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Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells

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Immunoglobulin class switching from immunoglobulin M (IgM) to IgG and IgA is central to immunity against viruses and requires the activation of B cells by T cells via CD154 (CD40 ligand) and cytokines. These molecules limit their signaling activity in immune cells by turning on negative feedback proteins, including IκB and SOCS. We show here that negative factor (Nef) protein, an immunosuppressive human immunodeficiency virus 1 protein expressed and released by infected cells, penetrates B cells both *in vivo* and *in vitro*. Nef suppressed immunoglobulin class-switch DNA recombination by inducing IκBα and SOCS proteins, which blocked CD154 and cytokine signaling via NF-κB and STAT transcription factors. Thus, human immunodeficiency virus 1 may evade protective T cell–dependent IgG and IgA responses by 'hijacking' physiological feedback inhibitors in B cells via Nef.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS, a systemic disorder characterized by recurrent opportunistic infections due to severe impairment of both cellular and humoral immune responses[1]. Although T cell defects have a prominent function, B cell defects are also important and include nonspecific hypergammaglobulinemia as well as impaired immunoglobulin G (IgG) and IgA responses to pathogens and vaccines[2]. HIV-1 is thought to attenuate antigen-specific antibody production by inducing progressive loss of CD4$^+$ T cells.

CD4$^+$ T cells activate B cells via CD154 (CD40 ligand) and cytokines, including interleukin 4 (IL-4) and IL-10 (ref. 3). After entering the germinal centers of secondary lymphoid organs, these activated B cells undergo immunoglobulin heavy-chain class-switch recombination (CSR) and somatic hypermutation[4]. Class switching substitutes the heavy-chain constant region (C_H) of IgM and IgD with that of IgG, IgA or IgE, thereby endowing antibodies with new effector functions that enhance the clearance of pathogens[5]. Somatic hypermutation introduces point mutations in the variable genes encoding the antigen-binding region of immunoglobulins, thereby providing the structural correlate for selection by antigen of higher-affinity mutants[6]. Ultimately, germinal center B cells differentiate into memory B cells and antibody-secreting plasma cells.

Unlike IgM, class-switched IgG and IgA can neutralize viruses both systemically and at portal sites of entry, including the respiratory, intestinal and genital mucosae[3]. Like other viruses, HIV-1 has developed many strategies to evade T cell–dependent switching to IgG and IgA. In particular, HIV-1 promotes depletion of CD4$^+$ T cells and degeneration of follicular dendritic cells[7], which are essential for maintaining a normal germinal center architecture[4]. Although important, these mechanisms are not sufficient to explain humoral defects arising at an early stage of HIV-1 infection, nor can they account for the intrinsically poor responsiveness of B cells to CD4$^+$ T cell help[2,8].

HIV-1-induced B cell defects are due at least in part to viral factors, as antiretroviral therapy substantially improves T cell–dependent antibody responses both *in vivo* and *in vitro*[8,9]. Given its inability to infect B cells[10], HIV-1 must alter their function in an indirect way. Some viruses suppress the activation of B cells by CD4$^+$ T cells via soluble proteins. This strategy is exemplified by measles virus, which inhibits CD40-dependent IgG and IgA production by engaging inhibitory Fcγ receptor IIB (FcγRIIB) on bystander B cells via a nucleocapsid protein[11]. Like that protein, the HIV-1 negative factor (Nef) protein has been linked to immunosuppression and can be released into the extracellular milieu by infected cells[12–14].

Nef is an early HIV-1 protein required for efficient *in vivo* viral replication and pathogenicity[15,16]. The mechanisms whereby Nef favors the development of AIDS remain elusive. By altering the endocytotic machinery[17], Nef downregulates expression of surface major histocompatibility complex (MHC) class I, thereby protecting infected cells from destruction by cytotoxic T lymphocytes[18]. In addition, Nef downregulates CD4 (ref. 19), thereby preventing the interaction of budding virions on infected cells with CD4. Furthermore, Nef manipulates signaling via multiple intracellular kinases, thereby enabling infected dendritic cells and macrophages to attract

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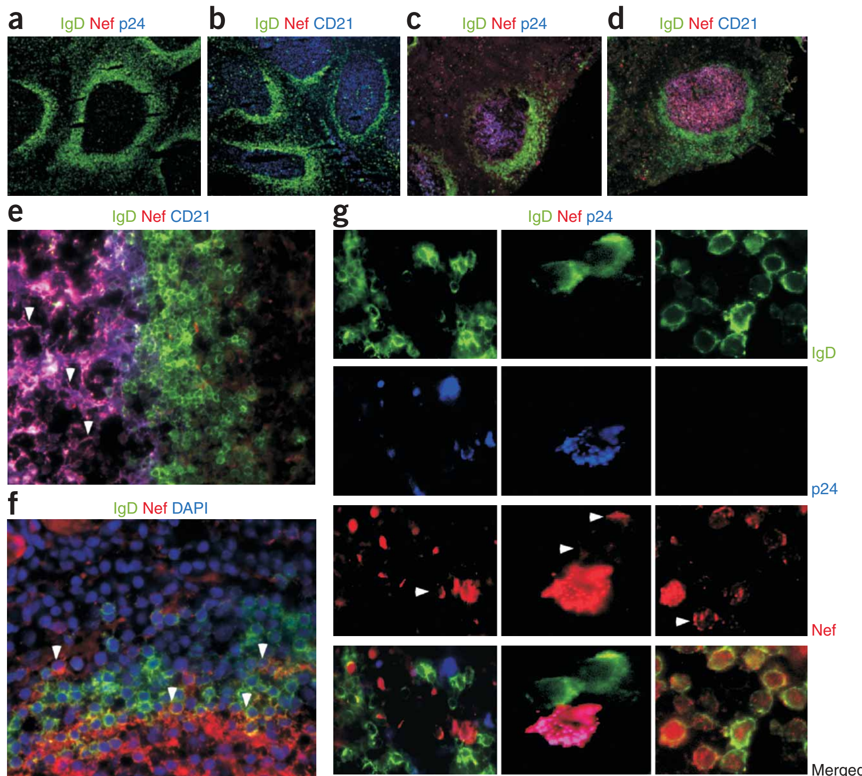

Figure 1 B cells accumulate Nef *in vivo*. (**a**–**d**) Immunofluorescence analysis of HIV-1⁻ (**a**,**b**) and HIV-1⁺ (**c**,**d**) lymphoid tissue stained for IgD (green), Nef (red), p24 or CD21 (blue). Original magnification, ×5. (**e**) Nef in the germinal center of an HIV-1⁺ follicle; arrowheads indicate CD21⁺ follicular dendritic cells with a granular Nef pattern in the membrane. Original magnification, ×20. (**f**) Nef in the follicular mantle and interfollicular area of an HIV-1⁺ follicle; arrowheads indicate IgD⁺ B cells containing Nef. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride; blue) stains nuclei. Original magnification, ×20. (**g**) Nef accumulation in IgD⁺ B cells from HIV-1⁺ follicles. Arrowheads indicate Nef-containing IgD⁺ B cells that lack p24 (left, middle and right), interact with infected macrophages (middle) and have a punctuate Nef membrane pattern (right). Original magnification, ×20 (left), ×60 (middle) and ×40 (right). Images represent one of five HIV-1⁻ and ten HIV-1⁺ samples yielding similar results.

and subsequently infect permissive CD4⁺ T cells[20–22]. Finally, Nef protects infected cells from apoptosis[23].

In addition to containing large amounts of Nef in the cytoplasm and membrane, HIV-1-infected cells release Nef into the extracellular environment[12,13]. In infected patients, the concentration of Nef in the serum ranges from 1 to 10 ng/ml (ref. 13), and its concentration may be even higher in the germinal center, where virion-trapping follicular dendritic cells and virion-infected CD4⁺ T cells and macrophages are densely packed[7,24]. These cells communicate with each other and with B cells through immune synapses, which probably contain abundant Nef. After entering neighboring uninfected immune cells[25–30], including B cells, Nef could perturb T cell–dependent signals required for IgG and IgA CSR. In a similar way, B cells would capture transactivator of transcription (Tat), an early HIV-1 protein that exits infected cells and alters the functionality of bystander cells similar to Nef[31,32].

