Effects of the HIV-1 protein Nef on the stromal cells of mouse peripheral lymph nodes and on mouse keratinocytes

Clémence Meunier

Division of Experimental Medicine
McGill University, Montreal

June 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

Abstract

The HIV-1 protein Nef plays an important role in HIV-1 pathogenesis, both in humans and mouse models. Indeed, transgenic (Tg) mice expressing Nef under the control of the human CD4 promoter develop many phenotypes that closely resemble those of AIDS patients. Using these Tg mice, we have studied the effects of Nef on lymph node stroma, namely on fibroblastic reticular cells (FRCs), and blood and lymphatic endothelial cells (BECs and LECs, respectively). In human patients, a severe and irreversible loss of LN structure and function is observed, and this is correlated with the depletion of FRCs. We show that, in our model, Nef does not significantly change the size of the FRC population, nor does it affect its functions in resting lymph nodes (LNs). We hypothesized that FRCs are supported by lymphoid tissue inducer cells, which prevent their depletion. No fibrosis or loss of structure could be observed in the LNs of Tg mice, contrary to what is seen in human AIDS patients.

Nef did, however, cause a localized expansion of BECs and LECs in medullary blood vessels and the subcapsular sinus, respectively, sometimes to the point of completely obstructing these structures. The mechanism driving this expansion is still under investigation.

We also studied an unexpected effect of Nef expression on skin keratinocytes. This expression led to the development of an atopic dermatitis-like disease. Atopic dermatitis is one of the most prevalent skin diseases associated with AIDS but, to our knowledge, no HIV-1 protein had previously been directly linked to it. We show here that Nef causes this atopic dermatitis-like disease by inhibiting the Notch1 signalling pathway in keratinocytes. Thus, our data adds to the list of known Nef effects, and provides potential new insights for therapy.

Résumé

La protéine Nef du VIH-1 joue un rôle important dans la pathogenèse de ce virus, chez les humains et chez des modèles murins. En effet, des souris transgéniques (Tg) exprimant Nef sous le contrôle du promoteur CD4 humain développent des phénotypes très similaires à ceux retrouvés chez les patients atteints du SIDA. Nous avons étudié, chez ce modèle murin, les effets de Nef sur le stroma des ganglions lymphatiques (GL), c'est-à-dire sur les cellules réticulaires fibroblastiques (CRFs) et les cellules endothéliales vasculaires et lymphatiques (respectivement CEVs et CELs). Chez les humains infectés par le VIH-1, une perte sévère et irréversible de la structure et de la fonction des GLs est observée et est corrélée avec la déplétion des CRFs. Nous montrons ici que, dans notre modèle, Nef ne change pas significativement la taille de la population de CRFs et n'affecte pas ses fonctions. Nous proposons que la population de CRFs est maintenue par les cellules inductrices de tissu lymphoïde. Aucune fibrose ou perte de structure n'a été observée dans les GLs des souris Tg, contrairement à ce qui se retrouve chez les patients humains.

Par contre, Nef cause une expansion localisée des CEVs et des CELs dans les vaisseaux sanguins médullaires et dans le sinus subcapsulaire, respectivement, parfois au point de complètement obstruer ces structures. Le mécanisme de cette expansion est à l'étude.

Nous avons également étudié des effets inattendus de l'expression de Nef dans les kératinocytes de la peau. Cette expression a provoqué le développement d'une maladie similaire à la dermatite atopique. La dermatite atopique est l'une des maladies de peau les plus fréquentes chez les patients atteints du SIDA. Cependant, à notre connaissance, aucune protéine du VIH-1 n'y avait été directement associée à ce jour. Nous montrons ici que Nef cause cette maladie en inhibant la voie de signalisation de Notch1 dans les kératinocytes. Ainsi, nos résultats identifient de nouveaux effets de Nef et fournissent de nouvelles perspectives potentielles pour des thérapies.

Table of Contents

Acknowledgements

I would like to thank Dr Paul Jolicoeur first, for giving me this opportunity and trusting me with these projects. I thank him for his guidance and for teaching me scientific rigor and what it means to lead a research project. I believe I have received a very good training, and I owe it to him.

A very special thank you to everyone in Dr Jolicoeur's laboratory and in the technological platforms at the IRCM for their advice and patience when teaching me new techniques. I did my best!

I would also like to mention my colleagues and fellow students in the lab for their support, good mood, and sense of humour. It was great discussing anything from cooking recipes to astronomy around lunch. They made these past years a very pleasant experience!

Last but certainly not least, my deepest thanks to my family and friends on both sides of the Atlantic Ocean for their encouragements and unwavering support.

Introduction

Chapter 1: HIV-1 and AIDS

1.1 Epidemiology

The Human Immunodeficiency Virus (HIV) is a retrovirus of the *Lentivirinae* subfamily. These viruses are characterized by an RNA genome and a reverse transcriptase (Gonda et al, 1986). HIV is responsible for the development of the acquired immunodeficiency syndrome (AIDS) in humans. The United Nations Organization estimates that, in 2011, 34 million people around the world lived with HIV. Although these figures have been steadily declining in the past decade, about 2,5 million adults and children were newly infected in 2011. HIV-1 is mainly transmitted sexually, from mother to child during pregnancy, delivery, and breastfeeding, between drug users exchanging syringes and needles, and by transfusion of contaminated blood (UNAIDS World AIDS Day Report, 2012; www.unaids.org/).

Infection leads to a progressive loss of immune cells. An individual is considered to be suffering from AIDS when their CD4+ T cell count drops below the threshold value of 200 cells/mm^3 of blood (Pantaleo et al 1993a). By then, the immune system is severely compromised, which allows for the development of neoplasms and opportunistic infections. The UNAIDS 2012 report states that there were 1,7 million AIDS-related deaths in 2011.

1.2 Disease progression

The evolution of the disease follows three main phases that are defined by the CD4+ T cell count: primary infection or acute phase, latency or chronic stage, and progression to AIDS. In the absence of therapy, this process can take about

ten to fifteen years (Gonda et al, 1986, Pantaleo et al, 1993a). Figure 1 summarizes the different steps of the disease.

The acute phase is characterized in most patients by non-specific symptoms resembling mononucleosis that arise 3 to 6 weeks post-infection (Gonda et al, 1986; Tindal and Cooper, 1991). Plasma virus titers rise to about 10^6 to 10^7 virions/mL (Clark et al, 1991) as CD4 lymphocyte counts drop drastically (Gaines et al, 1990). It is during this phase that lymphoid organs are seeded and become reservoirs of virions (Pantaleo et al, 1993b). The plasma viral load then begins decreasing with the onset of specific humoral and cell-mediated immune responses (Tindal and Cooper, 1991; Koup et al, 1994; Safrit et al, 1994; Pantaleo et al 1995). This control of the infection allows for a partial or almost-complete reconstitution of the population of CD4+ T cells (Pantaleo et al, 1993b). This marks the transition into the chronic phase.

The chronic phase, or latent phase, has a median length of ten years and is mostly asymptomatic (Pantaleo et al, 1993a). However, the viral load increases steadily and there is active HIV replication in the reservoir lymphoid organs, accompanied by a progressive loss of CD4+ T cells (Pantaleo et al, 1993c). As the disease progresses, the structure of the lymphoid organs is also lost, which further precipitates the deterioration of the immune system (Pantaleo et al, 1993b, 1995).

The threshold for entry into the AIDS phase of the infection has been set to 200 CD4+ T cells/mm^3. The viral load continues to rise, and neoplasms and opportunistic infections appear, that can lead to the patient's death (Cheng-Mayer et al, 1988; Pantaleo et al, 1993a).

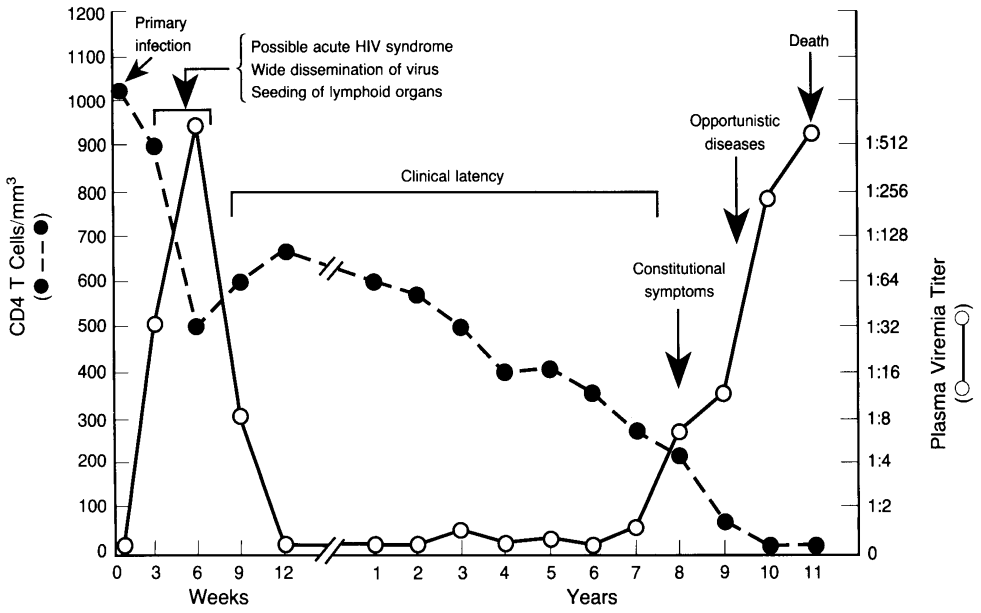


Figure 1: Evolution of CD4+ T cell counts and plasma viremia during HIV-1 infection. Adapted from Pantaleo et al, 1993a. The acute phase appears in the first weeks after infection and is characterized by a marked increase in viremia and decrease in CD4+ T cell counts. The specific immune response that develops allows the host to regain control of the viremia, which allows the partial reconstitution of the CD4+ T cell population. This latent, asymptomatic phase can last several years and sees a progressive loss of CD4+ T cells. Eventually, T cell counts become too low to control the infection, and the patient progresses to AIDS, during which the plasma viremia increases and the patient becomes susceptible to opportunistic infections that can lead to their death.

1.3 Mechanism of infection

1.3.1 Tropism

The main receptor for HIV-1 is the CD4 molecule (Dalgleish et al, 1984). The HIV-1 protein gp120 binds to CD4, but entry into the target cell also requires the expression of co-receptors. Two molecules preferentially fill this role: CCR5 and CXCR4 (Paxton et al, 1996; Berger et al, 1999). Other co-receptors have been described and can be classified into two categories: chemokine receptors and C-type lectins (Pollakis and Paxton, 2012). This array of co-receptors allows the virus to infect a large panel of cells of the immune system. Naïve and memory

CD4+ T cells (Centlivre et al, 2011), monocytes, macrophages, dendritic cells, Langerhans cells (Almodóvar et al, 2007; Boy et al, 2009; Vázquez et al, 2010), plasmacytoid dendritic cells (Centlivre et al, 2011), and mast cells (Steinsvoll et al, 2004) have all been shown to carry HIV-1. The virus has also been found in epithelial cells of the skin and digestive tract (Steinsvoll et al, 2004).

During transmission and primary infection, HIV-1 virions use exclusively CCR5 as co-receptor and CXCR4-using strains appear later during the course of the disease (Rieder et al, 2011). The affinity of the envelope of the virus and the expression patterns of both co-receptors can explain this preference. For instance, high levels of CCR5 have been observed in mucosae of entry sites of the virus (McKinnon et al, 2011; Prodger et al, 2012; Meditz et al, 2012). The other chemokine receptors that can be used as co-receptors includes CCR1, CCR2b, CCR3, CCR4, CCR6, CCR8, APJ, CXCR6, CXCR3 or CXCR7 (Pollakis and Paxton, 2012). As is the case for CCR5 and CXCR4, their expression patterns through time and in the body determine which organs and cell types can be infected. These co-receptors allow the entry of HIV-1, but also of HIV-2 and the Simian Immunodeficiency Virus (SIV; Pollackis and Paxton, 2012).

The other category of HIV-1 co-receptors is C-type lectins. These molecules bind the virus and thus increase the possibility of infection through the CD4/CCR5 or CXCR4 pathway (Pollakis and Paxton, 2012). C-type lectins such as DC-SIGN, L-SIGN, or DC-SIGNR play a role in the dendritic cell trafficking. They also promote the formation of immunological synapses (Pöhlmann et al, 2011; Soilleux, 2003). This facilitates the dissemination of the virus through the body as well as the infection in *trans* of target cells (Wichukchinda et al, 2007; Hijazi et al, 2011).

1.3.2 Replication cycle

Once the gp120 protein binds CD4 and a co-receptor, gp41, the other envelope protein of HIV, undergoes conformational changes that lead to the fusion of the viral membrane with that of the host cell (Chan and Kim, 1998). The

virus can also enter the cell by endocytosis followed by fusion of the viral membrane with the endosome membrane (Goto et al, 1998). Following this fusion, the viral RNA and the viral enzymes (reverse transcriptase, integrase, ribonuclease, and protease) are released into the cell's cytoplasm (Chan and Kim, 1998) and the RNA is transcribed into double-strand DNA by the reverse transcriptase.

The double-strand DNA translocates to the nucleus in association with viral and host proteins, forming the preintegration complex. The viral integrase is part of this complex and inserts the viral DNA into the host cell's genome, forming the provirus (Asante-Appiah and Skalka, 1999; Craigie, 2001). The viral DNA can then be transcribed. The viral DNA contains a promoter and an enhancer sequence, but they are weakly active in the basal state. Efficient elongation requires host transcription factors such as P-TEFb, which phosphorylates the C-terminal domain of the host RNA polymerase II and favor elongation (Garber and Jones, 1999; Zheng et al, 2005). Purcell and colleagues (1993) have reported that one full-length, 9kb, mRNA can be spliced into 46 products, some single-spliced at 4,4 kb, others multiply-spliced (2,2 kb). The spliced mRNA are then exported into the nucleus, which again requires host proteins such as CRM1 and RanGTP, and translated in the cytoplasm (Cullen, 2003).

The viral proteins are assembled into virions in glycolipid-enriched lipid rafts of the cellular membrane (Nguyen and Hildreth, 2000), and the viral particles are released.

1.4 The HIV-1 genome

The HIV-1 genome consists of two identical copies of a plus strand of linear RNA. Each copy contains nine open reading frames (ORFs) that encode 15 proteins. The HIV-1 genes can be separated into structural genes, regulatory genes, and accessory genes (Frankel and Young, 1998).

1.4.1 Structural genes (gag, env, pol)

The *gag, pol,* and *env* genes are common to all retroviruses. They are first transcribed and translated as polyproteins, Gag-Pol and Env, and then cleaved. Gag is synthetized as a precursor, $Pr55^{Gag}$, that contains four protein domains and localizes to the cell membrane for virion assembly. The newly assembled virion is released as a non-infectious particle. Cleaving of $Pr55^{Gag}$ into p24 (capsid), p17 (matrix), p7 (nucleocapsid), and p6 is necessary for the maturation of the virion. It acquires a condensed and conical core typical of an infectious virion (Ono et al, 2000).

The matrix protein of Gag is required for the targeting of Gag and Gag-Pol polyproteins to the cell membrane. Its N-terminal domain is myristoylated, which allows it to bind the cell membrane and anchor the virion during assembly (Hill et al, 1996). In addition to this, it also plays a role in the incorporation of the Env proteins gp41 and gp120 into viral particles (Freed and Martin, 1996).

The full length, genomic viral mRNA is delivered to the assembling virion by the nucleocapsid protein bound to its ψ locus (Clever and Parslow, 1997). The nucleocapsid protein also coats it in the mature viral particle (Laughrea M et al, 1997; Frankel and Young, 1998). It also binds single stranded nucleic acids present in a non-specific way to coat the genomic RNA and protect it from the nucleases of the host cell (Frankel and Young, 1998).

In the assembled virion, the genomic RNA is localized within the core of the particle composed of about 2,000 capsid proteins (Frankel and Young, 1998).

The last Gag protein is p6, which mediates an efficient particle release and incorporates Vpr into the virions (Huang et al, 1995).

The *env* gene encodes a precursor protein, gp160, that transits through the endoplasmic reticulum to be glycosylated. It then moves on to the Golgi apparatus where is cleaved into gp120 and gp41 by host proteases. Both of these proteins subsequently undergo glycosylation modifications (Earl et al, 1991). These modifications play an important part in virus infectivity, as they are recognized and bound by DC-SIGN and other lectin receptors (Turville et al, 2002). Gp120

and gp41 remain linked by non-covalent bonds, and eventually transit to the plasma membrane for virion assembly (Corbitt et al, 1990).

gp120 is the first viral protein to interact with the target cell. As mentioned earlier, it primarily binds CD4, for which it has a high affinity (K_D ~ 4nM; Luciw, 1996). This binding leads to conformation changes of both gp120 and gp41, which facilitate co-receptor binding and entry into the target cell (Clapham, 1997).

gp41 binds the co-receptors, usually CCR5 or CXCR4, and mediates the fusion of the viral membrane with that of the target cell (Frankel and Young, 1998).

The third gene common to all retroviruses is *pol*. It encodes the enzymes necessary to the viral life cycle.

The first of these enzymes is the reverse transcriptase, which catalyzes both RNA-dependent and DNA-dependent DNA synthesis in order to generate a viral double stranded DNA (Telesnitsky and Goff, 1997). This process occurs in three steps: first, the enzyme synthetizes cDNA from the genomic RNA. It then degrades the RNA strand, and generates the second DNA strand (Huber et al, 1989; Fuentes et al, 1996). The reverse-transcription of the viral RNA requires the host $tRNA^{Lys3}$ that is used as a primer (Varmus and Swanstrom, 1991).

The viral DNA is then integrated into the host cell's genome by the viral integrase, which catalyzes the two steps of the process. While still in the cytoplasm of the cell, the integrase binds the 3' end of the viral DNA and cleaves it to expose its hydroxyl group. Once in the nucleus, it acts as a ligase to join the 3' end of the viral DNA to the 5' end of the host's cleaved DNA. The integrase is also responsible for cleaving the cell's DNA (Bushman and Craigie, 1991).

The third enzyme is the viral protease. During assembly, the polyproteins Gag and Gag-Pol are used, instead of their cleaved products. Thus, the virion that is released from the host cell is immature and non-infectious. The protease catalyzes the cleaving of these polyproteins, which triggers conformational rearrangements and gives rise to the fully mature virus. As it is part of Pol, its own activity depends on Gag-Pol levels and the rate of autoprocessing (Zybarth

and Carter, 1995). The protease cleaves Gag into the matrix (p17), capsid (p24), nucleocapsid (p7), and p6 proteins, and Pol into the reverse transcriptase, the integrase, and more protease (Oroszlan and Luftig, 1990).

1.4.2 Regulatory genes (tat, rev)

Initiation of transcription of the HIV-1 provirus depends on host factors such as NF-κB or TBP, which can bind the HIV-1 promoter found in the 5' long terminal repeat (LTR) of the viral DNA (Jones and Peterlin, 1994). Still, even in the presence of these factors, the transcription of the viral genome is inefficient and blocked at the elongation phase. The transactivation protein Tat enhances the activity of RNA polymerase II. It can increase the production of viral mRNA by 100-fold. To do so, Tat binds the transcription complex and recruits factors, such as TFIIH, cyclin T and CDK9, to phosphorylate the RNA polymerase II C-terminal domain (Parada and Roeder, 1996; Cujec et al, 1997; García-Martínez et al, 1997; Karn, 1999).

The protein Rev has been implicated in the export of viral RNAs out of the nucleus. It binds viral mRNAs via the Rev-Response Element (RRE) present on unspliced *gag* and *gag-pol* mRNA, and on single-spliced *env* mRNA. This allows for export into the cytoplasm through the interaction of Rev with the CRM-1-mediated nuclear export pathway (Ullman et al, 1997; Grewe and Uberla, 2010). This increases the production of Gag, Gag-Pol, and Env polyproteins in the cytoplasm, as well as genomic RNA packaging, all necessary for virion assembly. In the absence of Rev, most viral mRNAs are doubly spliced and encode Tat, Nef, and Rev (Frankel and Young, 1998; Groom et al, 2009).

1.4.3 Accessory genes (vif, vpu, vpr, nef)

The virion infectivity factor (Vif) plays a role in the production of infectious virions. Viral particles containing Vif mutants show defects in

infectivity, but only in certain cell types, which suggests that some cell populations produce a factor that has similar effects on virions (Cohen et al, 1996). Vif also counters some of the cell's defenses, as it blocks the antiretroviral effects of APOBEC proteins. In the absence of Vif, these proteins are encapsidated in the viral particles and restrict and disrupt the RNA reverse transcription by cytidine to uridine editing and by deaminase-dependent mechanisms, respectively. Vif prevents the encapsidation of certain APOBEC proteins, such as APOBEC3G and APOBEC3F, by inducing their ubiquitination, which leads to their degradation (Goila-Gaur and Strebel, 2008).

The viral protein U (Vpu) also counters cellular defenses. Specifically, it antagonizes the effects of the host protein Tetherin. Tetherin is a glycosylated transmembrane protein that cross-links virions to the plasma membrane. It has been shown to have similar effects on many enveloped viruses, including retroviruses, filoviruses (Ebola and Marburg viruses), paramyxoviruses (Nipah virus), and Kaposi Sarcoma Herpes Virus (Neil et al, 2006; Van Damme et al, 2008; Jouvenet et al, 2009; Mansouri et al, 2009; Sakuma et al, 2009), thus indicating that it does not need specific interactions with the virions to exert its effects. Vpu induces a downregulation of Tetherin at the cell surface by increasing its degradation in lysosomes and sequestering it in the trans Golgi network, thus facilitating the release of viral particles from the cell surface (Van Damme et al, 2008).

The other role of Vpu is to induce CD4 downregulation from the plasma membrane. Vpu binds the cytoplasmic domain of newly-synthetized CD4 in the endoplasmic reticulum (Willey et al, 1992; Bour et al, 1995) and targets it for degradation by the ubiquitin/proteasome pathway (Margottin et al, 1998). This depends on Vpu phosphorylation (Schubert and Strebel, 1994). The degradation of CD4 prevents it from binding gp160 as it traffics to the plasma membrane, which increases the efficiency of the production of viral proteins and of their assembly into virions. In addition, Vpu also promotes MHC I degradation, which can protect the infected cells from cytotoxic T lymphocytes (Kerkau et al, 1997).

Vpr, the viral protein R, is present in HIV-1 viral particles (Bachand et al, 1999). It has been implicated in the migration to the nucleus of the preintegration complex (Cohen et al, 1996). It can also induce an arrest of the cell cycle in G2 phase, prevent entry into mitosis (Emerman, 1996), and promotes apoptosis. In addition, Vpr has been shown to modulate the transcription of the viral DNA (Sawaya, 2000) and to reduce its mutation rate (Mansky, 1996).

The last regulatory protein encoded by the HIV-1 genome is Nef. It plays a crucial role in HIV-1 pathology as humans and rhesus macaques infected with a Nef-deficient strain of HIV-1 or SIV, respectively, show a very slow progression of the disease (Kestler et al, 1991; Daniel et al, 1992; Deacon et al, 1995). The best-characterized effects of Nef are the downregulation of CD4 and of MHC I, which leads to their degradation, as well as its disruption of the TcR signaling pathway in CD4+ T cells (Mangasarian and Trono, 1997; Simmons et al, 2001, reviewed in Foster and Garcia, 2008). The structure of Nef allows it to interact with many host proteins and to mediate a great panel of effects. We are going to detail these, as well as the above-mentioned ones, in the next chapter. Figure 2 represents the organization of the HIV-1 genome, as well as the localization of the different viral proteins in the virion.

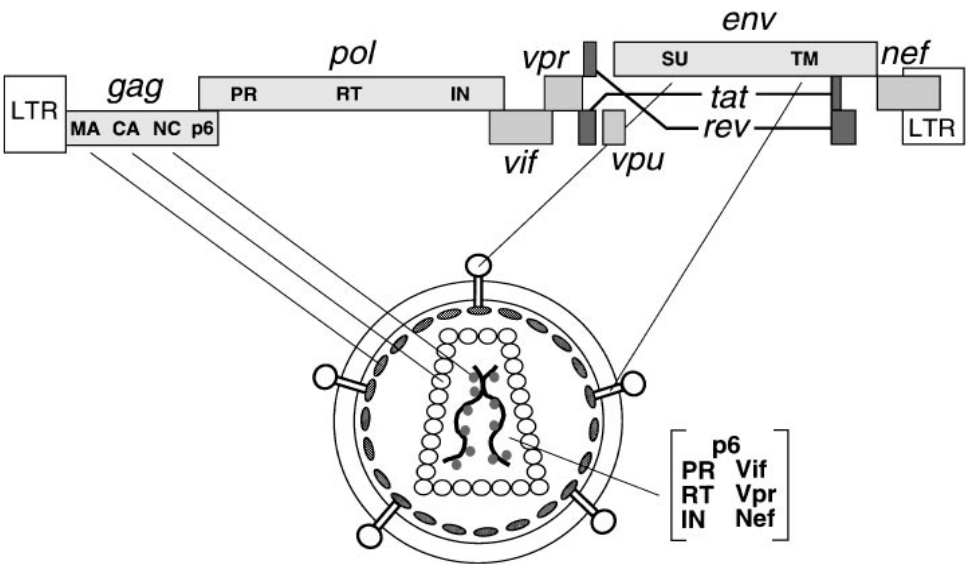


Figure 2: Organization of the HIV-1 genome and viral particle. Adapted from Frankel and Young, 1998. The HIV-1 genome comprises 9 genes, which encode 15 proteins. LTR: Long Terminal Repeat; MA: p17 (Matrix); CA: p24 (Capsid); NC: p7 (Nucleocapsid); PR: Protease; RT: Reverse transcriptase; IN: Integrase; SU: gp120 (Surface); TM: gp41 (Transmembrane).

Chapter 2: The HIV-1 protein Nef

2.1 Structure of Nef

Nef is a 27-35 kDa protein found in HIV and SIV, and is one of the first viral proteins to be expressed after infection. Its structure has been resolved by NMR and X-ray crystallography. Numerous conserved motives have been identified in its sequence, as well as a wide array of interacting partners that allow it to mediate its many effects: enhancement of virion infectivity, support of viral replication, downregulation of CD4 and MHC I from the cell surface, activation of Pak kinases, and disruption of the TcR signaling pathway (Geyer et al, 2001).

Nef possesses an N-terminal arm of ~70 residues, a core domain of ~120 residues, and a C-terminal tail of 10-30 residues present only in HIV-2 and SIV. The core domain has the most conserved sequence, which suggests that it contains the different interaction domains necessary to Nef function (Arod and Baur, 2001). Table 1 summarizes the different motifs found in the sequence of Nef and their known interaction partners. The signification and consequences of these interactions is discussed below.

Nef also receives post-transcriptional modifications. It has a myristoylation motif in its N-terminal arm that is highly conserved. This indicates that Nef is targeted to the cellular membranes and that this association is critical to its function. In fact, N-myritoylation of Nef has been shown to be required for all of its biological effects (Harris, 1995), except the activation of the Hematopoietic Cellular Kinase, Hck (Briggs et al, 2001).

Table 1: Conserved motifs of HIV-1 Nef. Adapted from Geyer et al, 2001. Reviewed in Geyer et al, 2001, and Arold and Baur, 2001.

Motif in HIV-1 Nef (NL4-3)	Interacting protein
Protein Modification	
$MGxxxS_{(1)}$	N-myrisoyl transferase
$CAW\downarrow LEA_{(55)}$	HIV-1 protease
Signaling	
$PxxPxR_{(72)}$	SH3 domains of Src family kinases (Hck, Lck, Src, Fyn); TcR ζ
$RR_{(105)}$	PAK 1 and PAK2
$DDPxxE_{(174)}$	cRaf-1 kinase
Trafficking	
$WL_{(57)}$, $L_{(110)}$	Cytoplasmic tail of CD4
$EEEE_{(62)}$	PACS-1
$FPD_{(121)}$	Human thioesterase
$EE_{(154)}$	β-COP
$ExxxLL_{(160)}$	Adaptor proteins AP-1, AP-2, and AP-3
$DD_{(174)}$	V1H

2.2 Functions of Nef

As shown in Table 1, Nef has a wide array of interaction partners. Foster and colleagues (2011) propose that Nef forms ternary complexes that result in abnormal associations between host proteins. This explains how Nef is able to mediate effects as different as the enhancement of virion infectivity and replication, the downregulation of CD4 and MHC I from the cell surface, the activation of Pak kinases, and the disruption of the TcR signaling pathway. All of these phenotypes, however, have one thing in common, in that they contribute to

the persistence of the HIV-1 infection (Geyer et al, 2001). We are now going to further detail the main effects of Nef.

2.2.1 Enhancement of viral infectivity

A functional *nef* gene is necessary to maintain a high viral load, for virus replication, and for progression to AIDS (Kestler et al, 1991; Aldrovandi and Zack, 1996). Kimpton and Emmerman (1992) have demonstrated that Nef is also required for virion infectivity. This process is very complex and not yet fully understood, but several of the implicated mechanisms have been identified (Foster et al, 2011).

Inhibition of lysosome and proteasome function can enhance HIV-1 infectivity, which suggests that Nef acts by stabilizing the pre-integration complex (Wei et al, 2005). Functional PxxPxR and ExxxLL domains, that bind SH3 domains and adaptor proteins, respectively, are also required to enhance virion infectivity. Thus, it appears that the modifications of cell signaling pathways and of protein trafficking are also involved in this process (Madrid et al, 2005; Coleman et al, 2006). In addition, the downregulation and degradation of CD4 mediated by Nef also play a part. CD4 binds the HIV Env protein. It can therefore bind budding virions, thus preventing their release. CD4 can also accumulate into virions, which could prevent the incorporation of the Env protein during virion assembly (Lama et al, 1999; Lundquist et al, 2002). Nef counters these effects and enhances the release of viral particles.

2.2.2 CD4 downregulation

CD4 downregulation is one of the best well-known effects of Nef, and the best studied. Nef binds the cytoplasmic tail of CD4 via its $WL_{(57)}$, $L_{(110)}$ motif (K_D ~ 1 µM; Grzesick et al 1996; Preusser et al, 2001) and recruits AP-2 to mediate the internalization of CD4 (Chaudhuri et al, 2009). CD4 is then targeted to the lysosome for degradation (da Silva et al, 2009).

Of note, Vpu has also been shown to induce CD4 downregulation at the cell surface, but this HIV-1 protein acts by blocking its traffic to the cell membrane (Piguet et al, 1999).

As CD4 is the primary receptor for HIV-1 particles, its downregulation at the cell surface prevents over-infection of the target cell, which would overload protein synthesis capacity without leading to the production of more viral particles (Harris, 1999). As mentioned earlier, it also facilitates the release of virions. In addition, the downregulation of CD4 disrupts its interaction with the Lymphocyte-specific protein tyrosine Kinase (Lck) involved in the TcR signaling pathway. This leads to an accumulation of Lck to endosomes, which affects the activation of the TcR signaling pathway and the formation of efficient immunological synapses when CD4+ T cells come into contact with antigen-presenting cells (Thoulouze et al, 2006).

2.2.3 MHC I downregulation

MHC I downregulation presents similarities with CD4 downregulation. The proposed mechanism has Nef binding the cytoplasmic tail of MHC I and recruiting AP-1, thus leading to MHC I internalization (Noviello et al, 2008). MHC I first accumulates in endosomes, then transits to trans-Golgi vesicles from where it is targeted to lysosomes and degraded (Piguet et al, 1999 and 2000). In addition, newly-synthetized MHC I can also be rerouted to lysosomes instead of trafficking to the cell surface (Schaefer et al, 2008). This downregulation of MHC I prevents the display of HIV-specific antigens at the cell surface and protects the infected cell from recognition and lysis by cytotoxic T lymphocytes (Marsh, 1999). This enhances viral persistence in infected individuals.

2.2.4 Cellular activation

The main function of Nef is to modify the cellular environment to promote HIV replication and persistence. Activation of infected T cells is necessary for

virus replication, and has been linked to disease progression (Hazenberg et al, 2003). Indeed, activated T cells divide rapidly and die, most likely through activation-induced cell death, which accelerates the transition to AIDS (Meyaard et al, 1992). Nef has been shown to mediate such an effect (Simmons et al, 2001). Simmons and colleagues studied the changes in gene expression induced by Nef expression and crosslinking of CD3 in T cells, and found a striking 97% overlap. Activated T cells express factors such as NFAT and NF-κB, which can bind the viral promoter and thus upregulate the transcription of the provirus (Zack et al, 1990).

In addition, Nef enhances Lck activity in thymocytes, which mimics a constitutively active TcR signaling pathway. However, this limits the reactivity of the cells to TcR stimulus and the capacity of CD4 to contribute to TcR signaling (Chrobak et al, 2010), as well as disrupting the maturation of the thymocytes. Nef has also been shown to inhibit the formation of immune synapse (Thoulouze et al, 2006). Thus, while an activated state of infected cells promotes viral replication, it also increases the perturbation of the immune system.

2.2.5. Apoptosis and cell survival

While the state of chronic inflammation that results from HIV-1 infection leads to cell death by exhaustion, Nef itself influences survival and apoptosis of both infected and virus-free cells. Nef can bind the ζ chain of the TcR, which leads to Fas-ligand expression at the cell surface (Xu et al, 1999). It can bind the Fas receptor of a cytotoxic T lymphocyte (CTL), and thus induce the cell's apoptosis before the CTL could recognize, and kill, the infected cell (Mueller et al, 2001). This, combined with the downregulation of MHC I, leads to evasion of the immune system and survival of the infected cell. In addition, it contributes to the general immune disruption induced by HIV infection, and prevents the development of an efficient HIV-specific immune response.

In addition to enhancing immune evasion, Nef promotes the survival of the infected cells by preventing their own apoptosis. Nef associates with and blocks

the activity of the Apoptosis Signaling Regulating Kinase 1, ASK 1, which is involved in Fas and TNF-receptor signaling pathways. By doing so, Nef effectively inhibits the induction of apoptosis through these pathways (Fackler and Baur, 2002).

Nef has also been shown to bind both the PAK2 kinase and the PI-3 kinase simultaneously, which leads to the activation of PAK2. The Nef-PAK2-PI-3 complex then phosphorylates Bad (Wolf et al, 2001). The pro-apoptotic Bad forms heterodimers with the anti-apoptotic Bcl-2. Upon phosphorylation of Bad, Bcl-2 is released and promotes cell survival (Gross et al, 1999).

2.2.6 Remodelling of actin cytoskeleton

Nef has also been shown to interact with the actin cytoskeleton of infected cells, with different outcomes. It remodels the cortical actin barrier to facilitate the entry of the viral proteins and genome into the cell (Campbell et al, 2004). There is also evidence that Nef, through its interaction with PAK2 and the host Rho GTPase exchange factor Vav, both involved in actin cytoskeleton dynamics, enhances trafficking of the viral core to the microtubule network, where reverse transcription happens and which then transports the viral elements to the nucleus (Anderson and Hope, 2005).

In addition to their role in intracellular traffic, the interactions of Nef with the actin cytoskeleton also modulate the motility of the infected cells. Nef is necessary and sufficient to induce the formation of filopodia in HIV-infected T cells. Virions were shown to accumulate in these filopodia, which also established contact with neighboring T cells, infected or not. This likely has a role in virus dissemination (Nobile et al, 2010). *In vitro*, the infected T cells showed a reduced intrinsic motility and chemotaxis towards CXCL12, CCL19, and CCL3, although not because of reduced receptor levels at the cell surface (Nobile et al, 2010). This would affect T cell migration to and through secondary lymphoid organs. In the case of T cells infected directly in lymph nodes, defects in migration could

explain their sequestration to the organ, as seen in chronic HIV infection (Lederman and Margolis, 2008).

Immune system evasion and cellular activation are also modulated by actin remodelings mediated by Nef. As is the case with intracellular trafficking, these effects depend on the interaction of Nef with Vav and PAK2 (Niederman et al, 1993; Fackler et al, 1999 and 2000).

Taken together, these results show that Nef is a major determinant of pathogenicity, and that it modulates the cellular environment to enhance viral survival and dispersal through the host's body.

Part 1: Effects of the HIV-1 protein Nef on the stroma of peripheral lymph nodes

Chapter 3: Peripheral lymph nodes

3.1 Organogenesis of lymph nodes

Lymph nodes (LNs) are part of the secondary lymphoid organs (SLOs). They filter lymph and exist only in mammals (Lammerman and Sixt, 2008). In humans, about 450 LNs are spread throughout the body, while mice comprise 22 identifiable LNs (Willard-Mack, 2006). In mice, LN development starts at around embryonic day (E) 10.5 with the formation of lymph sacs that result from endothelial cell budding from the larger veins. Connective tissue then invades the lymph sacs, thus forming the anlagen of LNs (Mebius, 2003). The first cells to be recruited to the LN anlagen are the CD45+ CD4+ CD3– Lymphoid Tissue Inducer cells (LTi), between E12.5 and E13.5 (Yoshida et al, 2002). These cells originate in the fetal liver and their development is RORγt-dependent (Eberl et al, 2004; Spits and Di Santo, 2011).

LTi recruit Lymphoid Tissue Organizing cells (LTo) to the developing LN. LTo are stromal cells of mesenchymal origin that express both VCAM-1 and ICAM-1 (Yoshida et al, 2002). Signaling through both the IL-7 receptor and the TRANCE receptor induces the expression of Lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) at the surface of LTi (Mebius, 2003) – although only TRANCE-receptor signaling seems necessary for LN development (Yoshida et al, 2002). $LT\alpha_1\beta_2$ expression triggers LTβ receptor signaling in the LTo, which upregulates their expression of chemokines such as CCL19, CCL21, CXCL12, and CXCL13, as well as that of VCAM-1 (Ngo et al, 1999; Yilmaz et al, 2003). LTi bind LTo via integrin-$\alpha_4\beta_1$, and the close contact between the two cell types further increases the production of chemokines (Finke et al, 2002). These chemokines then attract hematopoietic cells. T and B cells also express $LT\alpha_1\beta_2$ (Ansel et al, 2000), and therefore sustain

a positive-feedback loop that results in the accumulation of more immune cells to the nascent LN. High endothelial venules, blood vessels specialized in lymphocyte transmigration, develop in parallel, also in a $LT\alpha_1\beta_2$-dependent way (Mebius, 2003).

The segregation of B and T cells into B follicles and the T zone, respectively, is mediated by CCL19, CCL21, CXCL12, and CXCL13 (Cyster, 1999; Okada et al, 2002). The follicular stromal cells present in B follicles and the fibroblastic reticular cells of the T zone express these chemokines (Ngo et al, 1999). This secretion of chemokines is sustained by the expression of $LT\alpha_1\beta_2$ by both types of lymphocytes, but this is not required for the maintenance of the compartmentalization in the adult mouse, as the $LT\alpha_1\beta_2$-expressing LTi seem to fulfill this position (Mebius, 2003).

Of note, it has been shown that mesenteric LNs can develop in LTβ-deficient mice and in the absence of CXCL13 and CCR5, contrary to peripheral LNs (pLNs), which indicates that different LNs follow somewhat different development programs (Alimzhanov et al, 1997; Ansel et al, 2000).

3.2 Lymph nodes structure and function in the adult mouse

3.2.1 Resting lymph nodes

3.2.1.1 Lymph node compartments

In adult mice and humans, LNs are responsible for draining the lymph from all tissues and organs of the body. They represent a crossroad where antigen-presenting cells (APCs), T and B lymphocytes, and lymph-borne antigens all meet, and play an active role in their homeostasis. This concentration of immune cells increases the chances that a naïve CD4+ T cell will recognize its cognate antigen and start an appropriate immune response. The structure of LNs is key to this function. They are divided into clearly marked compartments, each characterized by the immune and stromal cells found there (Figure 3).

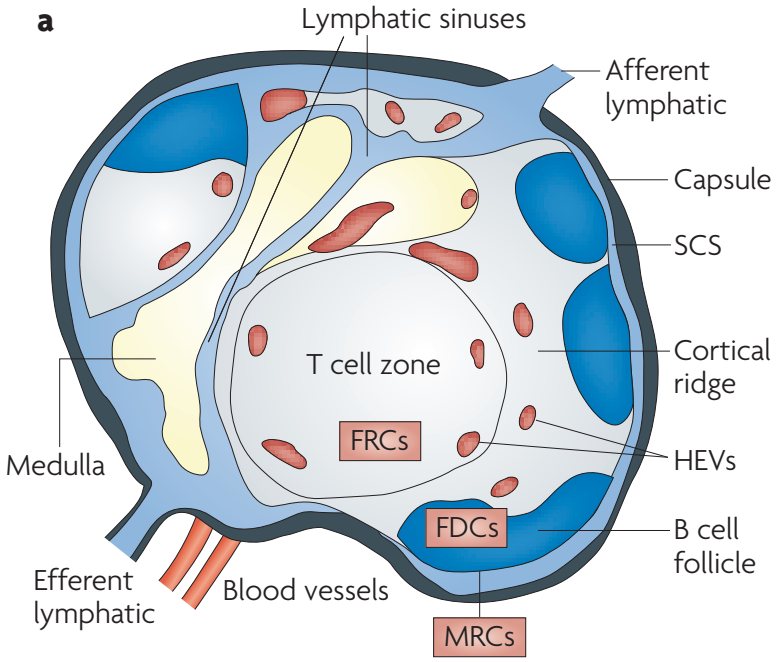


Figure 3: Organization of a peripheral lymph node. Adapted from Mueller and Germain, 2009. APCs and lymph-borne antigens enter the LN via the afferent lymphatic vessel that opens into the subcapsular sinus (SCS). T and B lymphocytes enter via the high endothelial venules (HEVs), located at the periphery of T zones, before homing to their respective areas. Non-resident cells exit the LN via the lymphatic sinuses present in the medulla. FRCs: fibroblastic reticular cells; FDCs: follicular dendritic cells; MRCs: marginal reticular cells.

Lymph, lymphocytes, APCs, antigens, and signaling molecules all reach the LN via the afferent lymphatic vessels. The lymphatic endothelial cells (LECs) present in the vessels secrete CCL21, a chemoattractant for both T cells and dendritic cells (Cueni and Detmar, 2008). The afferent vessels open into the subcapsular sinus (SCS), which is lined with LECs and macrophages that capture lymph-borne antigens (Mueller and Germain, 2009).

Under the SCS, in the cortex of the LN, are the B follicles, where B lymphocytes home. CXCR5-expressing B cells are attracted to the follicles by follicular dendritic cells (FDCs) that secrete CXCL13 (Gunn et al, 1998a). FDCs express Fc receptors (CD16, CD23, and CD32), complement receptors (CD21 and CD35), and complement components (such as C4; Mueller and Germain, 2009).

They can capture and present unprocessed antigens to the B cells, especially antigens in the form of immune complexes. In addition, FDCs produce a dense network of desmin+ collagen+ fibers that supports B cell motility (Mueller and Germain, 2009).

A specific subset of stromal cells, the marginal reticular cells (MRCs), is found between the SCS and the B follicles (Katakai et al, 2008). They express many markers in common with FDCs and the fibroblastic reticular cells found in the T zone (see below), but specifically express TRANCE (also known as RANKL, Katakai et al, 2008). They also build a conduit network that channels antigens from the SCS to the B follicle (Roozendal et al, 2008).

