutation). Consistently, activation of JNK by transient expression of hep^{CA}, encoding a constitutively active form of JNKK, also did not affect *Dpt* gene expression in either infected or uninfected larvae (see Fig. S3E in supplementary material). These data demonstrate that Nef inhibits *Drosophila* innate immune responses independent of JNK activation.

Discussion

In the current study, we have investigated the in vivo functions of the HIV-1 Nef protein by analysing flies carrying a transgenic version of the *nef* gene. In *Drosophila*, because most signaling pathways are well conserved and because genetic and histological studies can be conveniently conducted, we reasoned that using *nef*-transgenic flies would provide a better and simpler way to understand the molecular mechanisms behind Nef-dependent AIDS pathogenesis. Indeed, by observing its intracellular effects on apoptosis and immune responses, and by analysing its genetic interactions with various signaling components, we were able to gain valuable insights into the in vivo roles of Nef.

Nef induces caspase-dependent apoptosis in a cell-autonomous manner

Apoptosis has been suggested to be responsible for the T-cell depletion observed in AIDS patients, and so many efforts have been directed towards elucidating the function of HIV-1 proteins in apoptosis. Other HIV-1 proteins such as Tat, Vpr and Env are also known to induce apoptosis in lymphoid cells (Roshal et al., 2001). However, previous observations that Nef harbors a major disease determinant

(Hanna et al., 1998) and that functional Nef is crucial for AIDS pathogenesis (Kirchhoff et al., 1995; Xu and Screaton, 2001) suggest that Nef-dependent induction of apoptosis might play a central role in the progression to AIDS in HIV-infected patients.

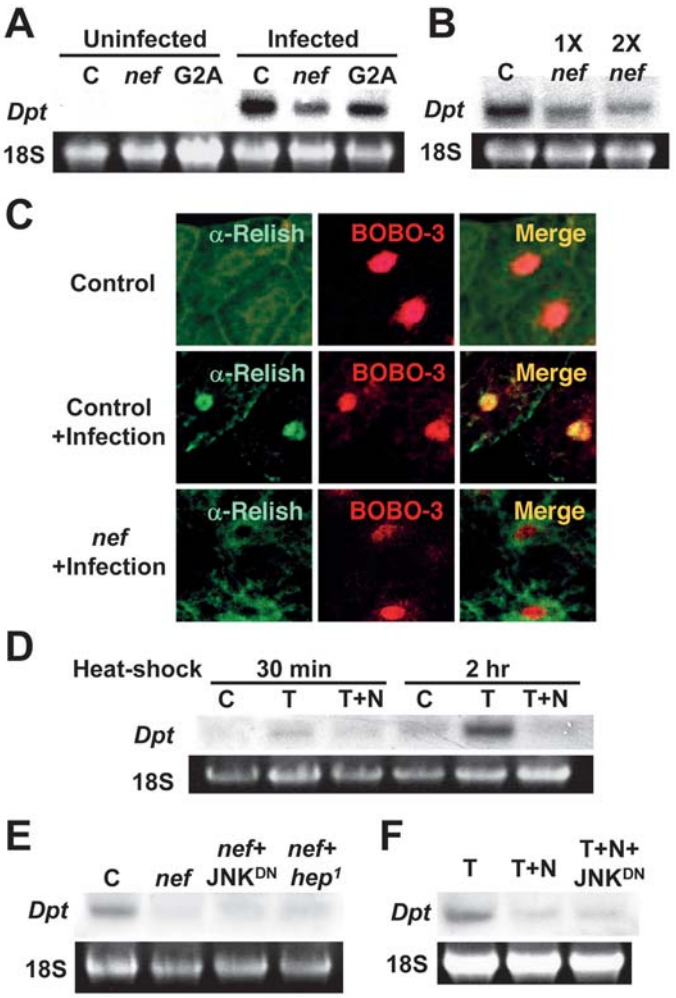

Fig. 5. Inhibition of immune responses by Nef. (A,B) Induction of *Dpt* mRNA by bacterial infection was determined by northern-blot analysis (top). 18S rRNA was used as a loading control (bottom). (C) Intracellular localization of Relish was determined by immunostaining with anti-Relish antibody (left). BOBO-3 iodide was used to visualize the nuclear structure (middle). Merged images (right) are shown. (D) Induction of *Dpt* mRNA by DTRAF2 overexpression was determined by northern-blot analysis (top). 18S rRNA was used as a loading control (bottom). (E,F) Induction of *Dpt* mRNA by bacterial infection (E) or *DTRAF2* overexpression (F) was determined by northern-blot analysis (top). 18S rRNA was used as a loading control (bottom). Fly genotypes were as follows. C: Control, *hsp70*-GAL4/+. *nef* or 1×*nef*: *hsp70*-GAL4/+; UAS-*nef*/+. 2×*nef*: *hsp70*-GAL4/+; UAS-*nef*/UAS-*nef*. G2A: *hsp70*-GAL4/UAS-*nef*-G2A. *nef*+JNK^{DN}: UAS-*JNK^{DN}*/+; *hsp70*-GAL4/+; UAS-*nef*/+. *nef*+*hep^{1}*: *hep^{1}*/*hep^{1}*; *hsp70*-GAL4/+; UAS-*nef*/+. T: EP(X)1516/X; *hsp70*-GAL4/+. T+N: EP(X)1516/X; *hsp70*-GAL4/+; UAS-*nef*/+. T+N+JNK^{DN}: EP(X)1516/UAS-*JNK^{DN}*; *hsp70*-GAL4/+; UAS-*nef*/+. To induce ectopic gene expression, third-instar larvae were heat shocked for 30 minutes at 37°C and subsequently incubated for 90 minutes at 25°C [A,B,D (left three lanes),E] or heat shocked for 2 hours at 37°C [C,D (right three lanes),F].