Here we report that B cells accumulated Nef *in vivo* and internalized Nef *in vitro*. Nef impaired T cell–dependent CSR and antibody production by upregulating the expression of inhibitor of nuclear factor-κB (IκB) and suppressor of cytokine signaling (SOCS). These feedback inhibitors prevented CD154 and IL-4 from activating germline downstream IgH constant-region gene segments via the transcription factors NF-κB and STAT6, respectively. Thus, HIV-1 may evade protective T cell–dependent antibody responses by 'pirating' physiological negative feedback pathways in bystander B cells via Nef.

RESULTS

Nef accumulates in B cells *in vivo*

Lymphoid follicles are a chief site of HIV-1 infection and deposition[7] and thus are hypothesized to contain abundant Nef. Follicles not infected with HIV-1, from healthy donors, comprised a well defined mantle zone filled with 'preswitched' IgD⁺ B cells as well as a germinal center encompassing a regular meshwork of CD21⁺ follicular dendritic cells (**Fig. 1a**,**b**). Uninfected follicles were negative for Nef, the viral capsid protein p24 and the viral matrix protein p17 (**Fig. 1a**,**b** and **Supplementary Fig. 1** online). HIV-1-infected follicles comprised a more irregular mantle zone and germinal centers with a rather disorganized follicular dendritic cell meshwork (**Fig. 1c**). Consistent with published data[24], germinal centers and scattered interfollicular cells of infected follicles were positive for Nef, p24 and p17 (**Fig. 1c** and **Supplementary Fig. 1** online). Nef localized together with CD21⁺ follicular dendritic cells and CD21⁻ cells (**Fig. 1d**), including CD3⁺ T cells, CD11c⁺ dendritic cells and CD68⁺ macrophages (data not shown). In CD21⁺ follicular dendritic cells, Nef had a punctuate pattern on the membrane and a more diffuse pattern in the cytosol (**Fig. 1e**). A variable proportion of IgD⁺ B cells at the edge of the germinal center and in the interfollicular area contained Nef (**Fig. 1f**). Although they contained Nef, IgD⁺ B cells were unlikely to be infected by HIV-1, because they lacked p24 (**Fig. 1g**) and p17 (**Supplementary Fig. 1** online). Notably, some Nef-positive IgD⁺ B cells were proximal to HIV-1-permissive macrophages containing Nef, p24 and p17, suggesting that follicular B cells internalize Nef after interaction with infected cells. Finally, IgD⁺ B cells accumulated Nef in the membrane, the cytoplasm and, occasionally, the nucleus. Of note, Nef associated with the membranes of IgD⁺ B cells was characterized by a punctuate pattern (**Fig. 1g** and **Supplementary Fig. 1** online). Thus, IgD⁺ B cells are exposed to Nef-containing cells and can accumulate Nef *in vivo*.

Nef penetrates in B cells *in vitro*

B cells from HIV-1 patients are unlikely to accumulate Nef as a result of endogenous synthesis, because they lack p24, p17 and, as shown before[10], viral RNA. One interpretation is that B cells internalize Nef from an exogenous source. Consistent with that idea, Nef is released in the extracellular environment by infected cells[12] and can reach a concentration of 1–10 ng/ml in the serum of infected patients[13]. Given its ability to penetrate in myeloid cells[25–30], we hypothesized that Nef penetrates in B cells as well. We incubated purified IgD⁺ B cells with a recombinant myristoylated Nef protein that resembled native Nef. Like IgD⁺ B cells exposed to Nef *in vivo*, IgD⁺ B cells exposed to Nef *in vitro* accumulated Nef in the cytosol (**Fig. 2a**). Nef

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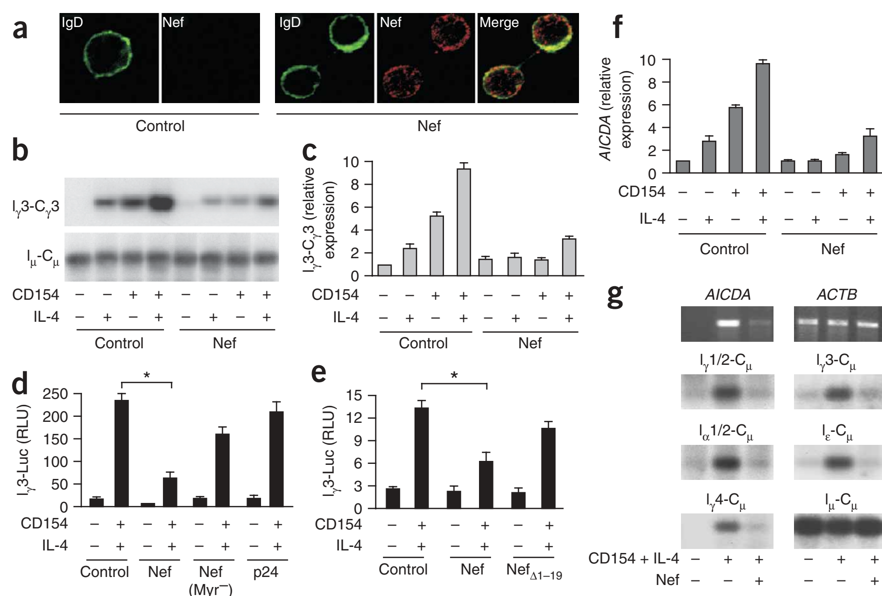

Figure 2 Nef penetrates in B cells *in vitro* and inhibits induction of CSR by CD154 and IL-4. (**a**) Confocal microscopy of peripheral blood IgD$^+$ B cells incubated for 30 min with BSA (control) or Nef and then stained for IgD (green) and Nef (red). Original magnification, ×60. (**b**) RT-PCR amplification of $I_\gamma3$-$C_\gamma3$ and I_μ-C_μ transcripts from peripheral blood IgD$^+$ B cells exposed for 4 d to CD154, IL-4 and/or Nef and hybridized to a radiolabeled C_μ oligonucleotide probe. Control, no Nef. (**c**) Real-time RT-PCR of $I_\gamma3$-$C_\gamma3$ mRNA from peripheral blood IgD$^+$ B cells cultured as described in **b**. $I_\gamma3$-$C_\gamma3$ mRNA is normalized to *ACTB* mRNA. (**d**) Luciferase activity of IgD$^+$ 2E2 B cells transfected with $I_\gamma3$-Luc and cultured for 48 h with or without CD154 and IL-4 in the presence of PBS (Control), Nef, nonmyristoylated Nef (Nef Myr$^-$) or p24. RLU, relative light units. (**e**) Luciferase activity of IgD$^+$ 2E2 B cells transfected with $I_\gamma3$-Luc plus empty pcDNA3.1 (Control), wild-type Nef-pcDNA3.1 (Nef) or mutant Nef$_{\Delta1-19}$-pcDNA3.1 (Nef$_{\Delta1-19}$) and cultured with or without CD154 and IL-4. (**f**) Real-time RT-PCR of *AICDA* mRNA from peripheral blood IgD$^+$ B cells cultured as described in **b**. *AICDA* mRNA is normalized to *ACTB* mRNA. (**g**) RT-PCR amplification of *AICDA*, *ACTB*, $I_\gamma1/2$-C_μ, $I_\gamma3$-C_μ, $I_\gamma4$-C_μ, $I_\alpha1/2$-C_μ, I_ε-C_μ and I_μ-C_μ transcripts from peripheral blood IgD$^+$ B cells cultured as described in **b**. Circle transcripts were hybridized with a radiolabeled C_μ oligonucleotide probe. Error bars indicate s.d.; *, $P < 0.005$. Data represent one of three experiments yielding similar results (**a**,**b**,**g**) or a summary of four experiments (**c**–**f**).

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internalization was associated with a punctuate membrane pattern similar to that in IgD$^+$ B cells from HIV-1-infected follicles and occurred in the presence of as little as 1 ng/ml of Nef and as early as 5 min after exposure of IgD$^+$ B cells to Nef (**Supplementary Fig. 2** online). These studies provide an *in vitro* model for 'dissecting' the function of B cell–associated Nef in T cell–dependent class switching and antibody production.