The fibroblastic reticular cells (FRCs) are present in the paracortical T zone of the LN. They attract CCR7-expressing CD4+ and CD8+ T cells and dendritic cells by secreting the chemokines CCL21 and CCL19 (Gunn et al, 1998b). Like the FDCs, FRCs build a reticular network of fibers (RN) that spreads throughout the T zone. Lymphocytes and dendritic cells use these fibers as a physical support for their migration through the LN. FRCs also surround the collagen-rich fibers, thus forming a conduit network that connects to the SCS and the HEVs. Small molecules carried to the LN by the lymph can enter this network from the SCS and quickly reach the T zone or the HEVs (Gretz et al, 2000). This allows for the rapid delivery of signal molecules coming from the organs and tissues drained by LNs. Molecules over 70 kDa cannot enter the conduits and are therefore trapped in the SCS, where they are sampled by the resident macrophages or drain through the efferent lymphatic vessels (Gretz et al, 2000). FRCs have a third important function in LN homeostasis, in that they secrete IL-7, a critical survival factor for naïve T cells.

3.2.1.2 Lymphocyte migration through lymph nodes

The majority of T cells and B cells enter the LN via the high endothelial venules (HEVs) found at the periphery of the T zone (Anderson and Anderson, 1975; Pfeiffer et al, 2008). These specialized blood vessels are the prolongation of

an afferent arteriole that enters the LN at its hilum (Willard-Mack, 2006). The blood endothelial cells (BECs) that line them have a distinct cuboidal morphology (Anderson and Anderson, 1976) – hence the name "high" endothelial venules – and express CCL21, thus attracting the immune cells, as well as specific adhesion molecules, such as the peripheral lymph node addressin PNAD in pLNs, or the mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) in mesenteric LNs (Cueni et al, 2008). Blood vessels are also found in the medulla: they are prolongations of the HEVs leaving the LN and converging into an efferent venule, and HEVs themselves forming a loop reaching farther than the T zone (Pfeiffer et al, 2008). While their preferred way of entry into the LNs is undeniably via the T zone HEVs, B and T lymphocytes have been shown to also extravasate out of the HEVs present in the medulla (Pfeiffer et al, 2008).

About 25% of lymphocytes exit the blood circulation once they find themselves in a HEV. In pLNs, the first step of the extravasation is the binding of PNAD, expressed by BECs, by their L-selectin. In mesenteric LNs, the lymphocytes recognize and bind MadCAM-1 through their $\alpha_4\beta_7$-integrin (Kraal and Mebius, 1997, Butcher et al, 1999). In both cases, this causes the lymphocytes to slow down and roll along the HEV endothelium. This leads to binding of ICAM-1 and ICAM-2, at the surface of BECs, by the β_2-integrin lymphocyte function-associated antigen 1 (LFA-1) expressed by the lymphocytes, which activates it (Dustin and Springer, 1989). The lymphocytes then stop and migrate between adjacent BECs and through the basement membrane of the HEV to reach the T zone (Kraal and Mebius, 1997, Butcher et al, 1999).

Interestingly, HEVs specifically allow the extravasation of lymphocytes, and, possibly, plasmacytoid dendritic cells (Cella et al, 1999). L-selectin, however, is expressed by other leukocytes, as is LFA-1. Granulocytes, for example, have been observed rolling along the endothelium of HEVs, but they failed to exit the blood circulation at this level (Warnock et al, 1998). This suggests that other factors act on lymphocytes to enable their extravasation. One of these factors is CCL21, which is expressed by BECs in HEVs. This chemokine is found on the luminal surface of HEVs (Gunn et al, 1998b; Vassileva et al,

1999), activates LFA-1, and increases its binding to ICAM-1 and MadCAM-1 (Campbell et al, 1998; Tangemann et al, 1998; Pachynski et al, 1998). CCL19, which is produced by the FRCs of the T zone, is also transported to the lumen of HEVs via the conduit network (Baekkevold et al, 2001). Knockout mice lacking both chemokines show marked defects in T cell migration, which demonstrates that they are indeed important for lymphocyte extravasation (Förster et al, 1999). CXCL12 has also been shown to increase LFA-1 binding to ICAM-1 (Campbell et al, 1998). One team has suggested that CXCR5-signaling triggered by CXCL13 had no effect on B cell migration (Okada et al, 2002), as they failed to detect this chemokine in HEVs, but this was contradicted by another team (Ebisuno et al, 2003). This second study showed that CXCL13 was present on the luminal side of about 50% of HEVs and that B cell migration was partially impaired in CXCL13-deficient mice.

Once inside the LN, lymphocytes home to their respective compartments following a gradient of the corresponding chemokines.

FRCs secrete high levels of CCL21 and CCL19, which provide guiding clues to migrating T cells (Bajénoff et al, 2008) and retain them into the T zone (Cyster, 2005). T cells connect with the reticular network of fibers built by the FRCs and move along it in a seemingly random manner (Bajénoff et al, 2006, 2008). They have been shown to spend 12 hours on average in a given LN before recirculating (Cyster, 2005).

If no immune response is started, the non-resident cells exit the LN via the lymphatic sinuses located in the medulla of the LN, which converge into an efferent lymphatic vessel. This requires the expression of the sphingosine 1-phosphate receptor on the lymphocytes and of its ligand, sphingosine 1-phosphate, which is secreted into the lymph by LECs (Pappu et al, 2007; Grigorova et al, 2009). T cells gradually increase their expression of sphingosine 1-phosphate receptor while in the LN, until it overcomes the attraction and retention mediated by CCL21 (Lo et al, 2005).

Like T cells, B cells enter the LN through the HEVs. Attracted by CXCL13 produced by FDCs, they migrate through the T zone on the reticular

network fibers to reach the B follicles (Bajénoff et al, 2006). And, like T cells, they upregulate sphingosine 1-phosphate receptor levels at their surface to exit the LN (Matloubian et al, 2004).

Recirculating lymphocytes re-enter the blood circulation via the thoracic duct. Exit from the LN, however, happens through the lymphatic sinuses present in the medulla.

3.2.2 Lymph nodes during inflammation

As mentioned earlier, LNs are the crossroad where APCs, antigen, and lymphocytes meet, and this concentration of cells facilitates the development of an adaptive immune response. LN structure and function is modified after infection to optimize this immune response. Exit of cells from the organ is shut down for several days by the release of interferon-α/β, which lowers the expression level and sensitivity of sphingosine 1-phosphate receptor (Shiow et al, 2006). Cell migration to the LN increases in parallel, to increase the pool of naïve lymphocytes sampling the antigens presented by APCs (Sodeberg et al, 2005). This accounts for the swelling of LNs observed during inflammation (Hall and Morris, 1965).

In order to attract more lymphocytes to the LN, the volume of the afferent arterioles increases. FRCs upregulate their production of vascular endothelial growth factor (VEGF), which induces the proliferation of BECs in HEVs and the growth of more blood vessels. BECs also upregulate CCL21 expression to enhance the migration of lymphocytes to the LN (Anderson et al, 1975; Webster et al, 2006). In addition, VEGF-A expression by B cells leads to the expansion of the lymphatic sinuses to increase the recruitment of APCs from surrounding tissues (Angeli et al, 2006). The FRC and FDC populations expand and mediate the expansion of the RN to accompany the increase in LN size (Katakai et al, 2004; Peduto et al, 2009).

Inside the LN, the production of inflammatory chemokines such as CCL2 and CCL5 is upregulated. Activated T cells decrease their expression of CCR7,

while FRCs downregulate their secretion of CCR7 ligands in an interferon-γ-dependent way. Similarly, FDCs express less CXCL13 (Mueller et al, 2007). This could facilitate the exit of effector cells from the LN (Mueller and Germain, 2009).

Interaction between activated T cells and B cells is also facilitated by the development of a new compartment within B follicles, the germinal center (GC), which contains proliferating B cells, macrophages, CD4+ T cells, and FDCs. The main function of GCs is to generate antibody-producing cells to carry out a humoral response to an invading pathogen (MacLennan, 1994). Activated T cells upregulate their expression of CXCR5 and migrate to the B follicles, attracted by CXCL13 produced by FDCs. Once in the GC, they adopt a follicular-helper T cell (T_{fh}) phenotype (Breitfeld et al, 2000; Schaerli et al, 2000). They secrete IL-4 and IL-21 and upregulate CD40-ligand (Nurieva et al, 2008; Vogelzang et al, 2008), which induces LTαβ in antigen-specific B cells, which in turn increases LTβ-receptor signaling in FDCs. As a result to this, FDCs aggregate and form a niche optimal for antigen-capture and B cell help (Vu et al, 2008). The interaction between a T_{fh} cell and a B cell is specific, as it involves the T cell's TcR and the B cell's peptide-MHC II complex. Ligation of CD28 on the T cell by CD80 and CD86 on the B cell also plays a part (Zotos and Tarlinton, 2012).

GC B cells have three possible fates: apoptosis, further proliferation and somatic hypermutation, or differentiation into antibody-expressing memory B cells or plasma cells (Zotos and Tarlinton, 2012). Apoptosis occurs if a B cell fails to receive T cell help (Vinuesa et al, 2010). In addition, T_{fh} cells perform a positive selection of high-affinity B cells. GC B cells with a high affinity for a given antigen may be more efficient at capturing, processing, and presenting it and, indeed, T_{fh} cells concentrate on the B cells expressing the highest levels of antigen-MHC II complexes (Oprea and Peterson, 1997; Allen et al, 2007). Differentiation into plasma cells seems to be biased in favor of high-affinity B cells, whereas they can differentiate into memory cells regardless of MHC II affinity (Smith et al, 2000; Phan et al, 2006).

Somatic mutations, which result in the modification of the immunoglobulin variable region of the B cell receptor to increase its affinity, and class-switch recombination, which sees the activated B cell go from producing IgM or IgD to producing IgA, IgG, or IgE – depending on the characteristics required for the clearance of a particular antigen – also take place in GCs (Gowthaman et al, 2010).

FRCs are heavily involved in T cell homeostasis in resting LNs, and also play a part in the immune response. Indeed, they can negatively regulate the T cell response after infection. They increase their iNOS activity and secrete nitric oxide (NO) in response to interferon-γ and TNFα (Siegert et al, 2011). Kasic and colleagues (2011) have proposed that NO inhibits the T cell response by nitrosylating diverse amino acid residues, leading to the downregulation of the TcR complex. In addition, NO may prevent STAT5 phosphorylation and thus inhibit IL-2 signaling (Bingisser et al, 1998; Bogdan, 2011). Thus, FRCs decrease the proliferation of both CD4+ and CD8+ T cells (Siegert et al, 2011). Interestingly, crosstalk between dendritic cells and FRCs in the presence of activated T cells upregulates iNOS expression in both cell types (Siegert and Lutther, 2012). Inhibiting the T cell response after an infection seems counterintuitive, but it may help to limit tissue damage associated with acute inflammation or autoimmunity (Siegert and Luther, 2012).

Chapter 4: Effects of HIV-1 infection on lymph nodes

4.1 Effects of HIV-1 infection on lymph node structure and function

Because CD4+ T cells, the primary targets of HIV-1, home to LNs, these organs represent an important reservoir of virus. They are seeded during the acute infection phase, and thus represent a reservoir of HIV DNA and virions. Their structure and function also progressively degenerate as the disease progresses (Pantaleo et al, 1993b). We will review here what changes appear in LNs of infected patients.

4.1.1 Establishment of an HIV reservoir

As mentioned earlier, the HIV-1 virus manipulates the expression of surface markers, such as MHC I and Fas-ligand, by the infected cell, so that it will escape the immune system. While activation is necessary for virion production, the majority of infected T cells enters a quiescent state and persists for years in the patient's body (Chun et al, 1997a, b). The formation of this reservoir is one of the main reasons why the complete eradication of the virus has so far been unsuccessful (Chun et al, 1997a, b; Finzi et al, 1997; Wong et al, 1997).

CD4+ memory T cells are a major component of the HIV-1 reservoir. In fact, they are the first, and the most profoundly, depleted CD4+ T cell subset during the acute phase of the infection (Chomont et al, 2009).

T_{fh} cells, present in GCs, expand during the acute phase of HIV-1 infection. Perreau and colleagues (2013) even demonstrated that the expanding T_{fh} cells were predominantly HIV-specific. Furthermore, they showed that the proportion of these cells in LNs is actually correlated with the evolution of plasma viremia levels. This indicates that there is a connection between the two.

HIV-specific cells have been shown to be preferential targets of infection (Douek et al, 2002). T_{fh} cells are susceptible to SIV infection (Brenchley et al, 2012), and Perreau and colleagues found HIV DNA in about 5,1% of them, more

than twice the proportion of infected CD4+ memory T cells. Thus, T_{fh} cells represent the majority of cells in the viral reservoir. T_{fh} cells also had the highest levels of HIV replication and production, consistent with their activated state. T_{fh} cells are activated every time an adaptive immune response is started. This means that HIV replication and production would be reactivated each time as well, thus contributing to the maintenance and progression of the disease. In addition, macrophages and dendritic cells can be infected by the virus and are also part of the latent reservoir (Stevenson, 2003).

4.1.2 Effects on lymphocytes

The onset of an HIV-specific immune response following infection leads to activation of the immune system. This state of inflammation persists even into the chronic phase of the disease. At the cellular level, LNs in the chronic stage of infection contain fewer CD4+ T cells than non-infected controls, as well as fewer plasmacytoid and myeloid dendritic cells (Biancotto et al, 2007). As previously mentioned, memory T cells are severely depleted while the proportion of activated and effector T cells increases. The proportion of naïve T cells among total CD4+ T cells remains similar in patients and controls (Bianocotto et al, 2007; Perreau et al, 2013). In CD8+ T cells as well, the proportion of the effector subset increases (Biancotto et al, 2007). Biancotto and colleagues also found a strong negative correlation between the frequencies of activated T cells and the absolute CD4 cell count.

It has been known for well over a decade (Meyaard and Miedema, 1995; Finkel et al, 1995) that non-infected T cells also die. This can be attributed to the consequences of HIV infection on lymph node structure degeneration (see below, Schacker et al, 2006), and also to an impaired thymic function (decreased output of both CD4+ and CD8+ T cells, Douek et al, 2001), increased turnover not accompanied by proliferation (Fleury et al, 2000) and a lack of homeostatic proliferation of peripheral T cells (Sieg et al, 2005). As they also observed that naïve T cells expressed activation markers such as CD38, Biancotto and

colleagues propose that dysregulated activation underlies the expansion failure of circulating naïve T cells.

In the LN, the activation of T cells is accompanied by a profound perturbation of cytokine expression, as drastically increased levels of IL-1β, IL-2, IL-10, IL-12, and IL-15 were observed, accompanied by a significant decrease of MIP-1α, SDF-1β, and IP-10 levels. IL-2 levels, for example, can be multiplied by over 100 in HIV+ LNs compared to controls. Furthermore, these changes in cytokine secretion appear to drive the activation of T cells. They also contribute to an enhanced attraction to and retention of effector T cells in the LN, where they could further promote the activation of new cells (Biancotto et al, 2007).

In summary, HIV-1 infection induces a massive activation of LNs, and this has been showed to precipitate T cell depletion (Hazenberg et al, 2000, 2003; McCune, 2001).

4.1.3 Effects on lymph node structure

The highly inflamed state of LNs causes the organ's architecture to evolve and progressively degenerate during the course of the disease. Fibrosis, characterized by collagen deposits, is a major factor in this. In the LNs of HIV-infected individuals, the collagen deposits are first observed in HEVs and eventually spread to the T zone while B follicles remain mostly spared (Schacker et al, 2002). The main consequence of this is the complete disruption, and subsequent loss, of the T zone network of fibers (Zeng et al 2011, 2012a, b, and c). Therefore, cell migration through the LN is greatly impaired. Fibrosis also prevents contact between CD4+ T cells and LN stroma, namely FRCs. The lymphocytes thus lose access to survival factors such as IL-7, which further compromises T cell homeostasis. The FRCs also lose contact with an important source of $LT\alpha_1\beta_2$, which blocks their function, and are depleted as well. Thus, the development of fibrosis in the LN creates a feedback loop that precipitates the loss of a functional immune system (Zeng et al, 2011, 2012a, b, c).

Moreover, as it is irreversible, fibrosis limits the effects of antiretroviral therapy (Zeng et al, 2012c). Indeed, in the T zone, the extent of collagen deposition is inversely correlated with CD4+ T cell numbers (Schacker et al, 2002). Furthermore, it is also negatively correlated with the recovery of the CD4+ T cell population after therapy (Zeng et al, 2011). While CD4+ T cells counts can be restored in the blood, the depletion is still evident in fibrotic LNs. Therefore, successful therapy depends on the time of initiation: the sooner the replication and propagation of the virus is stopped, the less damage lymphoid tissue will have sustained and, thus, the more effective the treatment will be.

Blocking the development of fibrosis itself is also essential to improve the effectiveness of existing therapies. In SIV-infected rhesus macaques, transforming growth factor (TGF)-β1 by regulatory T cells drive the production of collagen (Estes et al, 2007). TGF-β inhibitors such as pirfenidone exist and have been tested in the context of pulmonary fibrosis (Schacker, 2008). More need to be developed and tested for HIV patients.

Taken together, these results show that HIV-1 not only affects immune cells, but also mediates a complete degeneration of lymphoid organs. Preserving the structure of these organs is paramount in ensuring a complete reconstitution of the immune function during antiretroviral therapy.

4.2 The CD4C/HIV^{Nef} mouse model

4.2.1 Generation of the CD4C/HIV^{Nef} mouse

The transgenic mouse used in this project has been generated in Dr Paul Jolicoeur's laboratory, at the Institut de Recherches Cliniques de Montréal. It expresses the HIV-1 protein Nef under the control of the human CD4 promoter fused to the murine CD4 enhancer (Hanna et al, 1998). The transgene contains all HIV-1 genes, but the open reading frames of all genes except Nef have been interrupted (Figure 4).

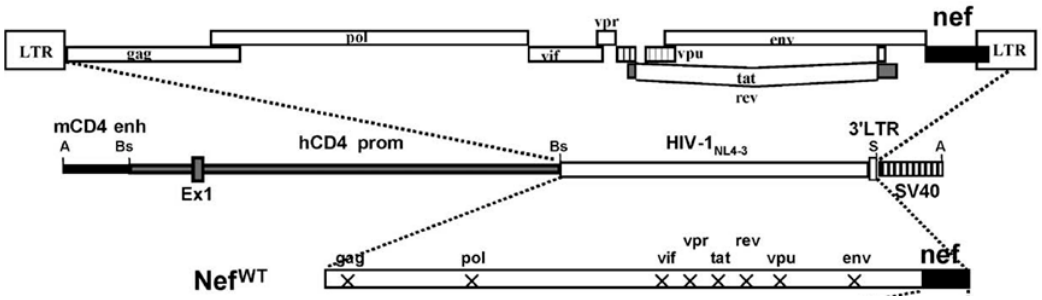


Figure 4: Structure of the CD4C/HIV^{Nef} transgene. Adapted from Hanna et al, 2006. The expression of the transgene is regulated by the murine enhancer sequence (mCD4 enh) and the human CD4 promoter (hCD4 prom). It contains all genes of HIV-1^{NL4-3}, but the open reading frames of all genes except Nef have been interrupted (symbolized by an X). The 3' long terminal repeat (LTR) of the HIV-1 genome and the polyadenylation sequence of the simian virus 40 (SV40) is also present.

4.2.2. Phenotypes displayed

Expression of the transgene, as well as the presence of the Nef protein itself, has been reported in CD4+ CD8+ thymocytes, mature CD4+ T cells, dendritic cells, and macrophages (Hanna et al, 1998).

The CD4C/HIV^{Nef} mice have a survival rate of 50% at 6 months of age. They present severe wasting and atrophy of lymphoid organs (thymus, spleen, and LNs). At the cellular level, they show severe depletion of thymocytes and peripheral T cells, defects in the maturation of thymocytes (Chrobak et al, 2010), downregulation of CD4, defective proliferation of peripheral T cells, increased activation and apoptosis of CD4+ T cells, loss of CD8+ T cells (Hanna et al, 1998). Non-lymphoid organs are also affected: the lungs present an interstitial pneumonitis, and tubulo-interstitial nephritis and glomerulosclerosis were observed in kidneys (Hanna et al, 1998; Zuo et al, 2006). In mice that survive more than 4 months, damage to the liver in the form of necrotizing granulomas can also be seen (Hanna et al, 1998). Focal myocytolysis and coronary arteriospasm were also observed in the heart of these mice (Kay et al, 2002).

The development of these phenotypes in the presence of only Nef highlighted the fact that this protein is a major determinant of pathogenicity. Of

note, an inducible mouse model was also generated in our laboratory to ensure that the phenotypes could not be attributed to developmental defects caused by the expression of Nef in embryos. Induction of Nef expression in adult mice caused similar phenotypes to those seen in the CD4C/HIV^{Nef} mouse (Rahim et al, 2009).

In addition, the phenotypes seen in the presence of Nef are very reminiscent of those observed in human AIDS patients. As such, the CD4C/HIV^{Nef} mouse represents a good model to study the roles of Nef in the progression of AIDS, notably pediatric AIDS, as the mice constitutively express the protein throughout life.

4.2.3 Rationale for Master's project and hypothesis

4.2.3.1 Rationale

As previously mentioned, HIV-1 infection induces a gradual loss of LN architecture. Fibrosis first develops in HEVs and spreads to the T zone (Schacker et al, 2002). As the disease progresses, the structure of the reticular network of fibers built by the FRCs is gradually lost, and the cells are depleted (Zeng et al, 2011). FRCs play a very central role in adaptive immunity as they regulate CD4+ T cell homeostasis and facilitate their encounters with APCs. Their loss would therefore precipitate the progression to AIDS.

Humans infected with a strain of HIV-1 lacking a functional Nef gene have no, or very slow, disruption of the immune system (Daniel et al, 1992; Deacon et al, 1995). This shows that Nef is necessary for the development of AIDS. This is also found in rhesus monkeys infected with SIV, as Nef is essential for the maintenance of high viral loads and disease progression (Kestler et al, 1991). The CD4C/HIV^{Nef} mouse model has shown that, at least in mice, Nef is sufficient to induce most of the AIDS phenotypes (Hanna et al, 1998). We therefore asked whether Nef could also affect FRCs and cause the degeneration of LN architecture.

4.2.3.2 Hypothesis

Nef affects FRCs, thereby contributing to the perturbation of the immune system.

4.2.3.3 Objectives

The first objective of this work was to discover, in CD4C/HIV^{Nef} mice, whether FRCs express Nef. We then studied the FRC population itself, and assessed two of its main functions: building the reticular network of fibers, and secreting chemokines and survival factors regulating the homeostasis of CD4+ T cells. We also evaluated the structure of the T zone.

Because HEVs are disrupted by HIV-1 infection and are supported by FRCs secreting VEGF, we also studied the endothelial cell populations of LNs.

We centered our work on skin-draining peripheral LNs (pLNs). Differences exist between these pLNs and mesenteric LNs, for example in T cell homing. We focused on the skin-draining pLNs in order to work on more homogenous samples. Moreover, as AIDS is a progressive disease, we used 5 month-old mice to ensure that phenotypes would have time to develop.

Chapter 5: Material and Methods

5.1 Material

Mice

The CD4C/HIV^{Nef}, CD4C/GFP x CD4C/HIV^{Nef}, CD4C/HIV $^{Nef, Rev, Env}$, and CD4C/HIV$^{\Delta Nef}$ mice were all generated on a wild type C3H background (Harlan Laboratories) and kept in a sterile, specific pathogen free facility, according to the recommendations of the Canadian Council on Animal Care. All manipulations were approved by the Committee for Animal Protection (Comité de Protection des Animaux) of the Institut de Recherches Cliniques de Montréal.

Antibodies and reagents

Table 2 lists the different antibodies that were used for each experiment. In **flow cytometry**, CD31-biotin was coupled with streptavidin-APC. 7-Aminoactinomycin-D (7AAD) was used as a viability marker to distinguish live and dead cells. Lymph nodes were digested with DNAse 25 and collagenase IV.

In **immunofluorescence**, the nuclei of the cells were stained with 4',6'-diamino-2-phenylindol (DAPI; Vector Laboratories, Inc.).

The primers used for **Reverse Transcription Polymerase Chain Reaction (RT-PCR)** are listed in table 3.

The cells were always lysed in MLB buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1% Triton X-100, 2mM EDTA) to which we added 4 mM of sodium orthovanadate (Na_3VO_4), 10 mM of sodium fluoride (NaF), 1 mM of phenylmethylsulfonylfluoride (PMSF) and protease inhibitors.

Table 2: Antibodies used. The working dilutions are indicated.

Antibodies (species)	Dilution	Company (catalog #)
Primary antibodies		
Flow cytometry		
CD45.2-Pacific Blue	1/100	Molecular Probes (MCD4528)
CD45.2-FITC (cell sorting)	1/100	Molecular Probes (MCD4501)
gp38 (podoplanin)-PE	1/100	eBioscience (12-5381)
CD31-biotin	1/400	BD Pharmingen (553371)
CD4-APC	1/100	Cedarlane (CL013APC)
CD3-PE	1/100	BD Pharmingen (553063)
CD11c-FITC	1/100	BD Pharmingen (553801)
CD25-PE	1/200	Cedarlane (CL8925PE)
Human CD4	1/20	BD Pharmingen (555346)
Immunofluorescence		
gp38 (clone 8.1.1, hamster)	1/150	Hybridoma
Desmin (mouse)	1/80	Dako (M0760)
ERTR7 (rat)	1/50	Hycult Biotechnology (HM1086)
CCL21 (goat)	1/300	R&D Systems (AF457)
CD31 (clone MEC13.3, rat)	1/100	BD Pharmingen (550274)
α-smooth muscle actin (clone 1A4, mouse)	1/200	Sigma (A 2547)
von Willebrand Factor (rabbit)	1/200	Dako (A 0082)
LYVE-1-Alexa Fluor 488	1/150	eBioscience (53-0443)
Western blot		
Desmin (clone D33, mouse)	1/500	Dako (M0760)
Actin (rabbit)	1/500	Sigma (A 2066)
Anti-Nef rabbit serum 3116 C/F	1/500	Developed by Dr Jolicoeur's laboratory.
Secondary antibodies		
Immunofluorescence		
Goat anti-hamster IgG Alexa Fluor 488	1/250	Molecular Probes (A-21110)
Goat anti-mouse IgG Alexa Fluor 555	1/400	Molecular Probes (A-21422)
Goat anti-rat IgG Alexa Fluor 488	1/300	Molecular Probes (A-11006)
Goat anti-rat IgG Alexa Fluor 568	1/250	Molecular Probes (A-11077)
Donkey anti-goat IgG Alexa Fluor 488	1/250	Molecular Probes (A-11055)
Western blot		
Goat anti-mouse IgG Alexa Fluor 680	1/2500	Molecular Probes (A-21057)
Goat anti-rabbit IgG Alexa Fluor 680	1/2500	Molecular Probes (A-21076)

Table 3: Primers used for RT-PCR.

Gene	Sense primer (5' to 3')	Anti-sense primer (5' to 3')
CCL21	TGAGCTATGTGCAAACCCTGAGGA	TAGCTCCCTCTTTGCCTGTGAGTT
CCL19	GTGCTAATGATGCGGAAGACTGCT	CCTTCTGGTGCTGTTGCCTTTGTT
IL-7	TGGGAGTGATTATGGGTGGTGAGA	TGATCATCAGTACACTCTCATATGCT
LTβ	CCTGGTGACCCTGTTGTT	CCTGGAAGCATTGGATCTCT
Nef	GCACGGCAAGAGGCGAGGG	CTAATCGAATGGATCTGTCTCTG
HPRT	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC

5.2 Methods

Flow cytometry

Skin-draining peripheral lymph nodes were collected, pooled, and digested in ISCOVE medium with DNAse (DN25, Sigma-Aldrich, #9003-98-9) and type IV collagenase (Worthington Biochemical, #LS004188) for 30 minutes at 37°C. They were then homogenized with a pipette and the cell suspension was filtered on a nylon mesh and transferred to a STOP solution (ISCOVE medium + 25% inactivated foetal bovine serum (FBSI) to stop the digestion process. Cells were resuspended in 1 mL of blocking buffer (phosphate buffered saline (PBS) 1X + 20% FBSI) for 30 minutes at 4°C and counted. They were then transferred to a 96-well plate at a concentration of 0.5-1x10^6 cells/well, resuspended in 100 µL of FACS buffer (PBS 1X + 2% FBSI) and stained for 30 minutes at 4°C. After incubation, the cells were washed once in 100 µL of FACS buffer. Staining with streptavidin-APC and 7AAD was then performed in 100 µL of FACS buffer, for 10-15 minutes at 4°C. The cells were then washed once in FACS buffer, resuspended in 100 µL of FACS buffer, transferred into micro test tubes (Bio-Rad, catalog #223-9391) and diluted with an additional 200 µL of FACS buffer.

Samples were run on a CyAn flow cytometer (Dako). The obtained data was analyzed with Flow Jo software (version 9.4.8, Tree Star, Inc.).

The combinations of markers used to identify the cell populations studied in this project were: **FRCs**: 7AAD– CD45.2– CD31– gp38+; **BECs**: 7AAD– CD45.2– CD31+ gp38–; **LECs**: 7AAD– CD45.2– CD31+ gp38+ (FRCs, BECs, and LECs were stained as described by Link and colleagues, 2007. Figure 7 shows the gating strategy, represented by the pink rectangle); **CD4+ T cells**:

7AAD– CD45.2+ TcR+ CD4+; **LTi**: 7AAD– CD45.2+ CD11c– CD4+ CD3- (gating strategy shown on Figure 13, represented by the pink rectangle) and **Treg**: 7AAD– CD45.2+ CD4+ CD25+ (gating strategy shown on Figure 16, represented by the pink rectangle).

C*ell Sorting*

Lymph nodes were harvested and processed as for flow cytometry. After blocking, all the cells were stained with 7AAD, CD45.2-FITC, gp38-PE, and CD31-biotin coupled with streptavidin-APC as for flow cytometry, in 15mL-Falcon tubes (Fisher Scientific).

The cells were sorted using a MoFlo sorter (Beckam Coulter). After sorting, they were transferred into 2-mL Eppendorf tubes and centrifugated 5 minutes at 1200 rpm at 4°C. The supernatant was discarded and the pellets were snap-frozen in liquid nitrogen. They were stored at -80°C until further use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA extraction

RNA was extracted from sorted FRCs, BECs, and LECs using the RNEasy Plus Micro kit (QIAgen, #74034) and following the manufacturer's instructions. At the final step, RNA was eluted with 16 µL of 0,2% diethylpyrocarbonate (DEPC) water and stored at -20°C. Because of the small yield, RNA integrity was not assessed.

Reverse Transcription (RT)

The purified RNA was reverse-transcripted into cDNA in RT buffer (1X reverse transcriptase buffer, 5 mM dNTP, 0,1 M dithiothreitol (DTT), 1 mM random hexamers (pdN6), 2-5 µg RNA, mixed in DEPC water). The RNA was denaturated 5 minutes at 65°C. An RNA inhibitor (RNA guard) was then added. The enzyme used was Moloney Murine Leukemia Virus (MMLV) reverse-transcriptase (Invitrogen Life Technologies, #28025-013). The RT mix was

incubated 2 hours at 42°C, and the process was stopped by heating the samples 5 minutes at 99°C. The samples were cooled on ice and stored at -20°C.

Polymerase Chain Reaction (PCR)

The cDNA was amplified by PCR. The mix contained 1X PCR buffer, 1,25 mM dNTP, 5 pmol of each primer, and 2 μL of cDNA, mixed in nanopure water. The primers used for each gene are listed in table 3. The enzyme used was a Taq enzyme produced in Dr Jolicoeur's laboratory. The amplified DNA was then run on a 1,5% agarose gel and stained with ethidium bromide. Pictures were taken with an AlphaImager (Alpha Innotech) and analysed with Image Quant software (version 5.0, Molecular Dynamics).

Immunofluorescence and microscopy

Skin-draining peripheral lymph nodes were harvested, embedded into OCT compound (Sakura Tissue-Tek), and frozen by submerging them in 2-methylbutane cooled with dry ice. The blocks obtained were stored at -80°C.

Cryosections were performed by Dominique Lauzier of the Institut de Recherches Cliniques de Montréal histology facility and stored at -80°C. Prior to use, the sections were fixed in cold acetone for 20 minutes at -20°C, then left to dry at room temperature. All steps after this were performed at room temperature.

The slides were first rehydrated in PBS 1X for 10 minutes. When necessary, permeabilization was done with a 5-minute incubation in 0,1% Triton X-100 in PBS 1x (0,4% Triton X-100 in the case of CD31 staining), then washed in PBS 1X.

The slides were blocked in PBS 1X + 20% inactivated foetal calf serum (FCSI) for at least 1 hour. Primary antibodies were diluted in PBS 1X + 3% FCSI and incubated for 60-90 minutes. The slides were then washed twice 5 minutes in PBS 1X. Secondary antibodies were diluted in PBS 1X + 3% FCSI and incubated for 60 minutes. The slides were once again washed twice 5 minutes in PBS 1X, then mounted using Vectashield with DAPI (Vector Laboratories) and stored in

the dark at 4°C. They were imaged within 48 hours with a Leitz DMRB microscope (Leica) using Northern Eclipse software (version 7.0, EMPIX).

Western blot

Skin-draining peripheral lymph nodes were harvested and homogenized in 1mL of lysis buffer. The lysate was centrifuged at 12,000 rpm at 4°C for 15 minutes and the supernatant was transferred into a new tube. Samples were diluted in 4X loading buffer and run on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Immobilon-P, #IPVH00010).

The membranes were blocked in Tris Buffer Saline (TBS) 1X + 5% bovine serum albumin (BSA) for 1 hour at room temperature or overnight at 4°C. All subsequent steps were performed at room temperature. Primary antibodies were diluted in TBS 1X + 1,25% BSA and incubated for 1 to 4 hours (when blotting Nef) in the dark. The membranes were then washed twice 5 minutes in TBS 1X + 0,1% Tween 20, in the dark. Secondary antibodies were diluted in TBS 1X + 1,25% BSA and incubated for 45 minutes in the dark. The membranes were then washed again twice 5 in TBS 1X + 0,1% Tween 20, and a third time in TBS 1X. They were kept in TBS 1X in the dark at 4°C until they were scanned.

Scanning was done with the Odyssey Infrared Imaging system (LI-COR) using Odyssey software (version 3.0, LI-COR).

Hematoxylin and eosin staining

The hematoxylin and eosin staining was performed on frozen sections of lymph nodes by Dominique Lauzier, of the Institut de Recherches Cliniques de Montréal histology facility. The slides were kept at room temperature and imaged using an Axiophot microscope (Zeiss) using Northern Eclipse software (version 7.0, EMPIX).

Results

Chapter 6: Results

6.1 The population of FRCs in Nef-expressing mice

Dr Ashley Haase's team has extensively documented the changes that occur in the pLNs of rhesus macaques and human patients in the presence of SIV-1 and HIV-1 infections (Schaker et al, 2006; Zeng et al, 2011). They describe the development of fibrosis triggered by the activation of regulatory T cells, which stimulates the production and deposition of collagen by fibroblasts in pLNs. This leads to a disruption of the reticular network (RN), which prevents contact between CD4+ T cells and fibroblastic reticular cells (FRCs). Thus, both cell populations lose access to survival factors produced by the other cell type, which precipitates their depletion. This process is summarized in Figure 5. As our CD4C/HIV^{Nef} mouse model recapitulates many phenotypes seen in AIDS patients, we expected to find a phenomenon similar to the one described by Dr Haase's team in the pLNs of these mice: a loss of FRCs and of their functions triggered by the onset of fibrosis. The aim of this work was to study FRCs in the presence of Nef to verify this hypothesis.

6.1.1 Expression of Nef by FRCs

Previous studies have demonstrated the infection of FRCs by the Ebola virus (Davis et al, 1997) and the lymphocytic choriomeningitis virus (LCMV; Mueller et al, 2007). LCMV infection, for instance, results in the disruption of the conduit and reticular networks, which contributes to the development of immunosuppression by LCMV (Mueller et al, 2007). These infections are associated with severe immune defects. However, to our knowledge, the possibility of a direct infection of FRCs by HIV-1 has not yet been studied. The CD4C/HIV^{Nef} mouse developed in our laboratory presents many phenotypes that

closely resemble those of human AIDS patients, especially pediatric AIDS (Hanna et al, 1998). We asked whether FRCs express Nef in this model. Because of the small numbers of FRCs present in pLNs that would require that we pool several mice for an RT-PCR study, we decided to first test the activity of the CD4C promoter in FRCs in two reporter lines, the CD4C/GFP x CD4C/HIV^{Nef} line and the CD4C/hCD4-CCR5 line.

In the first mouse line, FRCs appeared clearly GFP-positive (fig. 6A), as GFP levels were comparable to those of the hematopoietic (CD45.2+) cells used as positive controls. In double-Tg mice, the presence of Nef induced no variation in the intensity of the GFP signal. The second reporter line expresses the human CD4 (hCD4) and CCR5 molecules under the control of the CD4C promoter. We stained for hCD4 and FRCs were, again, clearly positive (fig. 6B). These results show that the CD4C promoter is indeed active in FRCs. They should therefore express Nef in the CD4C/HIV^{Nef} model.

We extracted RNA from sorted FRCs from pooled transgenic (Tg) CD4C/HIV^{Nef} mice and their wild type littermates. RT-PCR showed that FRCs from Tg mice do express Nef mRNA (fig 6C). Because of the number of mice that would have had to be pooled to perform a Western blot analysis, the presence of the Nef protein in FRCs could not be assessed.

These results show that FRCs express Nef RNA and that the human CD4 promoter is active in them. It is therefore likely that their human counterparts express CD4, which would mark them as potential direct targets for HIV-1 infection. The expression of the co-receptors CXCR4 and CCR5 needs to be assessed to confirm or infirm this.

6.1.2 The FRC population

We then investigated whether the expression of Nef in Tg mice affected the population of FRCs itself. FRCs can easily be identified by flow cytometry by a very simple staining procedure, as described by Link and colleagues (2007). No differences in the percentage of FRCs or their absolute numbers in pLNs were observed (fig. 7A-B). The CD4+ T cells population was also measured in the same mice to make sure that Nef was indeed active in these mice (fig. 7C). These results show that despite inducing a severe depletion of CD4+ T cells, Nef does not affect the size of the population of FRCs.

The FRC population was also studied by immunofluoresence by staining for gp38 and imaging the T zone of pLNs (fig. 8A). There was no difference in the gp38 signal area between Tg and control mice, which suggests that FRCs spread normally throughout the T zone even in Nef-expressing pLNs (fig. 8B). This also confirms that the cells are not depleted. A statistically significant decrease in the intensity of the signal was observed (fig. 8B). However, it is a 1.45-fold decrease, which seems too small to be biologically significant. Furthermore, this decrease was not seen by flow cytometry. Thus, it is probably an artifact of the immunostaining.

Taken together, these results suggest that Nef does not affect the size of the FRC population and its distribution in resting pLNs.

6.1.3 Production of reticular network fibers

As Nef did not seem to affect the population of FRCs itself, we asked whether it could disrupt their function. The main functions of FRCs are to build the reticular network of fibers (RN) that T lymphocytes and non-resident dendritic cells use as support to migrate through pLNs, and to secrete chemoattractants and survival factors for these immune cells (Katakai et al, 2004; Link et al, 2007).

Immunostaining of desmin and of the yet-unknown antigen to the ERTR7 antibody (hereafter ERTR7), two components of the RN fibers, was performed

and the T zone was imaged (fig. 9A). Both desmin and ERTR7 signals presented no differences in signal intensity or area. This shows that FRCs of Tg mice produce quantities of desmin comparable to those of their wild type, control littermates, and that the architecture of the RN is maintained (fig. 9B). Western blotting of desmin on the protein extract of total pLNs of Tg mice and control, wild type littermates confirmed that there is no difference in the quantity of desmin produced in Tg mice (fig. 9C).

These results show that RN-building by FRCs is preserved even in the presence of Nef.

6.1.4 Production of CCL21

In murine pLNs, FRCs are the main source of CCL21, a chemoattractant for CD4+ T cells and dendritic cells (Link et al, 2007). We stained frozen sections of pLNs for CCL21 and imaged the T zone. Link and colleagues (2007) have reported that most CCL21 is located in the conduit system controlled by FRCs, which we see in our model as conduits appear clearly stained in both Nef– and Nef+ pLNs (fig. 10A). A 1.1-fold decrease in the signal intensity was observed but is likely too small to have any biological significance (fig. 10B). The area of the signal remained unchanged, which suggests that CCL21 localizes normally in Tg mice (fig. 10B). This result was confirmed by RT-PCR on RNA extracted from total pLNs (fig. 10C). No differences were found in the Tg animals compared to wild type controls. Therefore, Nef does not affect the production of CCL21 by FRCs, nor does it block its secretion and spread through the pLN.

6.1.5 Production of survival factors

FRCs also are the main sources of CCL19 and IL-7, two survival factors key to CD4+ T cells homeostasis, especially naïve CD4+ T cells (Link et al, 2007). The production of these two molecules was assessed by RT-PCR on RNA extracted from total pLNs (fig 11A). Given previous results, we expected no

differences between Tg pLNs and wild type controls and, indeed, we found that levels of CCL19 and of IL-7 mRNA remained unchanged in Tg mice compared to controls (fig 11B).

Taken together, these results show that, although FRCs express Nef, the cells are not depleted and their main functions are preserved. While FRCs play a crucial role in CD4+ T cell homeostasis and in acquired immunity, they have also been shown to depend on Lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) for their own survival and function (Katakai et al, 2004). In murine pLNs, CD4+ T cells are the main source of $LT\alpha_1\beta_2$ (Ohshima et al, 1999). As $LT\alpha_1\beta_2$ is plasma membrane-bound and not secreted, contact between the two cell types is needed for signal transduction, via the LTβ-receptor expressed by FRCs (Katakai et al, 2004). We have shown that, in the CD4C/HIV^{Nef} mouse model, Nef induces a severe depletion of peripheral CD4+ T cells (Hanna et al, 1998). We therefore expected to see a loss of FRCs and of their functions, as they would lose contact with $LT\alpha_1\beta_2$. However, this was not observed, which suggests that FRCs either manage to come in contact with enough $LT\alpha_1\beta_2$ to ensure their functioning, or that other survival factors can compensate for the loss of $LT\alpha_1\beta_2$.

6.1.6 $LT\alpha_1\beta_2$ levels in pLNs

We performed RT-PCR on RNA extracted from total pLNs of Tg mice and of their wild type littermates to measure the production of $LT\alpha_1\beta_2$ in the organ (fig. 12A). Lymphotoxin exists in two forms: soluble $LT\alpha_3$, a homotrimer of LTα molecules, and plasma membrane-bound $LT\alpha_1\beta_2$ formed by one LTα molecule bound to two transmembrane LTβ molecules (Ohshima et al, 1999). To make sure that we measured the expression of the plasma membrane-bound form, we amplified only the LTβ gene.

This revealed that, in Tg animals, LTβ RNA is produced in amounts comparable to those of wild type controls (fig. 12B). Presumably, the levels of LTβ, and $LT\alpha_1\beta_2$, proteins found at the cell surface in Tg animals would also be

similar to those found in controls, although we did not directly measure the quantity of cytokine produced. This result suggests that the population of FRCs and their functions are preserved in Tg mice because they do have access to enough $LT\alpha_1\beta_2$ to trigger sufficient LTβ-receptor signaling. This $LT\alpha_1\beta_2$ could come from the remaining CD4+ T cells or be provided by another cell population. The most likely candidate is the tissue-inducer cell population (LTi).

6.1.7 The LTi population

LTi are CD45.2+, CD11c-, CD4+, CD3- cells that play a major part in LN development (reviewed in Mebius, 2003). In the adult, they activate the production of chemokines by stromal cells such as FRCs and sustain CD4+ T cell memory (Kim et al, 2003). Flow cytometry analysis (fig. 13A) revealed that while the percentage of LTi is preserved in pLNs of CD4C/HIV^{Nef} Tg mice, there is a significant 3.3-fold loss in the absolute number of these cells (fig. 13B). The proportion of LTi among total CD4+ cells also drastically increased in Tg mice, from 0.68% to 4.45%, which represents a 6.5-fold increase (fig. 13C). As this population does not seem to suffer as much as CD4+ T cells from Nef expression, it is conceivable that the remaining LTi, probably in association with the remaining CD4+ T cells, produce enough $LT\alpha_1\beta_2$ to support the FRC population. However, their specific production of $LT\alpha_1\beta_2$ still has to be assessed.