Despite much previous evidence that Nef induces apoptosis both in a host cell and its surrounding cells, many of the recent studies have focused primarily on apoptosis in the surrounding cells (Geleziunas et al., 2001; Xu and Screaton, 2001), through induction of Fas ligand from the *nef*-expressing host cell. In this study, we clearly demonstrated that wild-type Nef drives apoptosis only in a cell-autonomous manner (Fig. 4). The results demonstrate that cell-autonomous apoptosis by Nef is a plausible mechanism for explaining the T-cell depletion observed in AIDS patients, although cell-to-cell recognition and the intracellular signaling pathways activated or inhibited by Nef might function differently in human lymphoid cells and in *Drosophila* wing cells.

Nef interacts with two distinct intracellular signaling pathways: JNK and NFκB

Using genetic approaches, we revealed that the JNK signaling pathway (consisting of MKK7 and JNK) is tightly correlated with Nef-dependent apoptosis in wings, and also that myristoylation of Nef is crucial for cell-autonomous JNK activation. However, to understand how Nef activates the JNK pathway, the upstream components of the JNK pathway that interact directly with Nef remain to be identified. Interestingly, previous reports have suggested that Nef activates JNK activity through interaction with membrane-localized Vav and PAK (NAK) proteins, and that PAK2 is involved in Nef-dependent apoptosis (Fackler et al., 1999; Fackler et al., 2000; Roshal et al., 2001). This implicates these molecules in Nef-dependent JNK activation to induce cell-autonomous apoptosis. Vav and PAK are well conserved between *Drosophila* and mammals (Harden et al., 1996; Hing et al., 1999; Dekel et al., 2000). Therefore, further genetic studies investigating interactions between these molecules and Nef in *Drosophila* should be pursued, if relevant mutants are available.

Furthermore, we have shown that *nef* expression inhibited *Drosophila* innate immune responses including Relish NF-κB activation, concomitant induction of antimicrobial-peptide production and the melanization reaction in which Toll receptor and its downstream NF-κB pathway are thought to be involved (Hoffmann and Reichhart, 2002; Ligoxygakis et al., 2002). The NF-κB pathway in *Drosophila* is crucial for the *Drosophila* immune system and the signaling components of the pathway are highly conserved in mammals (Hoffmann and Reichhart, 2002). Judging from our data and the fact that NF-κB activity is required in the mammalian system for proper functioning of the immune system, such as T-helper-cell development and immunoglobulin- and cytokine-gene activation (Ghosh et al., 1998), it is highly possible that the inhibition of NF-κB by Nef also contributes to the lowered immune responses observed in AIDS patients.

Finally, we demonstrated that the inhibition of *Drosophila* innate immune responses by Nef is independent of the Nef-induced JNK activation. Our results indicate that Nef might regulate the NF-κB and JNK signaling pathways separately by interacting with different upstream molecules for each pathway at the plasma membrane. Taken together, we cautiously suggest a possible model for in vivo signaling cascades initiated from the Nef protein that results in both cell-autonomous apoptosis and inhibition of immune responses (Fig. 6). Our *nef*-transgenic model flies will be useful in future studies

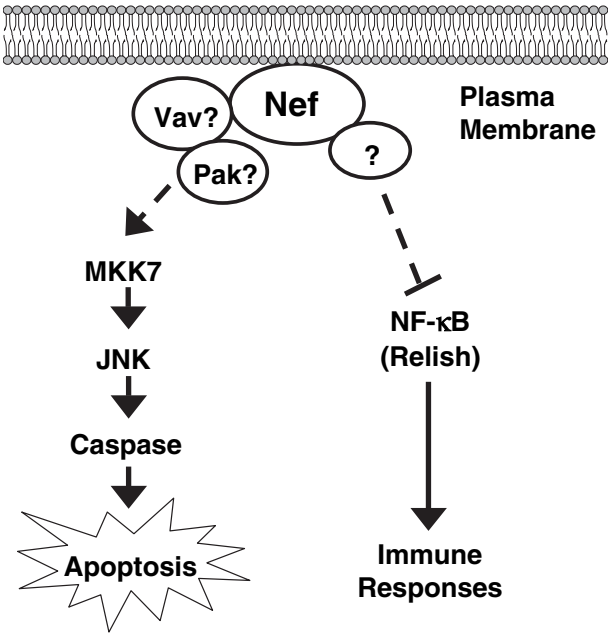

Fig. 6. Intracellular signals initiated from Nef. Nef localized to the plasma membrane activates MKK7 and JNK to induce caspase-dependent apoptosis, and also inhibits Relish NF-κB activity to negatively modulate immune responses.

addressing the detailed molecular mechanism behind Nef-dependent AIDS pathogenesis.

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