Nef inhibits initiation of CSR by CD40 ligand and IL-4

B cells from HIV-1-infected patients respond poorly to CD4$^+$ T cell help[8]. As Nef accumulates in the germinal center, where CD4$^+$ T cells signal CSR to B cells, we hypothesized that Nef interferes with T cell–dependent immunoglobulin class switching. CSR from C_μ to $C_\gamma3$, $C_\gamma1$, $C_\alpha1$, $C_\gamma2$, $C_\gamma4$, $C_\alpha2$ and C_ε genes is guided by switch (S) regions 5′ of each C_H gene and 3′ of an intronic (I_H) exon[5]. CSR is preceded by germline I_H-S-C_H transcription, a key event that requires activation of the I_H promoter flanking each I_H exon[5]. In addition to yielding 'sterile' I_H-C_H transcripts[3], germline IgH gene transcription enables recruitment of the CSR machinery, including the B cell–specific enzyme activation-induced cytidine deaminase (AID), to the targeted S region[6].

To confirm that exogenous Nef prevents T cell–dependent stimuli from initiating germline IgH gene transcription, we primed IgD$^+$ B cells for 3 h with recombinant myristoylated Nef. Nef inhibited the induction of germline $I_\gamma3$-$C_\gamma3$ transcripts by CD154 and/or IL-4, but did not affect germline I_μ-C_μ transcripts (**Fig. 2b**,**c**), which are constitutively expressed by IgD$^+$ B cells. Unlike the viral control p24 protein, Nef impaired the CD154- and IL-4-induced transcriptional activation of the $I_\gamma3$ promoter in IgD$^+$ human 2E2 B cells (**Fig. 2d**), a subclone of the CL-01 cell line[33]. Consistent with published studies indicating that myristoylation is associated with Nef signaling[23,34,35], nonmyristoylated Nef had a smaller inhibitory effect than did myristoylated Nef. Similarly, overexpression of wild-type *Nef* inhibited CD154- and IL-4-induced $I_\gamma3$ activation more than overexpression of a mutated *Nef* with no myristoylation signal sequence (**Fig. 2e**). Notably, both exogenous recombinant and endogenous plasmid-encoded Nef proteins also inhibited $I_\gamma1$, I_α and I_ε activation by CD154 and cytokines (data not shown). Thus, Nef inhibited a key pre-CSR event in IgD$^+$ B cells exposed to T cell–dependent stimuli.

Nef inhibits induction of CSR by CD154 and IL-4

Immunoglobulin CSR generates an extrachromosomal reciprocal switch DNA recombination product known as the 'switch circle', which includes the I_H promoter 5′ of the targeted C_H gene, the DNA segment between S_μ and the targeted S region, and C_μ[3]. Under the influence of the I_H promoter, the switch circle transcribes a chimeric I_H-C_μ product referred to as the 'switch circle transcript'[36]. Together with germline I_H-C_H transcripts and AID-encoding *AICDA* transcripts, switch circle transcripts constitute molecular markers of ongoing immunoglobulin CSR[36]. To further confirm that exogenous Nef inhibits T cell–dependent immunoglobulin CSR, we assessed the presence of *AICDA* transcripts and switch circle transcripts in IgD$^+$ B cells stimulated by CD154 and IL-4 in the presence or absence of Nef. Because of the high sequence homology of $I_\gamma1$ and $I_\gamma2$ regions and $I_\alpha1$ and $I_\alpha2$ regions, we amplified $I_\gamma1$-C_μ and $I_\gamma2$-C_μ circle transcripts as well as $I_\alpha1$-C_μ and $I_\alpha2$-C_μ circle transcripts using common $I_\gamma1/2$ and $I_\alpha1/2$ primers, respectively[36]. Recombinant myristoylated Nef attenuated the induction of *AICDA* transcripts and $I_\gamma1/2$-C_μ, $I_\gamma3$-C_μ, $I_\gamma4$-C_μ, $I_\alpha1/2$-C_μ and I_ε-C_μ switch circle transcripts in IgD$^+$ B cells exposed to CD154 and/or IL-4 (**Fig. 2f**,**g**). Nef did not affect the expression of germline I_μ-C_μ transcripts, which were constitutively expressed by IgD$^+$ B cells. Notably, as little as 1 ng/ml of Nef was sufficient to inhibit the induction of *AICDA* and switch circle transcripts by CD154 and IL-4 (**Supplementary Fig. 2** online). Thus, exogenous Nef inhibited CSR in IgD$^+$ B cells exposed to T cell–dependent stimuli.

Nef inhibits induction of IgG, IgA and IgE by IL-10

Together with CD154 and IL-4, IL-10 drives the differentiation of human B cells into antibody-producing cells. Recombinant myristoylated Nef considerably reduced IgG and IgA production and nearly completely abrogated IgE production in IgD$^+$ B cells exposed to

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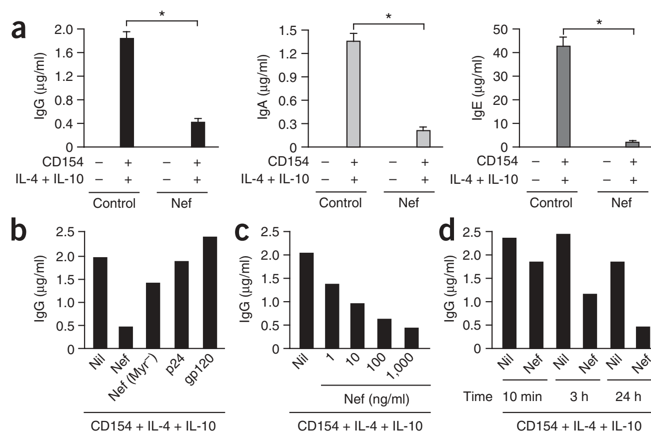

Figure 3 Nef inhibits the production of IgG, IgA and IgE in B cells exposed to CD154 and IL-4. (**a**) Enzyme-linked immunosorbent assay of IgG, IgA and IgE in the supernatant of peripheral blood IgD$^+$ B cells cultured for 8 d in the presence or absence of CD154, IL-4 and IL-10 and with or without Nef. IgG and IgA are less than 0.05 mg/ml and IgE is less than 0.5 ng/ml in IgD$^+$ B cells incubated with medium alone or Nef alone. Control, no Nef. Error bars indicate s.d. of four experiments; *, $P < 0.005$. (**b**) IgG secretion by peripheral blood IgD$^+$ B cells exposed for 7 d to CD154, IL-4 and IL-10, plus PBS (Control), Nef, nonmyristoylated Nef (Myr$^-$), p24 or envelope protein gp120. (**c**) IgG secretion by peripheral blood IgD$^+$ B cells exposed for 7 d to CD154, IL-4 and IL-10, plus 0, 1, 10, 100 or 1,000 ng/ml of Nef. (**d**) IgG secretion by peripheral blood IgD$^+$ B cells cultured as described in **c**. B cells were preincubated with Nef for 10 min, 3 h or 24 h before the addition of CD154, IL-4 and IL-10. Nil, no viral protein (**b**) or no Nef (**c**,**d**). Data represent one of four experiments yielding similar results (**b**–**d**).

CD154, IL-4 and IL-10 (**Fig. 3a**). In contrast, nonmyristoylated Nef or viral control p24 and envelope gp120 proteins did not substantially decrease IgG production (**Fig. 3b**). As little as 1 ng/ml of Nef was sufficient to attenuate IgG and IgA production by IgD$^+$ B cells that had been purified by either positive or negative selection (**Fig. 3c** and **Supplementary Fig. 2** online). Optimal inhibition of IgG production required priming of IgD$^+$ B cells with Nef for 3–24 h (**Fig. 3d**), suggesting that Nef-mediated impairment of T cell–dependent immunoglobulin class switching requires first synthesis of intracellular CSR-inhibiting factors. Thus, exogenous Nef inhibits production of IgG, IgA and IgE in IgD$^+$ B cells exposed to T cell–dependent stimuli.