6.1.8 pLN phenotypes in other transgenic mouse strains

Another possibility to explain the preservation of the FRC population and functions is that Nef is not sufficient to induce the phenotypes seen in human AIDS patients. Our laboratory developed a transgenic mouse expressing all HIV-1 genes under the control of the human CD4 promoter, the CD4C/HIV^{WT} mouse (Hanna et al, 1998). The pLNs of these mice showed fibrosis and a disrupted architecture, which tells us that HIV-1 can induce perturbations in murine pLNs. To investigate whether Nef was sufficient, or required at all, to trigger these

phenotypes, we analyzed pLNs of two other mouse strains also developed in our laboratory. The first strain, the CD4C/HIV$^{\Delta Nef}$ mouse, expresses all HIV-1 genes except Nef under the control of the human CD4 promoter. Sections were stained with hematoxylin and eosin (fig. 14A). The transgenic pLNs were indistinguishable from the wild type ones. This suggests that Nef is required, but not sufficient, to induce the phenotypes seen in the CD4C/HIV^{WT} mouse. To confirm this result, we looked at hematoxylin and eosin-stained sections of pLNs from CD4C/HIV$^{Nef,\ Rev,\ Env}$ mice, which express Nef, Rev, and Env of HIV-1 under the control of the human CD4 promoter. These mice show a clear hypocellularity and a potentially disrupted architecture (fig. 14B), while sections of CD4C/HIV^{Nef} pLNs also presented hypocellularity and no perturbation of the architecture (fig. 14C), which confirms previous results. However, Env and Rev are not known to have any functions that would increase the effects of Nef (Hanna et al, 1998; Vicenzi and Poli, 2013). We therefore hypothesized that the differences between CD4C/HIV^{Nef} and CD4C/HIV$^{Nef,\ Rev,\ Env}$ pLNs were due to a difference in levels of expression of Nef between these two mouse strains.

To test this hypothesis, we performed a Western blot analysis on protein extracts from total thymi of these mice. We chose to use the thymus because transgene expression has been shown to be much higher in this organ compared to pLNs (Hanna et al, 1998). It has also been shown, in an inducible system of Nef expression that also uses the CD4C promoter, that the CD4+ T cells that remain in the pLNs after several months of induction, present no CD4 downregulation (Rahim et al, 2009). These cells probably expressed very low levels of Nef compared to the CD4+ T cells that were depleted and showed CD4 downregulation, and a CD4^{high} T cell population has also been observed in CD4C/HIV^{Nef} mice (Rahim et al, 2009). Thus, we measured the amount of Nef in the thymus by Western blot (fig. 15A). It revealed no significant difference between the two mouse strains (fig. 15B). The phenotypes caused by Nef tend to evolve faster in the CD4C/HIV$^{Nef,\ Rev,\ Env}$ mice than in the CD4C/HIV^{Nef} mice (Hanna et al, 1998). We show here that this is not caused by a difference in the levels of protein expressed. The two mouse lines were generated on the same

background, which therefore cannot account for the differences observed. They could be due to a founder effect. A more detailed comparison of the two mouse lines in regard to FRCs would be needed to answer this question. For example, the state of the RN fibers was not assessed in CD4C/HIV$^{Nef, Rev, Env}$ mice.

6.1.9 Increase in the proportion of regulatory T cells among total CD4+ cells

A third hypothesis to explain the preservation of the FRC population is that fibrosis fails to develop in pLNs of Nef-expressing mice. In this event, the structure of the RN would be maintained and the contact between FRCs and the cells providing them with $LT\alpha_1\beta_2$, be it the remaining CD4+ T cells or LTi, would also be preserved. In rhesus macaques infected with SIV and in human AIDS patients, the onset of fibrosis is triggered by activated regulatory T cells (Treg) that increase the collagen deposition of fibroblasts in a TGFβ1-dependent manner (Zeng et al, 2011). We analyzed the Treg population by flow cytometry (fig. 16A). No significant differences in percentage or absolute numbers of Treg were observed (fig. 16B), but a clear 4-fold increase of the proportion of Treg among total CD4+ cells appeared (fig. 16C). This has been previously reported in Dr Jolicoeur's laboratory by Pavel Chrobak (Chrobak et al, under review), as well as in human patients (Cao et al, 2009). TGFβ1 levels were not measured in our study, but an elevated Treg proportion has been linked with disease progression, particularly in fast-progressing patients (Cao et al, 2009). Our result suggests that fibrosis could in theory develop in pLNs of CD4C/HIV^{Nef} mice. Further research is needed to discover which step of this process is blocked and why, while Nef is responsible for many AIDS phenotypes on its own, it does not induce the expected loss of FRCs and of their functions.

6.2 Effects of Nef on endothelial cells (BECs and LECs)

Other stromal cells essential to pLN function are the endothelial cells. T and B cells enter the pLN by extravasation across the high endothelial venules

(HEVs) located at the periphery of the T zone (Bajénoff et al, 2007). Blood endothelial cells (BECs) regulate this process by their expression of ICAM-1, which binds CD62L (also called L-selectin) on T cells (Bajénoff et al, 2007). For example, ICAM-1 expression is upregulated during inflammation and this has been linked to increased extravasation of lymphocytes across HEVs (Chen et al, 2006). Dendritic cells (DCs) and macrophages can enter the pLN via the subcapsular sinus (SCS; reviewed in Förster et al, 2012) and all non-resident immune cells exit the pLN via the lymphatic sinuses. BECs and lymphatic endothelial cells (LECs) have also been shown to express CCL21 to direct the migration of the immune cells (Martín-Fontecha et al, 2003). Moreover, FRCs support them as they are the main source of VEGF in murine pLN (Chyou et al, 2008; Kataru et al, 2011). Human patients infected with HIV-1 are at higher risk of suffering from coronary heart disease (Vittecoq et al, 2003) and acute myocardial infarction (Triant et al, 2007). Previous observations made in our laboratory have revealed an accumulation of immature dendritic cells in the SCS of Tg CD4C/HIV$^{Nef, Rev, Env}$ mice (Poudrier et al, 2003). Because of this and of their link with FRCs, studying the BEC and LEC populations in the CD4C/HIV^{Nef} mouse appeared crucial.

6.2.1 Effects of Nef on blood endothelial cells (BECs)

CD31 and αSMA staining revealed that, in the T zone, there are no significant differences in the intensity or area of both signals between Nef-expressing animals and their wild type littermates (fig. 17A and B). This suggested that, in pLNs, Nef does not perturb the structure of the HEVs. To confirm this, we examined every HEV imaged separately and counted the ones that appeared abnormally structured. An HEV was considered abnormally structured if it presented more than one layer of endothelial cells or if it did not include a clear αSMA signal – this would indicate a defect in pericyte recruitment to the perivascular sheath. As expected, we found no difference in the percentage of abnormally structured HEVs among total HEVs between transgenic and wild

type animals (fig. 17C). It is likely that some of these abnormally structured HEVs are in fact artifacts of the harvesting, cutting, and staining procedure.

The blood vessels of the medulla were imaged in parallel (fig. 18A). While there was no significant variation in the αSMA signal, we did find a significant 2.4-fold increase of the CD31+ area (fig. 18B). The lumen of some of these vessels appeared partially, or even completely, obstructed by CD31+ cells, as seen on fig. 14A (white arrow). We counted the abnormally structured vessels using the same criteria as for HEVs (fig. 18C). There were 2.7 times more abnormally structured vessels in the medulla of Nef+ mice than in that of Nef– mice. As for HEVs, some of these may be artifacts of the procedure. However, the significant difference suggests that there is indeed a defect in the structure of medullary blood vessels in the pLNs of Nef+ mice, mostly because of an expansion of CD31+ cells. Since not all medullary blood vessels seemed affected, we propose that this is a focal phenotype.

To see whether the CD31+ cells were BECs, we performed a von Willebrand Factor (vWF, also known as Factor VIII) and CD31 co-staining on slides from these same pLNs (fig. 19A). We found medullary blood vessels that were obstructed by CD31+ cells. These cells were also vWF+, which suggests that they are indeed BECs (fig. 19B). There was no difference in the vWF signal area and, to our surprise, the increase in the CD31+ area could not be reproduced (fig. 19C). This is an argument in favor of a very local expansion of CD31+ cells, as it would not systematically be seen on all pLN sections. Of course, the possibility that the expansion was in fact merely an artifact of the procedure remained, and more experiments were needed to confirm or infirm it.

A study of the BEC population by flow cytometry (fig. 20A) revealed no increase or decrease of this population in Nef+ mice compared to their wild type littermates, be it in percentage or absolute number of cells (fig. 20B). In this experiment, we gated on CD45.2– 7AAD– CD31+ gp38– cells, as described previously (Link et al, 2007). The data was then reanalyzed by gating on CD45.2– CD31+ gp38– cells to see what percentage of BECs were 7AAD+ (fig. 20C). 7AAD (7-Aminoactinomycin-D) does not normally pass through the plasma

membrane; only cells with compromised membranes (i.e. dead cells) will be stained. It is therefore an indicator of cell viability. We found a significant 2.5-fold decrease in 7AAD+ BECs in Tg mice, which suggests a defect in apoptosis in these cells, or at least a better survival. A 3.3-fold decrease of the absolute number of 7AAD+ cells was also observed but was not significant (p-value = 0.087, Student's T test). Thus, we propose that Nef can induce a local expansion of the BEC population, and that this expansion happens through preventing apoptosis of the cells. However, this data is preliminary and these results must be confirmed by staining for actual apoptosis and proliferation markers. It will also be important to investigate why only the BECs of the medullary blood vessels, and not those of the HEVs, are affected, and also whether this expansion of BECs concerns all the medullary blood vessels, or only a portion of them.

6.2.2 Expression of Nef by BECs

The expression of Nef by BECs in CD4C/HIV^{Nef} mice was also assessed. As is the case with the FRCs described previously, BECs are present in small numbers in pLNs. Therefore, we decided to first test the activity of the CD4C promoter in two reporter mouse lines. Like for the study of the FRCs, we used the CD4C/GFP x CD4C/HIV^{Nef} line and the CD4C/hCD4-CCR5 line.

By flow cytometry, BECs of the CD4C/GFP x CD4C/HIV^{Nef} line appeared to be weakly GFP+ (fig. 21A). A staining for hCD4 in BECs from CD4C/hCD4-CCR5 mice also marked them as hCD4^{low} (fig. 21B). These results indicate that the CD4C promoter is indeed active in these cells, albeit weakly. We therefore performed an RT-PCR on sorted BECs from pooled Nef– or Nef+ mice. BECs isolated from Nef-expressing animals were all clearly Nef+ (fig. 21C). However, because of the small number of cells obtained even after pooling the pLNs of several mice, the presence of the Nef protein in BECs could not be assessed. Taken together, these results nevertheless suggest that BECs do express Nef.

6.2.3 Effects of Nef on lymphatic endothelial cells (LECs)

To ensure that the expanding CD31+ cells seen in immunofluorescence were not LECs, a LYVE-1 and CD31 co-staining was performed, as LECs are CD31+ and LYVE-1+ (Podgrabinska et al, 2002; fig. 22A). This staining revealed no difference in the signal intensity or signal area of LYVE-1 (fig. 22B), which indicates that the expanding cells of the medulla are not LECs.

The same staining revealed a sometimes extensive expansion of CD31+ LYVE-1+ cells in the subcapsular sinus (SCS, fig. 23A). This expansion varied greatly from one pLN to another and only affected localized areas of the SCS (fig. 23B). The quantification of the LYVE-1+ and of the CD31+ area in the SCS revealed significant 1.58- and 1.78-fold increases in Nef+ animals compared to their wild type littermates, respectively. These increases may seem too small to be biologically significant but, since we included the area measurements from all pictures and not just the ones showing an expansion, they most likely reflect the fact that the observed expansions are focal and never seem to affect the entire SCS of a given pLN.

To confirm this result, we analyzed the LEC population by flow cytometry (fig. 24A). CD45.2– 7AAD– CD31+ gp38+ cells were considered to be LECs, as described previously (Link et al, 2007). This failed to show any significant change in the percentage or absolute number of LECs in pLNs (fig. 24B). The data was analyzed a second time by gating on CD45.2– CD31+ gp38+ cells to see whether we could observe any difference in the number of 7AAD+ LECs. None was found (fig. 24C).

Taken together, these results suggest that Nef may induce an expansion of LECs specific to the SCS. This expansion would be too localized to be measurable by flow cytometry and, since there are no differences in the percentage or number of dead LECs between Nef– and Nef+ mice, it could be caused by a proliferation of the cells rather than a defect in apoptosis, contrary to what we found in BECs. However, as for BECs, these results need to be confirmed by staining for specific proliferation and apoptosis markers. More

experiments are also needed to discover why LECs in the SCS and not in the medullary sinuses are affected, and why this expansion is so localized.

6.2.4 Expression of Nef by LECs

A first step to answering these questions was to investigate whether the LECs themselves express Nef. As was the case for FRCs and BECs, we first tested the activity of the CD4C promoter in LECs on our two reporter mouse lines, the CD4C/GFP x CD4C/HIV^{Nef} line and the CD4C/hCD4-CCR5 line. By flow cytometry, we gated on CD45.2– 7AAD– CD31+ gp38+ LECs from pLNs of CD4C/GFP x CD4C/HIV^{Nef} mice (fig. 25A). These cells were GFP^{low}. The apparent heterogeneity of the GFP signal in this population may be due to the low number of cells obtained from each pLN. This result was confirmed by staining LECs for hCD4 in CD4C/hCD4-CCR5 mice (fig. 25B). The cells also expressed low levels of hCD4. These data indicate that the CD4C promoter is active in LECs. As expected, an RT-PCR performed on RNA extracted from sorted LECs showed that the cells do express Nef mRNA (fig. 25C).

While the low cell yield prevented us from directly measuring the amount of Nef protein in LECs, all the results mentioned here are consistent. It is therefore safe to say that, in our CD4C/HIV^{Nef} mouse model, LECs express Nef.

In summary, we have demonstrated that, in the CD4C/HIV^{Nef} mouse model developed in our laboratory, FRCs, BECs, and LECs express Nef. To our knowledge, this is the first time that the expression of an HIV-1 protein is reported in FRCs. Contrary to what is seen in CD4+ T cells, for example, this expression of Nef does not appear to have direct effects on FRCs in resting pLNs. The cell population is maintained, as are its main functions.

Nef does seem to affect BECs and LECs by causing a localized expansion of these two cell populations. However, these results need to be confirmed. It is also unclear whether this expansion is a direct effect of Nef or whether other factors come into play.

Figures

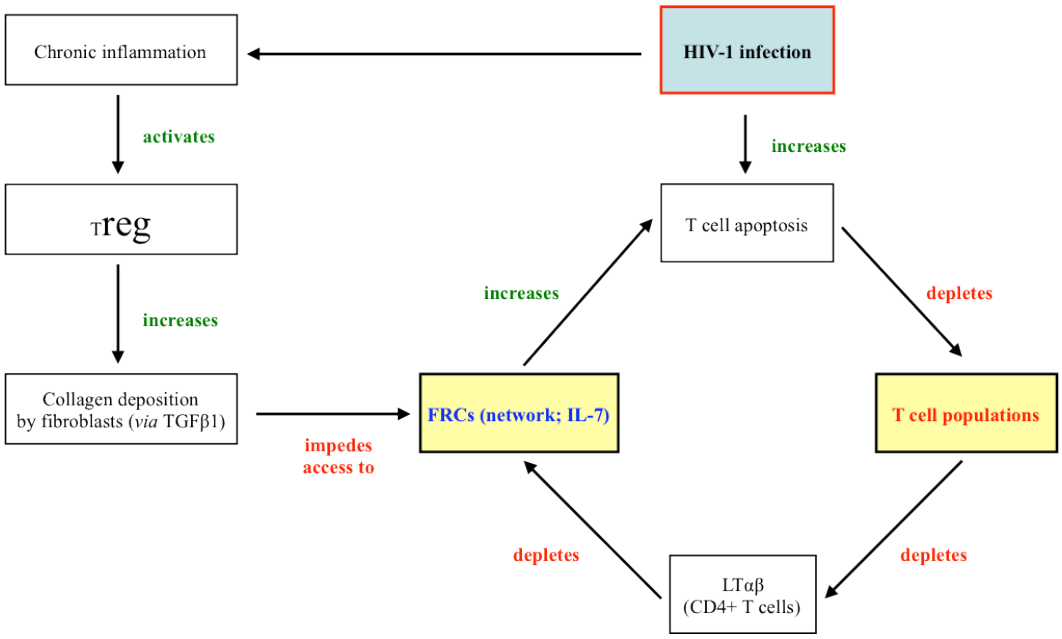


Figure 5: HIV-1 infection leads to loss of CD4+ T cells and FRCs, as well as development of fibrosis. By precipitating the apoptosis of CD4+ T cells, HIV-1 deprives FRCs of Lymphotoxin-αβ (LTαβ), which leads to their own depletion. CD4+ T cells therefore lose access to IL-7, which further accelerates their depletion. In parallel, chronic inflammation induces the development of fibrosis, thus further blocking contact between CD4+ T cells and FRCs. Adapted from: Zeng et al (2011), *Journal of Clinical Investigation*, 121(3):998-1008, supplemental Figure 1A.

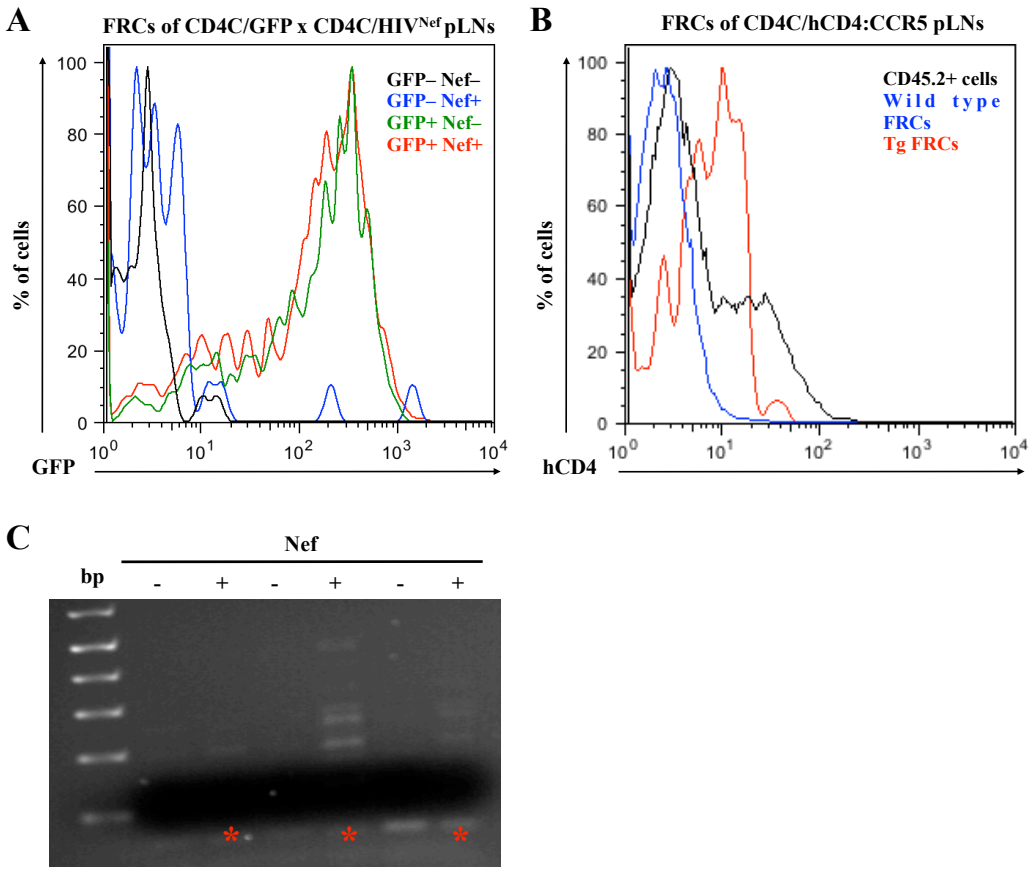


Figure 6: Expression of Nef in FRCs. . A cell suspension from lymph nodes of CD4C/GFP x CD4C/HIV^{Nef} reporter mouse line was analyzed (A). The flow cytometry profile shows the GFP signal in FRCs from wild type mice (black line), from Nef+ only mice (blue line), from GFP+ mice (green line), and from GFP+ Nef+ mice (red line). CD45.2– CD31– gp38+ cells were considered to be FRCs. The CD4C/hCD4-CCR5 mouse line was also used to verify the activity of the CD4C promoter in FRCs (B). A representative flow cytometry profile is shown (n=3) with the hCD4 signal in wild type C3H lymph node cell suspension (blue line, negative control), in CD45.2+ cells (black line, positive control), and in FRCs (red line). To assess the expression of Nef in FRCs, an RT-PCR on sorted FRCs was performed (C). Red stars indicate samples containing Nef. All come from Nef-expressing animals. bp: DNA base pair ladder.

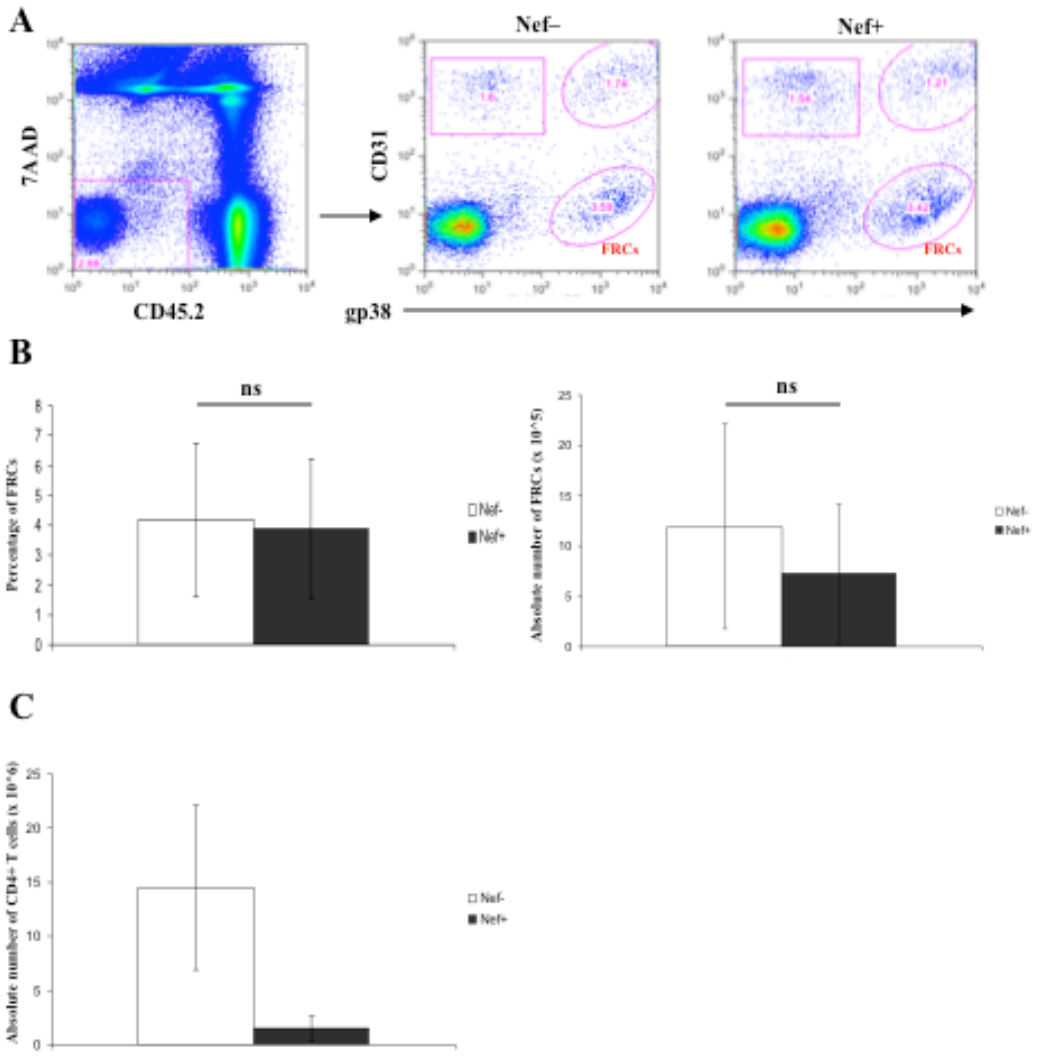


Figure 7: Population of FRCs in pLN. The FRC population was analyzed by flow cytometry on total pLNs (A). CD45.2- CD31- gp38+ cells were considered to be FRCs, as described by Link and colleagues (2007). The profiles shown are representative of three separate experiments (n = 9 Nef– and 9 Nef+ animals). No differences in percentage or absolute number of cells were observed (B), despite a clear loss of CD4+ T cells in these same animals (C). ns: non significant difference, as determined by paired Student t test.

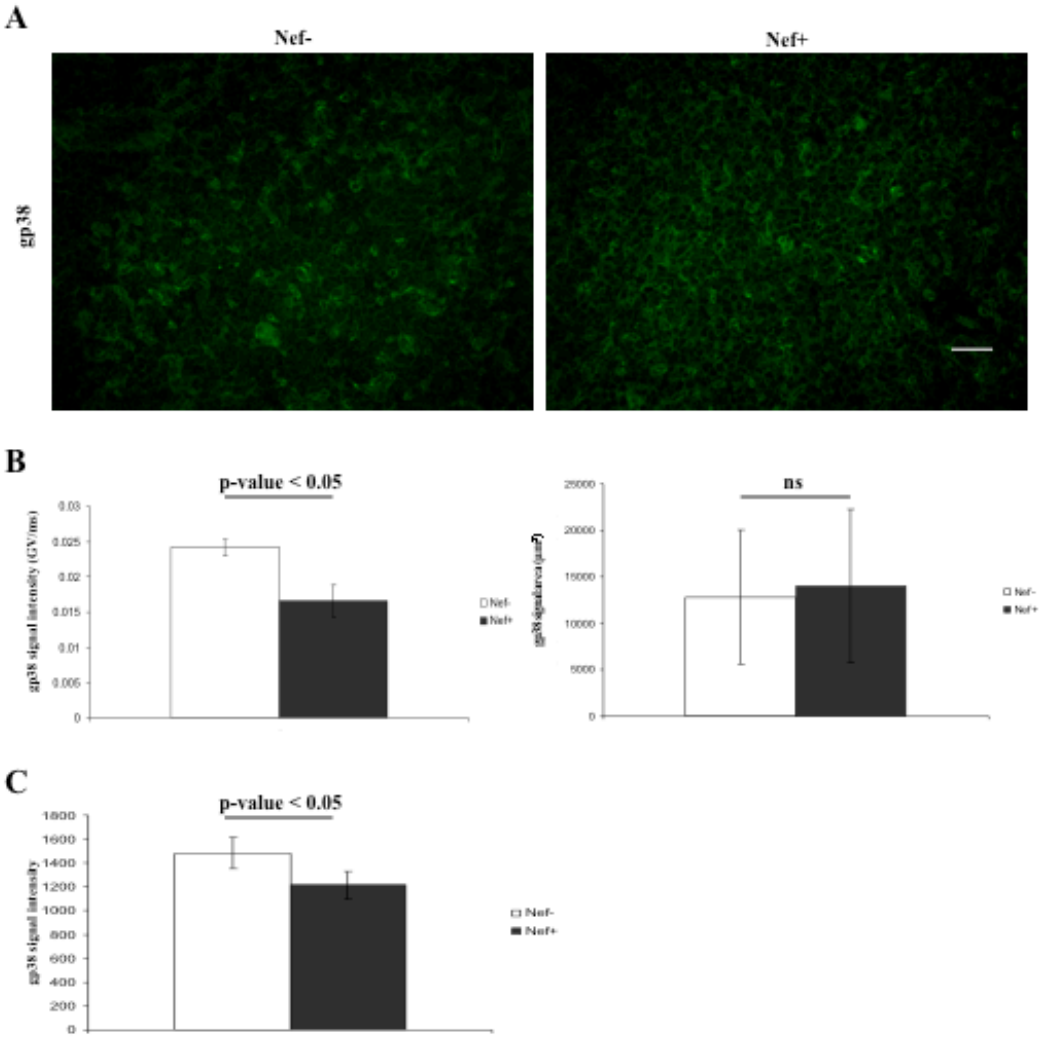


Figure 8: FRCs spread normally in pLNs of Tg animals. gp38 immunofluorescent staining was performed on 5-µm thick sections of frozen pLNs and the T zone was imaged (A). Signal intensity and area were then measured (B). The signal intensity presents a significant 1.45-fold decrease by immunohistochemistry and a 1.22-fold decrease by flow cytometry (C), as determined by a paired Student t test. However, this decrease is probably too small to be biologically significant. No differences in signal area were observed by immunohistochemistry. Scale bar: 25 µm. ns: non significant.

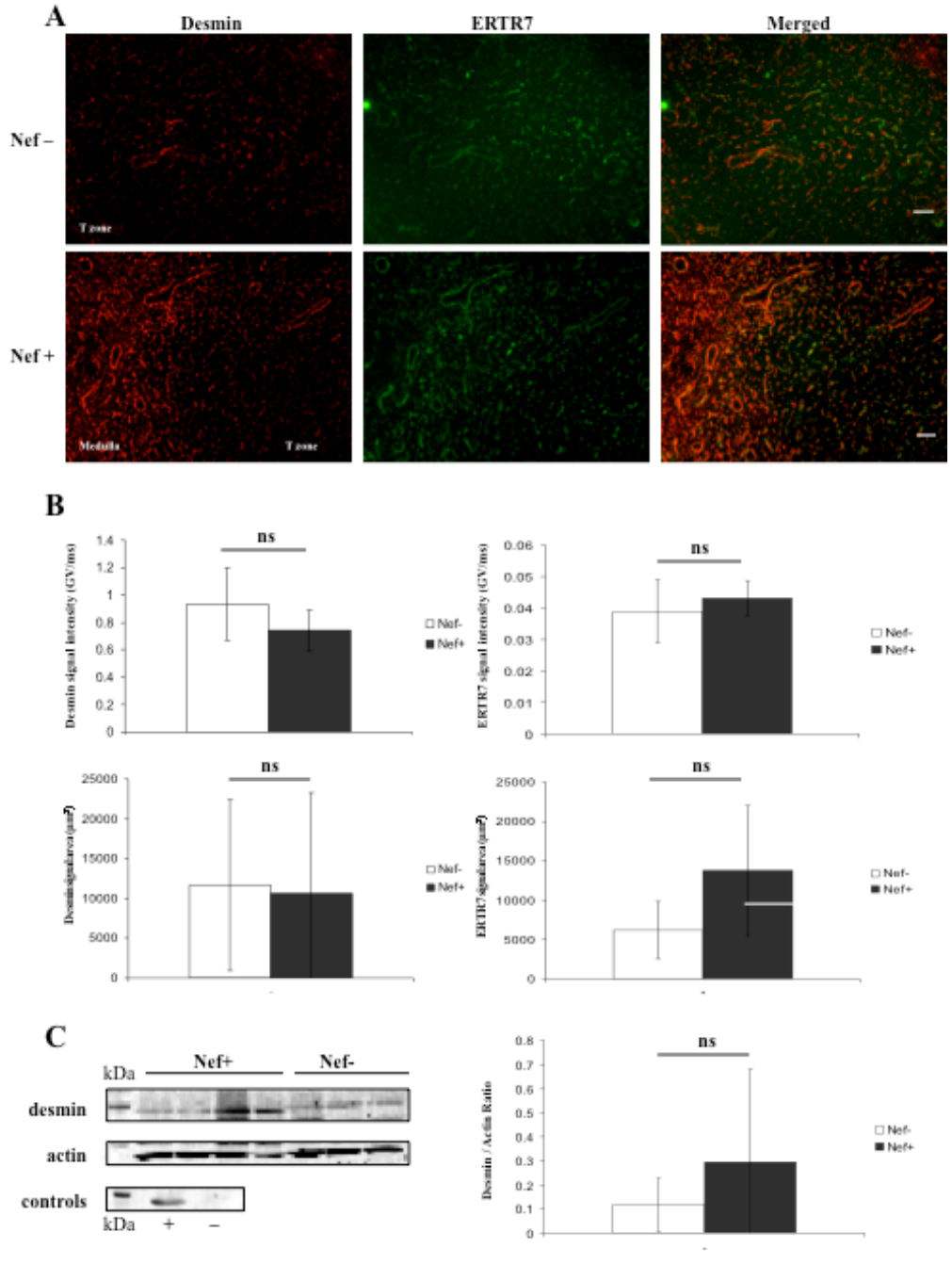


Figure 9: Production of RN fibers by FRCs. 5-µm thick sections from frozen pLNs were stained for desmin and the unknown antigen to the ERTR7 antibody (thereafter ERTR7) and the T zone was imaged (A). The intensity and area of both signals were measured (B). There were no significant differences in desmin or ERTR7 signal, as determined by a paired Student t test. This result was confirmed by Western blot on protein extract from total pLNs (C). Actin was used as a normalizing control. Scale bar: 100µm. kDA: molecular weight marker. ns: non significant.

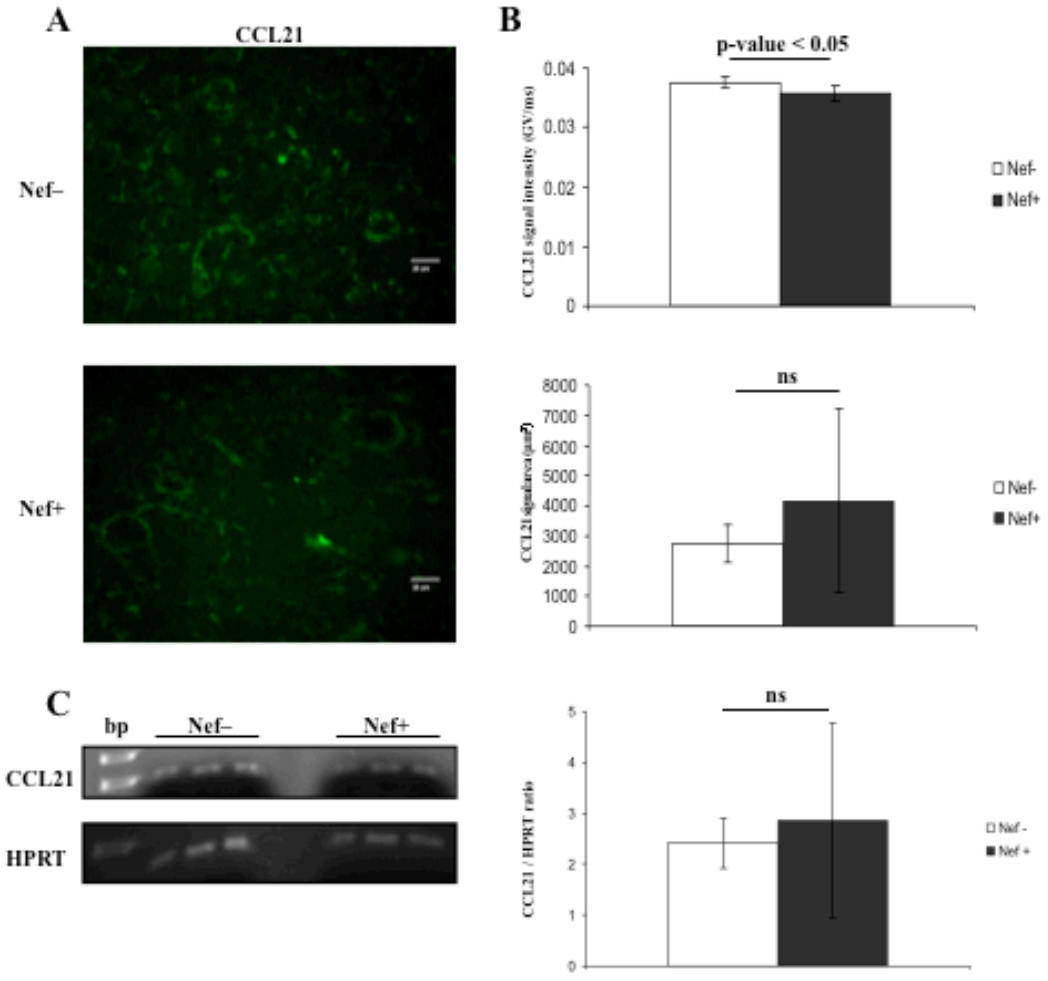


Figure 10: Production of chemoattractants. The production of CCL21 by FRCs was assessed by immunohistochemistry on 5µm-thick frozen sections of pLNs (A). The images are representative of the T zones of pLNs from Nef– (n=6) and Nef+ (n=6) animals.The difference observed in signal intensity (B) is likely too small to be biologically significant (1.1-fold difference). There is no difference in signal area (B). This result was confirmed by RT-PCR on RNA extracted from total pLNs of 3 Nef– mice and 3 Nef+ mice. The RNA was reverse-transcripted to cDNA and the CCL21 gene was amplified (C). HPRT was used as a normalizing control. ns: non significant difference, as determined by a paired Student t test. bp: DNA base pair ladder.

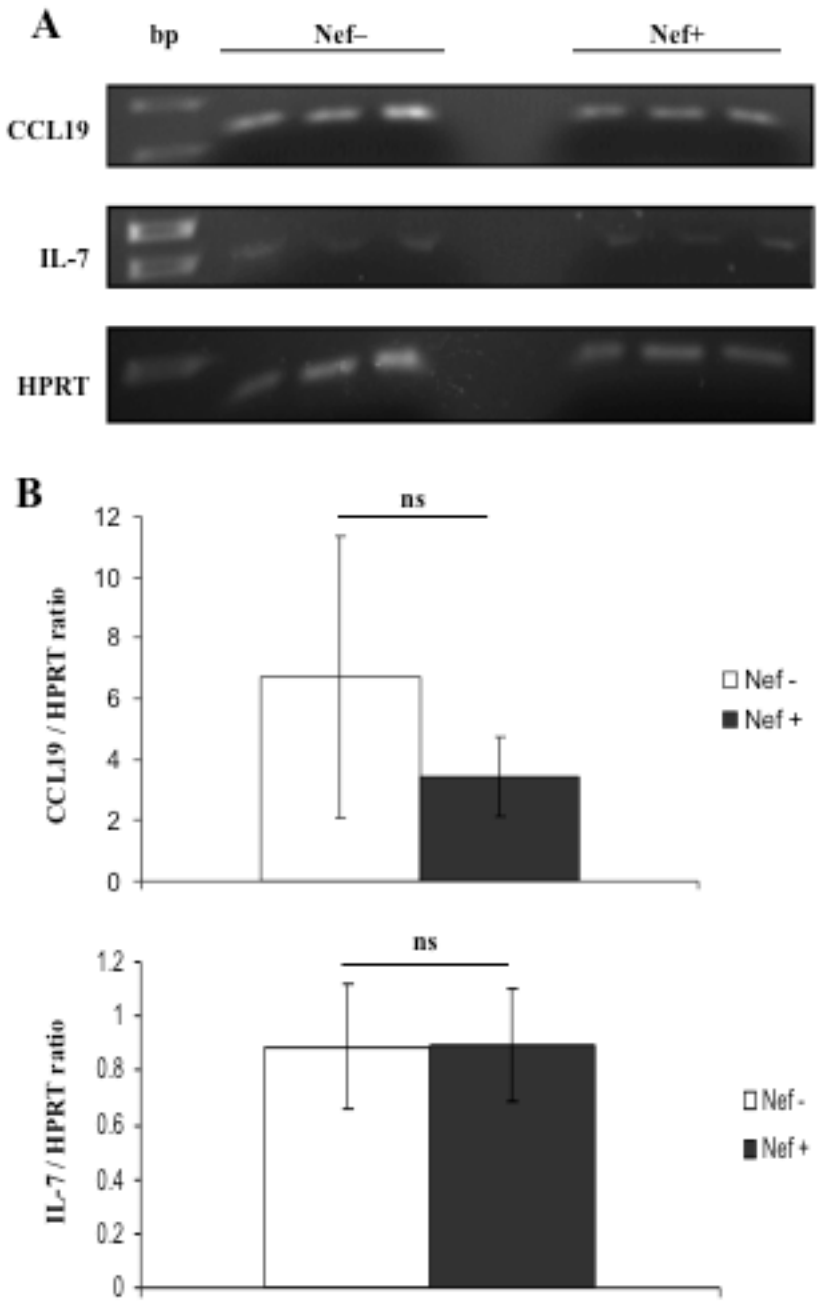


Figure 11: Production of survival factors. The production of CCL19 and IL-7 was assessed by RT-PCR on RNA extracted from total pLNs from 3 Nef– mice and 3 Nef+ mice. The RNA was reverse-transcripted to cDNA and the CCL19 and IL-7 genes were amplified (A). HPRT was used as a normalizing control. No differences were found in the quantities of mRNA produced for the two genes (B), as determined by a paired Student t test. bp: DNA ladder. ns: non significant.

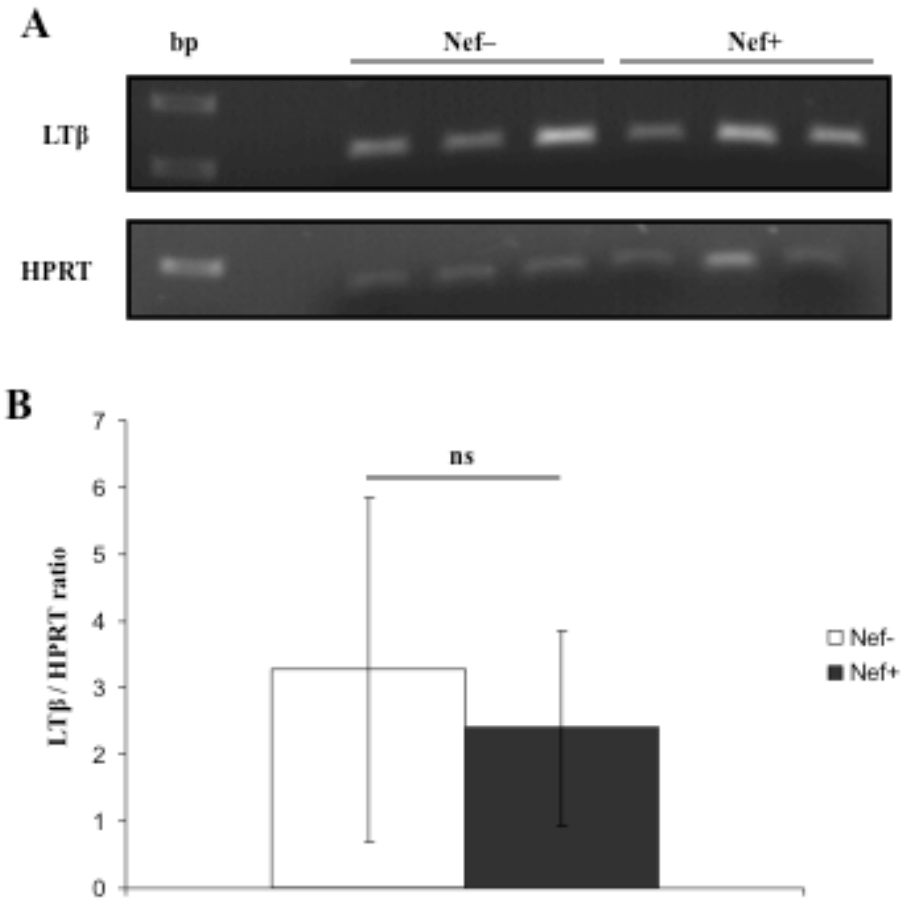


Figure 12: $LT\alpha_1\beta_2$ production. The production of $LT\alpha_1\beta_2$ was assessed by RT-PCR on RNA extracted from total pLNs from 3 Nef– mice and 3 Nef+ mice. The RNA was reverse-transcripted to cDNA. As LTα also exists as a soluble homotrimer, only the LTβ gene was amplified (A). HPRT was used as a normalizing control. No differences were found in the quantities of mRNA produced (B), as determined by a paired Student t test. bp: DNA base pair ladder. ns: non significant.

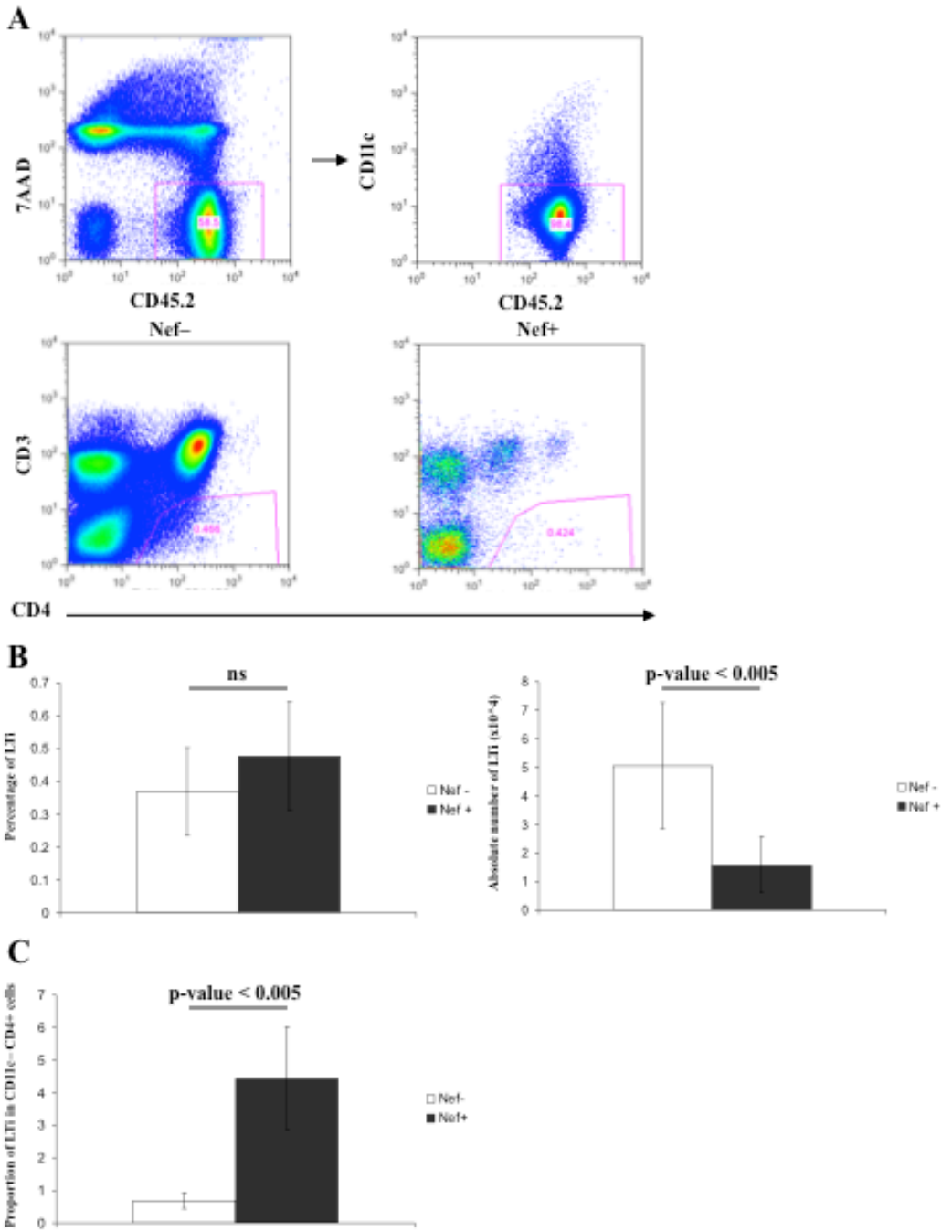


Figure 13: Possible alternative source of $LT\alpha_1\beta_2$. The LTi were likely candidates to explain the unchanged production of $LT\alpha_1\beta_2$. We analyzed CD45.2+ CD11c– CD4+ CD3– cells by flow cytometry (A). While there was no difference in the percentage of these cells, a significant 3.2-fold decrease in absolute numbers was observed (B). However, the proportion of LTi among total CD11c– CD4+ cells increased by 6.53 folds (C). Significance was assesed by a paired Student t test. ns: non significant.

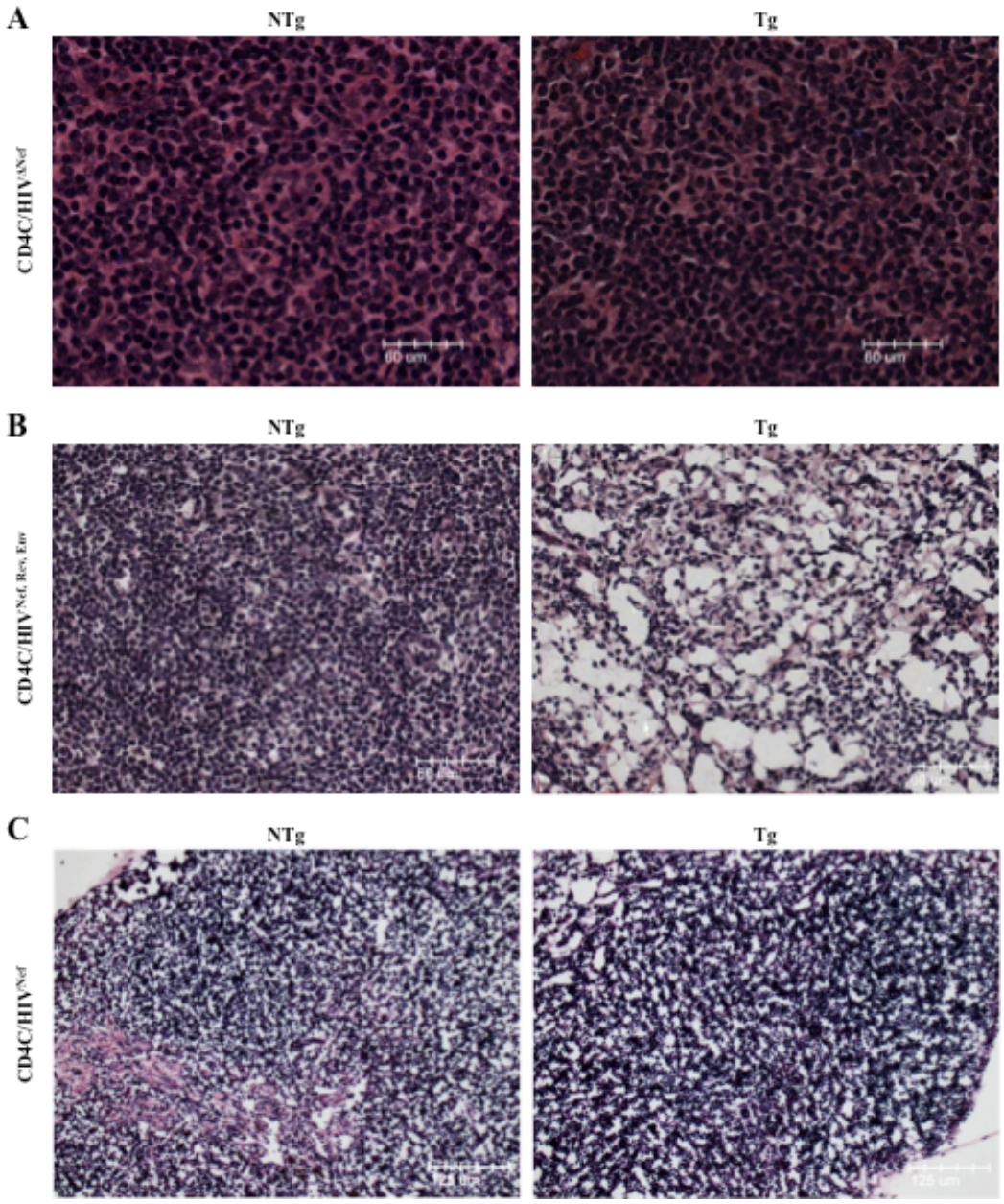


Figure 14: pLN phenotypes in other transgenic (Tg) mouse strains. 5µm-thick sections from frozen pLNs of CD4C/HIV$^{\Delta Nef}$ (A), CD4C/HIV$^{Nef, Rev, Env}$ (B), and CD4C/HIV^{Nef} (C) mice were stained with hematoxylin and eosin. Representative images of the T zones of these pLNs are shown (the medulla also appears at the lower left of the NTg CD4C/HIV^{Nef} image). Non-transgenic (NTg) and transgenic (Tg) pLNs from CD4C/HIV$^{\Delta Nef}$ mice are practically indistinguishable, while a clear hypocellularity and a possibly disrupted RN appear in Tg CD4C/HIV$^{Nef, Rev, Env}$ mice. Hypocellularity is also apparent in the Tg CD4C/HIV^{Nef} image, but no modification of the RN is visible.

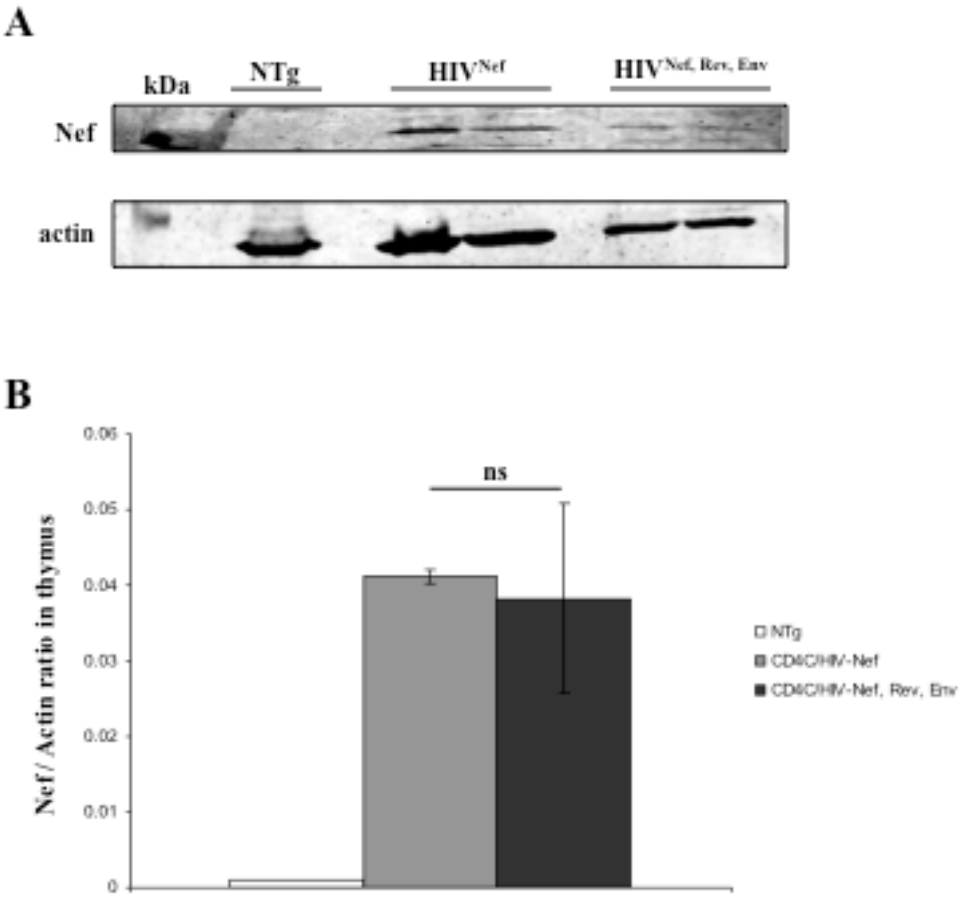


Figure 15: Comparison between levels of expression of Nef in CD4C/HIV^{Nef} and CD4C/HIV$^{Nef,\ Rev,\ Env}$ mice. Thymi were harvested and meshed. The resulting cell suspension was then lysed and Western blot analysis was performed on the protein extract (A). Actin was used as a normalizing control. There was no significant difference in the levels of Nef in the two mouse strains (B), as determined by a paired Student t test. NTg: wild type control. ns: non significant.

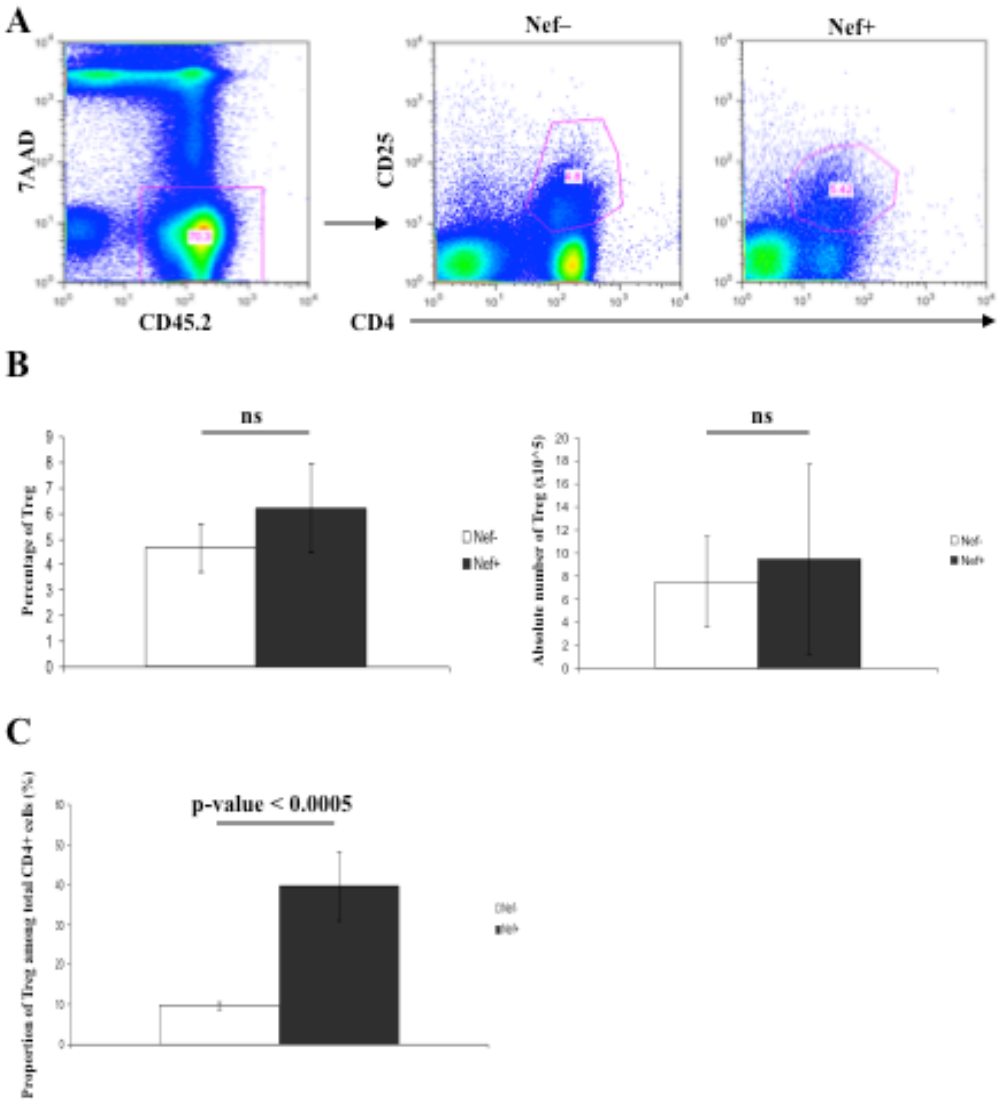


Figure 16: Population of Treg in pLN. The Treg population was analysized by flow cytometry on total pLNs. CD45.2+ CD4+ CD25+ cells were considered to be Treg. The profiles shown are representative of Nef– (n=6) and Nef+ animals (n=6) (A). No differences in percentage or absolute number of cells were observed (B), but the proportion of Treg among total CD4+ cells was increased 4-fold (C). ns: non significant difference as determined by paired Student t test.

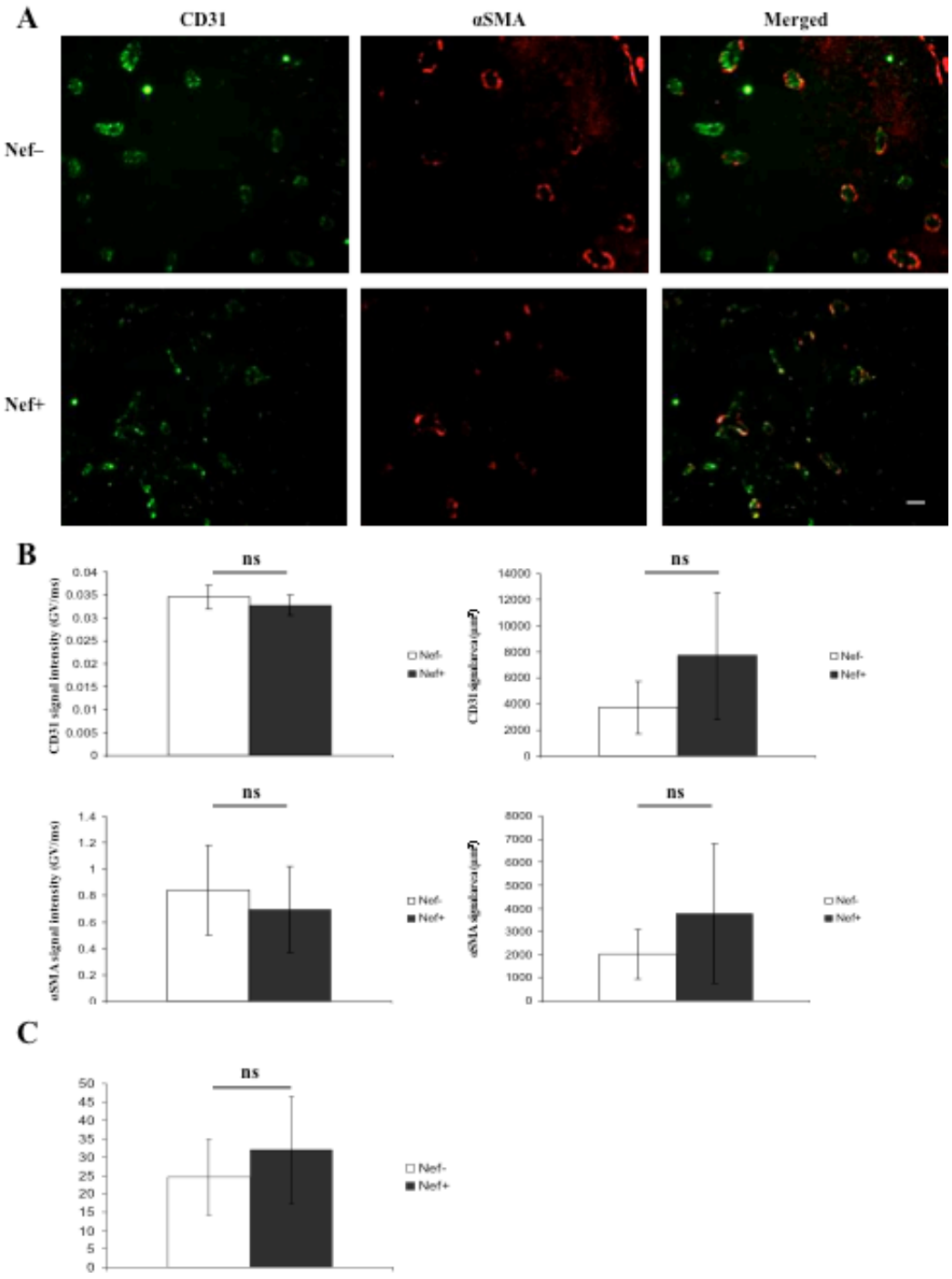


Figure 17: BECs of the HEVs of the T zone. Immunohistochemistry was performed on 5µm-thick sections of frozen pLNs. Representative images of the HEVs found in the T zone are shown (A). The intensity and the area of the CD31 and αSMA signals were measured (B). No significant differences were observed. The percentage of abnormally structured HEVs was also evaluated (C). There was no significant difference between Nef– and Nef+ animals. Scale bar 50 µm. ns: non significant, as determined by a paired Student t test.

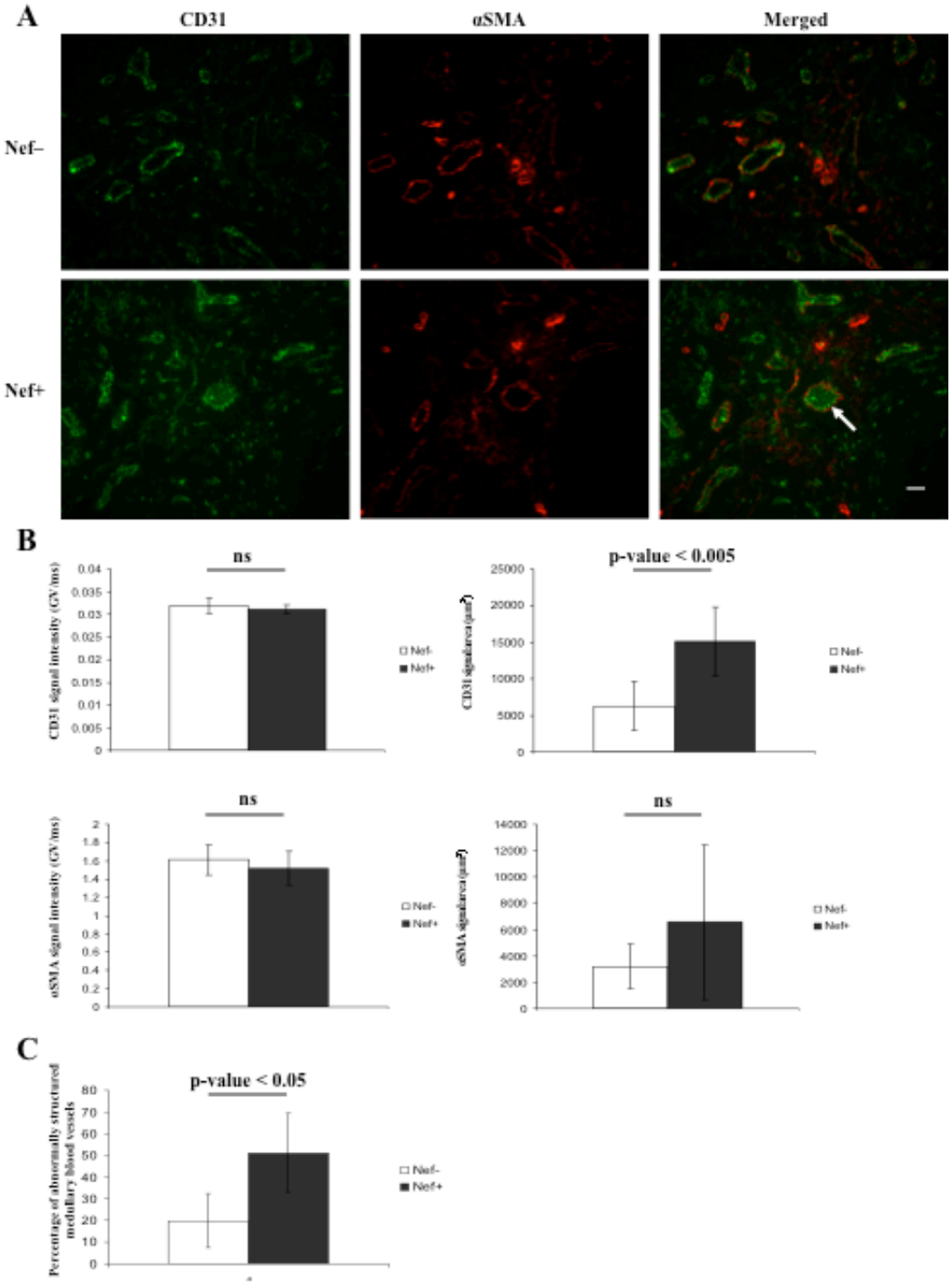

A
CD31
αSMA
Merged
Nef–
Nef+
B
ns
p-value < 0.005
CD31 signal intensity (GV/ms)
CD31 signal area (μm²)
Nef-
Nef+
ns
ns
αSMA signal intensity (GV/ms)
αSMA signal area (μm²)
C
p-value < 0.05
Percentage of abnormally structured medullary blood vessels

Figure 18: BECs of the medullary blood vessels. Immunohistochemistry was performed on 5μm-thick sections of frozen pLNs. Representative images of the medulla are shown (A). The white arrow indicates a vessel with a lumen completely obstructed by CD31+ cells. The intensity and the area of the CD31 and αSMA signals were measured (B). No significant difference was observed in the intensity of the signal, but a significant 2.4-fold increase was found in the CD31+ area (paired Student t test). We also found a significant 2.7-fold increase in abnormally structured vessels (C). Scale bar 50 um. ns: non significant, as determined by a paired Student t test.

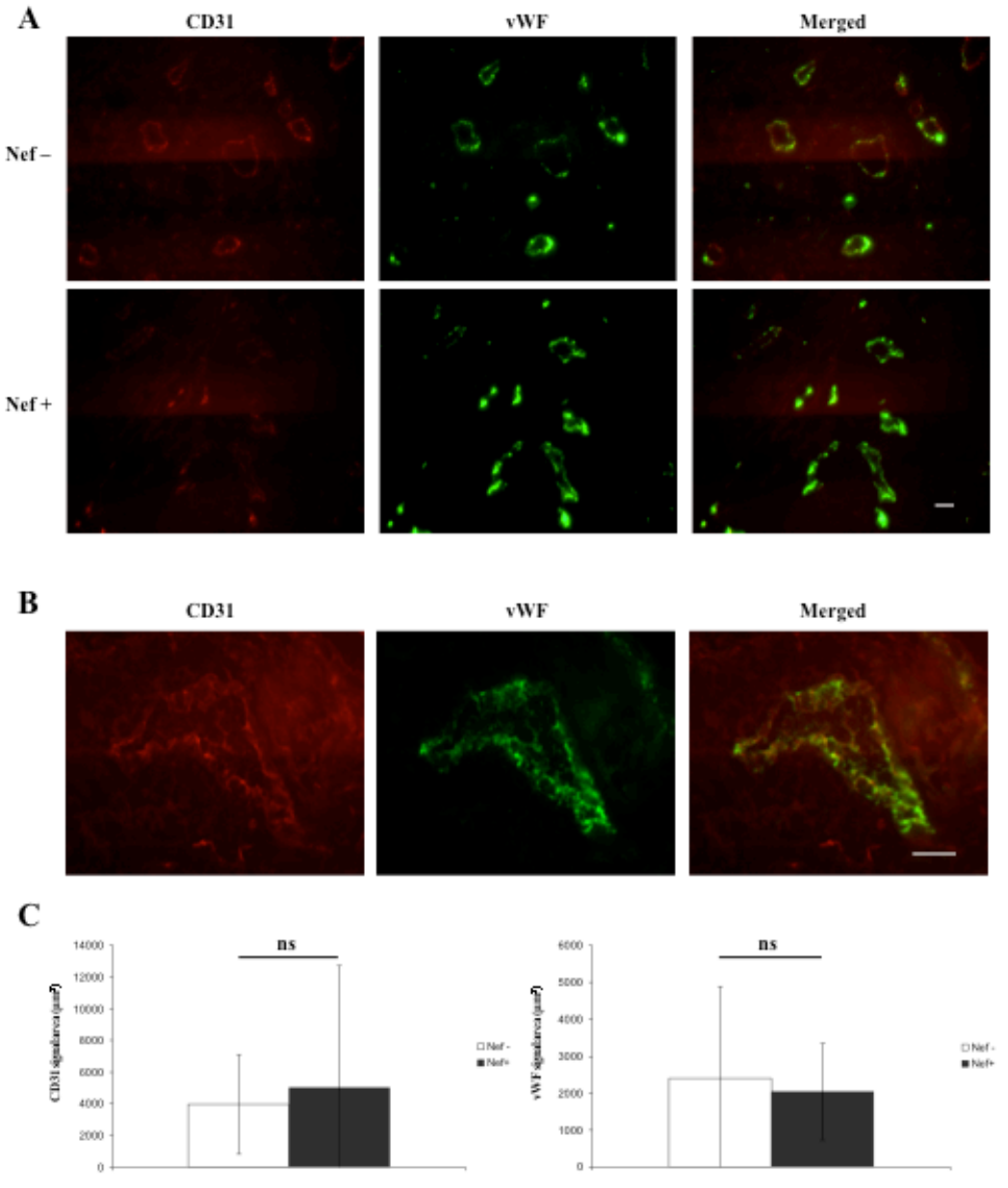


Figure 19: The expanding cells of the medullary blood vessels are BECs. vWF stains BECs specifically, and not LECs. We stained 5µm-thick sections of frozen pLNs and imaged the medulla (A). Representative images of Nef– and Nef+ animals are represented. Scale bar: 50µm. Expanding cells in a medullary blood vessel were cleary CD31+ vWF+ (B). Scale bar: 25µm. However, contrary to previous results, no significant difference was observed in CD31+ or vWF+ area (C). ns: non significant, as determined by a paired Student t test.

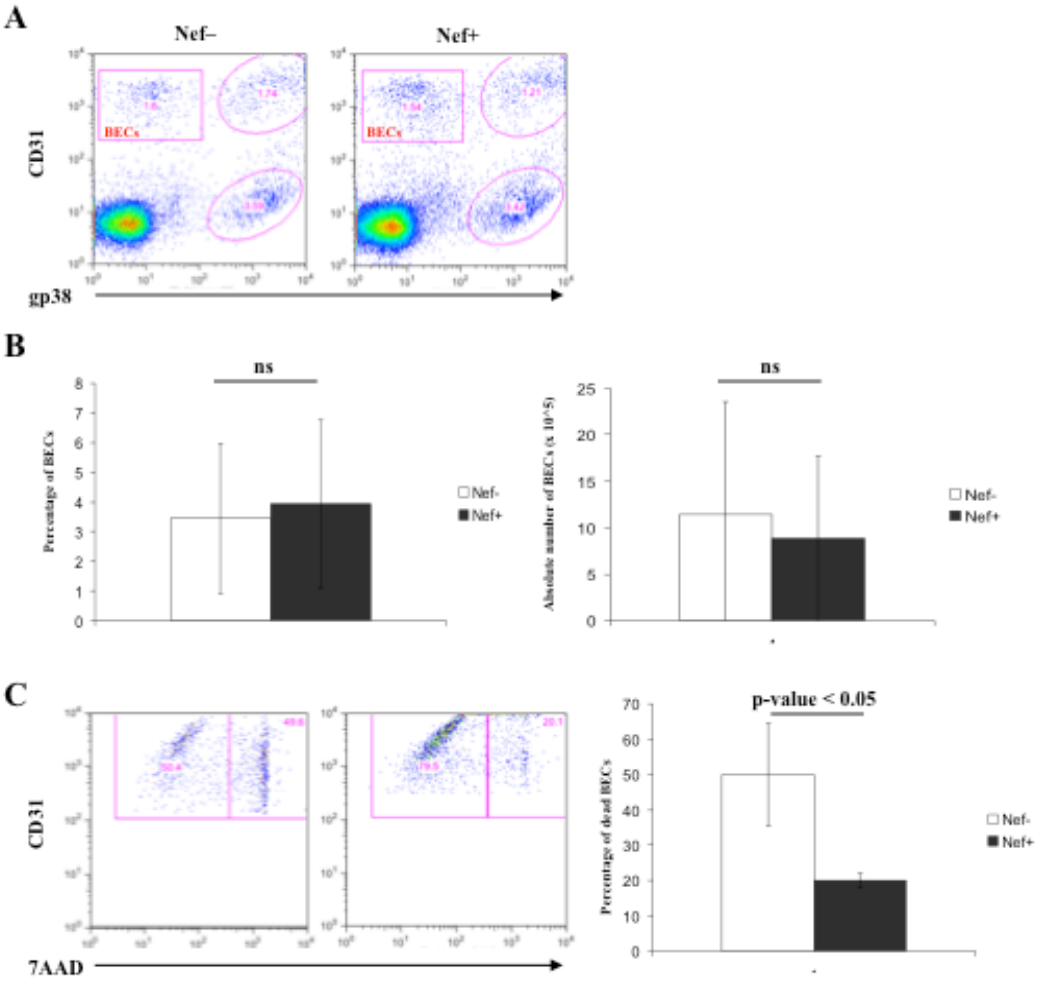


Figure 20: Expansion of BECs. A flow cytometry analysis on total pLNs was performed to confirm the immunohistochemistry results (A). CD45.2– CD31+ gp38– cells were considered BECs, as described previously (Link et al, 2007). The flow cytometry profiles shown are representative of Nef– (n=6) and Nef+ (n=6) animals. No significant difference in percentage or absolute number of BECs in pLNs was found (B). A second analysis of the same data revealed that there are about 2.5 times less 7AAD+ BECs in Nef+ mice than in control animals (C), which suggests a defect in or protection from apoptosis of these cells in transgenic mice. ns: non significant, as determined by a paired Student t test.

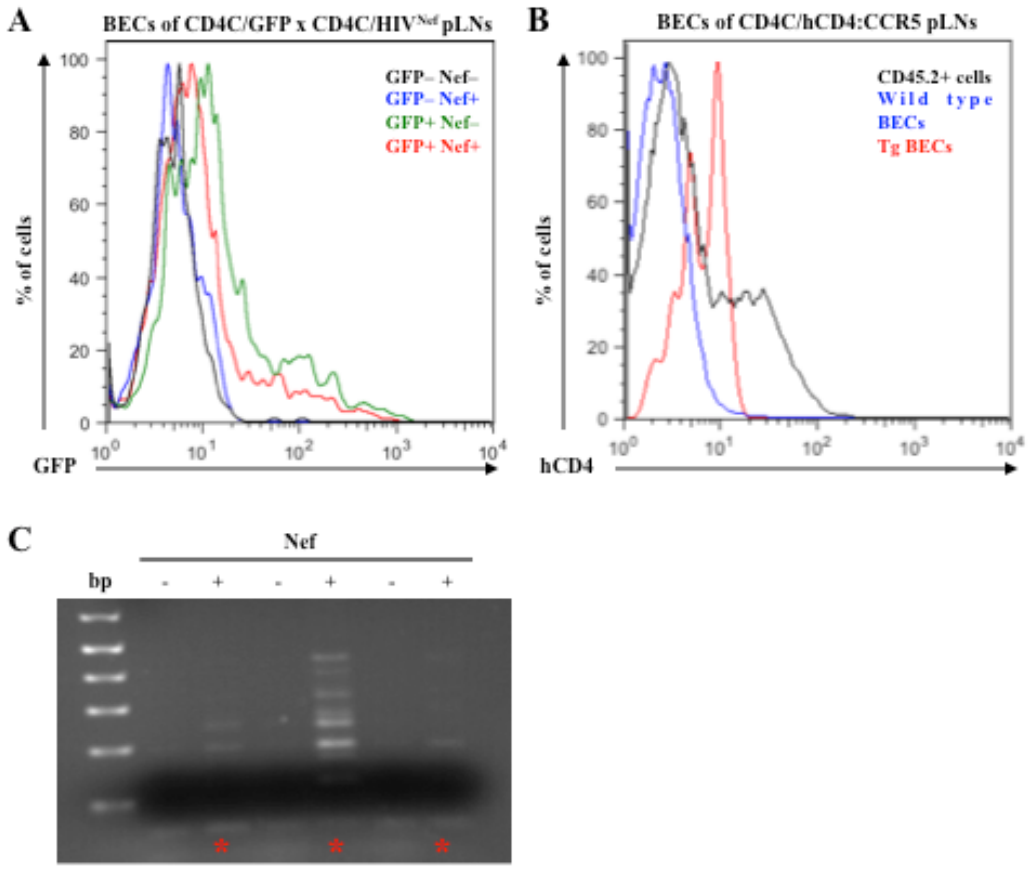


Figure 21: Expression of Nef in BECs. A cell suspension from lymph nodes of CD4C/GFP x CD4C/HIV^{Nef} reporter mouse line was analyzed (A). The flow cytometry profile shows the GFP signal in BECs from wild type mice (black line), from Nef+ only mice (blue line), from GFP+ mice (green line), and from GFP+ Nef+ mice (red line). As mentioned previously, CD45.2– CD31+ gp38– cells were considered to be BECs. The CD4C/hCD4-CCR5 mouse line was also used to verify the activity of the CD4C promoter in BECs (B). A representative flow cytometry profile is shown (n=3) with the hCD4 signal in wild type C3H lymph node cell suspension (blue line, negative control), in CD45.2+ cells (black line, positive control), and in BECs (red line). To assess the expression of Nef in BECs, an RT-PCR on sorted BECs was performed (C). Red stars indicate samples containing Nef. All come from Nef-expressing animals. bp: DNA base pair ladder.

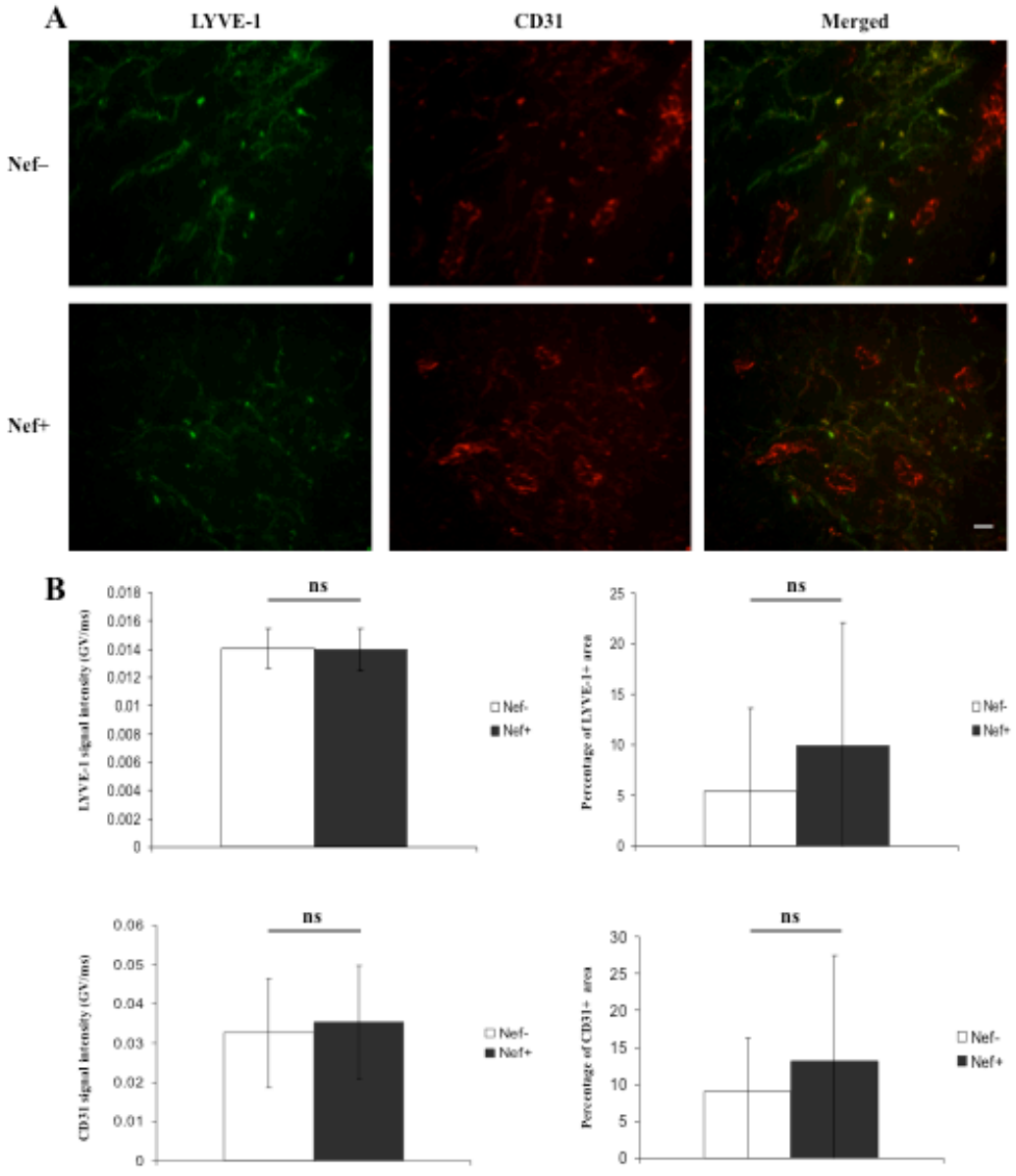


Figure 22: LEC population in the medulla. To identify the LECs of the medullary sinuses, a LYVE-1 and CD31 co-staining was performed on 5µm-thick sections of frozen pLNs and the medulla was imaged (A). Representative images of Nef– (n=7) and Nef+ (n=10) animals are shown. The intensity and area for both the LYVE-1 and CD31 signals were measured (B). No significant differences were seen, as determined by a paired Student t test. Scale bar: 50 µm. ns: non significant.