Nef does not affect CD40, IL-4 and IL-10 receptors

Given that endogenous Nef downregulates expression of CD4, MHC class I and class II, CD80 and CD86 on infected cells[18,19,37,38], we hypothesized that exogenous Nef inhibits T cell–dependent CSR by decreasing CD40 and cytokine receptor expression on IgD$^+$ B cells. Recombinant myristoylated Nef downregulated expression of MHC class I and class II on IgD$^+$ B cells as well as expression of CD4, MHC class I and class II and, to a lesser extent, CD80 and CD86 on macrophages (**Fig. 4a**), a cell type that efficiently internalizes exogenous Nef *in vitro*[26,28]. In contrast, Nef did not modulate CD19, CD40, IL-4 receptor and IL-10 receptor on IgD$^+$ B cells and CD14 on macrophages. As endogenous Nef can trigger apoptosis[14,23], we tested whether exogenous Nef affects IgD$^+$ B cell survival. Recombinant myristoylated Nef did not affect the proliferation and survival of IgD$^+$ B cells exposed to CD154 and/or IL-4 plus IL-10 (**Fig. 4b**,**c**). Thus, the CSR-inhibiting activity of exogenous Nef was not due to the downregulation of CD40 and cytokine receptors on IgD$^+$ B cells or to the decreased proliferation and survival of IgD$^+$ B cells.

Nef inhibits CD154 signaling through NF-κB

CD154 induces CSR through NF-κB[3,5]. In resting B cells, the cytoplasmic p65-p50, p50-p50 and p50-c-Rel NF-κB dimers are kept in an inactive form by IκBα[39]. CD40 engagement by CD154 elicits phosphorylation of an IκB kinase (IKK) complex comprising two α- and β-catalytic subunits and one γ-regulatory subunit (also called NEMO). Phosphorylation of IκBα by IKK is followed by degradation of IκBα and nuclear translocation of NF-κB[40], which initiates CSR by binding to κB sites on I_H promoters[3,5]. Given its ability to inhibit NF-κB activation in drosophila[41], we hypothesized Nef would inhibit NF-κB activation in human IgD$^+$ B cells as well. Recombinant myristoylated Nef attenuated the phosphorylation of cytoplasmic IKKβ, IKKγ and IκBα as well as the degradation of cytoplasmic IκBα in IgD$^+$ B cells exposed to CD154 (**Fig. 5a–d**). These effects were specific, as Nef did not inhibit but instead enhanced the phosphorylation of cytoplasmic p38 (**Fig. 5e**), a mitogen-activated protein kinase linked to many B cell functions[3]. Nef inhibited the upregulation of nuclear p50, p65 and c-Rel in IgD$^+$ B cells stimulated with CD154 (**Fig. 5f–h**). In contrast, Nef enhanced CD154-induced upregulation of nuclear B cell–specific activator protein (BSAP; **Fig. 5i**), a key transcriptional regulator of many B cell genes[3]. In addition, Nef did not affect the constitutive expression of nuclear Octamer 1 (Oct1), another important regulator of B cell gene expression[3]. As little as 1 ng/ml of Nef attenuated CD154-induced binding of nuclear p50, p65 and c-Rel to a κB site on the $I_{\gamma}3$ promoter (**Fig. 5j** and **Supplementary Fig. 3** online). In contrast, Nef augmented CD154-induced binding of nuclear BSAP to an homonymous site on the $I_{\gamma}3$ promoter (**Fig. 5j** and **Supplementary Fig. 3** online). Unlike Nef, viral p24 and gp120 proteins did not impair CD154-induced signaling through NF-κB (**Supplementary Fig. 3** online). Thus, exogenous Nef inhibited CD40 signaling through the IKK–NF-κB pathway in IgD$^+$ B cells.

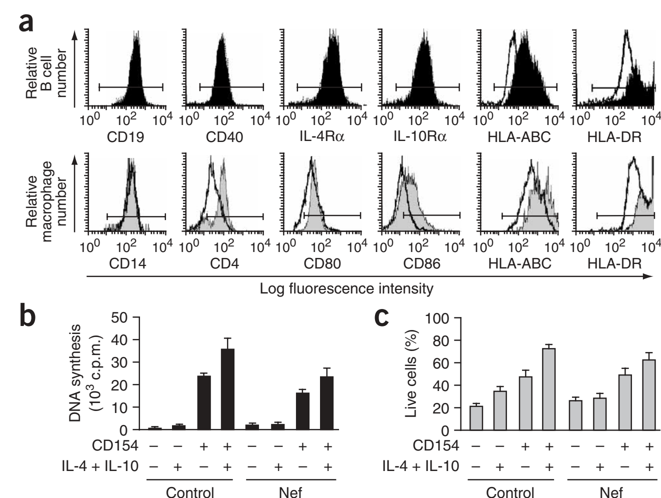

Figure 4 Nef does not downregulate CD40, IL-4 and IL-10 receptors on B cells. (**a**) Flow cytometry of CD19, CD40, IL-4 receptor α-chain (IL-4Rα), IL-10 receptor α-chain (IL-10Rα), HLA-ABC (MHC-I), HLA-DR (MHC-II), CD14, CD4, CD80 and CD86 on peripheral blood IgD$^+$ B cells or monocyte-derived macrophages incubated for 48 h with Nef (open histograms) or without Nef (filled histograms). Data represent one of four experiments yielding similar results. (**b**) [^{3}H]thymidine incorporation in peripheral blood IgD$^+$ B cells cultured for 4 d in the presence or absence of CD154, IL-4 and IL-10, with or without Nef. Control, no Nef. (**c**) Viability of peripheral blood IgD$^+$ B cells cultured as described in **b**. Data represent a summary of three experiments (**b**,**c**); error bars indicate s.d.

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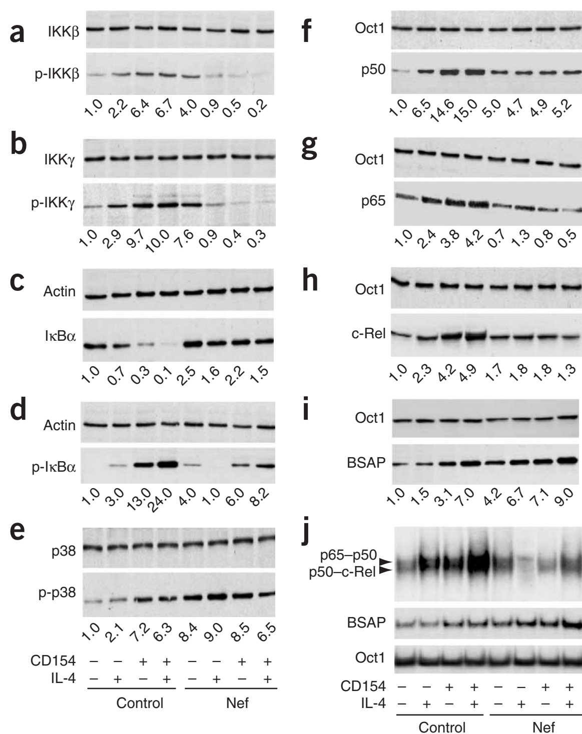

Figure 5 Nef inhibits CD154 signaling through NF-κB in B cells. (**a**–**e**) Immunoblots of relevant cytoplasmic proteins from tonsillar IgD+ B cells cultured with CD154 and/or IL-4 and in the presence or absence of Nef for 5 min (IKKs and p38) or for 2 h (IκBα). Phosphorylated bands (p-IKK-β, p-IKKγ, IκBα, p-IκBα and p-p38) were quantified (below lanes) after normalization to the IKKβ, IKKγ, actin and p38 loading controls. The value corresponding to IgD+ B cells incubated with medium alone (far left lane in each) was arbitrarily set as 1. (**f**–**i**) Immunoblots of relevant nuclear proteins from tonsillar IgD+ B cells cultured for 2 h as described in **a**. The p50, p65, c-Rel and BSAP bands were quantified (below lanes) after normalization to Oct1, a constitutively expressed nuclear protein used as a loading control. (**j**) EMSA of nuclear NF-κB–I_γ3 DNA, BSAP–I_γ3 DNA and Oct1–DNA complexes from tonsillar IgD+ B cells cultured for 2 h as described in **a**. **Supplementary Fig. 3** online presents supershift assays identifying these complexes. Control, no Nef. Data (**a**–**j**) represent one of three experiments yielding similar results.