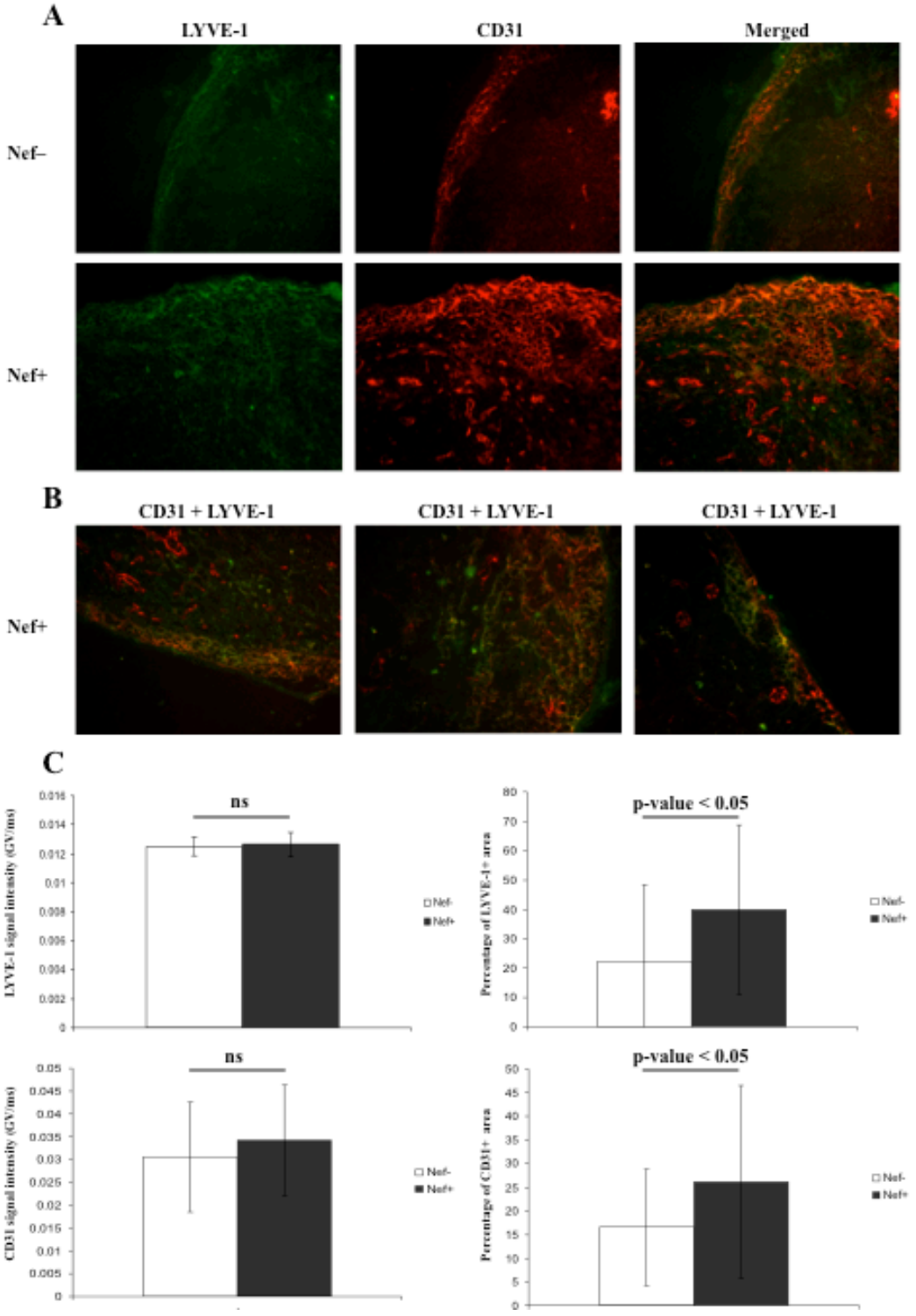

A
LYVE-1
CD31
Merged
Nef–
Nef+
B
CD31 + LYVE-1
CD31 + LYVE-1
CD31 + LYVE-1
Nef+
C
ns
LYVE-1 signal intensity (GV/ms)
Nef-
Nef+
p-value < 0.05
Percentage of LYVE-1+ area
Nef-
Nef+
ns
CD31 signal intensity (GV/ms)
Nef-
Nef+
p-value < 0.05
Percentage of CD31+ area
Nef-
Nef+

Figure 23: LEC population of the subcapsular sinus. A CD31 and LYVE-1 co-staining was also used to study the LECs present in the SCS of pLNs (A). Representative images of Nef– (n=5) and Nef+ (n=8) animals are shown. This revealed an expansion of LECs in Nef+ animals compared to controls. This expansion varied greatly from one pLN to another, as shown in (B). There was no significant difference in the intensity of signal of CD31 and LYVE-1, but a significant 1.78- and 1.58-fold increase in LYVE-1 and CD31 signal area, respectively (paired Student t test). ns: non significant.

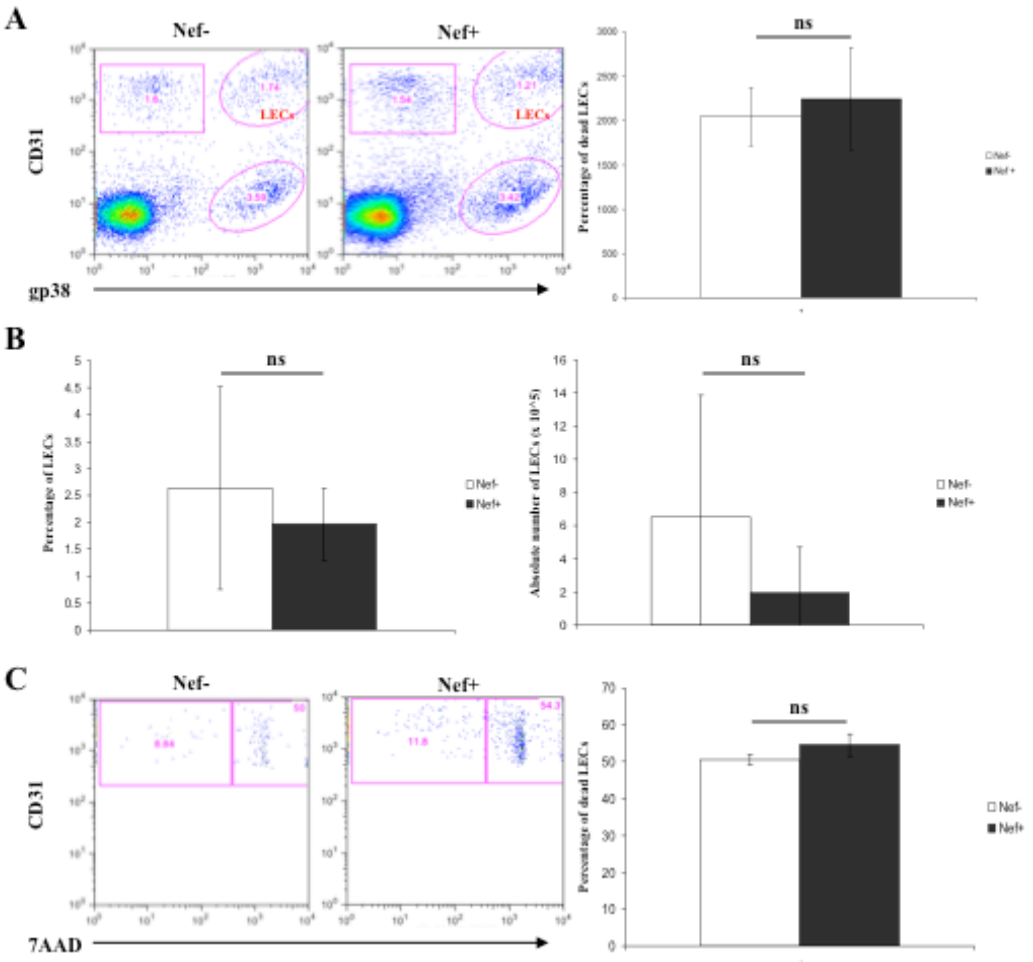


Figure 24: Expansion of LECs. A flow cytometry analysis on total pLNs was performed to confirm the immunohistochemistry results (A). CD45.2– CD31+ gp38+ cells were considered to be LECs, as described previously (Link et al, 2007). The flow cytometry profiles shown are representative of Nef– (n=9) and Nef+ (n=9) animals. No significant difference in percentage or absolute number of LECs in total pLNs was found (B). A second analysis of the same data revealed that there is no difference in the percentage of 7AAD+ LECs in Nef+ mice compared to control animals (C), which suggests that the expansion of the population, if confirmed, could be due to the proliferation of the cells. ns: non significant, as determined by a paired Student t test.

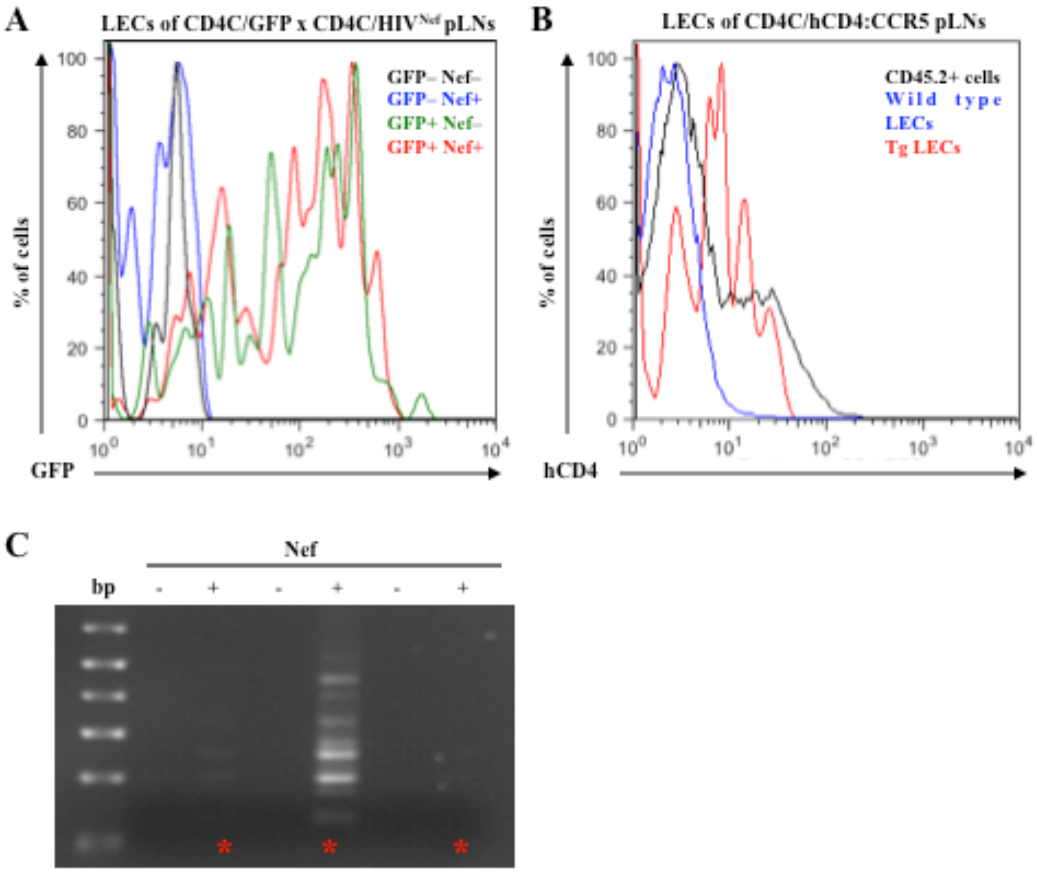


Figure 25: Expression of Nef in LECs. A cell suspension from lymph nodes of CD4C/GFP x CD4C/HIV^{Nef} reporter mouse line was analyzed (A). The flow cytometry profile shows the GFP signal in LECs from wild type mice (black line), from Nef+ only mice (blue line), from GFP+ mice (green line), and from GFP+ Nef+ mice (red line). As mentioned previously, CD45.2– CD31+ gp38+ cells were considered to be LECs. The CD4C/hCD4-CCR5 mouse line was also used to verify the activity of the CD4C promoter in LECs (B). A representative flow cytometry profile is shown (n=3) with the hCD4 signal in wild type C3H lymph node cell suspension (blue line, negative control), in CD45.2+ cells (black line, positive control), and in LECs (red line). To assess the expression of Nef in LECs, an RT-PCR on sorted LECs was performed (C). Red stars indicate samples containing Nef. All come from Nef-expressing animals. bp: DNA base pair ladder.

Discussion

Chapter 7: Discussion

7.1 Effects of Nef on FRCs

Our laboratory has developed a mouse model, the CD4C/HIV^{Nef} mouse, that presents many phenotypes associated with AIDS in humans, especially pediatric AIDS: among others, they die prematurely and present an increased activation and apoptosis of peripheral CD4+ T cells that leads to the depletion of these cells (Hanna et al, 1998; Weng et al, 2004). These phenotypes could at least partially be explained by defects in stromal cell function in lymph nodes, as they play key roles in CD4 T cell homeostasis. As such, we decided to study three of these populations – FRCs, BECs, and LECs – in the pLNs of this mouse model.

FRCs express Nef but this does not affect their functions in resting pLNs.

In our model, FRCs express Nef. The consistency of results between FACS analysis of different mouse lines and RT-PCR on sorted cells show that the human CD4 promoter is active in FRCs. To our knowledge, this is the first time that the expression of an HIV-1 protein has been reported in these cells.

Surprisingly, however, we observed no loss of FRCs or of their functions in pLNs of transgenic CD4C/HIV^{Nef} mice. The population is maintained, both in percentage and absolute number of cells. The structure of the reticular network is also preserved, as is the production of chemokines and of IL-7. This suggests that, in FRCs, Nef does not interact with proteins in a significant way or that, if it does, redundancy of function can compensate for this perturbation. To this day, few direct interaction partners of Nef are known. The only host protein that has been shown to bind to Nef with high affinity is the kinase Hck (Lee et al, 1996). Nef

mediates most of its effects, such as the downregulation of CD4 or MHC I, by forming complexes that cause abnormal interactions between host proteins to favor virus replication (Foster et al, 2011). While CD4 and MHC I downregulation are well understood, the components of these complexes are mostly unknown. Hck is expressed in monocytes and would therefore not be present in FRCs. Another protein, the p21-activated kinase 2 (PAK2), is also known to interact with Nef and is ubiquitously expressed (Jaffer and Chernoff, 2002). The interaction of PAK2 with Nef leads to an increased autophosphorylation of the kinase. This Nef-mediated activation of PAK2 has been shown to decrease apoptotic cell death and to promote the release of viral particles (Wolf et al, 2001). Nef alone might not induce significant changes in FRCs but, in the context of an HIV-1 infection, PAK2 activation could promote the survival of infected FRCs, which would then become a reservoir of virions. Because of their close contacts with them, this could increase infection of CD4+ T cells in *trans*.

Furthermore, Nef is known to induce a downregulation of MHC I at the cell surface. FRCs are involved in the induction of antigen-specific T cell tolerance, as they present various peripheral tissue-restricted antigens to naïve CD8+ T cells and induce their proliferation (Fletcher et al, 2010). While this was not tested here, Nef could perturb this particular function of FRCs, leading to defects in peripheral tolerance.

Nef is myristoylated post-transcriptionnally and is therefore localized to membranes, at least transiently. In fact, all of its functions, except the activation of Hck, are dependent of this myristoylation (Foster et al, 2011). A recent study by Kammula et al (2012) identified new membrane proteins that interact with Nef. Their study focused on targets found in the brain, but they did identify integrin β1, which is also expressed by FRCs (Fritz and Gommerman, 2011). However, the consequences of this interaction were not clear.

Another aspect to consider is that this study was done on resting pLNs, as the phenotypes mentioned above develop with the expression of only one HIV-1 gene, with no virus present, and the absence of an immune response against HIV-

1 (Hanna et al, 1998). It has been shown that, upon immunization with OVA, CD4C/HIV$^{Nef,\ Env,\ Rev}$ mice fail to develop germinal centers and an antigen-specific antibody maturation (Poudrier et al, 2001), which shows defects in the development of acquired immune responses. It may be that Nef affects the FRCs of CD4C/HIV^{Nef} mice only in the event of an immune response. This was not studied here, but assuredly needs to be. For example, FRCs upregulate MHC I upon immune stimulation (Fletcher et al, 2010). This could be hampered by Nef.

The active human CD4 promoter also marks FRCs as potential direct targets of HIV-1 infection in humans. Expression of the co-receptors CCR5 and CXCR4 was not assessed, but a dysfunction of FRCs would have severe consequences on CD4+ T cell homeostasis and on the development of acquired immune responses. Zeng and colleagues (2012c) have already highlighted that a damaged reticular network, normally produced by FRCs, limits the effectiveness of HAART therapy. As such, investigating whether or not they can indeed be infected by HIV-1 is of utmost importance, and could lead to changes in therapy.

Fibrosis does not develop in the pLNs of CD4C/HIV^{Nef} mice, despite an increased proportion of Treg.

In Rhesus macaques infected with SIV, the increase of the proportion of Treg among total CD4+ cells leads to the development of fibrosis, which disrupts the RN. This prevents contact between CD4+ T cells and FRCs and blocks their access to survival factors: CD4+ T cells do not have access to the IL-7 produced by the FRCs while the stimulation of the LTβ-receptor of FRCs by the $LT\alpha_1\beta_2$ present at the surface of CD4+ T cells is impeded (Zeng et al, 2011 and 2012a). In our mouse model, a very severe loss of CD4+ T cells and a 4-fold increase of the Treg proportion in pLNs are present. However, no fibrosis seems to develop and the structure of the RN is maintained.

Zeng and colleagues have proposed a model in which Treg produce TGF-β1, which activates collagen production by fibroblasts of the pLN (2011). In our Tg mice, this process appears to be blocked. Infection with HIV-1, or SIV, leads

to chronic inflammation, which promotes the development of fibrosis. The CD4C/HIV^{Nef} mice do not present this chronic inflammation (Hanna et al, 1998). This could explain why fibrosis does not develop in their pLNs. In addition, if FRCs are indeed infected by HIV-1, they could be affected by viral proteins other than Nef, which could trigger their loss or dysfunction, therefore "leaving room" for the development of fibrosis triggered by an increase of TGF-β1 levels. TGF-β1 levels in pLNs were not measured in this study, and this hypothesis has yet to be investigated. Determining whether Nef is responsible for the development of fibrosis is important as fibrosis creates permanent lesions in pLNs of human AIDS patients and limits the efficiency of anti-retroviral therapy (Zeng et al, 2012c).

$LT\alpha_1\beta_2$ levels are maintained despite the severe loss of CD4+ T cells.

FRCs depend on $LT\alpha_1\beta_2$ to function correctly. Stimulation of the LTβ-receptor by $LT\alpha_1\beta_2$ is required for their production of RN and of CCL19 and CCL21 (Ngo et al, 1999). The absence of apparent disruption of the FRC population raised the question of their access to this key factor. $LT\alpha_1\beta_2$ is produced mainly by CD4+ T cells and FRCs gain access to it when they come into contact with T cells migrating through the pLN, and depletion of CD4+ T cells by blocking antibodies, HIV-1 infection, and other immunodeficiency conditions lead to a loss of FRCs and RN (Zeng et al, 2012a). As CD4+ T cells are severely depleted in transgenic mice, we expected to see much lower levels of $LT\alpha_1\beta_2$ in their pLNs. However, RT-PCR on cDNA obtained from total pLNs revealed no difference in $LT\alpha_1\beta_2$ levels in Tg mice compared to wild type controls. In five-month old transgenic mice, about 90% of CD4+ T cells have been depleted. It is unlikely that the remaining cells are able to produce enough $LT\alpha_1\beta_2$, and come into contact with enough FRCs, to support the stromal cells. This suggests that another cell population is able to compensate for the loss of CD4+ T cells and provides the FRCs with sufficient $LT\alpha_1\beta_2$ to maintain their function.

Lymphoid Tissue Inducer cells (LTi) also express $LT\alpha_1\beta_2$, both in the embryo and in the adult, and have been shown to interact with VCAM-1+ stromal cells (Kim et al, 2007b). Like in the embryo, stimulation of the LTβ-receptor present on the stromal cells by the $LT\alpha_1\beta_2$ present on LTi has been shown to stimulate the production of chemokines by the stromal cells to attract T and B lymphocytes and maintain their proper segregation (Kim et al, 2007b). The LTi Kim and colleagues observed were located mainly at the interface between the T zone and B follicles, but were also found in the T zone itself. The VCAM-1+ stromal cells are therefore likely to be FDCs (at the T zone / B follicle interface) and FRCs (in the T zone), as both cells types are VCAM-1+ (Fritz and Gommerman, 2011).

Thus, LTi appeared to be very likely candidates. While no difference in the percentage of LTi was found in Tg mice, a significant 3.3-fold decrease of their absolute numbers was observed. Despite this loss, the proportion of LTi among total CD4+ CD11c– cells also drastically increased in Tg mice, from 0.68% to 4.45% (6.5-fold increase). It is possible that the remaining LTi produce enough $LT\alpha_1\beta_2$ to support the FRC population. Moreover, the LTi population in murine pLNs is heterogeneous, with about two thirds of the cells being CD4–, although no difference in function has been observed between these two types (Kim et al, 2007a). These particular CD4– LTi were not studied in our model. It is conceivable that they would also contribute to the production of $LT\alpha_1\beta_2$. RT-PCR on sorted CD4– and CD4+ LTi is needed to confirm this hypothesis. Kim and colleagues located both CD4– and CD4+ LTi at the T zone / B follicle interface and in the T zone (2007a), but their exact location in pLNs of our Tg mice should also be assessed, to determine whether a significant interaction between FRCs and LTi is possible. In addition to CD4+ T cells and LTi, lymphotoxins are expressed by activated B lymphocytes and NK cells (Fu et al, 1999; Ware, 2005; Heilenwalder et al, 2008). However, it is unlikely that these cells can support the FRCs: B cells only come into contact with them when they migrate to B follicles, and NK cells represent barely 0,2%-0,4% of the total cells in resting pLNs

(Bajénoff et al, 2006). Therefore, in the absence of CD4+ T cells, LTi are the designated source of $LT\alpha_1\beta_2$ in pLNs.

Indeed, Ngo and colleagues (2001) have demonstrated that production of CCL19 and CCL21 in lymph nodes, which is induced by $LT\alpha_1\beta_2$ (Ngo et al, 1999), does not depend on T cells or B cells. Gp38 expression also occurred independently of B cells. The authors used BCR-/- and LTα-deficient mice, which do not develop lymph nodes, when measuring gp38-expression. They therefore could not conclude on the production of gp38 in the absence of T cells, but our results indicate that, in pLNs, T cells are not required either.

Furthermore, in pLNs, the production of CCL19 and of CCL21 is provided by FRCs. The results Ngo and colleagues obtained also suggest that the function of the FRCs is maintained even in the absence of lymphocytes. This is an argument in favor of LTi being able to support them.

LTi are potential HIV-1 targets in humans.

In humans, all LTi appear to be CD4– or $CD4^{low}$ (Kim et al, 2009). However, they have been shown to express CXCR5 mRNA (Kim et al, 2009) and murine CD4+ LTi also express CXCR5 and CCR4 (Lane et al, 2008). Human LTi are therefore a potential direct target of HIV-1, although their actual infection by the virus has not yet been reported.

Because of the central role they play in maintaining pLN architecture, their loss or a perturbation of their function would only precipitate the depletion of FRCs, and of CD4+ T cells, observed in humans. Human LTi being CD4– means that the human CD4 promoter would not be active in the LTi of our Tg mice. This population may therefore be preserved by not expressing Nef in our model, thus preventing the loss of FRCs. However, that some human LTi are $CD4^{low}$ could explain why we still see a depletion of about 30% of CD4+ LTi in our mice: the human CD4 promoter could be moderately active in these cells, and Nef expression could lead to their death. The next step in explaining the

preservation of FRCs in Tg pLNs would be to assess the specific expression of $LT\alpha_1\beta_2$ by CD4+ and CD4– LTi, as well as remaining CD4+ T cells.

Whether LTi express Nef in our model should also be investigated. And, like for FRCs, it is critical to discover whether or not LTi are indeed infected by HIV-1 in humans, as this would have drastic consequences on pLN functions and the immune system.

Nef is required, but possibly not sufficient, to induce a perturbation of the pLN structure in mice.

Our laboratory also developed a CD4C/HIV^{WT} mouse, which expressed all of the HIV-1 genes. Its LNs presented hypocellularity and a disrupted architecture (Hanna et al, 1998). This shows that murine LNs can be affected by HIV-1 when it is expressed under the control of the human CD4 promoter. While there is a clear loss of CD4+ T cells, the normal structure of the pLNs of CD4C/HIV^{Nef} mice suggests that Nef alone is not sufficient to induce these phenotypes. It may not even be involved at all in the disruption of the pLN architecture.

We could find no structural differences between wild type and Tg CD4C/$HIV^{\Delta Nef}$ mice, which suggests that Nef is indeed required for the development of LN structure disruption. However, since it is not seen in CD4C/HIV^{Nef} mice, we hypothesized that Nef may be required, but not sufficient. Our laboratory also developed a CD4C/$HIV^{Nef, Rev, Env}$ mouse. These mice develop a severe disease and die faster than CD4C/HIV^{Nef} mice. It is unlikely that Rev and Env would directly contribute to the progression of the disease. We therefore asked whether levels of Nef in these mice differed from those of HIV^{Nef} mice, and the difference in phenotypes were dose-dependent. To our surprise, there was no significant difference in the amount of Nef protein in these two mouse lines. This discrepancy is not yet explained. It could be an artifact due to increased hypocellularity that would make RN fibers appear more disrupted in CD4C/$HIV^{Nef, Rev, Env}$ mice, or a founder effect. In fact, preliminary data (not shown) obtained by flow cytometry on total pLNs of CD4C/$HIV^{Nef, Rev, Env}$ mice

were inconclusive: the first group (three Tg mice and two controls) showed a decrease in absolute numbers of FRCs as well as endothelial cells, but no difference in the percentage of these cells in Tg pLNs. The second group (again three Tg mice and two controls that were the same age as the mice of the first group) showed an increase in percentage of FRCs and endothelial cells that was only significant for the latter, and no difference in total numbers of cells. Thus, the pLN stroma could also be preserved in these mice. Understanding why the disease progresses faster in CD4C/HIV$^{Nef, Rev, Env}$ mice than in CD4C/HIV^{Nef} mice is crucial to our comprehension of the exact effects of Nef in these models.

Of note, the HIV-1 Env gene encodes the precursor protein gp160 that is then cleaved by a host protease into gp120 and gp41 (Willey et al, 1988). It would be of interest to investigate whether this cleavage occurs in our transgenic mice, as gp120 has been shown to induce endothelial cell apoptosis and to increase the permeability of blood vessels (Mu et al, 2007). These effects could explain, in part, the differences seen between CD4C/HIV^{Nef} and CD4C/HIV$^{Nef, Rev, Env}$ mice mentioned above.

In summary, we propose here that Nef does not affect the FRC population in CD4C/HIV^{Nef} mice despite being present in these cells. This could be explained by the preservation of LTi that produce enough $LT\alpha_1\beta_2$, and thus trigger sufficient LTβ-receptor signaling in FRCs, to support them. In human patients, LTi are most likely infected by HIV-1 and depleted, which, in addition to the depletion of CD4+ T cells, leads to the loss of stromal cells and RN observed. Nevertheless, we cannot exclude that defects in FRC functions could arise after immunization of the mice, and this needs to be tested.

7.2 Effects of Nef on endothelial cells

BECs and LECs both express Nef RNA.

We found that both BECs and LECs express Nef RNA. Infection of BECs by HIV-1 has already been reported in the vessels of several organs: brain capillaries (Moses et al, 1993), kidney glomeruli (Green et al, 1991), hepatic sinusoid (Lafon et al, 1993) and bone marrow (Moses et al, 1996). Nef has also been linked to the development of pulmonary arterial hypertension in AIDS patients, although, in this case, BECs seem to uptake circulating Nef instead of being infected by HIV-1 (Voelkel et al, 2008). Thus, the infection of BECs depends greatly on the organ considered and on the specific features of the associated vasculature. To this day, there is no evidence of a direct infection of BECs in the pLN. While not definite, our results suggest that this could be the case, as they imply that human BECs express CD4.

Direct infection of LECs by HIV-1 has not been reported, although Tat has been reported to stimulate angiogenesis (Rusnati and Presta, 2002) and increases leaking from lymphatic vessels (Zhang et al, 2012). Our results suggest that, like BECs, they could be infected. It is also possible that their susceptibility to HIV-1 infection would vary according to the organ considered.

The expression of Nef by BECs and LECs of pLNs leads to their expansion.

IHC stainings have shown expansion of both BECs and LECs in pLNs of Tg mice, but only in specific areas of the organ. For BECs, this expansion is localized to the medullary blood vessels. In the case of LECs, only the subcapsular sinus seems concerned. Moreover, only parts of these structures are affected, which indicates that these modifications are focal.

Endothelial cell proliferation associated with HIV-1 infection has been mostly studied in the context of Kaposi's sarcoma. However, the development of

this cancer depends on infection with Human Herpesvirus 8 (Moore and Chang, 1998), which is not present in our mice. Moreover, Kaposi's sarcoma induces a reprogramming of the affected endothelial cells: they acquire an intermediary phenotype, with BECs developing LEC characteristics, and vice versa (Hong et al, 2004; Liu et al, 2010). The expanding cells we observed in CD4C/HIV^{Nef} mice seem to retain their respective characteristics, as BECs remain vWF+ and LECs remain LYVE-1+. These specific markers are both downregulated in Kaposi's sarcoma cells (Hong et al, 2004; Liu et al, 2010). The lesions that develop in the pLNs of these Tg mice are therefore of another type.

Nef has been shown to induce plexiform-like vascular lesions in the lungs of SHIV infected macaques (Marecki et al, 2006) similar to those seen in HIV-related pulmonary hypertension. These lesions include endothelial cell proliferation that obstructs the lumen, which resembles what we found in the medulla of our Tg mice. The cells that Marecki and colleagues observed obstructing the lumen were Factor VIII (vWF) positive, as are the cells that we saw. Marecki and colleagues propose that the presence of Nef in BECs coupled with loss of immune function leads to the apparition of dysfunctional, apoptosis-resistant BECs that secrete increased amounts of cytokines and growth factors, which leads to their proliferation. Our results suggest that there is indeed a defect in apoptosis in BECs in the pLNs of Nef-expressing mice, although we were not able to verify whether these abnormal BECs actually proliferate or express Nef. Marecki and colleagues also propose that BECs uptake Nef. We could not assess the presence of the protein in BECs because of an insufficient number of cells, but it is entirely conceivable that an uptake of Nef could happen. Our results indicate that, in addition to this, BECs also directly express Nef RNA. Nef could therefore affect BECs by different processes. If they can happen simultaneously, they could greatly increase the perturbations induced by Nef in these cells.

Interestingly, two other HIV-1 proteins have been linked with BEC dysfunction. Gp120 and Tat can both induce apoptosis of BECs in the human umbilical vein (Huang et al, 2001; Jia et al, 2001), the brain microvasculature (where they alter the blood-brain barrier, Kanmogne et al, 2002; Kim et al, 2003),

and in the lung (Kanmogne et al, 2005; Park et al, 2001), as well as increase the permeability of blood vessels. Gp120 can act as a soluble factor, virion-associated or membrane-bound (Huang et al, 2001), while Tat is secreted by infected cells and taken up by normal ones. Tat also stimulates growth of Kaposi's sarcoma cells and angiogenesis (Mu et al, 2007). Thus, Nef contributes to the endothelial disruption that occurs during HIV-1 infection. While Marecki and colleagues (2006) showed that it has effects on BECs in the lung, we show that it also acts in pLNs.

Like BECs, LECs have been mostly studied in the context of HIV-1 infection for their involvement in Kaposi's sarcoma, which is not the type of lesion we observed. While still preliminary, our results suggest that the LECs of the pLNs of Tg mice do not have a defect in apoptosis. Thus, their expansion could be due to proliferation.

In normal pLNs, endothelial cell proliferation depends on vascular endothelial growth factors (VEGFs, Webster et al, 2006), with FRCs being the main source of both VEGF-A and VEGF-C (Chyou et al, 2008, Malhotra et al, 2012). Based on previous results, we would expect the production of VEGFs by FRCs to not be affected in Nef-expressing pLNs. However, this has to be confirmed. The integrin α 7-expressing pericytes (IAPs) are cells found around blood vessels of the T zone and the medulla that also express VEGF, although at lower levels than FRCs. They are also strongly positive for α-smooth muscle actin (Malhotra et al, 2012). Interestingly, when identifying abnormally structured vessels in the medulla of Tg pLNs, we observed that some of them presented a dim or not-continuous α-smooth muscle actin signal surrounding the endothelial cells. Nevertheless, the vessels that presented an expansion of endothelial cells did not always present this defect in α-smooth muscle actin signal. Little is known about the function of the IAPs and it would be interesting to study them in greater detail, as our results show that they may be affected in the presence of Nef, but other factors must be at play.

Another possible way to induce a proliferation of endothelial cells would be through the activation of the Notch signaling pathway. This phenomenon is

seen in Kaposi's sarcoma, for example (Liu et al, 2010), and Nef may interfere with Notch signaling to cause HIV-associated nephropathy (Zuo et al, 2006; Sharma et al, 2010 and 2013).

Nef is sufficient to induce HIV-associated nephropathy (Hanna et al, 1998). In a transgenic rat model of this disease, Sharma and colleagues observed phenotypes similar to those known to be caused by Nef, such as proliferation and dedifferentiation of podocytes (Sharma et al, 2010; Zuo et al, 2006). They also showed that the Notch signaling pathway was activated in these cells. *In vitro*, podocytes treated with Nef also proliferated. Notch inhibitors were able to block this effect (Sharma et al, 2013). These results suggest that Nef and the Notch signaling pathway may interact to induce HIV-associated nephropathy.

Moreover, another study performed in our laboratory strongly supports the idea that Nef may interact with the Notch1 pathway (see part 2 of this thesis). Thus, it appears that Nef may interfere with the Notch signaling pathway, which is known to induce endothelial cell proliferation (reviewed in Chappell and Bautch, 2010). Notch signaling should therefore be measured in our Tg mice, along with VEGF-A and VEGF-C levels.

The expansion of BECs and LECs is focal.

It is striking that the abnormal, expanding BECs seem to appear in the medullary blood vessels but not in the HEVs of the T zone. HEVs are specialized in the extravasation of lymphocytes and display specific adhesion markers, such as PNAd (von Adrian and Mempel, 2003). Lymphocyte entry into the pLN from the medulla is also possible, but much less frequent, and medullary blood vessels mostly comprise afferent arterioles and efferent venules (von Adrian and Mempel, 2003; Pfeiffer et al, 2008). Our results indicate that, in addition to these functional differences, structural ones exist between these two blood vessel types. However, few studies comparing them exist.

Pfeiffer and colleagues have analyzed the junctions found between endothelial cells of pLNs (Pfeiffer et al, 2008). Apart from PNAd expression, the

only difference they found between HEVs and medullary blood vessels was in the expression of claudins. All claudins were absent from HEV, while medullary blood vessels expressed only claudin 5. Claudins are required for the formation of tight junctions (Furuse and Tsukita, 2006). Their absence suggests that HEVs lack tight junctions and is in agreement with their support of the paracellular diapedesis of lymphocytes. Medullary blood vessels express claudin 5, which would indicate that the BECs of these vessels form a tighter barrier for lymphocytes. This is understandable as these vessels are not a site of entry or exit from the pLN for lymphocytes (reviewed in von Adrian and Mempel, 2003). Interestingly, Pfieffer and colleagues observed PNAd+ vessels in the medulla of pLNs, which marks them as HEVs. They showed that both B and T lymphocytes could enter the pLN through these medullary HEVs, albeit less efficiently than through T zone HEVs. Our own observations revealed that only certain vessels of the medulla presented expanding cells, and T zone HEVs never seemed affected. It is therefore possible that the preserved vessels of the medulla were these medullary HEVs, and that the affected vessels were either the afferent arterioles or efferent venules. A PNAd staining is needed to confirm this hypothesis. While they give us an idea why only some blood vessels were affected in the pLNs of Nef-expressing mice, these results do not explain how BECs started expanding.

We have shown that BECs express low levels of Nef RNA. It could be that the cells expressing higher levels of the HIV-1 protein would be affected and start behaving in a way similar to what Marecki and colleagues described to form plexiform-like lesions. This phenotype could be cell-autonomous or involve a paracrine effect. A protocol to isolate these cells is needed to study them in greater detail.

In the pLNs of Nef-expressing mice, LECs expansion was localized to the SCS. In the most severe cases, expanding cells reached into the medulla and no clear separation between the two compartments could be observed, so we cannot completely exclude that LECs of the medulla may also proliferate. Nevertheless, the expansion started in the SCS. Our laboratory previously reported an accumulation of dendritic cells in the SCS of CD4C/HIV$^{Nef, Rev, Env}$ mice, which

reflects a defect in cell migration through the pLN (Poudrier et al, 2003). An obstruction of the lumen of the SCS by expanding LECs could explain this defect. Since BEC expansion indicates that there are differences between HEVs and medullary blood vessels, the pattern of LEC expansion reveals differences between the SCS and the medullary sinuses. LECs from the SCS are the first endothelial cells encountered by migrating dendritic cells when they arrive at the pLN via the afferent lymphatic vessels. They also allow for sampling of lymph-borne antigens by the macrophages located just beneath the SCS, and the conduits surrounded by FRCs connect to the SCS (Ushiki et al, 1995). Meanwhile, the medullary sinuses specialize in the egress of immune cells (reviewed in von Adrian and Mempel, 2003). The different functions of the two compartments suggest different patterns of expression of chemokines and adhesion proteins. However, the study by Pfeiffer and colleagues (2008) revealed no differences in the cell-cell junctions.

Like BECs, LECs of CD4C/HIV^{Nef} mice express Nef RNA. It is conceivable that only the cells expressing higher levels of Nef would start expanding. As for BECs, this could be cell-autonomous, or the expanding cells could stimulate the expansion of their neighbors by a paracrine effect. We need to assess the production of growth and proliferation factors such as VEGFs in the total pLN and in these particular cells to evaluate this. It seems unlikely that this phenotype would be triggered by the interaction of the endothelial cells with Nef-expressing T cells, as HEVs and medullary sinuses, where this contact is more frequent, are not affected.

The CD4C/HIV^{Nef} mice display cardiac and renal diseases (Hanna et al, 1998). It would be informative to investigate whether the blood and lymphatic vessels in these organs also present an expansion of endothelial cells, or whether it is confined to pLNs. Interestingly, while the blood vessels themselves were not studied at the time, the lesions observed in both cardiomyocytes and glomeruli were also focal (Kay et al, 2002; Zuo et al, 2006). Understanding why Nef affects some cells of a given population, but not all, would be key to understanding the HIV-1-associated organ diseases.

Thus, the exact causes of the expansion of BECs and LECs in CD4C/HIV^{Nef} mice remain unknown. Blocked vessels and sinuses would disrupt the migration of immune cells to and from the lymph node, thereby hindering their access to survival factors and preventing contact between them. In HIV-infected patients, this would further compromise the survival of these immune cells, as well as the development of acquired immune responses.

Part 2: Expression of the HIV-1 protein Nef in basal keratinocytes induces the development of an atopic dermatitis-like disease.

Introduction

Chapter 8: Organization of murine skin

8.1 Structure of adult mouse skin

The second part of this project focuses on the effects of Nef on another organ, the skin. Mouse skin greatly resembles that of humans. It is composed of three layers: the hypodermis, dermis, and epidermis. The hypodermis, the innermost layer, consists mostly of adipose tissue. The dermis contains fibroblasts and a specialized extracellular matrix made of collagens, elastin, proteoglycans, and fibronectin, among other components. This matrix confers its mechanical properties to the skin. Blood and lymphatic vessels are also present in the dermis, as well as sensory nerve endings. The main cells found in the epidermis, the outermost layer, are keratinocytes, which act as a barrier between the body and its environment. Melanocytes and Langerhans cells can also be found in the epidermis. Lastly, sebaceous and sweat glands, as well as hair follicles, grow from the epidermis into the dermis (Wong and Chang, 2009). Figure 26 represents the anatomy of the skin.

Like the skin itself, the epidermis can be divided into several layers: the stratum basale, the innermost layer, the stratum spinosum, the stratum granulosum, and the stratum corneum. It is a self-renewing tissue. It comprises stem cells that are found in the bulge of hair follicles. These stem cells give rise to the epidermis, but also to hair follicles and sebaceous glands (Taylor et al, 2000; Oshima et al, 2001). The daughter cells they produce migrate up the hair follicle and populate the stratum basale, or basal layer, of the epidermis. These basal keratinocytes undergo a limited number of divisions before starting to

differentiate. They migrate to the next epidermis layer, the stratum spinosum or spinous layer, which causes them to stop proliferating and to undergo terminal division (Jones and Watt, 1993; Jones et al, 1995). The cells in the uppermost layer, the stratum corneum, are dead and enucleated cells that are eventually shed from the skin. Regulation of the production of new cells and of differentiation is therefore essential to maintain a healthy, functioning epidermis (Fuchs and Raghavan, 2002). Figure 27 represents this stratification of the epidermis. Keratins are intermediate filaments that can make up to 85% of fully differentiated keratinocytes (Fuchs, 1995). They attach to desmosomes to form cell-cell junctions and provide mechanical strength to the cells, and therefore to the skin (Green et al, 1992; Fuchs, 1995).

The different layers of keratinocytes can be distinguished by their expression of keratins. The basal layer expresses keratins 5 and 14, which are restricted to cells that maintain their ability to proliferate (Byrne et al, 1994). As they move up the layers of the epidermis and differentiate, keratinocytes downregulate keratins 5 and 14 and start expressing keratins 1 and 10. (Fuchs and Green, 1980). Keratinocytes of the upper epidermal layers also express other differentiation markers such as involucrin and loricrin (Dotto, 1999).

The Wnt and Notch signaling pathways are both implicated in the differentiation of the epidermis, with Wnt being required for the separation of the hair lineage, which will give rise to hair follicles, and the epidermal lineage (Powell et al, 1998; Millar et al, 1999; Fuchs and Ranghavan, 2002). The importance of Notch signaling in the skin will be detailed below.

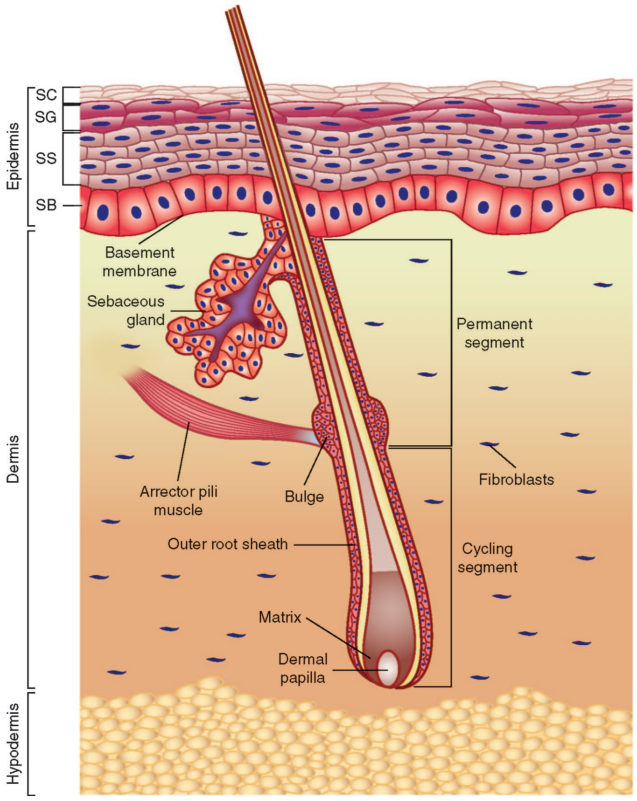


Figure 26: Anatomy of the skin. Adapted from Wong and Chang, 2009. The epidermis itself is divided into four layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC).

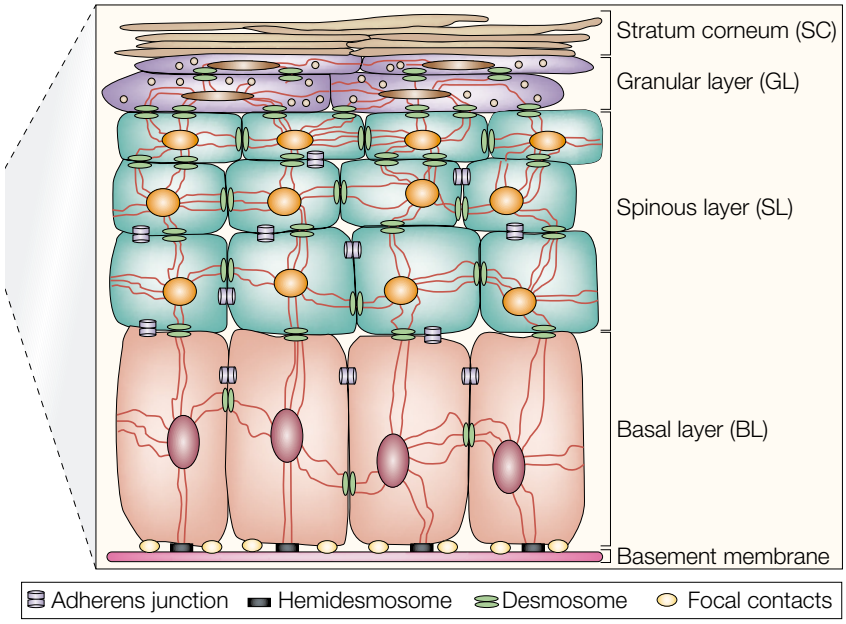


Figure 27: Stratification of the epidermis. Adapted from Fuchs and Raghavan, 2002. The epidermis is separated from the dermis by a basement membrane. The cells of the basal layer divide a limited number of times before undergoing differentiation and moving to the upper layers of the epidermis. When they reach the stratum corneum, they are dead and denucleated, and are eventually shed from the skin.

8.2 The Notch signaling pathway in mouse skin

8.2.1 The Notch signaling pathway

The Notch signaling pathway is remarkably conserved in all metazoans (Artavanis-Tsakonas et al, 1999). In mammals, this family of genes encodes four type 1 transmembrane receptors, named Notch1 to 4. Five ligands have been identified: Jagged 1 and 2, and Delta-like 1, 3, and 4 (Artavanis-Tsakonas et al, 1999). Activation of this pathway is regulated by cell-cell interactions, in that the receptors present on one cell are activated by the ligands expressed by a juxtaposed cell. This triggers cleavage of the receptors. First, an ADAM metalloprotease releases the extracellular domain of Notch (Brou et al, 2000). Then, the γ-secretase cleaves the receptor within its transmembrane domain. The

Notch intracellular domain is then released into the cytoplasm and translocates to the nucleus. Once there, it associates with RBP-Jκ, named CBF1 in mammals. CBF-1 is a ubiquitous DNA-binding protein normally associated with a corepressor complex. It dissociates from this complex upon binding of the Notch intracellular domain. The two proteins then form a transcription activator complex and modulate the expression of Notch target genes such as the transcription factors of the HES and Hey (also called HERP) families (Iso et al, 2003; Lubman et al, 2004; Mammucari et al, 2005).

Of note, the extracellular domain of Notch also plays a role after its cleavage. Indeed, it can remain bound to the ligand and be internalized into the ligand-expressing cell by trans-endocytosis. It then participates in the activation of signaling pathways, possibly by inducing conformational changes of the ligand (Parks et al, 2000; Hansson et al, 2010).

8.2.2 Notch can act as an oncogene and as a tumor suppressor

Notch receptors are widely expressed in mammals and are heavily implicated in cell-fate decisions (stem cell maintenance and differentiation), proliferation, and apoptosis (Radtke and Raj, 2003). For example, Notch1 is necessary to T-cell lineage commitment and T cell maturation, as loss of its signaling leads to the generation of double positive T cells stuck in this developmental stage (Radtke et al, 1999). In addition, overexpression of Notch1 is involved in T-cell leukemia (Reynolds et al, 1987). Notch signaling is also abnormally activated in many neoplasms, such as mouse mammary tumors (Dievart et al, 1999), human breast cancers (Weijzen et al, 2002), colon adenocarcinomas and cervical carcinoma (Zagouras et al, 1995), and non-small cell lung cancer (Guo et al, 2012).

In the skin, Notch1 acts as a tumor suppressor, as mice with Notch1-deficient epithelium spontaneously develop basal cell carcinoma-like lesions (Nicolas et al, 2003). These lesions are caused by the downregulation of p21 and by the aberrant expression of Gli2, a downstream component of the Sonic-

hedgehog signaling pathway normally inhibited by Notch1 (Nicolas et al, 2003). Moreover, activation of Notch1 signaling reduces the growth of neuroendocrine tumors such as medullary thyroid tumors *in vitro* (Kunnimalaiyaan and Chen, 2007), and also induces cell-cycle arrest in small cell lung cancer cells (Sriuranpong et al, 2001). Thus, Notch signaling can have either tumor promoting or tumor suppressing functions. As mentioned, its role in the skin falls into the second category. We will now discuss it in further detail.

8.2.3 Role of the Notch signaling pathway in the epidermis

In mice, Notch 1 and Notch 2 are expressed in the spinous and granulous layers of the epidermis, as are their ligands Jagged 1 and Jagged 2 (Rangarajan et al, 2001). In humans, however, Notch1 is expressed in all epidermal cells. Notch2 is restricted to the basal layer, and Notch3 is found in the spinous and granulous layers (Lowell et al, 2000; Nickoloff et al, 2002). Despite these differences in expression patterns, Notch signaling plays the same role in both murine and human skin, and that is to induce differentiation (Lowell et al, 2000; Rangarajan et al, 2001, Nickoloff et al, 2002).

Notch signaling, and in particular Notch1 signaling, inhibits the proliferation of keratinocytes by upregulating the expression of p21 (WAF1, Rangarajan et al, 2001) and activating NF-κB (Nickoloff et al, 2002). It also increases the expression of differentiation markers such as keratin 1 and involucrin.

Thus, in the skin, Notch acts as a tumor suppressor. As mentioned above, mice with Notch1-deficient epithelial cells spontaneously develop basal cell carcinoma-like lesions because of aberrant Sonic-hedgehog signaling (Nicolas et al, 2003). In addition, Notch1 also inhibits Wnt signaling, which is involved in epidermal stem cell renewal and lineage selection (Zhu and Watt, 1996, 1999), and is implicated in the development of basal-cell carcinoma (Boonchai et al, 2000; Lo Muzio et al, 2002). Therefore, Notch1 signaling also promotes differentiation.

The Notch1 signaling pathway is activated by ligand-binding (Artavanis-Tsakonas et al, 1999), but Dotto (1999) has reported that calcium levels play an important role in keratinocyte growth and differentiation control. It has been known for decades that adding 0.12 to 2 mM of calcium to the culture medium induces keratinocyte differentiation *in vitro* (Hennings et al, 1980). I*n vivo*, an extracellular calcium gradient exists in the epidermis and shows an increase in concentration toward the stratum corneum (Menon et al, 1992; Forslind et al, 1997). Increased extracellular calcium concentrations induce the upregulation of p21, and promotes withdrawal from the cell cycle (Missero et al, 1995; Prowse et al, 1997). This effect is mediated by the calcium/Calmodulin dependent phosphatase Calcineurin. It dephosphorylates the nuclear factors of activated T cells (NFATs, Crabtree and Olson, 2002), which then induce the transcription of the *p21* gene (Santini et al, 2001).

Mammucari and colleagues (2005) have demonstrated that mice lacking the CnB1 Calcineurin subunit have significantly lower p21 mRNA levels after Notch1 stimulation compared to wild type mice. They also show that Notch1 directly activates this pathway, as Notch1 signaling leads to the inhibition of Calcipressin, a Calcineurin inhibitor, in a HES-1-dependent manner (Mammucari et al, 2005). Thus, through Calcineurin, calcium levels and Notch1 signaling are linked in the control of growth arrest and differentiation of mouse keratinocytes.

Chapter 9: HIV-1 skin pathogenesis

9.1 Skin diseases in AIDS patients

In addition to the effects it has on the immune system, HIV-1 infection is also associated with the development of many skin disorders. These disorders cause often-disfiguring lesions, and can thus severely undermine the patient's self-esteem and induce depression (Zalia et al, 1992). Moreover, some of them, like Kaposi's sarcoma, present a direct threat to the patient's survival. Table 4 recapitulates the most common skin diseases that develop in AIDS patients. The primary manifestations listed appear to be directly related to HIV-1 infection, while the secondary manifestations require infection with another pathogen.

By inducing the restoration of the immune system, highly active antiretroviral therapy has caused a decrease in the incidence of the secondary manifestations (Uthayakumar et al, 1997; Maurer et al, 2004; Maurer, 2005). The frequency of primary manifestations, however, did not change after the introduction of therapy (Maurer et al, 2004).

Moreover, a direct link between HIV-1 gene products and the development of these diseases has yet to be found (Cedeno-Laurent et al, 2011). Several factors are thought to be implicated in this process: the loss of a functional immune system (Tschachler et al, 1996), the shift to a Th2 cytokine profile (Amerio et al, 2001), and mimicry and expression of super-antigens and xenobiotics (Yarwood et al, 2000; Perl, 2003).

The study of these primary manifestations is complicated by the lack of animal models that faithfully reproduce the phenotypes seen in human patients (Cedeno-Laurent et al, 2011). Reid and colleagues have developed a transgenic rat model that does present skin lesions, such as hyperkeratosis and infiltration of mononuclear cells (Reid et al, 2001). Cedeno-Laurent and colleagues further studied this model and reported that these lesions developed into epidermal hyperplasia with hemorrhage and necrosis of keratinocytes (Cedeno-Laurent et al, 2009). They also recently reported that a newly-developed humanized mouse

model may prove useful to study HIV-associated skin diseases (Cedeno-Laurent et al, 2011), but more work is needed to fully elucidate the link between HIV-1 and these diseases.

Table 4: Skin diseases associated with HIV-1 infection. Adapted from Cedeno-Laurent et al, 2011. The percentages in parenthesis indicate the prevalence of primary manifestations in HIV-1/AIDS patients, when available.

Primary manifestations	Secondary manifestations	
	Infections	**Neoplasms**
- Seborrheic dermatitis (40% - 80 %) - Xerosis (20%) - Atopic dermatitis (30% -50%) - Eosinophilic folliculitis (seen almost exclusively in AIDS patients) - Psoriasis (2%, but more severe than in seronegative patients) - HIV-1 related pruritus - Drug induced	- Herpes simplex - Varicella-Zoster - HPV infection - Molluscum contagiosum - *S. aureus* infections - folliculitis - bullous impetigo - echtyma - Mycobacterial cutaneous infection - Bacillary angiomatosis - *P. aeruginosa* cutaneous infection - Candidiasis - Dermatophyte infection - Histoplasmosis - Cryptococosis - Pneumocystis	- Kaposi's sarcoma - T cell lymphoma - Basal cell carcinoma - Squamous cell carcinoma

9.2 Involvement of the Notch1 signaling pathway in skin diseases associated with HIV-1 infection

As previously mentioned, the inhibition of Notch1 signaling in the epidermis leads to the spontaneous development of basal cell carcinoma (Nicolas et al, 2003). Mice lacking epidermal Notch1 signaling also develop squamous cell carcinomas (Proweller et al, 2006). HIV-1-infected individuals have a higher risk of developing these cancers (Cedeno et al, 2011). In addition, Notch signaling is also decreased in psoriasis, which is characterized by aberrant keratinocyte

proliferation and lack of differentiation, as well as infiltration of leukocytes (Thélu et al, 2002). Atopic dermatitis is also prevalent in AIDS patients. Dumortier and colleagues (2010) were able to induce an atopic dermatitis-like disease in adult mice by inhibiting Notch1 and Notch2 signaling in basal keratinocytes.

These results suggest that inhibition of the Notch signaling pathways could occur in HIV-1-infected individuals, and cause some of the skin pathologies observed. A possible interaction between Notch and HIV-1 proteins has previously been reported in HIV-associated nephropathy. Indeed, the HIV-1 protein Nef causes a proliferation and dedifferentiation of podocytes (Zuo et al, 2006). Sharma and colleagues have showed that the Notch signaling pathway is activated in this disease *in vivo* (Sharma et al, 2010). *In vitro*, cultured podocytes treated with Nef also proliferated and presented in active Notch pathway. The use of Notch signaling inhibitors blocked the effects of Nef on the cells (Sharma et al, 2013). These results suggest that Nef could interact with Notch to induce HIV-associated nephropathy. Tat is also known to interact with the EGF-like repeats present in the extracellular domain of Notch receptors of mice and humans, and could modulate their activation (Shoham et al, 2003).

Given the many roles of Notch in the skin and other organs, studying the interactions between HIV-1 proteins and the Notch signaling pathways could therefore offer new insights into HIV-1 pathogenesis.

9.3 The rThy1/rtTA x TRE/HIV^{Nef} mouse

Our laboratory generated an rThy1/rtTA x TRE/HIV^{Nef} mouse. Nef expression is induced in the presence of doxycycline when the tetracycline-controlled transactivator (rtTA) protein, expressed under the control of the rat Thy1 promoter, binds the tetracycline response element (TRE). This system also has the advantage of being reversible (Mansui et al, 1998). Our laboratory has developed other inducible systems to study the effects of Nef on adult mice (Rahim et al, 2009). Our other transgenic mice expressed the protein before birth,

and an inducible construct ensured that the phenotypes observed were not caused by an effect of Nef on the development of the embryo (Rahim et al, 2009).

The induction of Nef expression led to the development of unexpected skin phenotypes, as described in the Results section (chapter 11).

9.4 Rationale for Master's project and hypothesis

9.4.1 Rationale

A group of rThy1/rtTA x TRE/HIV^{Nef} mice was exposed to doxycycline diluted in their drinking water. They developed an extensive hair loss and thickening of the skin. They also presented enlarged lymph nodes and spleens. Closer inspection of the skin revealed an atopic dermatitis-like disease. Such a disease can be induced in mice by inhibiting the Notch signaling pathway in basal keratinocytes (Dumortier et al, 2010). This unexpected result led us to think that we had in fact generated a mouse model of HIV-1-related atopic dermatitis. We sought to further characterize the phenotypes observed.

9.4.2 Hypothesis

Nef inhibits the Notch signaling pathway in basal keratinocytes, which leads to the development of an atopic dermatitis-like disease.

9.4.3 Objectives

Our first objective was to further characterize the rThy1/rtTA x TRE/HIV^{Nef} mouse model. We studied the skin phenotypes as well as peripheral ones. We compared our mice with the model developed by Dumortier and colleagues (2010) to ensure that the rThy1/rtTA x TRE/HIV^{Nef} mice did indeed develop an atopic dermatitis-like disease.

The second objective was to demonstrate that Nef does inhibit Notch signaling in basal keratinocytes.

Chapter 10: Material and Methods

10.1 Material

Mice

The rThy1/rtTA x TRE/HIV^{Nef} mice were generated on a wild type C3H background (Harlan Laboratories) and kept in a sterile, specific pathogen free facility, according to the recommendations of the Canadian Council on Animal Care. Doxycycline was added to their drinking water to induce Nef expression, at 2mg/mL. The mice were exposed to it for one or three months.

All manipulations were approved by the Committee for Animal Protection (Comité de Protection des Animaux) of the Institut de Recherches Cliniques de Montréal.

Cells

We used 293T cells for the transfection and infection experiments. The cell line was derived from human embryonic kidney cells and are known to be easily transfected (Graham et al, 1977). The cells were kept in 10 mm Petri dishes in a sterile incubator at 37°C with 5% CO_2. The culture medium was DME medium (Gibco) + 5% inactivated foetal bovine serum (FBSI) + 1% penicillin-streptomycin (at 10,000 units/mL and 10,000 μg/mL, respectively). 293T cells stably transfected with the full-length Notch1 receptor were a kind gift from Dr Katarzyna Jesien, then a PhD candidate in Dr Jolicoeur's laboratory.

Antibodies and reagents

The antibodies used for this project are listed in table 5, along with their working dilutions.

10.2 Methods

Hematoxylin and eosin staining

The hematoxylin and eosin staining was performed on frozen sections of skin by Dominique Lauzier, of the Institut de Recherches Cliniques de Montréal histology facility. The slides were kept at room temperature and imaged with an Axiophot microscope (Zeiss) using Northern Eclipse software (version 7.0, EMPIX).

Table 5: Antibodies used. The working dilutions are indicated.

Antibodies (species)	Dilution	Company (catalog #)
Primary antibodies		
Western blot		
Rabbit anti-Nef serum 3116 C/F	1/500	Developed by Dr Jolicoeur's laboratory.
Rabbit anti-Notch1 intracellular domain serum 1727-B2	1/500	Developed by Dr Jolicoeur's laboratory.
Immunofluorescence		
GFP (sheep)	1/100	Bio Genesis (4745-1057)
Flow cytometry		
CD4-APC	1/100	Cedarlane (CL013APC)
CD8-FITC	1/100	Cedarlane (CL169F)
TcR-biotin	1/100	Cedarlane (CL7200B)
B220-FITC	1/100	Cedarlane (CL8990F)
CD19-biotin	1/100	BD Pharmingen (553784)
CD11b-PE	1/100	Cedarlane (CL8941PE)
GR1-FITC	1/100	Cedarlane (CL8991F)
CD45.2-Pacific Blue	1/100	Molecular Probes (MCD4528)
gp38 (podoplanin)-PE	1/100	eBioscience (12-5381)
CD31-biotin	1/400	BD Pharmingen (553371)
Ly51-PE	1/100	BD Biosciences (553735)
Uea-1-FITC	1/100	Vector Laboratories (FL-1061)
Notch1-PE	1/100	eBioscience (12-5765)
Immunoprecipitation		
HA-tag	1/100	GenScript (A01244-100)
Secondary antibodies		
Western blot		
Goat anti-rabbit IgG Alexa Fluor 680	1/2500	Molecular Probes (A-21076)

Immunofluorescence

Donkey anti-sheep IgG Alexa Fluor 488	1/250	Molecular Probes (A-11015)

Western blot

Keratinocytes were purified as previously described (Dlugosz et al, 1995) and lysed in lysis buffer 30 minutes on ice. The lysate was centrifuged at 12,000 rpm at 4°C for 15 minutes and the supernatant was transferred into a new tube. Samples were diluted in 4X loading buffer and run on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Immobilon-P, #IPVH00010).

The blotting procedure was performed as described on page 48, but the membrane was blocked overnight at 4°C. The anti-Nef serum was incubated for 4 hours at room temperature. The secondary antibody was incubated for 60 minutes at room temperature.

Scanning was also done with the Odyssey Infrared Imaging system (LI-COR) using Odyssey software (version 3.0, LI-COR).

Subcutaneous injection of a viral vector expressing Nef-GFP or GFP only

A Moloney viral vector with an Eco envelope expressing either Nef-GFP or GFP only was generated by Ginette Massé and Benoît Laganière, both from Dr Jolicoeur's laboratory. Its ability to infect cells was tested by infecting 293T cells and NIH 3T3 cells. Three days following infection, the cells were fixed in 1% paraformaldehyde for 1 hour and their GFP expression was assessed by flow cytometry. About 75% of the 293T cells infected with the vector containing GFP only were GFP+, as were 50% of those infected with the vector expressing Nef-GFP. In the case of the NIH 3T3 cells, 85-90% of the cells were GFP+ for each vector (data not shown).

Following this test, wild type C3H mice were injected subcutaneously with the virus expressing either Nef-GFP or GFP only, as previously described (Okimoto and Fan, 1999). Ginette Massé did the infection. The mice were kept in

a confinement level 2 laboratory. Two mice infected with the Nef-GFP vector, and two infected with the GFP-only vector were euthanized 1 month post-infection. The skins were collected and hair was removed with a hair removal cream (Veet). They were fixed for 1 hour in 4% paraformaldehyde, then overnight in a 10% sucrose solution in PBS 1X. The next morning, they were incubated in a 20% sucrose solution in PBS 1X for 4 hours, then frozen in OCT compound (Sakura Tissue-Tek). The blocks obtained were stored at -80°C. This process was repeated 2 months after infection.

Cryosections were performed by Dominique Lauzier and stored at -80°C.

Immunofluorescence and confocal microscopy

Immunostaining was performed as described on page 47. As keratin is strongly autofluorescent, we stained the slides for GFP to minimize the background signal. The cells were permeabilized in 0,4% Triton X-100 in PBS 1X. The slides were imaged with a LSM 710 confocal microscope (Zeiss) using Zen software (2009 version, Zeiss).

Flow cytometry

Peripheral and mesenteric lymph nodes, spleen, and thymus were harvested. Lymph nodes were digested as described on page 45. The spleen was meshed and red blood cells were lysed in Gey's solution. Thymi were first meshed to collect the thymocytes. They were then digested with DNase and Liberase in RPMI medium (Gibco) to free the epithelial cells.

All cell suspensions were filtered to remove debris. The cells were then incubated in blocking buffer for at least 30 minutes on ice. The staining procedure was as described on page 45. The Ly51 and Uea-1 antibodies were diluted in HBSS medium (Gibco). We used 0.5-1x10^6 cells in each well. The cells were fixed overnight in 0.5% paraformaldehyde in PBS 1X at 4°C.

Samples were run the next morning on a CyAn flow cytometer (Dako). The obtained data was analyzed with Flow Jo software (version 9.4.8, Tree Star, Inc.).

The combination of markers was as follows. In both peripheral and mesenteric lymph nodes: **CD4+ T cells**: CD4+ TcR+; **CD8+ T cells**: CD8+ TcR+; **B lymphocytes**: B220+ CD19+, **granulocytes**: CD11b+ Gr1+, **FRCs**: CD45.2- CD31- gp38+; **BECs**: CD45.2-, CD31+ gp38-, **LECs**: CD45.2- CD31+ gp38+. In the thymus: CD4+ single positive T cells: CD4+ TcR+; **CD8+ single positive T cells**: CD8+ TcR+; **double positive thymocytes**: CD4+ CD8+ TcR+, **medullary thymic epithelial cells (mTECs)**: CD45.2- $Ly51^{low}$ Uea-1+, **cortical thymic epithelial cells (cTECs)**: CD45.2- Ly51+ Uea-1-. In the spleen: **immature myeloid cells**: CD11b+ $GR1^{int}$; **mature myeloid cells**: CD11b+ $GR1^{high}$. B lymphocytes, granulocytes, and myeloid cells were stained using the same markers as Dumortier and colleagues (2010).

Infection of 293 T cells expressing full length Notch1

Two clones (6 and 17) of 293T cells stably transfected with full-length Notch1 were infected with a Moloney virus with a VSV envelope expressing Nef-GFP. Ginette Massé, from Dr Jolicoeur's laboratory, performed the infection in a Containment Level 2 laboratory. Three days post infection, the cells were stained for Notch1 then fixed in 1% paraformaldehyde for 1 hour. The samples were analysed on a CyAn flow cytometer (Dako) and the data obtained was analyzed with Flow Jo software (version 9.4.8, Tree Star, Inc.).

Transfection of 293 T cells with Notch1 intracellular domain and Nef

We used a pcDNA3 vector containing the Nef gene or sequence coding for a HA-tagged Notch1 intracellular domain. We used $8x10^6$ cells per transfection, in 100 mm Petri dishes. The cells were transfected with one vector, or both. 10 µg of DNA were mixed in 1 mL of sterile water containing 124 mM $CaCl_2.2H_2O$. It was then precipitated by mixing it with 1 mL of HBS solution (2X solution: 50 mM HEPES pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, and 1.5 mM $Na_2HPO_4.7H_2O$).

The cells were first treated with 25 μM of chloroquine added to their culture medium. The DNA mix was then added, and incubation was continued for 8 hours before the culture medium was changed to remove the chloroquine.

Immunoprecipitation

The cells were harvested 48h after transfection and lysed in 1 mL of lysis buffer. The anti-HA-tag antibody was added an incubated for 90 minutes at 4°C with agitation. G protein coupled to agarose beads was then added and incubated for 90 minutes at 4°C. The samples were centrifuged at 1,000 rpm for 30 seconds and washed in lysis buffer 3 times to prevent non-specific interactions. The samples were mixed with loading buffer and loaded on a 10% polyacrylamide gel. They were then transferred to a PVDF membrane (Immobilon-P, #IPVH00010) and blotted to reveal Nef. The efficiency of the immunoprecipitation was also assessed by blotting for the Notch1 intracellular domain.

Results

Chapter 11: Results

11.1 Skin phenotypes of transgenic rThy1/rtTA x TRE/HIV^{Nef} mice

The rThy1/rtTA x TRE/HIV^{Nef} double transgenic mice (hereafter rThy1 mice) were originally generated by our laboratory to study the kidney disease that develops in Nef-expressing mice. A rThy1/rtTA x TETO/GFP reporter mouse predicted expression in thymus, pLN, and spleen, as well as kidney, lung, and heart (this work was done by Nathalie Bouchard, former Research Assistant in Dr Jolicoeur's laboratory).

When a first group of rThy1/rtTA x TRE/HIV^{Nef} mice was put on doxycycline (DOX) for three months, double transgenic (double Tg) animals showed, as their most striking feature, an extensive hair loss, especially on the back and head, as well as a thickening of the skin (fig. 28A). The hair loss originated in the back of the neck and was clearly visible after less than a month on DOX (fig. 28A). Double Tg mice also had enlarged skin-draining pLNs. Their spleen was also slightly bigger than that of control mice (fig. 28B).

A hematoxylin and eosin staining of sections of the skin of double Tg mice revealed a thickened epidermis with an expansion of keratinocytes and the formation of cysts in the dermis and the adipose tissue (fig. 28C). The keratin layer at the surface of the skin also appeared thicker.

11.2 Nef is responsible for the phenotypes seen in the skin of double Tg mice

To see whether Nef could be directly involved in the development of these phenotypes, we purified keratinocytes of double Tg mice and their control littermates (either wild type or having inherited only one of the two transgenes) as

previously described (Andrzej et al, 1995). Keratinocytes from double Tg mice clearly expressed Nef (fig. 29).

We then injected wild type C3H mice subcutaneously with a Moloney murine leukemia virus vector expressing either Nef-GFP or GFP only, as described by Okimoto and colleagues (1999). We hoped to infect keratinocytes and recreate phenotypes similar to those of the rThy1 mice. Skins were collected and analyzed one week and one month post-infection (fig. 30). No skin phenotype was visible a month after infection, and we failed to detect infected, GFP+ cells (fig. 30A and B). It may have been masked by keratin autofluorescence, but there was also no visible hair loss of thickening of the skin in the mice that received Nef-GFP (fig. 30C). Therefore, the vector probably failed to infect keratinocytes. However, since we had no control over how the vector spread after the injection, it could simply be that the skin sections we used did not include any infected cells. We need to perform a deeper analysis, using whole mounts of skin, to investigate this. Nevertheless, we could still see no external phenotypes (hair loss or thickening of the skin) three months after injection (data not shown), which suggests that either the phenotypes take much longer to develop than in the rThy1 double Tg mice or, more likely, the infection failed.

11.3 The skin phenotypes of rThy1 double Tg mice are reminiscent of those seen when inhibiting Notch1 signaling in keratinocytes

11.3.1 Consequences of the inhibition of Notch1 signaling in keratinocytes

By reviewing the literature to find how Nef could cause the phenotypes seen in double Tg rThy1 mice, we found that a mouse model developed by Dr Freddy Radtke's group presented skin phenotypes very similar to those of our mice. In this model, an inhibition of the Notch1 and Notch2 signaling pathways was induced by Cre recombination under the control of the Keratin5 promoter (Dumortier et al, 2010). This promoter is expressed in keratinocytes of the basal

cell layer of the epidermis (Lane et al, 1985). In adult mice, specific inhibition of the Notch1 signaling pathway (N1K5 mice) induced a hyperproliferation of the basal cell layer of the epidermis, the formation of epidermal cysts, and hair loss. Inhibiting only the Notch2 (N2K5 mice) signaling pathway did not have any visible effect (no difference with control animals), but the simultaneous ablation of both Notch1 and Notch2 signaling (N1N2K5 mice) led to loss of subcutaneous fat, dermal hypercellularity, epidermoid cysts, thickening and hyperkeratinization of the epidermis, and hair loss (Dumortier et al, 2010). These phenotypes are highly reminiscent of those seen in our rThy1 double Tg mice (fig. 31A).

Dumortier and colleagues also describe a number of systemic phenotypes that develop in the absence of Notch1 signaling in the skin. They observed a recruitment of mast cells and eosinophils to the dermis, which led to an atopic dermatitis-like disease, and a myeloproliferative disorder that affected mature and immature granulocytes in the spleen. The mice developed splenomegaly and also lymphadenopathy.

The mechanism they propose is summarized in Figure 31B. Briefly, both the atopic dermatitis-like disease and the myeloproliferative disorder are triggered by a massive increase in thymic stromal lymphopoietin (TSLP) production by suprabasal keratinocytes, in response to the loss of Notch1 signaling in basal keratinocytes.

Of note, another group has described that when Notch1 signaling is inhibited in the skin of newborn mice, a proliferation of immune cells is also observed, as well as splenomegaly and lymphadenopathy. However, it affected pre- and immature B cells instead of granulocytes (Demehri et al, 2008).

11.3.2 Systemic phenotypes of the rThy1 double Tg mice

The remarkable similarities between the skins of N1K5 mice, N1N2K5 mice, and the rThy1 double Tg mice led us to hypothesize that, in our model, Nef inhibits Notch1 signaling in keratinocytes. To further test this hypothesis, rThy1 mice were put on DOX for a month and their thymi, spleens, peripheral, and

mesenteric lymph nodes (pLNs and mLNs, respectively) were analyzed by flow cytometry. The markers and gating used were the same as those used by Dumortier and colleagues. The results are summarized in tables 6 to 9.

In pLNs (table 6), a significant 3.94-fold increase in absolute numbers of B220+ CD19+ B lymphocytes was observed. No significant differences in the percentage or the absolute numbers of CD11b+ GR1+ granulocytes were found. The N1N2K5 mice showed a clear lymphadenopathy. The total number of cells in the organs did increase in rThy1 double Tg mice (3.68 folds), but this was only a trend (p-value = 0.07, Student's t test).

The only significant differences found in mLNs (table 7) were a decrease in percentage of CD4+ and CD8+ T cells, while the percentage of B220+ CD19+ B lymphocytes increased. No increase in the total number of cells in the organ was observed. The FRC and LEC populations were also increased, but this was not significant.

In the thymus (table 8), a clear loss of CD4+ and CD8+ single positive thymocytes occurred (2.89- and 2.16-fold decrease of the absolute number of cells, respectively), while a significant 2.89-fold increase in the number of CD4+ CD8+ double positive thymocytes was observed. This suggests a block in the maturation of double positive thymocytes. There were no differences in the percentage or absolute numbers of stromal cells, but a significant 2.55-fold decrease of the number of total cells in the organ was found.

Finally, an expansion of the CD11b+ $GR1^{high}$ mature myeloid cells population, as well as of total myeloid cells, was observed in the spleen (table 9). Because of the large error bars, this did not translate to a significant increase in absolute cell numbers. No significant increase in the total number of cells in spleens was observed either.

These results indicate that there are similarities between the systemic phenotypes of the rThy1 double Tg mice and those of the N1N2K5 mice: the rThy1 double Tg mice show an increase in the B lymphocyte and myeloid cell populations, which further suggests that Nef does indeed interfere with Notch1 signaling in keratinocytes.

11.4 Interaction of Nef with Notch1

To verify whether Nef does actually inhibit the Notch1 signaling pathway in rThy1 double Tg mice, we first tried to extract RNA from purified keratinocytes to test the expression of Hes1, a target gene of Notch1 in the skin (Dumortier et al, 2010). We were not able to get usable RNA for this experiment. We therefore used an *in vitro* system to test the possible interactions of Nef with Notch1.

Nef has been shown to downregulate CD4 expression of proteins at the surface of CD4+ T cells (Garcia and Miller, 1991). It also interferes with the migration of proteins to the plasma membrane, notably Lck and MCH I (Chrobak et al, 2010). We hypothesized that Nef also caused a downregulation of Notch1 at the surface of keratinocytes. To test this hypothesis, we infected two clones of 293T cells expressing the full length Notch1 protein with a Moloney murine leukemia virus vector containing either Nef-GFP or GFP only. We then analyzed the Notch1 signal at the cell surface by flow cytometry, by gating on infected, GFP+ cells (fig. 32A). The flow cytometry profiles clearly show that there is no decrease of the Notch1 signal in the cells expressing Nef-GFP compared to those expressing only GFP (fig. 32B). Therefore, Nef does not induce a downregulation of Notch1 at the cell surface.

Nef is a myristoylated protein and localizes at the cytoplasmic side of the plasma membrane (Resh, 1999). We hypothesized that it may bind to the intracellular domain of Notch1 to prevent its cleavage or its migration to the nucleus. To test this hypothesis, we co-transfected 293T cells with an HA-tagged Notch1 intracellular domain (N1 IC) and Nef, then performed an immunoprecipitation of the HA-tagged N1 IC (fig. 33). While we did obtain a clear N1 IC signal, we could not detect Nef in the immunoprecipitate, which suggests that Nef does not bind directly to N1 IC. If it does indeed block Notch1 signaling, Nef could act on Notch1 via an intermediary protein. Alternatively, its target could also be further down the Notch1 signaling pathway.

Taken together, these results suggest that Nef could be directly implicated in the development of skin diseases in human AIDS patients, notably atopic dermatitis. We also show here that it very likely interacts with the Notch1 signaling pathway. This interaction could happen in different cell types in addition to keratinocytes, and have important consequences in the maturation and proliferation of certain cell populations or the development of cancers.

Figures

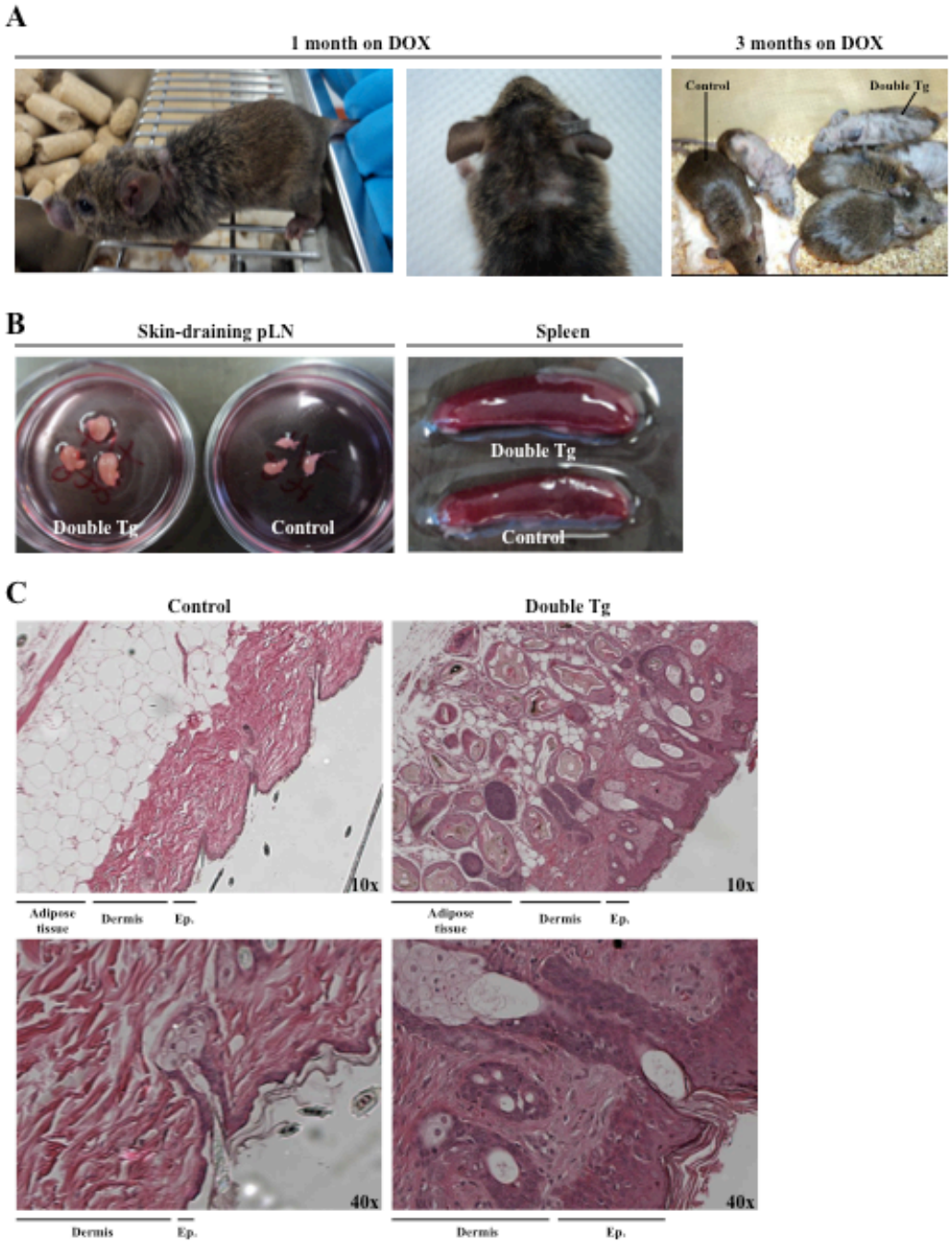


Figure 28: Phenotypes of the rThy1/rtTA x TRE/HIV mouse. Hair loss started developing on the back of the mice (A). It was clearly visible after one month on DOX and spread to the entire body as exposure to DOX continued. After one month of treatment, double Tg mice also presented enlarged lymph nodes and spleen (B). An hematoxylin and eosin staining of the skin revealed that double Tg mice had a thickened, hyperkeratinized epidermis and dermal cysts (C).

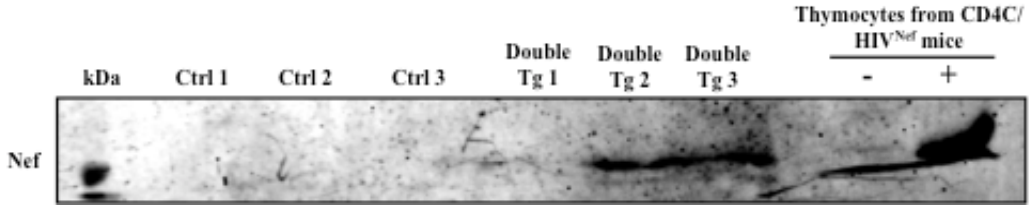


Figure 29: Expression of Nef in keratinocytes. Purified keratinocytes of double Tg rThy1 mice (n=3) and controls (n=3) were lysed and the protein extracts were analyzed by Western blot. Nef was clearly present in Tg double samples. The lower band that appears in the control lanes is in fact the migration front.

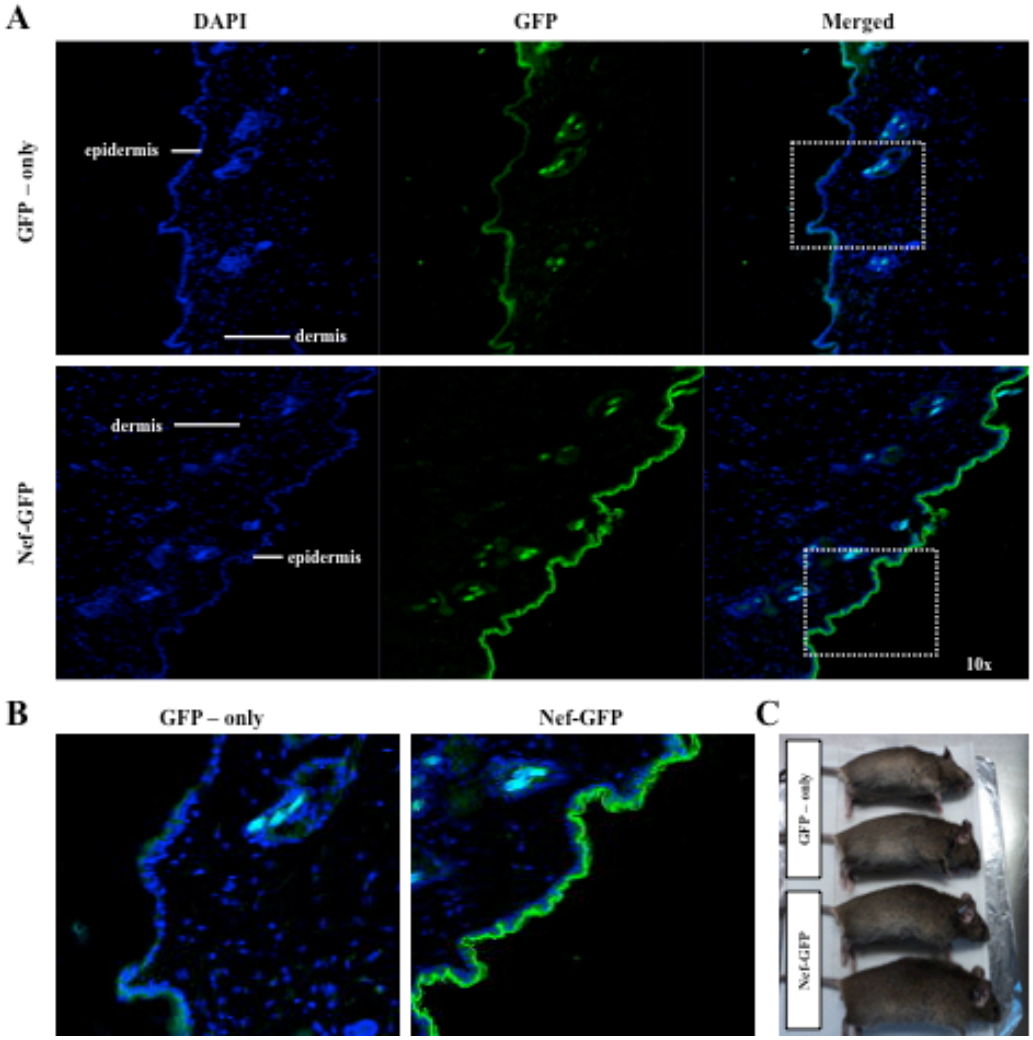
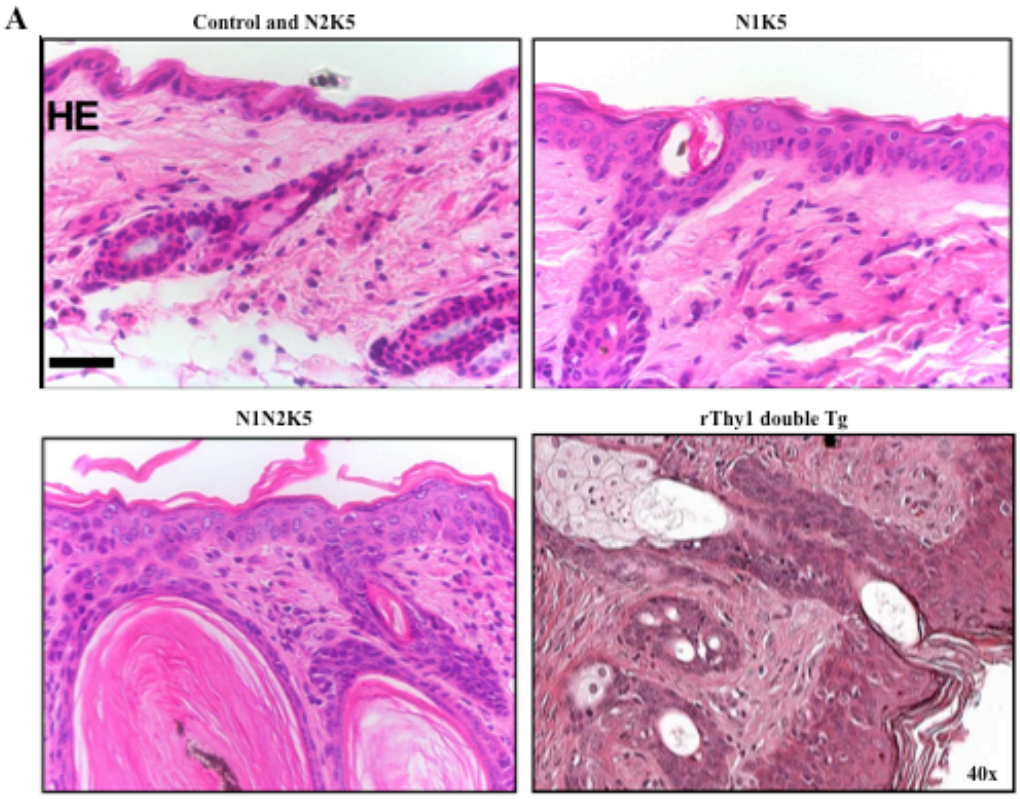


Figure 30: Infection of keratinocytes by a viral vector containing GFP only or Nef-GFP. Wild type C3H mice were injected subcutaneously in the flank, as previously described (Okimoto and Fan, 1999) and skins were harvested 1 month after injection. No infected, GFP+ cell could be found in the dermis or epidermis, and the green signal is in fact keratin autofluorescence (A). The dashed rectangles represent the area that was magnified in (B). No proliferation of keratinocytes was observed in the epidermis. As shown in (C), there was also no hair loss in control, GFP-only mice (upper two mice) or in Nef-GFP mice (bottom two mice).