Nef inhibits IL-4 and IL-10 signaling via STATs

IL-4 induces CSR via STAT6 (refs. 3,5). IL-4 receptor–induced phosphorylation of Janus kinase 1 (Jak1) and Jak3 is followed by phosphorylation, dimerization and nuclear translocation of STAT6 (ref. 42). Once in the nucleus, STAT6 initiates germline transcription of IgH constant-region genes by binding to an interferon-γ-activated sequence (GAS) on I_H promoters[3,5]. Similarly, IL-10 stimulates immunoglobulin production by activating STAT1 and STAT3 via Jak1 (ref. 43). Given its ability to inhibit CD154 signaling via NF-κB, Nef was hypothesized to inhibit IL-4 and IL-10 signaling via STATs as well. Recombinant myristoylated Nef attenuated IL-4-induced phosphorylation of Jak1, Jak3 and STAT6 (**Fig. 6a**–**c**). In addition, as little as 1 ng/ml of Nef attenuated IL-4-induced binding of STAT6 to the GAS site of the I_γ3 promoter (**Fig. 6d** and **Supplementary Fig. 3** online). These effects were specific, as Nef did not inhibit but instead augmented IL-4-induced phosphorylation of p38 as well as the expression and I_γ3-binding activity of BSAP (**Fig. 5e,i,j**). Unlike Nef, p24 and gp120 did not affect IL-4-induced binding of STAT6 to I_γ3 (**Supplementary Fig. 3** online). Finally, Nef decreased IL-10-induced phosphorylation of Jak1, STAT1 and STAT3 (**Fig. 6e**–**g**). Thus, exogenous Nef attenuates IL-4 and IL-10 signaling via the Jak-STAT pathway in IgD+ B cells.

Nef induces IκBα and SOCS feedback inhibitors

After exposure to CD154 and cytokines, immune cells synthesize IκBα and SOCS proteins[40,44]. By turning off NF-κB and Jak-STAT signaling, IκBα and SOCS provide negative feedback that limits cellular responses to CD154 and cytokines[40,44]. Hence, exogenous Nef was hypothesized to impair T cell–dependent CSR by inducing IκBα and SOCS. Peripheral blood IgD+ B cells upregulated *NFKBIA* transcripts (encoding IκBα) and *SOCS1* transcripts 5–10 min after exposure to recombinant myristoylated Nef but not after exposure to control viral p24 (**Fig. 7a,b**). *NFKBIA* and *SOCS1* transcripts peaked after 60 and 30 min, respectively, and although they progressively decreased, they did not return to the baseline throughout the subsequent 24 h. Expression of IκBα and SOCS1 proteins increased 5–10 min after exposure of IgD+ B cells to Nef and peaked after 3 h and 30 min, respectively (**Fig. 7c,d**). Expression of IκBα and SOCS1 proteins remained above baseline throughout the subsequent 24 h. IgD+ B cells upregulated *SOCS3* transcript and SOCS3 protein as early as 5 min after exposure to Nef. This expression progressively decreased in the subsequent 6 h, but increased

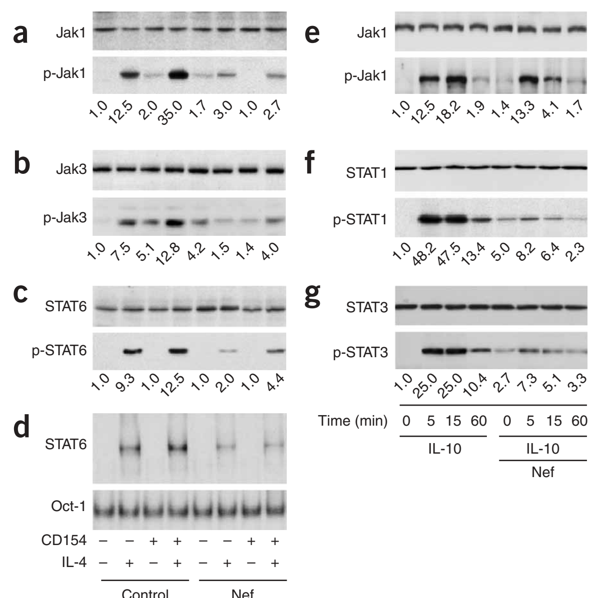

Figure 6 Nef inhibits cytokine signaling through the Jak-STAT pathway in B cells. (**a**–**c**) Immunoblots of relevant cytoplasmic proteins from tonsillar IgD+ B cells cultured for 5 min with CD154, IL-4 and/or Nef. Phosphorylated bands (p-Jak1, p-Jak3 and p-STAT6) were quantified (below lanes) after normalization to the Jak1, Jak3 and STAT6 loading controls. The value corresponding to IgD+ B cells incubated with medium alone (far left lane in each) was arbitrarily set as 1. Control, no Nef. (**d**) EMSA of nuclear STAT6–I_γ3 DNA and Oct1-DNA complexes from tonsillar IgD+ B cells cultured for 2 h as described in **a**–**c** (**Supplementary Fig. 3** online presents a supershift assay identifying the STAT6–I_γ3 DNA complex). (**e**–**g**) Immunoblots of relevant cytoplasmic proteins from tonsillar IgD+ B cells cultured with IL-10 for 0, 5, 15 or 60 min in the presence or absence of Nef. Phosphorylated bands (p-Jak1, p-STAT1 and p-STAT3) were quantified (below lanes) after normalization to the Jak1, STAT1 and STAT3 loading controls. Data (**a**–**g**) represent one of three experiments yielding similar results.