A
Control and N2K5
N1K5
HE
N1N2K5
rThy1 double Tg
40x

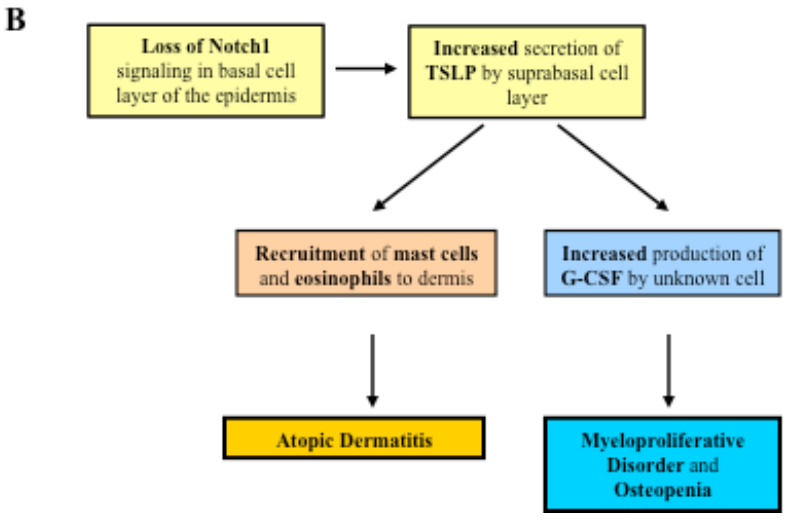

B
Loss of Notch1 signaling in basal cell layer of the epidermis
Increased secretion of TSLP by suprabasal cell layer
Recruitment of mast cells and eosinophils to dermis
Increased production of G-CSF by unknown cell
Atopic Dermatitis
Myeloproliferative Disorder and Osteopenia

Figure 31: The phenotypes seen in rThy1 double Tg mice are reminiscent of those observed when inhibiting Notch1 signalling in keratinocytes. This model was generated by Dumortier et al (2010) and presented severe skin phenotypes (A). Selective abolition of Notch1 signalling led to hair loss, proliferation of the basal cell layer of the epidermis, and formation of epidermal cysts. Inhibiting Notch1 and Notch2 simultaneously induced hair loss, hyperkeratinization of the epidermis, dermal hypercellularity and cysts, and loss of subcutaneous fat. Mice in which only the Notch2 pathway had been inhibited were virtually indistinguishable from controls. The phenotypes that develop in the skin of N1N2K5 mice are very similar to those found in the skin of rThy1 mice. Dumortier and colleagues propose that the inhibition of Notch1 signaling leads to increased secretion of TSLP, which in turn induces an atopic dermatitis-like disease as well as a myeloproliferative disorder (B). Scale bar: 50μm.

Table 6: Immune and stromal cell populations in pLNs of rThy1 mice. pLNs were harvested from double Tg (n=5) and control (n=5) mice and digested after a one-month treatment on DOX. The cells were then stained as described.

Peripheral LN	Percentage			Absolute number of cells (x10^5)		
Cell population	**Control**	**Double Tg**	**Significance**	**Control**	**Double Tg**	**Significance**
Immune cells						
CD4+ T cells (CD4+ TcR+)	32.56 ± 2.34	28.88 ± 5.32	ns	36.67 ± 18.40	127.23 ± 97.77	ns
CD8+ T cells (CD8+ TcR+)	21.34 ± 2.35	19.84 ± 2.87	ns	23.81 ± 11.38	83.63 ± 58.10	ns
B lymphocytes (B220+ CD19+)	30.84 ± 2.10	34.16 ± 4.61	ns	33.74 ± 13.48	133.05 ± 80.38	p < 0.05
Granulocytes (CD11b+ GR1+)	1.79 ± 1.55	2.75 ± 1.80	ns	2.04 ± 1.79	12.33 ± 12.04	ns
Stromal cells						
FRCs (CD45.2– CD31– gp38+)	3.25 ± 1.22	6.31 ± 2.89	ns	3.96 ± 3.38	20.60 ± 8.29	p < 0.005
BECs (CD45.2– CD31+ gp38–)	6.87 ± 1.23	7.15 ± 3.34	ns	7.67 ± 3.86	23.36 ± 10.49	p < 0.05
LECs (CD45.2– CD31+ gp38+)	2.24 ± 1.11	2.96 ± 1.41	ns	2.74 ± 2.45	10.06 ± 7.52	ns
Total number of cells in organ	-	-	-	111 ± 48.84	408.60 ± 273.42	ns

Table 7: Immune and stromal cell populations in mLNs of rThy1 mice. mLNs were harvested from double Tg (n=5) and control (n=5) mice and digested after a one-month treatment on DOX. The cells were then stained as described.

Mesenteric LN	Percentage			Absolute number of cells (x10^5)		
Cell population	**Control**	**Double Tg**	**Significance**	**Control**	**Double Tg**	**Significance**
Immune cells						
CD4+ T cells (CD4+ TcR+)	32.46 ± 3.79	26.02 ± 3.27	p < 0.05	14.46 ± 11.02	12.63 ± 13.40	ns
CD8+ T cells (CD4+ TcR+)	20.8 ± 1.64	14 ± 3.27	p < 0.005	9.31 ± 6.95	6.72 ± 6.68	ns
B lymphocytes (B220+ CD19+)	22.86 ± 6.26	37.94 ± 7.2	p < 0.01	11.70 ± 10.03	18.39 ± 21.89	ns
Granulocytes (CD11b+ GR1+)	0.96 ± 0.91	3.12 ± 2.89	ns	0.63 ± 0.63	2.41 ± 3.48	ns
Stromal cells						
FRCs (CD45.2– CD31– gp38+)	1.85 ± 1.67	6.33 ± 5.47	ns	1.25 ± 1.43	4.46 ± 6.22	ns
BECs (CD45.2– CD31+ gp38–)	5.97 ± 2.45	6.54 ± 2.4	ns	3.23 ± 3.09	3.60 ± 4.12	ns
LECs (CD45.2– CD31+ gp38+)	1.88 ± 1.92	3.55 ± 2.84	ns	1.30 ± 1.64	2.26 ± 2.88	ns
Total number of cells in organ	-	-	-	44.45 ± 33.33	48.30 ± 53.01	ns

Table 8. Lymphoid and stromal cell populations in thymi of rThy1 mice. Thymi were harvested from double Tg (n=5) and control (n=5) mice after one month of DOX exposure. They were first meshed to collect the thymocytes, then digested to free the epithelial cells. The cells were then stained as described. SP: single positive; DP: double positive; mTECs: medullary thymic epithelial cells; cTECs: cortical thymic epithelial cells.

Thymus	Percentage			Absolute number of cells (x10^5)		
Cell population	**Control**	**Double Tg**	**Significance**	**Control**	**Double Tg**	**Significance**
Lymphoid cells						
CD4 + SP T cells (CD4+ TcR+)	7.15 ± 1.67	6.79 ± 1.11	ns	30.08 ± 15.26	10.40 ± 3.91	p < 0.05
CD8 + SP T cells (CD8+ TcR+)	2.32 ± 0.50	2.84 ± 0.95	ns	9.43 ± 3.33	4.36 ± 2.21	p < 0.05
CD4+ CD8+ DP cells (CD4+ CD8+ TcR+)	87.86 ± 1.71	85.14 ± 5.38	ns	377.54 ± 153.67	130.48 ± 47.03	p < 0.05
Stromal cells						
mTECs(CD45.2− Ly51+ Ulea1−)	15.19 ± 16.91	18.53 ± 12.70	ns	12.50 ± 14.63	13.36 ± 11.68	ns
cTECs (CD45.2 − Ly51+ Ulea1−)	4.97 ± 5.06	4.85 ± 2.83	ns	1.26 ± 0.87	2.53 ± 2.47	ns
Total number of cells in organ	-	-	-	434 ± 176.86	169.95 ± 61.83	p < 0.05

Table 9. Myeloid cell populations in spleens of rThy1 mice. Spleens were harvested from double Tg (n=5) and control (n=5) mice after one month of DOX exposure. They were first meshed and red blood cells were lysed. The cells were then stained as described.

Spleen	Percentage			Absolute number of cells (x10^5)		
Cell population	**Control**	**Double Tg**	**Significance**	**Control**	**Double Tg**	**Significance**
Myeloid cells						
Immature myeloid cells (CD11b+ GR1int)	8.73 ± 4.49	9.55 ± 2.99	ns	72.72 ± 80.11	112.71 ± 79.66	ns
Mature myeloid cells (CD11b+ GR1high)	3.91 ± 1.47	9.95 ± 2.87	p < 0.005	35.61 ± 36.08	123.56 ± 102.42	ns
Total myeloid cells (CD11b+ GR1+)	12.63 ± 5.17	19.5 ± 3.94	p < 0.05	108.33 ± 113.80	237 ± 177.66	ns
Total number of cells in organ	-	-	-	775 ± 542.53	1131 ± 684.04	ns

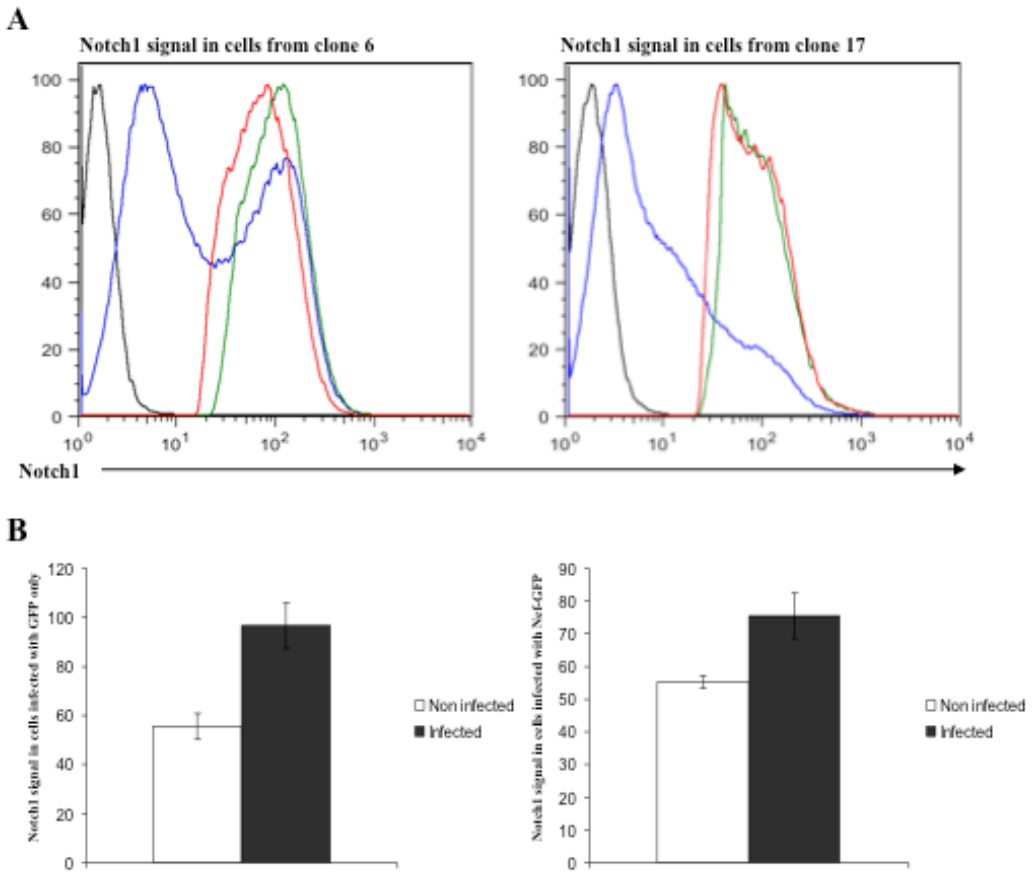


Figure 32: Nef expression does not lead to a downregulation of Notch1 at the cell surface. 293T cells expressing the full length Notch1 were infected with a Moloney virus containing either Nef-GFP or GFP only. Notch1 expression at the cell surface was assessed by FACS on infected, GFP+ cells (A). Two clones of Notch1-expressing cells were used in this experiment. Black line: unstained, uninfected cells; blue line: uninfected cells; green line: cells expressing GFP only; red line: cells expressing Nef-GFP. The fluorescence intensity of the Notch1 signal at the cell surface was then quantified in cells having received the GFP gene only (left, n = 2), or Nef-GFP (right, n = 2) (B). No downregulation is observed.

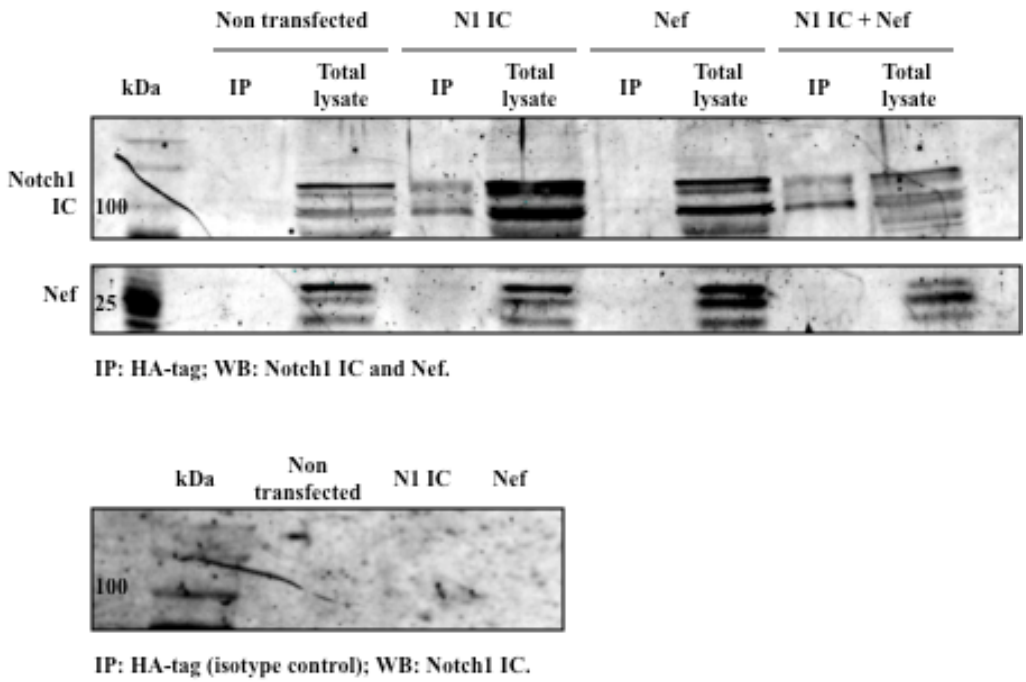


Figure 33: Nef does not bind directly to Notch1. 293T cells were transfected with plasmids containing Nef and/or an HA-tagged N1 IC. Immunoprecipitation of the HA-tag was performed, followed by immunoblotting of Nef and N1 IC. No Nef signal could be detected even when N1 IC had clearly been immunoprecipitated, which indicates that Nef does not bind Notch1 directly.

Discussion

Chapter 12: Discussion

Nef is responsible for the development of an atopic dermatitis-like disease in rThy1 double Tg mice.

The skin of mice expressing Nef in keratinocytes shows features very similar to those of human atopic dermatitis (AD). AD is one of the most prevalent skin diseases found in human AIDS patients. 30% to 50% of seropositive individuals develop it while it is present in 2% to 20% of the seronegative population (Cedeno-Laurent et al, 2011). We show here that Nef is involved in the development of an AD-like disease. In the N1N2K5 model, this disease was triggered by the inhibition of the Notch1 and Notch2 signaling pathways specifically in basal keratinocytes. We have shown that, in the rThy1 double Tg model, Nef is expressed in these cells. Thus, we propose that Nef acts directly to cause the disease. We need to investigate whether this is cell-autonomous or whether the Nef-expressing keratinocytes induce their neighbors to proliferate.

The expression of Nef in rThy1 double Tg mice induces a systemic expansion of B lymphocytes and granulocytes.

In the N1N2K5 model, the AD-like disease is caused by the loss of Notch1 and Notch2 signaling in keratinocytes. This leads to the drastic increase of TSLP levels in the blood, which induces the recruitment of mast cells and eosinophils to the dermis, thus contributing to inflammation and the development of the AD-like disease. High TSLP levels also trigger an increase in G-CSF by a yet-unknown cell, which causes the development of a myeloproliferative disease (Dumortier et al, 2010). While we did not measure TSLP production, the presence of systemic effects similar to those found in the N1N2K5 model suggests that the

same process takes place in our Tg mice. Moreover, while developing the N1N2K5 model, Dumortier and colleagues showed that the inhibition of Notch2 alone had no visible effect, while the specific inhibition of Notch1 triggered skin phenotypes that greatly resembled those of N1N2K5 mice. We therefore hypothesized that the expression of Nef in keratinocytes blocked the Notch1 signaling pathway in these cells, thus causing the AD-like disease.

The rThy1 promoter, however, is active in several cell types, not just keratinocytes. Therefore, Nef is expressed in other cells, which causes its own phenotypes and could counter the ones triggered by the absence of Notch1 signaling in the skin. For example, we observed an accumulation of CD4+ CD8+ thymocytes, which indicates a defect in their maturation to single positive thymocytes that Dumortier and colleagues did not report. The rThy1/rtTA x TETO/GFP reporter mouse showed that the rThy1 promoter is active in the thymus, and our result is consistent with the fact that Notch1 signaling promotes the maturation of double-positive thymocytes into both CD4+ and CD8+ single positive cells (Radtke et al, 1999; Deftos et al, 2000). Interestingly, a defect in the lineage-commitment of double positive thymocytes was also observed in transgenic CD4C/HIV^{Nef} mice (Chrobak et al, 2010). The Notch signaling pathway could also be targeted there.

The expansion of B220+ CD19+ B lymphocytes and of CD11b+ GR1+ granulocytes is consistent with the results obtained by Demehri and colleagues (2008), and Dumortier and colleagues (2010), respectively. Nevertheless, they appear much less drastic than what these teams observed, as they are not lethal. Our mice survived over three months on DOX while the N1N2K5 mice died within two months of Notch1 inhibition. Demehri and colleagues' mice survived a month post-inhibition. Both these teams used Cre recombination to completely abolish Notch1 and Notch2 signaling while our mice were exposed to DOX diluted in their drinking water. It probably led to a partial inhibition of Notch signaling only, sufficient to trigger an expansion of B cells and granulocytes, but not to render it lethal. We need to verify Notch1 signaling in these mice to

confirm this. Moreover, like mentioned before, the expression of Nef may have effects that counterbalance those of the inhibition of Notch signaling.

Animal models that faithfully reproduce the skin phenotypes seen in human AIDS patients are lacking (Cedeno-Laurent et al, 2011). In the case of AD, the rThy1/rtTA x TRE/HIV^{Nef} mouse model developed in our laboratory is a first answer to this. A mouse model expressing Nef specifically in basal keratinocytes, for example under the control of the K5 promoter, would be even more useful to investigate whether this is sufficient to induce an AD-like disease and proliferative disorders. If it is, we would be showing for the first time how HIV-1 could directly cause AD in human patients.

Nef inhibits the Notch1 signaling pathway in keratinocytes.

Perhaps the most striking discovery of this study was that Nef inhibits the Notch1 signaling pathway in keratinocytes. Previous studies suggest that there may be a link between Nef and the Notch signaling pathway in HIV-associated nephropathy. Indeed, Nef has been shown to induce a proliferation and dedifferentiation of podocytes, both of which are observed in HIV-associated nephropathy (Zuo et al, 2006). Another *in vivo* study by Sharma and colleagues revealed that Notch1 and Notch4 are activated in podocytes affected by this disease (Sharma et al, 2010). Cultured podocytes treated with Nef proliferated *in vitro* and also exhibited an activated Notch pathway (Sharma et al, 2013). These results suggest that Nef and Notch could interact to induce HIV-associated nephropathy. Our results strongly support the hypothesis that Nef can also inhibit Notch1 in keratinocytes. We are in the process of developing a triple transgenic reporter rThy1/rtTA x TRE/HIV^{Nef} x CBF-1/GFP mouse to directly visualize this phenomenon. We expect to observe a decrease of GFP signal in keratinocytes in animals expressing Nef, compared to controls.

It is interesting to see that the effect of Nef on Notch1 signaling depends on the organ considered. It may reflect the diversity of the effects of Notch1: for example, Notch1 acts as a tumor-suppressor in the skin by promoting the

differentiation of keratinocytes (Rangarajan et al, 2001), but a constitutively active Notch1 inhibits the maturation of CD4+ and CD8+ thymocytes (Izon et al, 2001) and leads to the development of T cell and B cell neoplasms in mice (Pear et al, 1996; Jundt et al, 2002, respectively). Thus, Nef may manipulate Notch signaling according to what would be more favorable to the replication and spread of HIV in the specific cell type considered. It will be important to take this into consideration when studying the etiology of AIDS-associated cancers.

Indeed, skin cancers such as basal cell carcinoma (BCC), squamous cell carcinoma (SCC), or Kaposi's sarcoma also appear frequently in AIDS patients (Cedeno-Laurent et al, 2011). Deletion of the Notch1 gene has been shown to cause the spontaneous development of BCC and to favor that of SCC and malignant papillomas (Nicolas et al, 2003). Moreover, BCC and SCC tumors present a marked decrease in Notch expression (Massi et al, 2006). In contrast, the activity of all four Notch receptors is increased in Kaposi's sarcoma, and the use of a γ-secretase inhibitor resulted in the apoptosis of primary and immortalized Kaposi's sarcoma cells (Curry et al, 2005). Kaposi's sarcoma is caused by the infection with Human Herpesvirus 8 (Moore and Chang, 1998), but the HIV-1 protein Tat has been shown to induce lesions in the dermis that resemble those of Kaposi's sarcoma (Vogel et al, 1988) and to stimulate the growth of Kaposi's sarcoma cells (Mu et al, 2007). It would be interesting to study whether Nef can also affect or facilitate the development of the disease, in a direct (by being taken up by malignant cells, for example) or indirect way.

While highly active antiretroviral therapy (HAART) generally lowers the risks of developing AIDS-associated neoplasms, this is not true for all cancers. In a cohort of over 300,000 people studied between 1980 and 2002, for example, Hodgkin's lymphoma incidence increased after the introduction of HAART (Biggar et al, 2006). This shows that, while HAART has undoubtedly greatly improved the lives of infected patients, more specific treatments are needed to control the development of HIV/AIDS-associated diseases.

Despite obvious effects on its signaling pathway, our results indicate that Nef does not interact directly with Notch1, nor does it trigger its downregulation.

Because of its localization at the cell membrane, Nef could act by preventing Notch1 cleavage by the γ-secretase. It could also inhibit the translocation of the intracellular domain of Notch1 to the nucleus, or its binding to CBF-1. More studies are needed to understand exactly how Nef and Notch1 interact in our model.

In summary, we show here that Nef interacts with the Notch1 signaling pathway in mouse keratinocytes. Its inhibition causes the development of an AD-like disease as well as an expansion of both B lymphocytes and granulocytes. This work identifies Nef as a possible agent of several diseases and neoplasms associated with HIV-1 infection in human patients. In addition, few animal models exist to study HIV-associated skin pathogenesis. The rThy1/rtTA x TRE/HIV^{Nef} mouse developed in our laboratory constitutes one such model and could be used to study the onset of AD in HIV-1-infected individuals.

General Conclusion

Preserving pLN functions is key to maintaining acquired immune responses, especially in the context of an immunodeficiency syndrome such as human AIDS. The consequences of an HIV-1 infection have been extensively studied for over thirty years. The accessory protein Nef has been identified as a major determinant of pathogenicity, as it single-handedly induces many phenotypes associated with HIV-1 infection in mice. Humans infected with HIV-1 strains lacking a functional Nef are able to control the infection and do not progress to AIDS. We present here the results of our study on its effects on stromal cells of mouse pLNs. We demonstrate that, in resting pLNs, Nef does not affect the population of FRCs, nor its functions. We hypothesize that the preservation of the LTi population is what maintains them. The survival of these stromal cells also seems to prevent the development of fibrosis, as none could be observed in our transgenic CD4C/HIV^{Nef} pLNs. In human patients, LTi are most likely infected and depleted which, along with the loss of CD4+ T cells, deprives FRCs of their survival factors. Fibrosis causes irreversible lesions that further hinder the contact between these three populations, which limits the effectiveness of HAART. Our results indicate that maintaining both LTi and FRCs is key to increasing the long-term success of HAART.

In addition to this comprehensive study on FRCs, we show that Nef causes the expansion of both BECs and LECs. This expansion can be so extensive that it completely obstructs the lumen of medullary blood vessels and of the SCS, respectively. If this were to occur in HIV-1-infected patients, it would greatly limit the circulation of cells through the pLNs, which would further compromise their survival and the development of acquired immune responses.

We have also studied an unexpected effect of Nef on keratinocytes. Its expression in mouse epidermis led to the development of an atopic dermatitis-like disease, as well as systemic expansion of both B lymphocytes and granulocytes. To our knowledge, Nef had not been linked to atopic dermatitis before. Thus, we

provide here a model to study the onset of atopic dermatitis in the context of HIV-1 infection. Moreover, these results suggest that Nef may interact with the Notch1 signaling pathway. This effect had already been described in the context of HIV-1-associated nephropathy. We describe here another setting in which this interaction occurs. Because of the role of Notch signaling in tumor development, this further identifies Nef as a potential agent for the onset of AIDS-associated neoplasms.

Taken together, our results add to the long list of known Nef-mediated effects in HIV-1 pathogenicity, which make it a critical target of therapy. Assays to identify Nef inhibitors exist (Emert-Sedlark et al, 2009), and our laboratory has collaborated in the characterization of a mouse expressing a single-domain anti-Nef antibody that already shows promise: it blocked the effects of Nef on CD4 downregulation, actin remodeling, thymocyte maturation and peripheral CD4+ T cell activation (Bouchet et al, 2011). These represent new avenues in antiretroviral therapy, as patients would certainly greatly benefit from the inhibition of Nef.

References

Aldrovandi GM and JA Zack (1996), Replication and Pathogenicity of Human Immunodeficiency Virus Type 1 Accessory Gene Mutants in SCID-hu Mice, *Journal of Virology*, 69(8):5045-5056.

Alimzhanov MB, DV Kuprash, MH Kosco-Vilbois, A Luz, RL Tureskaya, A Tarakhovsky, K Rajeewsky, SA Nedospasov and K Pfeffer (1997), Abnormal Development of Secondary Lymphoid Tissue in Lymphotoxin Beta-Deficient Mice, *Proceedings of the National Academy of Sciences USA*, 94(17):9302-9307.

Allen CS, T Okada, HL Tang and JG Cyster (2007), Imaging of Germinal Center Selection Events During Affinity Maturation, *Science*, 315(5811):528-531.

Almerio P, R Verdolini, G Proietto, C Feliciani, P Toto, G Shivji, F Loconsole, N Cassano, P Amerio, G Vena and DN Sauder (2001), Role of Th2 Cytokines, RANTES, and Eotaxin in AIDS-Associated Eosinophilic Folliculitis, *Acta Dermato-Venereologica*, 81:92-95.

Almodóvar S, M Del C Colón, IM Madonado, R Villafañe, S Abreus, I Meléndez, C Domínguez, W Cuevas, TM Collins and E Lorenzo (2007), HIV-1 Infection of Monocytes Is Directly Related to the Success of HAART, *Virology*, 369(1):35-46.

Anderson AO and ND Anderson (1975), Studies on the Structure and Permeability of the Microvasculature in Normal Rat Lymph Nodes, *American Journal of Pathology*, 80:387-418.

Anderson ND, AO Anderson and RG Wyllie (1975), Microvascular Changes in Lymph Nodes Draining Skin Allografts*, American Journal of Pathology*, 81:131-160.

Anderson AO and ND Anderson (1976), Lymphocyte Emigration from High Endothelial Venules in Rat Lymph Nodes, 31:731-748.

Anderson JL, TJ Hope (2005), Intracellular Trafficking of Retroviral Vectors: Obstacles and Advances, *Gene Therapy*, 12:1667-1678.

Angeli V, F Ginhoux, J Llodrà, L Quemeneur, PS Frenette, M Skobe, R Jessberger, M Merad and G Randolph (2006), B Cell-Driven Lymphangiogenesis in Inflamed Lymph Node Enhances Dendritic Cell Mobilization, *Immunity*, 24(2):203-215.

Ansel KM, VN Ngo, PL Hyman, SA Luther, R Förster, JD Sedgwick, JL Browning, M Lipp and JG Cyster (2000), A Chemokine-Driven Positive

Feedback Loop Organizes Lymphoid Follicles, *Nature*, 406(6793):309-314.

Arold ST and AS Baur (2001), Dynamic Nef and Nef Dynamics: How Structure Could Explain the Complex Activities of this Small HIV Protein, *TRENDS in Biochemical Sciences*, 26(6):356-363.

Asante-Appiah E and AM Skalka (1999), HIV-1 Integrase: Structural Organization, Conformational Changes, and Catalysis, *Advances in Virus Research*, 52:351-369.

Baekkevold ES, T Yamanaka, RT Palframan, HS Carlsen, FP Reinholt, UH von Andrian, P Brandtzaeg and G Haraldsen (2001), The CCR7 Ligand elc (CCL19), is Transcytosed in High Endothelial Venules and Mediates T Cell Recruitment, *Journal of Experimental Medicine*, 193(9):1105-1112.

Bajénoff M, JG Egen, LY Koo, JP Laugier, F Brau, N Glaichenhaus and RN Germain (2006), Stromal Cell Networks Regulate Lymphocyte Entry, Migration, and Territoriality in Lymph Nodes, *Immunity*, 25:989-1001.

Bajénoff M, B Breart, AYC Huang, H Qi, J Cazareth, VM Braud, RN Germain and N Glaichenhaus (2006), Natural Killer Cell Behavior in Lymph Nodes Revealed by Static and Real-Time Imaging, *Journal of Experimental Medicine*, 203(3):619-631.

Bajénoff M, JC Egen, H Qi, AYC Huang, F Castellino and RHN Germain (2007), Highways, Byways and Breadcrumbs: Directing Lymphocyte Traffic In The Lymph Node, *Trends in Immunology*, 28(8):346-352.

Bajénoff M, N Glaichenhaus and RN Germain (2008), Fibroblastic Reticular Cells Guide T Lymphocyte Entry Into and Migration Within the Splenic T Cell Zone, *Journal of Immunology*, 181:3947-3954.

Berger EA, PM Murphy and JM Farber (1999), Chemokine Receptors As HIV-1 Coreceptors: Roles in Viral Entry, Tropism, and Disease, *Annual Reviews in Immunology*, 17:657-700.

Biancotto A, JC Grivel, SJ Iglehart, C Vanpouille,A Lisco, SF Sieg, R Debernado, K Garate, B Rodriguez, LB Margolis and MM Lederman (2007), Abnormal Action and Cytokine Spectra in Lymph Nodes of People Chronically Infected with HIV-1, *Blood*, 109:4272-4279.

Bingisser RM, PA Tilbrook, PG Holt and UR Kees (1998), Macrophage-Derived Nitric Oxide Regulates T Cell Activation Via Reversible Disruption of the Jak3/STAT5 Signaling Pathway, *Journal of Immunology*, 160:5729–5734.

Bogdan C (2011), Regulation of Lymphocytes by Nictric Oxide, *Methods in*

Molucular Biology, 677:375-393.

Boonchai W, M Walsh, M Cummings and G Chenevix-Trench (2000), Expression of Beta-Catenin, a Key Mediator of the WNT Signaling Pathway, in Basal Cell Carcinoma, *Archives of Dermatology*, 136(7):937-938.

Bouchet J, SE Basmaciogullari, P Chrobak, B Stolp, N Bouchard, OT Fackler, P Cames, P Jolicoeur, S Benichou and D Baty, Inhibition of the Nef Regulatory Protein of HIV-1 by a Single-Domain Antibody, *Blood*, 117(13):3559-3568.

Bour S, U Schubert and K Strebel (1995), The Human Immunodeficiency Virus Type 1 Vpu Protein Specifically Binds the Cytoplasmic Domain of CD4: Implications for the Mechanism of Degradation, *Journal of Virology*, 69:1510-1520.

Boy SC, MB van Heerden, M Wolfaardt, R Cockeran, E Gema and van Heerden WF (2009), An Investigation of the Role of Oral Epithelial Cells and Langerhans Cells as Possible HIV Viral Reservoirs, *Journal of Oral Pathology and Medicine*, 38(1)114-119.

Breitfeld D, L Ohl, E Kremmer, J Ellwart, F Sallusto, M Lipp and R Forster (2000), Follicular B Helper T Cells Express CXC Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production, *Journal of Experimental Medicine*, 192(11):1545-1552.

Brenchley JM, C Vinton, B Tabb, XP Hao, E Connick, M Paiardini, JD Lifson, G Silvestri and JD Estes (2012), Differential Infection Patterns of CD4+ T Cells and Lymphoid Tissue Viral Burden Distinguish Progressive and Nonprogressive Lentiviral Infections, *Blood*, 120:4172-4181.

Briggs SD, B Scholtz, JM Jacque, S Swingler, M Stevenson and TE Smithgall (2001), HIV-1 Nef Promotes Survival of Myeloid Cells by a Stat3-Dependent Pathway, *Journal of Biological Chemistry*, 276(27):25605-25611.

Brou C, F Logeat, N Gupta, C Bessia, O LeBail, JR Doedens, A Cumano, P Roux, RA Black and A Israel (2000), A Novel Proteolytic Cleavage Involved in Notch Signaling: The Role of the Disintegrin-Metalloprotease TACE, *Molecular Cell*, 5:207-216.

Bushman FD and R Craigie (1991), Activities of Human Immunodeficiency Virus (HIV) Integration Protein In Vitro: Specific Cleavage and Integration of HIV DNA, *Proceedings of the National Academy of Sciences USA*, 88(4):1339-1343.

Butcher EC, M Williams, K Yougman, L Rott and M Briskin (1999), Lymphocte Trafficking and Regional Immunity, *Advances in Immunology*, 72:209-253.

Byrne C, M Tainsky and E Fuchs (1994), Programming Gene Expression in Developing Epidermis, *Development*, 120:2369-2383.

Cao W, BD Jamieson, LE Hultin, PM Hultin and R Detels (2009), Regulatory T Cell Expansion and Immune Activation During Untreated HIV Type 1 Infection Are Associated With Disease Progression, *AIDS Research and Human Retroviruses*, 25:183-191.

Campbell JJ, J Hedrick, A Zlotnik, MA Siani, DA Thompson and EC Butcher (1998), Chemokines and the Arrest of Lymphocytes Rolling Under Flow Conditions, *Science*, 279(5349):381-384.

Campbell EM, R Nunez and TJ Hope (2004), Disruption of the Actin Cytoskeleton Can Complement the Ability of Nef to Enhance Human Immunodeficiency Virus Type 1 Infectivity, *Journal of Virology*, 78:5745-5755.

Cedeno-Laurent F, J Bryant, R Fishelich, OD Jones, A Deng, ML ENg, AA Gaspari and JR Trujillo (2009), Inflammatory Papillomatous Hyperplasia and Epidermal Necrosis in Transgenic Rat for HIV-1, *Journal of Dermatological Science*, 53:112-119.

Cedeno-Laurent F, M Gómez-Flores, N Mendez, J Ancer-Rodríguez, JL Bryant, AA Gaspari and JR Trujillo (2011), New Insights into HIV-1 Primary Skin Disorders, *Journal of the Interational AIDS Society*, 14:5.

Cella M, D Jarrossay, F Facchetti, O Alebardi, H Nakajima, A Lanzavecchia and M Colonna (1999), Plasmacytoid Monocytes Migrate to Inflammed Lymph Nodes and Produce Large Amounts of Type 1 Interferon, *Nature*, 5(8):919-923.

Centlivre M, N Legrand, R Steingrover, R van der Sluis, ML GRijsen, M Bakker, S Jurriaans, B Berkhout, WA Paxton, JM Prins and G Pollakis (2011), Altered Dynamics and Differential Infection Profiles of Lymphoid and Myeloid Cell Subsets During Acute and Chronic HIV-1 Infection, *Journal of Leukocyte Biology*, 89(5):785-795.

Chan DC and PS Kim (1998), HIV Entry and Its Inhibition, *Cell*, 93(5):681-684.

Chappell JC and VL Bautch (2010), Vascular Development: Genetic Mechanisms and Links to Vascular Disease, *Current Topics in Developmental Biology*, 90:43-72.

Chaudhuri R, OW Lindwasser, WJ Smith, JH Hurley and JS Bonifacio (2007), Downregulation of CD4 by Human Immunodeficiency Virus Type 1 Nef is Dependent on Clathrin and Involves Direct Interaction of Nef with the AP2 Clathrin Adaptator, *Journal of Virology*, 81:3877-3890.

Chen Q, DT Fisher, KA Clancy, JM Gauguet, WC Wang, E Unger, S Rose-John, UH von Adrian, H Baumann and SS Evans (2006), Fever-Range Thermal Stress Promotes Lymphocyte Trafficking Across High Endothelial Venules Via An Interleukin 6 Trans-Signaling Mechanism, *Nature Immunology*, 7(12):1299-1308.

Cheng-Mayer C, D Seto, M Tateno and JA Levy (1988), Biologic Features of HIV-1 That Correlate With Virulence in the Host, *Science*, 240:80-82.

Chomont N, M El-Far, P Ancuta, L Trautmann, FA Procopio, B Yassine-Diab, G Boucher, MR Boulassel, G Ghattas, JM Brenchley, TW Schacker, BJ Hill, DC Douek, JP Routy, EK Haddad and RP Sékaly (2009), *Nature Medicine*, 15(8):893-900.

Chrobak P, MC Simard, N Bouchard, TM Ndolo, J Guertin, Z Hanna, V Dave and P Jolicoeur (2010), HIV-1 Nef Disrupts Maturation of CD4+ T Cells Through CD4/Lck Modulation, *Journal of Immunology*, 185:3948-3959.

Chrobak P, S Afkami, E Priceputu, J Poudrier, C Meunier, Z Hanna, T Sparwasser, and P Jolicoeur, HIV Nef expression favours the accumulation of CD4+ Treg cells which retain some important suppressive functions (paper under review).

Chun TW, L Carruth, D Finzi, X Shen, JA DiGuiseppe, H Taylor, M Hermankova, K Chadwick, J Margolick, TC Quinn, YH Kuo, R Brookmeyer, MA Zeiger, P Barditch-Crovo and RF Siciliano (1997a), Quantification of Latent Tissue Reservoirs and Total Body Viral Load in HIV-1 Infection, *Nature*, 387(6629):183-188.

Chun TW, L Stuyver, SB Mizell, LA Ehler, JA Mican, M Baseler, AL Lloyd, MA Nowak and AS Fauci (1997b), Presence of an Inducible HIV-1 Latent Reservoir During Highly Active Antiretroviral Therapy, *Proceedings of the National Academy of Sciences USA*, 94:13193-13197.

Chyou S, EH Ekland, AC Carpenter, TJ Tzeng, S Tian, M Michaud, JA Madri and TT Lu (2008), Fibroblast-Type Reticular Stromal Cells Regulate the Lymph Node Vasculature, *Journal of Immunology*, 181:3887-3896.

Clapham PR (1997), HIV and Chemokines: Ligands Sharing Cell-Surface Receptors, *Trends in Cell Biology*, 7:264–268.

Clever JL and TG Parslow (1997), Mutant Human Immunodeficiency Virus Type 1 Genomes With Defects in RNA Dimerization or Encapsidation, *Journal of Virology*, 71(5):3407-3414.

Cohen EA, RA Subbramanian and HG Göttlinger (1996), Role of Auxiliary Proteins in Retroviral Morphogenesis, *Current Topics in Microbiology and Immunology*, 214:219-235.

Coleman SH, R Madrid, N Van Damme, RS Mitchell, J Bouchet, C Servant, S Pillai, S Benichou and JC Guatelli (2006), Modulation of Cellular Protein Trafficking by Human Immunodeficiency Virus Type 1 Nef: Role of Acidic Residue in the ExxxLL motif, *Journal of Virology*, 80:1837-1849.

Corbitt G, AS Bailey and G Williams (1990), HIV Infection in Manchester, 1959, *Lancet*, 336(8706):51.

Crabtree GR and EN Olson (2002), NFAT Signaling: Choreographing the Social Lives of Cells, *Cell*, 109:S67-S79.

Craigie R (2001), HIV Integrase, a Brief Overview from Chemistry to Therapeutics, *Journal of Biological Chemistry*, 276:23213-23216.

Cueni LN and M Detmar (2008), The Lymphatic System in Health and Disease, *Lymphatic Research and Biology*, 6:109-122.

Cujec TP, H Cho, E Maldonado, J Meyer, D Reinberg and BM Peterlin (1997), The Human Immunodeficiency Virus Transactivator Tat Interacts with the RNA Polymerase II Holoenzyme, *Molecular Cell Biology*, 17(4):1817-1823.

Cullen BR (2003), Nuclear mRNA Export: Insights from Virology, *Trends in Biochemical Sciences*, 28:419-424.

Curry CL, LL Reed, TE Golde, L Miele, BJ Nickoloff and KE Foreman (2005), Gamma Secretase Inhibitor Blocks Notch Activation and Induces Apoptosis in Kaposi's Sarcoma Tumor Cells, *Oncogene*, 24:6333-6344.

Cyster JG (1999), Chemokines and Cell Migration in Secondary Lymphoid Organs, *Science*, 286:2098-2102.

Cyster JG (2005), Chemokines, Sphingosine-1-Phosphate, and Cell Migration in Secondary Lymphoid Organs, *Annual Reviews in Immunology*, 23:127-159.

Daniel MD, F Kirchhoff, SC Czajak, PK Sehgal and RC Desrosiers (1992), Protective Effects of a Live Attenuated SIV Vaccine With a Deletion in the Nef Gene, *Science*, 258(5090):1938-1941.

Dalgleish AG, PC Beverley, PR Clapham, DH Crawford, MF Greaves and RA Weiss (1984), The CD4 (T4) Antigen Is An Essential Component of the Receptor for the AIDS retrovirus, *Nature*, 312:763-767.

DaSilva LL, R Sougrat, PV Burgos, K Janvier, R Mattera and JS Bonifacio (2009), Human Immunodeficiency Virus Type 1 Nef Protein Targets CD4 to the Multivesicular Body Pathway, *Journal of Virology*, 83:6578-6590.

Davis KJ, AO Anderson, TW Geisbert, KE Steele, JB Geisbert, P Vogel, BM Connolly, JW Huggins, PB Jahrling and NK Jaax (1997), Pathology of Experimental Ebola Virus Infection in African Green Monkeys. Involvement of Fibroblastic Reticular Cells, *Archives of Pathology and Laboratory Medicine*, 121(8):805-819.

Deacon NJ, A Tsykin, A Solomon, K Smith, M Ludford-Menting, DJ Hooker, DA McPhee, AL Greenway, A Ellett, C Chatfield, VA Lawson, S Crowe, A Maerz, S Sonza, J Learmont, JS Sullivan, A Cunningham, D Dwyer, D Dowton and J Mills (1995), Genomic Structure of an Attenuated Quasi Species of HIV-1 from a Blood Transfusion Donor and Recipients, *Science*, 270(5238):988-991.

Deftos ML, E Huang, EW Ojala, KA Forbush and MJ Bevan (2000), Notch1 Signaling Promotes the Maturation of CD4 and CD8 SP Thymocytes, *Immunity*, 13:73-84.

Demehri S, Z Liu, J Lee, MH Lin, SD Crosby, CJ Roberts, PW Grigsby, JH Miner, AG Farr and R Kopan (2008), Notch-Deficient Skin Induces a Lethal Systemic B-Lymphoproliferative Disorder by Secreting TSLP, a Sentinel for Epidermal Integrity, *PLoS Biology*, 6(5):992-1005.