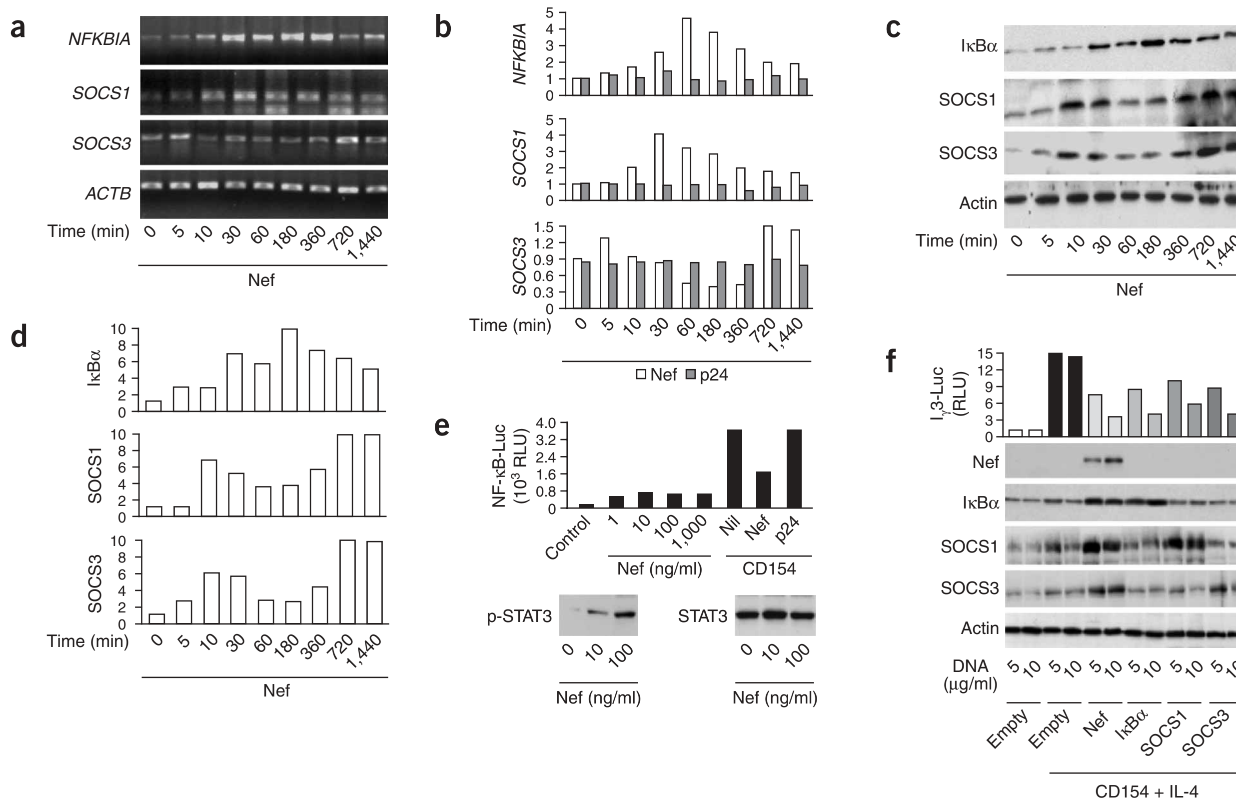

Figure 7 Nef induces IκBα, SOCS1 and SOCS3 in B cells. (**a**) RT-PCR of *NFKBIA*, *SOCS1*, *SOCS3* and *ACTB* transcripts from tonsillar IgD$^+$ B cells cultured with Nef for various times. (**b**) Real-time RT-PCR of *NFKBIA*, *SOCS1*, *SOCS3* mRNA from tonsillar IgD$^+$ B cells cultured with Nef or p24. Values are normalized to *ACTB* mRNA. (**c**,**d**) Immunoblots (**c**) and quantification (**d**) of Nef, IκBα, SOCS1, SOCS3 and actin protein from tonsillar IgD$^+$ B cells cultured as described in **a**. Relevant bands were quantified after normalization to actin. The control value (far left lane in each) was arbitrarily set as 1. (**e**) Top, luciferase activity of IgD$^+$ 2E2 B cells transfected with $\kappa B_{(2)}$-Luc and cultured for 48 h with Nef (1, 10, 100 or 1,000 ng/ml), CD154 and/or p24. Control, IgD$^+$ B cells cultured with medium alone. Bottom, immunoblots of STAT3 and phosphorylated STAT3 (p-STAT3) in IgD$^+$ 2E2 B cells exposed for 5 min to Nef (10 or 100 ng/ml). Nil, no viral protein. (**f**) Top, luciferase activity of IgD$^+$ 2E2 B cells transfected with $I_\gamma 3$-Luc plus 5 or 10 µg empty pcDNA3.1, Nef-pcDNA3.1, IκBα-pcDNA3.1, SOCS1-pcDNA3.1 or SOCS3-pcDNA3.1 and then cultured for 48 h with or without CD154 and IL-4. Bottom, immunoblots of IκBα, SOCS1, SOCS3 and actin proteins from cotransfected IgD$^+$ 2E2 B cells. Data (**a**–**f**) represent one of three experiments yielding similar results.

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again after 12 and 24 h. Thus, exogenous Nef rapidly induced expression of the IκBα, SOCS1 and SOCS3 negative feedback proteins in IgD$^+$ B cells.

IκBα and SOCS inhibit CD154- and IL-4-induced CSR

As CD154 and cytokines activate *NFKBIA* and *SOCS* genes via NF-κB and STAT[40,44], we sought to determine whether exogenous Nef activated NF-κB and STAT proteins in IgD$^+$ B cells. Myristoylated recombinant Nef activated NF-κB, STAT1 and STAT3 proteins in IgD$^+$ B cells, although this activation was less efficient than that induced by CD154 and cytokines (**Figs. 5a–h** and **6e–g**). In addition, as little as 1–10 ng/ml of Nef induced NF-κB-dependent gene transcription and STAT3 activation in IgD$^+$ 2E2 B cells, (**Fig. 7e**). These findings suggested that Nef upregulates feedback inhibitors of CD154 and cytokine signaling, such as IκBα and SOCS proteins, through NF-κB and STAT signals. Consistent with that possibility, IgD$^+$ 2E2 B cells incubated with Nef underwent CD154-induced NF-κB-dependent gene transcription less efficiently than did IgD$^+$ 2E2 B cells incubated with medium alone or control viral p24. Next, we assessed whether IκBα and SOCS proteins attenuate CSR. Overexpression of *Nef*, *NFKBIA*, *SOCS1* or *SOCS3* inhibited $I_\gamma 3$ activation in IgD$^+$ 2E2 B cells exposed for 2 d to CD154 and IL-4 (**Fig. 7f**). Notably, the amount of IκBα, SOCS1 and SOCS3 protein in *Nef*-transfected IgD$^+$ 2E2 B cells was similar to that in IgD$^+$ 2E2 B cells transfected with *NFKBIA*, *SOCS1* and *SOCS3*. Thus, exogenous Nef delivered relatively weak NF-κB and STAT signals that induced expression of negative feedback proteins with CSR-inhibiting activity in IgD$^+$ B cells (**Supplementary Fig. 4** online).

DISCUSSION

HIV-1 infection profoundly impairs IgG and IgA responses to T cell–dependent antigens. We have reported here that HIV-1 Nef penetrates B cells both *in vivo* and *in vitro*. Nef inhibited switching to IgG, IgA and IgE by inducing IκBα and SOCS negative feedback proteins, which blocked CD154 and cytokine signaling through NF-κB and STAT. Thus, Nef may suppress protective T cell–dependent antibody responses by a mechanism of 'molecular piracy' involving induction of physiological signal inhibitors in bystander B cells.

Antibodies are essential for protective immunity against viruses. In contrast to IgM, class-switched IgG and IgA are endowed with new effector functions that enhance the neutralization and clearance of viruses by the immune system[45]. This is exemplified by IgA, which can be released at key portals of entry, such as the respiratory, intestinal and genital mucosal surfaces, because of its ability to bind a polymeric immunoglobulin receptor on the side of epithelial cells away from the lumen[46]. Thus, it is not unexpected that viruses such as measles virus

have developed effective strategies to evade class switching. By producing a soluble nucleocapsid protein, measles virus engages a powerful inhibitory FcγIIB receptor on bystander B cells, thereby suppressing IgG and IgA production in response to CD154 and cytokines[11].

Nef shares sequence homology with the immunosuppressive retroviral protein p15E and has been linked to the development of AIDS[14,35]. We found abundant Nef in the germinal centers of infected lymphoid follicles, where protective IgG and IgA responses usually take place. Follicular B cells proximal to HIV-1-trapping follicular dendritic cells or HIV-1-infected cells contained Nef, but lacked p17, p24 and, as shown before[10], viral RNA. Thus, B cells might accumulate Nef not as a result of endogenous synthesis but instead as a result of internalization from the extracellular environment. Consistent with that, Nef accumulates in the serum of infected patients *in vivo*[13] and, as shown here, penetrates in B cells *in vitro*. Infected cells would release Nef through a nonclassical secretory pathway or after lysis[12,13]. Then, bystander cells could internalize Nef via endocytosis, pinocytosis or other unknown mechanisms[13,26–30]. In addition to Nef, B cells capture Tat[32], another early HIV-1 protein released by infected cells[31]. Given its ability to deliver suppressive signals[14,31], Tat may cooperate with Nef to alter B cell effector functions in the germinal center.

Endogenous Nef downregulates surface CD4, MHC class I and class II, CD80 and CD86 through a mechanism that involves anchoring of Nef to the membrane of infected cells via its myristoylated domain[17–19,35,37,38]. By showing that myristoylated recombinant Nef targeted the membranes of B cells and downregulated MHC class I and class II, our findings have extended published reports indicating that once it is internalized, exogenous Nef partially recapitulates the functions of endogenous Nef[25–30]. Exogenous Nef would suppress CD40-dependent IgG, IgA and IgE class switching in bystander B cells, thereby enabling HIV-1 to escape potentially protective T cell–dependent antibody responses. This immune evasion strategy could be particularly important at an early stage of HIV-1 infection, when $CD4^+$ T cells are relatively conserved[2].

Nef penetrated B cells and inhibited class switching at concentrations comparable to those found in the blood of HIV-1-infected patients[13]. Much higher concentrations of Nef may be present in germinal centers, which constitute the main site of HIV-1 deposition, replication and propagation[7]. In the germinal center, bystander B cells might internalize soluble Nef actively or passively released in the extracellular environment by infected cells[12,13]. Alternatively, HIV-1-infected cells might transfer Nef to bystander B cells while interacting with them through immune synapses and, perhaps, tunneling nanotubules[47].

Nef would turn off B cell responses independently of its positive effect in enhancing viral replication, as *Nef*-transgenic mice have severely disrupted germinal centers as well as impaired IgG and IgA responses to T cell–dependent antigens in the absence of active viral replication[48]. Notably, activated $CD4^+$ T cells from *Nef*-transgenic mice express less CD154 (ref. 48), suggesting that CD40 is key to Nef-induced immune defects. Consistent with that, we found that Nef blocked CSR and subsequent production of IgG, IgA and IgE by delivering CD40-inhibitory signals to B cells. Thus, Nef may impair T cell–dependent antibody production not only by downregulating CD154 on infected $CD4^+$ T cells but also by perturbing CD40 signaling in bystander B cells. In this way Nef would contribute to rendering B cells less responsive to $CD4^+$ T cell help[8].

Nef impaired CSR because of its ability to interfere with CD40 signaling through NF-κB. By attenuating CD40-mediated IκBα degradation and increasing IκBα synthesis, Nef inhibited the translocation of cytoplasmic NF-κB dimers to the nucleus, thereby impairing the binding of NF-κB to I_H promoters. Notably, Nef impairs protective immune responses in drosophila by interfering with the activation of Relish, an NF-κB family member[41]. Thus, Nef may weaken immune protection by targeting highly conserved NF-κB pathways in effector cells. Nef further inhibited CSR by dampening IL-4 signaling through STAT6. In particular, Nef attenuated IL-4 receptor–mediated Jak1 and Jak3 activation, thereby impairing phosphorylation, nuclear translocation and binding of STAT6 to I_H promoters. Finally, Nef inhibited IL-10 signaling through Jak1, STAT1 and STAT3, thereby preventing the differentiation of class-switched B cells into antibody-secreting cells. Notably, Nef did not inhibit but instead augmented CD154 and IL-4 signaling through p38 and BSAP, two key molecules in B cells[3]; this might explain why Nef dampens CSR and antibody production without affecting B cell proliferation and survival.

Nef inhibits T cell–dependent CSR through a specific mechanism, as p24 and gp120, two viral proteins abundant in infected follicles[7,24], do not affect CD40-dependent CSR. Like endogenous Nef in infected cells[35], exogenous Nef had to be myristoylated to effectively inhibit CSR in B cells. Although targeting the B cell membrane, Nef neither perturbed B cell proliferation and survival nor downregulated surface B cell CD40, IL-4 and IL-10 receptors. Instead, Nef upregulated feedback inhibitors of CD40, IL-4 and IL-10 receptor signaling, including IκBα, SOCS1 and SOCS3. In agreement with published studies[40,44], IκBα would block CD40 signaling by retaining NF-κB in the cytoplasm, whereas SOCS1 and SOCS3 would inhibit IL-4 and IL-10 receptor signaling by interfering with STAT activation via Jak. SOCS could also interfere with the activation of IKK[49] and therefore might cooperate with IκBα to inhibit CD40 signaling through NF-κB in Nef-containing B cells.

CD40 and cytokine receptors limit their own signaling activity by turning on genes encoding IκBα, SOCS1 and SOCS3 via NF-κB and STAT transcription factors[40,44]. Like myeloid cells[26,28], B cells activated NF-κB, STAT1 and STAT3 after Nef internalization. Subsequent IκBα and SOCS upregulation would render Nef-containing B cells less responsive to $CD4^+$ T cell help as provided by CD40 ligand, IL-4 and IL-10. Thus, HIV-1 may evade T cell–dependent class switching by 'pirating' negative feedback pathways in bystander B cells via Nef. Should this be the case, Nef-blocking agents might improve protective IgG and IgA responses to pathogens and vaccines in at least two ways: by attenuating the spread of HIV-1 to $CD4^+$ T helper cells[20–22] and by increasing the responsiveness of B cells to $CD4^+$ T cell help.

METHODS

Cells. Human B cells were negatively selected from peripheral blood and tonsillar mononuclear cells with a commercially available kit (Miltenyi Biotec). Buffy coats from healthy donors were purchased at the New York Blood Center and were used to isolate peripheral blood mononuclear cells. Tonsillar mononuclear cells were obtained from tissue specimens of patients undergoing tonsillectomy. The Institutional Review Board of Weill Medical College of Cornell University (New York, New York) approved the study and patients provided informed consent. IgD^+ B cells were magnetically sorted by incubation of total B cells with a biotinylated monoclonal antibody (mAb) to IgD (Southern Biotechnologies) and streptavidin MicroBeads (Miltenyi Biotec), which are specifically designed to avoid substantial receptor cross-linking. All sorting procedures were done on ice. In some experiments, B cells were negatively selected with a commercially available kit (Miltenyi Biotec). Positively and negatively selected IgD^+ B cells had comparable size, CD69 expression, phosphorylated phospatidylinositol-3-kinase content (data not shown) and CD40-dependent immunoglobulin secretion (**Supplementary Fig. 2** online). Monocytes were magnetically sorted with biotinylated mAb MCA596B to CD14 (Serotec) and streptavidin MicroBeads. Macrophages were obtained

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by incubation of monocytes for 7 d with RPMI medium supplemented with 10% FBS and 20 ng/ml of macrophage colony-stimulating factor (R&D Systems). 2E2 is a subclone of CL-01, a human IgD^+IgM^+ B cell line that initiates germline immunoglobulin gene transcription after exposure to appropriate stimuli[33].

Cultures and reagents. B cells were cultured in RPMI medium plus 10% FBS with or without 200 U/ml of IL-4, 200 ng/ml of IL-10 (Schering-Plough) and 0.5 μg/ml of soluble trimeric CD154 (Immunex). Recombinant myristoylated HIV-1 Nef from strain SF2 (Jena Bioscience) was generated as reported[50] and was used at a concentration of 100 ng/ml unless stated otherwise. Nef was incubated with B cells for at least 3 h before the addition of CD154 and cytokines. Recombinant nonmyristoylated Nef (National Institutes of Health AIDS Research & Reference Reagent Program, Bethesda, Maryland) was expressed in BL21 *Escherichia coli* with a pT7-7 plasmid encompassing a consensus *Nef* gene. Recombinant p24 from HIV-1 strain IIIB (Immuno Diagnostics) and recombinant glycosylated gp120 from HIV-1 strain BaL (National Institutes of Health AIDS Research & Reference Reagent Program) were used at a concentration of 100 ng/ml. All HIV-1 proteins were free of endotoxin (less than 0.03 U/ml), as determined by a Limulus assay, and did not contain cytotoxic contaminants, as determined by survival assays.

Flow cytometry. B cells and macrophages were stained with the following mAbs, conjugated to phycoerythrin or fluorescein isothiocyanate: Q4120, to CD4; UCHM1, to CD14; SJ25-c1, to CD19 (Sigma Aldrich); L307.4, to CD80; IT2.2, to CD86; L243, to HLA-DR (BD PharMingen); BE-1, to CD40; and 3F10, to HLA-ABC (Ancell). Biotin-conjugated polyclonal antibody BAF230 to IL-4 receptor α-chain (R&D Systems) and mAb 3F9 to IL-10 receptor α-chain (Serotec) were labeled with phycoerythrin-conjugated streptavidin (BD PharMingen). At least 1×10^4 viable cells were acquired with a FACScalibur analyzer (BD PharMingen). Histogram shifts were analyzed with the Kolmogorov-Smirnov test included in the CellQuest software (BD PharMingen) and were considered statistically significant at a p value of less than 0.001.

Enzyme-linked immunosorbent assay, survival assay and proliferation assay. IgG, IgA and IgE were detected as reported[36]. B cell survival was evaluated with the Trypan blue exclusion test. For measurement of B cell proliferation, 1×10^4 cells/200 μl were seeded in 96-well plates and were pulsed with 1 μCi [^{3}H]thymidine on day 4 of culture. After 18 h, cells were collected for measurement of [^{3}H]thymidine uptake.

Immunohistochemistry. Tonsil and lymph node tissue specimens were obtained from five healthy donors and ten symptomatic HIV-1 patients, respectively. The Institutional Review Board of Weill Medical College of Cornell University approved the study, and patients provided informed consent. Paraformaldehyde-fixed frozen tissue sections 5 μm in thickness were stained with fluorescein isothiocyanate–conjugated mouse mAb IA6-2 to IgD (BD PharMingen), unconjugated goat polyclonal antibody vA-19 to Nef (Santa Cruz), unconjugated mouse mAb 191 to p17 (Biodesign International), unconjugated mouse mAb Kal-1 to p24 (Dako) and/or unconjugated mouse mAb 1F8 to CD21 (BD PharMingen). Then, slides were incubated with secondary indodicarbocyanine-conjugated antibody to mouse (anti-mouse; Jackson ImmunoResearch Laboratories) and Alexa Fluor 456–conjugated anti-goat (Molecular Probes). Nuclei were visualized with 4′,6-diamidine-2′-phenylindole dihydrochloride (Boehringer Mannheim). Slides were analyzed with a Zeiss Axioplan 2 microscope (Atto Instruments).

Confocal microscopy. B cells were adhered to polylysine-coated glass slides, fixed and washed as described[36]. Mouse mAb IA6-2 to IgD (BD PharMingen) and goat polyclonal antibody vA-19 to Nef (Santa Cruz) were labeled with secondary Alexa Fluor 488–conjugated anti-mouse and Alexa Fluro 456–conjugated anti-goat, respectively. Coverslips were applied with Slow Fade reagent (Molecular Probes). Cells were visualized with a Zeiss LSM510 laser-scanning microscope (Carl Zeiss).

RT-PCR and Southern blot. cDNA was synthesized from total RNA as described[36]. Germline Iγ3-Cγ3 and I_μ-C_μ transcripts, I_γ1/2-C_μ, I_γ3-C_μ, I_γ4-C_μ, I_α1/2-C_μ and I_ε-C_μ circle transcripts, *AICDA* transcripts and *ACTB* transcripts (encoding β-actin) were amplified by RT-PCR for 25 cycles as reported[36]. I_γ3-C_γ3 transcripts were hybridized to a radiolabeled oligonucleotide probe encompassing a consensus Cγ sequence, whereas I_μ-C_μ transcripts and circle transcripts were hybridized to a radiolabeled probe recognizing C_μ (ref. 33). *NFKBIA* transcripts as well as *SOCS1* and *SOCS3* transcripts were amplified by RT-PCR for 25 cycles with the following forward and reverse primer pairs: *NFKBIA* forward, 5′-CTACACCTTGCCTGTGAGCAGGGCT-3′, and reverse, 5′-GCTCGTCCTCTGTGAACTCC GTGAA-3′; *SOCS1* forward, 5′-CCTTCCCCTTCCAGATTTGACC-3′, and reverse, 5′-AAGAGGT AGGAGG TGCGAGTTCAG-3′; and *SOCS3* forward, 5′-CTTCTCTCTGCAGAGCGATC-3′, and reverse, 5′-TGGTCCAGGAACTCCCGAAT-3′. The RT-PCR conditions have been described[33,36].

Quantitative real-time RT-PCR. Quantitative real-time PCR was done in triplicate with the iCycler iQ thermal cycler and detection system (BioRad) and the PCR Core Reagents kit (Applied Biosystems) with 500 nM primers; the final Mg^{2+} concentration was adjusted to 4 mM. Fourfold serial dilutions of cDNA were used to generate curves of log input amount versus threshold cycle, and comparable slopes for a given primer set were obtained for the group of cDNAs being tested, indicating comparable efficiencies of amplification. The amount of mRNA was normalized relative to the amount of *ACTB* mRNA. The generation of amplification products of only the correct size was confirmed by agarose gel electrophoresis. The following primer pairs were used: I_γ3-C_γ3 forward, 5′-GCCATGGGGTGATGC CAGGATGGGCAT-3′, and reverse, 5′-GAAGACCGATGGGCCCTTGGTGGA-3′; *AICDA* forward, 5′-CCTCCTAATGAGAGTATCTGGGTGAT-3′, and reverse, 5′-TTAAAACATAC AGC GCATGATTGG-3′; *NFKBIA* forward, 5′-TGCCTACACTTAGCCTC TATC-3′, and reverse, 5′-AGGTCCACTGCGAGGTGAAG-3′; *SOCS1* forward, 5′-TTGGAGGGAGCGGATGGGTGTAG-3′, and reverse, 5′-AGAGGTAGGAGG TGCGAGTTCAGGTC-3′; *SOCS3* forward, 5′-CACTCTTCAG CATCTC TGTCGGAAG-3′, and reverse, 5′-CATAGGAGTCCAGGTGGCCGTTGAC-3′; and *ACTB* forward, 5′-GGATGCAGAAGGAGATCACT-3′, and reverse, 5′-CGATCCACACGGAGTACTTG-3′.

Luciferase reporter assay. The 2E2 B cells (20×10^6 cells/ml) were transfected by electroporation with 40 μl plasmid DNA–Tris–EDTA solution, pH 8.0, containing 10 μg I_γ3-Luc or κ$B_{(2)}$-Luc reporter plasmid, expressing firefly luciferase, and 200 ng control pRL-TK reporter plasmid, expressing renilla luciferase under control of the thymidine kinase promoter (Promega). Firefly and renilla activity was measured after 48 h with the Dual-Luciferase Assay System (Promega). Luciferase activity is expressed as relative light units normalized to the pRL-TK control plasmid. The 2E2 B cells were also transfected with various amounts of pcDNA3.1 expression plasmids. Expression plasmids for consensus Nef and mutant Nef with deletion of the myristoylation signal sequence (Nef amino acids 1–19 ($Nef_{\Delta1-19}$)) were from M. Robert-Guroff (National Cancer Institute, Frederick, Maryland); expression plasmids for SOCS1 and SOCS3 were from D. Hilton (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). An expression plasmid for IκBα with mutant Ser 32 and Ser 36 was from E. Cesarman (Weill Medical College of Cornell University).

Immunoblots. Equal amounts of cytoplasmic or nuclear proteins were separated by 10% SDS-PAGE and were transferred to nylon membranes (BioRad). After being blocked, membranes were probed with primary polyclonal or monoclonal antibodies to p50 (C-19), p65 (C-20), c-Rel (B-6), IκBα (C-21), p38 (C-20), BSAP (C-20), Jak1 tryosine-phosphorylated at residues 1022 and 1023, Jak3 (C-21), Jak3 tryosine-phosphorylated at residue 980 (Tyr980), STAT1 (C-136), STAT3 (F-2), STAT6 (S-20), Oct1 (12F11), SOCS1 (C-20), SOCS3 (M-20), actin (I-19) and Nef (vC-19; Santa Cruz Biotechnologies). Membranes were also probed with primary polyclonal antibodies to Jak1; IKKβ; IKKβ-IKKα phosphorylated at serine residue 181; IKKγ; and IKKγ phosphorylated at serine residue 376; or with primary mAb 5A5, to IκBα phosphorylated at serine residues 26 and 36; 28B10, to p38 tryosine-phosphorylated at residues 180 and 182; 9H2, to STAT1 tryosine-phosphorylated at residue 701; 3E2, to STAT3 tryosine-phosphorylated at residue 705; or 5A4, to STAT6 tryosine-phosphorylated at residue 641 (Cell Signaling Technology).

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Membranes were then washed and were incubated with the appropriate secondary antibody (Santa Cruz). Proteins were detected with an enhanced chemiluminescence detection system (Amersham). Signal intensity was quantified by Quant1 software (Bio-Rad).

Electrophoretic mobility-shift assay (EMSA) and supershift assay. Oligonucleotides encompassing κB3, BSAP and GAS sites from the $I_{\gamma}3$ promoter and a control oligonucleotide encompassing an Oct1-binding site were end-labeled with [γ-P^{32}]ATP by T4 kinase and were used at approximately 30,000 c.p.m. in each EMSA[33,36]. Reaction samples were prepared as described[33,36] and were separated by 6% nondenaturing PAGE. The composition of DNA-bound protein complexes was determined by incubation of the reaction mixture with polyclonal antibody to p65 (C-20), c-Rel (B-6), Rel-B (C-19), p50 (C-19), BSAP (C-20) or STAT6 (S-20) (Santa Cruz) or with mAb to p52 (Upstate Biotechnologies) before the addition of radiolabeled probe[33,36].

Statistical analysis. For immunoglobulin secretion, proliferation, survival and reporter assays, values were calculated as mean ± standard deviation for at least three separate experiments done in triplicate. The significance of differences between experimental variables was determined with the paired Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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