Dievart A, N Beaulieu and P Jolicoeur (1999), Involvment of Notch1 in the Development of Mouse Mammary Tumors, *Oncogene*, 18:5973-5981.

Dlugosz AA, AB Glick, T Tennenbaum, WC Weinberg and SH Yuspa (1995), Isolation and Utilization of Epidermal Keratinocytes for Oncogene Research, *Methods in Enzymology*, vol 254: p3-20.

Dotto GP (1999), Signal Transduction Pathways Controlling the Switch Between Keratinocyte Growth and Differentiation, *Critical Reviews in Oral Biology & Medicine*, 10(4):442-457.

Douek CD, MR Betts, BJ Hill, SJ Little, R Lempicki, JA Metcalf, J Casazza, C Yoder, JW Adelsberger, RA Stevens, MW Baseler, P Keiser, DD Richman, RT Davey and RA Koup (2001), Evidence for Increased T Cell Turnover and Decreased Thymic Output in HIV Infection, *Journal of Immunology,* 167(11):6663-6668.

Douek DC, JM Brenchley, MR Betts, DR Ambrozak, BJ Hill, Y Okamoto, JP Casazza, J Kuruppu, Kunstman, S Wolinsky, Z Grossman, M Dybul, A Oxenius, DA Price, M Connors and RA Koup (2002), HIV Preferentially Infects HIV-Specific CD4+ T Cells, *Nature*, 417(6884):95-98.

Dumortier A, AD Durhman, M Di Piazza, S Vauclair, U Koch, G Ferrand, I Ferrero, S Demehri, LL Song, AG Farr, WJ Leonard, R Kopan, L Miele, D Hohl, D Finke and F Radtke (2010), Atopic Dermatitis-Like Disease and Associated Lethal Myeloproliferative Disorder Arise from Loss of Notch Signaling in the Murine Skin, *PLoS One*, 5(2):1-14.

Dustin ML and TA Springer (1989), T-Cell Receptor Cross-Linking Transiently Stimulates Adhesiveness Through LFA-1, *Nature*, 341:619-624.

Earl PL, B Moss and RW Doms (1991), Folding, Interaction with GRP78-BiP, Assembly, and Transport of the Human Immunodeficiency Virus Type 1 Envelope Protein, *Journal of Virology*, 65(4):2047-2055.

Eberl G, S Marmon, MJ Sunshine, PD Rennert, Y Choi and DR Littman (2004), An Essential Function for the Nuclear Receptor RORγt in the Generation of Fetal Lymphoid Tissue Inducer Cells, *Nature Immunology*, 5:64-73.

Ebisuno Y, T Tanaka, N Kanemitsu, H Kanda, K Yamaguchi, T Kaisho, S Akira and M Miyasaka (2003), Cutting Edge: the B Cell Chemokine CXC Chemokine Ligand 13/B Lymphocyte Chemoattractant Is Expressed in the High Endothelial Venules of Lymph Nodes and Peyer's Patches and Affects B Cell Trafficking Across High Endothelial Venules, *Journal of Immunology*, 171(4):1642-1646.

Edinger AL, JL Mankowski, BJ Doranz, BJ Margulies, B Lee, J Rucker, M Sharron, TL Hoffman, JF Berson, MC Zink, VM Hirsch, JE Clements and RW Doms (1997), CD4-Independent, CCR5-Dependent Infection of Brain Capillary Endothelial Cells By A Neurovirulent Simian Immunodeficiency Virus Strain, *PNAS*, 94(26):14742-14747.

Ehrich WE (2006), The Role of the Lymphocyte in the Circulation of the Lymph, *Annuals of the New York Academy of Sciences*, 46(8):823-857.

Emerman M (1996), HIV-1, Vpr and the Cell Cycle, *Current Biology*, 6(9):1096-1103.

Emert-Sedlak L, T Kodama, EC Lerner, W Dai, C Foster, BW Day, JS Lazo and TE Smithgall (2009), Chemical Library Screens Targeting an HIV-1 Accessory Factor / Host Cell Kinase Complex Identify Antiretroviral Compounds, *ACS Chemical Biology*, 4(11):939-947.

Estes JD, S Wietgrefe, T Schacker, P Southern, G Beilman, C Reilly, JM Milush, JD Lifson, DL Sodora, JV Carlis and AT Haase (2007), Simian Immunodeficiency Virus-Induced Lymphatic Tissue Fibrosis is Mediated by Transforming Growth Factor Beta 1-Positive Regulatory T Cells and Begins in Early Infection, *Journal of Infectious Diseases*, 195(4):551-561.

Fackler OT, W Luo, M Geyer, AS Alberts, BM Peterlin (1999), Activation of Vav by nef Induces Cytoskeletal Rearrangements and Downstream Effector Functions, *Molecular Cell*, 3(6):729-739.

Fackler OT, X Lu, JA Frost, M Geyer, B Jiang, W Luo, A Abo, AS Alberts and BM Peterlin (2000), p21-Activated Kinase 1 Plays A Critical Role in Cellular Activation by Nef, *Molecular Cell Biology*, 20(7):2619-2627.

Fackler OT and AS Baur (2002), Live and Let Die: Nef Functions Beyond HIV Replication, *Immunity*, 16:493-497.

Finke D, H Acha-Orbea, A Mattis, M Lipp and J Kraehenbuhl (2002), CD4+CD3- Cells Induce Peyer's Patch Development. Role of $\alpha_4\beta_1$ Integrin Activation by CXCR5, *Immunity*, 17:363-373.

Finkel TH, G Tudor-Williams, NK Banda, MF Cotton, T Curiel, C Monks, TW Baba, RM Ruprecht and A Kupfer (1995), Apoptosis Occurs Predominantly in Bystander Cells and Not in Productively Infected Cells of HIV- and SIV-Infected Lymph Nodes, *Nature Medicine*, 1(2):129-134.

Finzi D, M Hermankova, T Pierson, LM Carruth, C Buck, RE Chaisson, TC Quinn, K Chadwick, J Margolick, R Brookmeyer, J Gallant, M Markowitz, DD Ho, DD Richman and RF Siciliano (1997), Indentification of a Reservoir for HIV-1 in Patients on Highly Active Antiretroviral Therapy, *Science*, 278(5341):1295-1300.

Fletcher AL, V Lukacs-Kornek, ED Reynoso, SE Pinner, A Bellermare-Pelletier, MS Curry, AR Collier, RL Boyd and SJ Turley (2010), Lymph Node Fibroblastic Reticular Cells Directly Present Peripheral Tissue Antigen Under Steady-State and Inflammatory Conditions, *Journal of Experimental Medicine*, 207(4):689-697.

Fleury S, GP Rizzardi, A Chapuis, G Tambussi, C Knabenhans, E Simeoni, J Meuwly, J Corpataux, A Lazzarin, F Miedena and G Pantaleo (2000), Long-Term Kinectics of T Cell Production in HIV-Infected Subjects Treated with Highly Active Antiretroviral Therapy, *Proceedings of the National Academy of Sciences, USA*, 97(10):5393.5398.

Forslind B, M Lindberg, GM Roomans, I Pallon and Y Werner-Linde (1997), Aspects of the Physiology of Human Skin: Studies Using Particle Probe Analysis, *Micriscopy Research and Technique*, 38:373-386.

Förster R, A Schubel, D Breitfeld, E Kremmer, I Renner-Müller, E Wolf and M Lipp (1999), CCR7 Coordinates the Primary Immune Response by Establishing Functional Microenvironments in Secondary Lymphoid Organs, *Cell*, 99(1):23-33.

Förster R, A Braun and T Worbs (2012), Lymph Node Homing Of T Cells And Dendritic Cells Via Afferent Lymphatics, *Trends in Immunology*, 33(6):271-280.

Foster JL and JV Garcia (2008), HIV-1 Nef: At the Crossroads, *Retrovirology*, 5:84.

Foster JL, SJ Denial, BRS Temple and JV Garcia (2011), Mechanisms of HIV-1 Nef Function and Intracellular Signalling, *Journal of Neuroimmune Pharmacology*, 6:230-246.

Frankel AD and JAT Young (1998), HIV-1: Fifteen Proteins and an RNA, *Annual Reviews in Biochemistry*, 67:1-25.

Freed EO and MA Martin (1996), Domains of the Human Immunodeficiency Virus Type 1 Matrix and gp41 Cytoplasmic Tail Required for Envelope Incorporation into Virions, *Journal of Virology*, 70(1):341-351.

Fritz JH and JL Gommerman (2011), Cytokine/Stromal Cell Networks and Lymphoid Tissue Environments, *Journal of Interferon & Cytokine Research*, 31(3):277-289.

Fu YX, G Huang, Y Wang and DD Chaplin (1998), B Lymphocytes Induce the Formation of Follicular Dendritic Cell Clusters in a Lymphotoxin α-dependent Fashion, *Journal of Experimental Medicine*, 187(7):1009-1018.

Fuchs E and H Green (1980), Changes in Keratin Gene Expression During Terminal Differentiation of the Keratinocyte, *Cell*, 25:617-625.

Fuchs E (1995), Keratins and the Skin, *Annual Review of Cell and Developmental Biology*, 11:123-153.

Fuchs E and S Raghavan (2002), Getting Under the Skin of Epidermal Morphogenesis, *Nature Reviews Genetics*, 3(3):199-209.

Fuentes GM, L Rodriguez-Rodriguez, C Palaniappan, PJ Fay and RA Bambara (1996), Strand Discplacement Synthesis of the Long Terminal Repeats by HIV-1 Reverse Transcriptase, *Journal of Biological Chemistry*, 271:1966-1971.

Furuse M and S Tsukita (2006), Claudins In Occluding Junctions of Humans and Flies, *Trends in Cell Biology*, 16:181-188.

Gaines H, MA von Sydow, LV von Stedingk, G Biberfeld, B Böttinger, LO Hansson, P Lundberg, AB Sönnerborg, J Wasserman and OO Strannegaaed (1990), Immunological Changes in Primary HIV-1 Infection, *AIDS*, 4(10):995-999.

Garber ME and KA Jones (1999), HIV-1 Tat: Coping with Negative Elongation Factors, *Current Opinion in Immunology*, 11:460-465.

Garcia JV and AD Miller (1991) Serine Phosphorylation-Independent Downregulation of Cell-Surface CD4 by Nef, *Nature*, 350(6318):508-511.

García-Martínez LF, G Mavankal, JM Neveu, WS Lane, D Ivanov and RB Gaynor (1997), Purification of a Tat-Associated Kinase Reveals a TFIIH Complex that Modulates HIV-1 Transcription, *The EMBO Journal*, 16(10) :2836-2850.

Geyer M, OT Fackler and BM Peterlin (2001), Structure-Function Relationships in HIV-1 Nef, *EMBO Reports*, 2(7) :580-585.

Goila-Gaur R and K Strebel (2008), HIV-1 Vif, APOBEC, and Intrisic Immunity, *Retrovirology*, 5 :51.

Gonda MA, MJ Braun, JE Clemets, JM Pyper, F Wong-Staal, RC Gallo and RV Gilden (1986), Human T-Cell Lymphotropic Virus Type III Shares Sequence Homology With A Family of Pathogenic Lentiviruses, *Proceedings of the National Academy of Sciences USA*, 83(11):4007-4011.

Gowthaman U, SB Chodisetti and JN Agrewala (2010), T Cell Help to B Cells in Germinal Centers: Putting the Jigsaw Together, *International Reviews of Immunology*, 29(4):403-420.

Graham FL, J Smiley, WC Russell and R Nairn (1977), Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5, *The Journal of General Virology*, 36(1):59-74.

Green DF, L Resnick and JJ Bourgoignie (1991), HIV Infects Glomerular Endothelial and Mesangial But Not Epithelial Cells In Vitro, *Kidney International*, 41:956-960.

Green KJ, MLA Virata, GW Elgart, JR Stanley and DAD Parry (1992), Comparative Structural Analysis of Desmoplakin, Bullous Pemphogoid Antigen and Plectin: Members of New Gene Family Involved in Organization of Intermediate Filaments, *International Journal of Biological Macromolecules*, 14:145-153.

Gretz JE, CCNorbury, AO Anderson, AE Proudfoot, S Shaw (2000), Lymph-Borne Chemokines and Other Low Molecular Wieght Molecules Reach High Endothelial Venules Via Specialized Conduits While A Functional Barrier Limits Access to the Lymphocyte Microenvironments in Lymph Node Cortex, *Journal of Experimental Medicine*, 192:1425-1440.

Grewe B and K Uberla (2010), The Human Immunodeficiency Virus Type 1 Rev Protein: Ménage À Trois During the Early Phase of the Lentiviral Replication Cycle, *Journal of General Virology*, 91(8):1893-1897.

Grigorova IL, SR Schwab, TG Phan, TH Pham, T Okada and JG Cyster (2009), Cortical Sinus Probing, S1P1-Dependent Entry and Flow-Based Capture of Egressing T Cells, *Nature Immunology*, 10(1):58-65.

Groom, HC, EC Anderson and AM Lever (2009), Rev: Beyond Nuclear Export, *Journal of General Virology*, 90:1303–1318.

Gross A, JM McDonnell and SJ Korsmeyer (1999), BCL-2 Family Members and the Mitochondria in Apoptosis, *Genes & Development*, 13(15) :1899-1911.

Grzesick S, SJ Stahl, PT Wingfield and A Bax (1996), The CD4 Determinant for Downregulation by HIV-1 Nef Directly Binds to Nef. Mapping of the Nef Binding Surface by NMR, *Biochemistry*, 35 :10256-10261.

Gunn MD, VN Ngo, KM Ansel, EH Ekland, JG Cyster and LT Williams (1998a), A B-Cell-Homing CHemokine Made in Lymphoid Follcils Activates Burkitt's Lymphoma Receptor-1, *Nature*, 391:799-803.

Gunn MD, K Tangemann, C Tam, JG Cyster, SD Rosen and LT Williams (1998b), A Chemokine Expressed in High Endothelial Venules Promotes the Adhesion and Chemotaxis of Naive T Lymphocytes, *Proceedings of the National Academy of Sciences USA*, 95(1):258-263.

Guo J, L He, P Yuan, P Wang, Y Lu, F Tong, Y Wang, Y Yin, J Tian and J Sun (2012), ADAM10 Overexpression in Human Non-Small Cell Lung Cancer Correlates with Cell Migration and Invasion Through the Activation of the Notch1 Signaling Pathway, *Oncology Reports*, 28(5):1709-1718.

Hall JG and B Morris (1965), The Immediate Effect of Antigens on the Cell Output of a Lymph Node, *British Journal of Experimental Pathology*, 46:450-454.

Hanna Z, DG Kay, N Rebai, A Guimond, S Jothy and P Jolicoeur (1998), Nef Harbors A Major Determinant of Pathogenicity for an AIDS-like Disease Induced by HIV-1 in Transgenic Mice, *Cell*, 95:163-175.

Hanna Z, E Priceputu, C Hu, P Vincent and P Jolicoeur (2006), HIV-1 Nef Mutations Abrogating Downregulation of CD4 Affect Other Nef Funtions and Show Reduced Pathogenicity in Transgenic Mice, *Virology*, 346:40-52.

Hansson EM, F Lanner, D Das, A Mutvei, U Marklund, J Ericson, F Farnebo, G Stumm, H Stenmark, ER Anderson and U Lendahl (2010), Control of Notch-ligand Endocytosis by Ligand-Receptor Interaction, *Journal of Cell Science*, 123:2931-2942.

Harris M (1995), The Role of Myristoylation in the Interactions Between Human Immunodeficiency Virus Type 1 Nef and Cellular Proteins, *Biochemical Society Transactions*, 23:557-561.

Harris M (1999), HIV: A New Role for Nef in the Spread of HIV. *Current Biology*, 9:459-461.

Hazenberg MD, D Hamann, H Schuitemaker and F Miedema (2000), T Cell Depletion in HIV-1 Infection: How CD4+ T Cells Go Out of Stock, *Nature Immunology*, 1:285-289.

Hazenberg MD, SA Otto, H van Benthem, MT Roos, RA Coutinho, JM Lange, D Hamann, M Prins and F Miedema (2003), Persistent Immune Activation in HIV-1 Infection is Associated with Progression to AIDS, *AIDS*, 17:1881-1888.

Heikenwalder M, M PRinz, N Zeller, KS Lang, T Junt, S Rossi, A Tumanov, H Schmidt, J Priller, L Flatz, T Rülicke, AJ Macpherson, GA Höllander, SA Nedospasov and A Aguzzi (2008), Overexpression of Lymphotoxin in T Cells Induces Fulminant Thymic Involution, *American Journal of Pathology*, 172(6):1555-1570.

Hennings H, D Michael, C Cheng, P Steinert, K Holbrook and SH Yuspa (1980), Calcium Regulation of Growth and Differentiation of Mouse Epidermal Cells in Culture, *Cell* 19:245-254.

Hijzi K, Y Wang, C Scala, S Jeffs, C Longstaff, D Stieh, B Haggarty, G Vanham, D Schols, J Balzarini, IM Jones, J Hoxie, R Shattock and CG Kelly (2011), DC-SIGN Increases the Affinity of HIV-1 Envelope Glycoprotein Interaction With CD4, *PLoS One*, 6(12):1-14.

Hill CP, D Wirthylake, DP Bancroft, AM Christensen and WI Sundquist (1996), Crystal Structures of the Trimeric Human Immunodeficiency Virus Type-1 Matrix Protein: Implications for Membrane Assiciation and Assembly, *Proceedings of the National Academy of Sciences USA*, 93(7):3099-3104.

Hong YK, K Foreman, JW Shin, S Hirakawa, CL Curry, DR Sage, T Libermann, BJ Dezube, JD Fingeroth and M Detmar (2004), Lymphatic Reprogramming of Blood Vascular Endothelium by Kaposi Sarcoma-Associated Herpesvirus, *Nature Genetics*, 36:683-685.

Huang M, JM Orenstein, MA Martin and EO Freed (1995), p6Gag Is Required for Particle Production from Full-Length Human Immunodeficiency Virus Type-1 Molecular Clones Expressing Protease, *Journal of Virology*, 69(11):6810-6818.

Huang MB, M Khan, M Garcia-Barrio, M Powell and VC Bond (2001), Apoptotic Effects In Primary Human Umbilical Vein Endothelial Cell Cultures Caused By Exposure To Virion-Associated and Cell Membrane-Associated HIV-1 gp120, *Journal of Acquired Immune Deficiency Syndrome*, 27(3):213-221.

Huber HE, JM McCoy, JS Seehra and CC Richardson (1989), Human Immunodeficiency Virus 1 Reverse Transcriptase, *Journal of Biological Chemistry*, 264:4669-4678.

Iso T, L Kedes and Y Hamamori (2003), HES and HERP Families: Multiple Effectors of the Notch Signaling Pathway, *Journal of Cell Physiology*, 194:237-255.

Izon DJ, JA Punt, L Xu, FG Karnell, D Allman, PS Myung, NJ Boerth, JC Pui, GA Koretzky and WS Pear (2001), Notch1 Regulates Maturation of CD4+ and CD8+ Thymocytes by Modulating TCR Signal Strength, *Immunity*, 14:253-264.

Jaffer ZM and J Chernoff (2002), p21-Activated Kinases: Three More Join The Pak, *The International Journal of Biochemistry and Cell Biology*, 34(7):713-717.

Jia H, M Lohr, S Jezequel (2001), Cysteine-Rich and Basic Domain HIV-1 Tat Peptides Inhibit Angiogenesis and Induce Endothelial Cell Apoptosis, *Biochemical and Biophysical Research Communications*, 283:469-479.

Jones KA and BM Peterlin (1994), Control of RNA Initiation and Elongation at the HIV-1 Promoter, *Annual Reviews in Biochemistry*, 63:717-743.

Jones PH and FM Watt (1993), Separation of Human Epidermal Stem Cells from Transit Amplifying Cells on the Basis of Differences in Integrin Function and Expression, *Cell*, 73:713-724.

Jones PH, S Harper and FM Watt (1995), Stem Cell Patterning and Fate in the Human Epidermis, *Cell*, 80-83-93.

Jouvenet N, SJ Neil, M Zhadina, T Zang, Z Kratovac, Y Lee, M McNatt, T Hatziioannou and PD Bieniasz (2009), Broad-Spectrum Inhibition of Retroviral and Filoviral Particle Release by Tetherin, *Journal of Virology*, 83:1837-1844.

Jundt F, I Anagnostopoulos, R Förster, S Mathas, H Stein and B Dörken (2002), Activated Notch1 Signaling Promotes Tumor Cell Proliferation and Survival in Hodgkin and Anaplastic Large Cell Lymphoma, *Blood*, 99(9):3398-3403.

Kammula EC, J Mötter, A Gorgels, E Jonas, S Hoffmann and D Willbold (2012), Brain Transcriptome-Wide Screen for HIV-1 Nef Protein Interaction Partners Reveals Various Membrane-Associated Proteins, *PLoS One*, 7(12):1-12.

Kanmogne GD, RC Kennedy and P Grammas (2002), HIV-1 gp120 Proteins and gp160 Peptides Are Toxic To Brain Endothelial Cells and Neurons: Possible Pathways for HIV Entry Into The Brain and HIV-Associated Dementia, *Journal of Neuropathology and Experimental Neurology*, 61:992-1000.

Kanmogne GD, C Primeaux and P Grammas (2005), Induction of Apoptosis and Endothelin-1 Secretion In Primary Human Lung Endothelial Cells By HIV-1 gp120 Proteins, *Biochemical and Biophysical Research Communications*, 333(4):1107-1115.

Karn J (1999), Tackling Tat, *Journal of Molecular Biology*, 293(2):235-254.

Kasic T, P Colombo. C Soldani, C Wang, E Miranda, M Roncalli, V Bronte and A Viola (2011), Modulation of Human T-Cell Functions by Reactive Nitrogen Species, *European Journal of Immunology*, 41:1843-1849.

Katakai T, T Hara, M Sugai, H Gonda and A Shimizu (2004), Lymph Node Fibroblastic Reticular Cells Construct the Stromal Reticulum Via Contact With Lymphocytes, *Journal of Experimental Medicine*, 200(6):783-795.

Katakai T, H Suto, M Sugai, H Gonda, A Togawa, S Suematsu, Y Ebisuno, K Katagiri, T Kinashi and A Shimizu (2008), Organizer-Like Reticular Stromal Cell Layer Common to Adult Secondary Lymphoid Organs, *Journal of Immunology*, 181(9):6189-6200.

Kataru RP, H Kim, C Jang, DK Choi, BI Koh, M Kim, S Gollamudi, YK Kim, SH Lee and GY Koh (2011), T Lymphocytes Negatively Regulate Lymph Node Lymphatic Vessel Formation, *Immunity*, 34(1):96-107.

Kay DG, P Yue, Z Hanna, S Jothy, E Tremblay and P Jolicoeur (2002), Cardiac Disease in Transgenic Mice Expressing Human Immunodeficiency Virus-

1 Nef in Cells of the Immune System, *American Journal of Pathology*, 161(1):321-335.

Kenkau T, I Bacik, JR Bennink, JW Yewdell, T Húning, A Schimpl and U Schubert (1997), The Human Immunodeficiency Virus Type 1 (HIV-1) Vpu Protein Interferes with an Early Step in the Biosynthesis of Major Histocompatibility Complex (MHC) Class I Molecules, *Journal of Experimental Medicine*, 185(7):1295-1305.

Kestler HW, DJ Ringler, K Mori, DL Panicali, PK Sehgal, MD Daniel and RC Desrosiers (1991), Importance of the nef Gene for Maintenance of High Virus Loads and for Development of AIDS, *Cell*, 65(4):651-662.

Kim TA, HK Avraham, YH Koh, S Jiang, IW Park and S Avraham (2003), HIV-1 Tat-Mediated Apoptosis in Human Brain Microvascular Endothelial Cells, *Journal of Immunology*, 170:2629-2937.

Kim MY, Gaspal FM, HE Wiggett, FM McConnell, A Gulbranson-Judge, C Raykundalia, LS Walker, MD Goodall and PJ Lane (2003), CD4(+)CD3(-) Accessory Cells Costimulate Primed CD4 T Cells Through OX40 And CD30 At Sites Where T Cells Collaborate With B Cells, *Immunity*, 18(5):643-654.

Kim MY, G Anderson, A White, E Jenkinson, W Arlt, IL Martensson, L Erlandsson and PJ Lane (2005), OX40 Ligand And CD30 Ligand Are Expressed On adult But Not Neonatal CD4+ CD3- Inducer Cells: Evidence That IL-7 Signals Regulate CD30 Ligand but OX40 Ligand Expression, *Journal of Immunology*, 174:6686-6691.

Kim MY, S Rossi, D Withers, F McConnell, KM Toellner, F Gaspal, E Jenkinson, G Anderson and PJ Lane (2007a), Heterogeneity Of Lymphoid Tissue Inducer Cell Populations Present In Embryonic And Adult Mouse Lymphoid Tissues, *Immunology*, 124:166-174.

Kim MY, FM McConnell, FM Gaspal, A White, SH Glanville, V Bekiaris, LS Walker, J Caamano, E Jenkinson, G Anderson and PJ Lane (2007b), Function of CD4+ CD3- Cells in Relation To B- and T-Zone Stroma in Spleen, *Blood*, 109(4):1602-1610.

Kim MY, KS Kim, F McConnell and P Lane (2009), Lymphoid Tissue Inducer Cells: Architects of CD4 Immune Responses in Mice and Men, *Journal of Translation Immunology*, 157:20-26.

Kimpton J and M Emmerman (1992), Detection of Replication-Competent and Pseudotyped Human Immunodeficiency Virus with a Sensitive Cell Line on the Basis of Activation of an Integrated Beta-Galactosidase Gene, *Journal of Virology*, 66:2232-2239.

Kraal G and RE Mebius (1997), High Endothelial Venules: Lymphocyte Traffic Control and Controlled Traffic, *Advances in Immunology*, 65:347-395.

Kunnimalaiyaan M and H Chen (2007), Tumor Suppressor Role of Notch-1 Signaling in Neuroendocrine Tumors, *Oncologist*, 12(5):535-542.

Lafon ME, JL Gendrault, C Royer, A Kirn and AM Steffan (1993), Human Endothelial Cells Isolated From Hepatic Sinusoids and the Umbilical Vein Display a Different Permissiveness for HIV-1, *Research in Virology*, 144(1):99-104.

Lama J, A Mangasarian and D Trono (1999), Cell-Surface Expression of CD4 Reduces HIV-1 Infectivity by Blocking Env Incorporation in a Nef- and Vpu-Inhibitable Manner, *Current Biology*, 9(12):622-631.

Lammerman T and M Sixt (2008), The Microanatomy of T-Cell Responses, *Immunology Reviews*, 221:26-43.

Lane EB, J Bartek, PE Purkis and IM Leigh (1985), Keratin Antigens in Differentiating Skin, *Annals of the New York Academy of Sciences*, 455:241-258.

Lane P MY Kim, D Withers, F Gaspal, V Bekiaris, G Desanti, M Khan, F McConnell and G Anderson (2008), Lymphoid Tissue Inducer Cells In Adaptative CD4 T Cell Dependent Responses, *Seminars in Immunology*, 20(3):159-163.

Laughrea M, L Jetté, J Mak, L Kleiman, C Liang and MA Wainberg (1997), Mutations in the Kissing-Loop Hairpin of Human Immunodeficiency Virus Type 1 Reduce Viral Infectivity As Well As Genomic RNA Packaging and Dimerization, *Journal of Virology*, 71(5), 3397-3406.

Lederman MM and L Margolis (2008), The Lymph Node in HIV Pathogenesis, *Seminars in Immunology*, 20:187-195.

Lee CH, K Saksela, UA Mirza, BT Chait and J Kuriyan (1996), Crystal Structure of the Conserved Core of HIV-1 Nef Complexed With a Src Family SH3 Domain, *Cell*, 85(6):931-942.

Link A, TK Vogt, S Favre, MR Britschgi, H Acha-Orbera, B Hinz, JG Cyster and SA Luther (2007), Fibroblastic Reticular Cells In Lymph Nodes Regulate the Homeostasis of Naïve T Cells, *Nature Immunology*, 8(11):1255-1265.

Liu R, X Li, A Tulpule, Y Zhou, JS Scehnet, S Zhang, JS Lee, PM Chaudhary, J Jung and PS Gill (2010), KSHV-Induced Notch Components Render

Endothelial And Mural Cell Characteristics and Cell Survival, *Blood*, 115(4):887-895.

Lo CG, Y Xu, RL Proia and JG Cyster (2005), Cyclical Modulation of Sphingosine-1-Phosphate Receptor 1 Surface Expression During Lymphocyte Recirculation and Relationship to Lymphoid Organ Transit, *Journal of Experimental Medicine*, 201:291-301.

Lo Muzio L, G Pannone, S Staibano, MD Mignogna, M Grieco, P Ramires, AM Romito, G De Rosa and A Piattelli (2002), WNT-1 Expression in Basal Cell Carcinoma of Head and Neck. An Immunohistochemical and Confocal Study with Regard to the Intracellular Distribution of Beta-Catenin, *Anticancer Research*, 22(2A):565-576.

Lowell S, P Jones, I Le Roux, J Dunne and FM Watt (2000), Stimulation of Human Epidermal Differentiation by Delta-Notch Signaling at the Boundaries of Stem-Cell Clusters, *Current Biology*, 10(9):491-500.

Lubman OY, SV Korolev and R Kopan (2004), Anchoring Notch Genetics and Biochemistry; Structural Analysis of the Ankyrin Domain Sheds Light on Existing Data, *Molecular Cell*, 13:619-626.

Luciw PA (1996), in *Fundamental Virology*, ed. BM Fields, DM Knipe, PM Howley, pp845-916. Philadelphia: Lippincott-Raven. Third edition.

Lundquist CA, M Tobiume, J Zhou, D Unutmaz and C Aiken (2002), Nef-Mediated Downregulation of CD4 Enhances Human Immunodeficiency Virus Type 1 Replication in Primary T Lymphocytes, *Journal of Virology*, 76(9):4625-4633.

Madrid R, K Janvier, D Hitchin, J Day, S Coleman, C Noviello, J Bouchet, A Benmerah, J Guatelli and S Benichou (2005), Nef-Induced Alteration of the Early/Recycling Endosomal Compartment Correlates with Enhancement of HIV-1 Infectivity, *Journal of Biological Chemistry*, 280:5032-5044.

Malhotra D, AL Fletcher, J Astarita, V Lukacs-Kornek, P Tayalia, SF Gonzalez, KG Elpek, SK Chang, K Knoblich, ME Hemler, MB Brenner, MC Carroll, DJ Mooney, SJ Turley, Immunological Genome Project Consortium (2012), Transcription Profiling of Stroma from Inflamed and Resting Lymph Nodes Defines Immunological Hallmarks, *Nature Immunology*, 13(5):499-510.

Mammucari C, A Tommasi di Vignano, AA Sharov, J Neilson, MC Havrda, DR Roop, VA Botchkarev, GR Crabtree and GP Dotto (2005), Integration of Notch1 and Calcineurin/NFAT Signaling Pathways in Keratinocyte Growth and Differentiation Control, *Developmental Cell*, 8:665-676.

Mangasarian A and D Trono (1997), The Multifaceted Role of Nef, *Research in Virology*, 148(1):30-33.

Mansky LM (1996), The Mutation Rate of Human Immunodeficiency Virus Type 1 is Influenced by the vpr Gene, *Virology*, 222(2):391-400.

Mansoui IM, DG Winder, TM Moallem, M Osman, M Mayford, RD Hawkins, ER Kandel (1998), Inducible and Reversible Gene Expression wth the rtTA System for the Study of Memory, *Neuron*, 21:257-265.

Mansouri M, K Viswanathan, JH Douglas, J Hines, J Gustin, AV Moses and K Fruh (2009), Molecular Mechanism of BST2/Tetherin Downregulation by K5/MIR2 of Kaposi's Sarcoma-Associated Herpesvirus, *Journal of Virology*, 83:9672-9681.

Marecki JC, CD Cool, JE Parr, VE Beckey, PA Luciw, AF Tarantal, A Carville, RP Shannon, A Cota-Gomez, RM Tuder, NF Voelkel and SC Flores (2006), HIV-1 Nef Is Associated With Complex Pulmonary Vascular Lesions In SHIV-*nef*-infected Macaques, *American Journal of Respiratory and Critical Care Medicine*, 174:437-445.

Margottin F, SP Bour, H Durand, L Selig, S Benichou, V Richard, D Thomas, K Strebel and R Benarous (1998), A Novel WD Protein, h-beta TrCp, that Interacts with HIV-1 Vpu Connects CD4 to the ER Degradation Pathway Through an F-Box Motif, *Molecular Cell*, 1:565-574.

Marsh JW (1999), The Numerous Effector Functions of Nef, *Archives of Biochemistry and Biophysics*, 365:192-198.

Martín-Fontecha A, S Sebastiani, UE Höpken, M Uguccioni, M Lipp, A Lanzavecchia and F Sallusto (2003), Regulation of Dendritic Cell Migration to the Draining Lymph Node: Impact on T Lymphocyte Traffic and Priming, *Journal of Experimental Medicine*, 198(4):615-621.

Massi D, F Tarantini, A Franchi, M Paglierani, C Di Serio, S Pellerito, G Leoncini, G Cirino, P Geppetti and M Santucci (2006), Evidence for Differential Expression of Notch Receptors and Their Ligands in Melanocytic Nevi and Cutaneous Malignant Melanoma, *Modern Pathology*, 19(2):246-254.

Matloubian M, CG Lo, G Cinamon, MJ Lesneski, Y Xu, V Brinkmann, ML Allende, RL Proia and JG Cyster (2004), Lymphocyte Egress from Thymus and Peripheral Lymphoid Organs Is Dependent on S1P Receptor 1, *Nature*, 427(6972):355-360.

Maurer TA, LK Rodrigues, N Ameli, N Phanuphak, SJ Gange, J DeHovitz, AL Frenchm M Glesby, C Jordan, A Khalsa and NA Hessol (2004), The Effect of Highly Active Antiretroviral Therapy on Dermatologic Disease in Longitudinal Study of HIV Type 1-Infected Women, *Clinical Infectious Diseases*, 38:579-584.

Maurer TA (2005), Dermatologic Manifestations of HIV Infection, *Topics in HIV Medicine*, 13:149-154.

McCune JM (2001), The Dynamics of CD4+ T-cell Depletion in HIV Disease, *Nature*, 410:974-979.

McKinnon LR, B Nyanga, D Chege, P Izulla, M Kimani, S Huibner, L Gelmon, KE Block, C Cicala, AO Anzala, J Arthos, J Kimni and R Kaul (2011), Characterization of A Human Cervical CD4+ T Cell Subset Coexpressiong Multiple Markers of HIV Susceptibility, *Journal of Immunology*, 187(11):6032-6042.

Mebius RE (2003), Organigenesis of Lymphoid Tissues, *Nature Reviews Immunology*, 3:292-303.

Meditz AL, KL Moreau, S MaWhinney, WS Gozansky, K Melander, WM Kohrt, ME Wierman and E Connick (2012), CCR5 Expression Is Elevated On Endocervical CD4+ T Cells in Healthy Postmenopausal Women, *Journal of Acquired Immunodeficiency Syndrome*, 59(3):221-228.

Menon GK, PM Elias, SH Lee and KR Feingold (1992), Localization of Calcium in Murine Epidermis Following Disruption and Repair of the Permeability Barrier, *Cell Tissue Research*, 270:503-512.

Meyaard L, SA Otto, RR Jonker, MJ Mijnster, RPM Keet and F Miedema (1992), Programmed Death of T Cells in HIV-1 Infection, *Science*, 257:217-219.

Meyaard L and F Miedema (1995), Programmed Death of T Cells in the Course of HIV Infection, *Advances in Experimental Medicine and Biology*, 374:115-120.

Millar SE, K Willert, PC Salinas, H Roelink, R Nusse, DJ Sussman and GS Barsh (1999), WNT Signaling in the Control of Hair Growth and Structure, *Developmental Biology*, 207(1):133-149.

Missero C, E Calautti, R Eckner, J Chin, L Tsai, DM Livingstone and GP Dotto (1995), Involvement of the Cell-Cycle Inhibitor Cip1/WAF1 and the EIA-Associated p300 Protein in Terminal Differentiation, *Proceedings of the National Academy of Sciences USA*, 92:5451-5455.

Moore SP and Y Chang (1998), Kaposi's Sarcoma (KS), KS-Associated Herpesvirus, and the Criteria for Causality in the Age of Molecular Biology, *American Journal of Epidemiology*, 147(3):217-221.

Moses AV, FE Bloom, CD Pauza and JA Nelson (1993), Human Immunodeficiency Virus Infection of Human Brain Capillary Endothelial Cells Occurs Via A CD4/Galactosylceramide-independent Mechanism, *Proceedings of the National Academy of Sciences USA*, 90(22):10474-10478.

Moses AV, S Williams, ML Heneveld, J Strussenberg, M RArick, M Loveless, G Bagby and JA Nelson (1996), Human Immunodeficiency Virus Infection of Bone Marrow Endothelium Reduces Induction of Stromal Hematopoietic Growth Factors*, Blood*, 87:919-925.

Mu H, H Chai, PH Lin, Q Yao and C Chen (2007), Current Update on HIV-Associated Vascular Disease and Endothelial Dysfunction, *World Journal of Surgery*, 31:632-643.

Mueller YM, De Rosa SC, JA Hutton, J Witek, M Roederer, JD Altman and PD Katsikis (2001), Increased CD95/Fas-Induced Apoptosis of HIV-Specific CD8(+) T Cells, *Immunity*, 15(6):871-882.

Mueller SN, M Matloubian, DM Clemens, AH Sharpe, GJ Freeman, S Gangappa, CP Larsen and R Ahmed (2007), Viral Targeting of Fibroblastic Reticular Cells Contributes to Immunosuppression and Persistence During Chronic Infection, *Proceedings of the National Academy of Sciences USA*, 15430-15435.

Mueller SN, KA Hosiawa-Meagher, BT Konieczny, BM Sullican, MF Bachmann, RM Locksley, R Ahmed and M Matloubian (2007), Regulation of Homeostatic Chemokine Expression and Cell Trafficking During Immune Responses, *Science*, 317(5838):670-674.

Neil SJ, SW Eastman, N Jouvenet and PD Bieniasz (2006), HIV-1 Vpu Promotes Release and Prevents Endocytosis of Nascent Retrovirus Particles from the Plasma Membrane, PLoS Pathogene, 2:e39.

Ngo VN, H Komer, MD Gunn, KN Schmidt DS Rimiton, MD Cooper, JL Browning, JD Sedwick and JG Cyster (1999), Lymphotoxin Alpha/Beta and Tumor Necrosis Factor Are Required for Stromal Cell Expression of Homing Chemokines in B and T Cell Areas of the Spleen, *Journal of Experimental Medicine*, 189(2):403-412.

Ngo VN, RJ Cornall and JG Cyster (2001), Splenic T Zone Development Is B Cell Dependent, *Journal of Experimental Medicine*, 194(11):1649-1660.

Nguyen DH and JE Hildreth (2000), Evidence for Budding of Human Immunodeficiency Virus Type 1 Selectively from Glycolipid-Enriched Membrane Lipid Rafts, *Journal of Virology*, 74:3264-3272.

Nickoloff BJ, JZ Qin, V Chaturvedi, MF Denning, B Bonish and L Miele (2002), Jagged-1 Mediated Activation of Notch Signaling Induces Complete Maturation of Human Keratinocytes Through NF-kappaB and PPARgamma, *Cell Death and Differentiation*, 9(8): 842-855.

Nicolas M, A Wolfer, K Raj, JA Kummer, P Mll, M van Noort, CC Hui, H Clevers, GP Dotto and F Radtke (2003), Notch1 Functions as a Tumor Suppressor in Mouse Skin, *Nature Genetics*, 33(3):416-421.

Niederman TM, WR Hastings and L Ratner (1993), Myristoylation-Enhanced Binding of the HIV-1 Nef Protein To T Cell Skeletal Matrix, *Virology*, 197(1):420-425.

Nobile C, D Rudnicka, M Hasan, N Aulner, F Porrot, C Machu, O Renaud, MC Prévost, C Hivroz, O Schwartz and N Sol-Foulon (2010), HIV-1 Nef Inhibits Ruffles, Induces Filopodia, and Modulates Migration of Infected Lymphocytes, *Journal of Virology*, 84(5):2282-2293.

Noviello CM, S Benichou and JC Guatelli (2008), Cooperative Binding of the Class I Major Histocompatibility Complex Cytoplasmic Domain and Human Immunodeficiency Virus Type 1 Nef to the Endosomal AP-1 Complez via its mu subunit, *Journal of Virology*, 82:1249-1258.

Nurieva RI, Y Chung, D Hwang, XO Yang, HS Kang, L Ma, YH Wang, SS Watowich, AM Jetten, Q Tian and C Dong (2008), Generation of T Follicular Helper Cells Is Mediated By Interleukin-21 But Independent of T Helper 1, 2, or 17 Cell Lineages, *Immunity*, 29(1):138-149.

Ohshima Y, LP Yang, MN Avice, M Kurimoto, T Nakajima, M Sergerie, CE Demeure, M Sarfati and G Delespesse (1999), Naïve Human CD4+ T Cells Are a Major Source of Lymphotoxin α, *Journal of Immunology*, 162:3790-3794.

Okada T, VN Ngo, EH Ekland, R Förster, M Lipp, DR Littman and JG Cyster (2002), Chemokine Requirements for B Cell Entry to Lymph Nodes and Peyer's Patches, *Journal of Experimental Medicine*, 196(1):65-75.

Okimoto M and H. Fan (1999), Moloney Murine Leukemia Virus Infects Cells of the Developing Hair Follicle after Neonatal Subcutaneous Inoculation in Mice, *Journal of Virology*, 73(3):2509-2516.

Ono A, JM Orenstein, EO Freed (2000), Role of the Gag Matrix Domain in Targeting Human Immunodeficiency Virus Type 1 Assembly, *Journal of Virology*, 74(6):2855-2866.

Oprea M and AS Peterson (1997), Somatic Mutation Leads to Efficient Affinity Maturation When Centrocytes Recycle Back to Centroblasts, *Journal of Immunology*, 158:5155-5162.

Oroszlan S and Luftig RB (1990), Retroviral Proteases, *Current Topics in Microbiology and Immunology*, 157:153-185.

Oshima H, A Rochat, C Kedzia, K Kobayashi and Y Barrandon (2001), Morphogenesis and Renewal of Hair Follicles from Adult Multipotent Cells, *Cell*, 104:233-245.

Pachynski RK, SW Wu, MD Gunn and DJ Erle (1998), Secondary Lymphoid-Tissue Chemokine (SLC) Stimulates Integrin α4β7-Mediated Adhesion of Lymphocytes to Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) Under Flow, *Journal of Immunology*, 161:952–956.

Pantaleo G, C Graziosi and AS Fauci (1993a), The Immunopathogenesis of Human Immunodeficiency Virus Infection, *The New England Journal of Medicine*, 328(5):327-335.

Pantaleo G, C Graziosi, L Butini, PA Pizzo, SM Schnittman, DP Kopler and AS Fauci (1993b), Lymphoid Organs Function As Major Reservoirs for Human Immunodeficiency Virus, *Proceedings of the National Academy of Sciences USA*, 88:9838-9842.

Pantaleo G, C Graziosi, JF Demarest, L Butini, M Montroni, CH Fox, JM Orenstein, D PKotler, AS Fauci (1993c), HIV Infection Is Active and Progressive In Lymphoid Tissue During the Clinically Latent Stage of Disease, *Nature*, 362:355-359.

Pantaleo G and AS Fauci (1995), New Concepts in the Immunopathogenesis of HIV Infection, *Annual Reviews in Immunology*, 13:487-512.

Pappu R, SR Schwab, I Cornelissen, JP Pereira, JB Regard, Y Xu, E Camerer, YW Zheng, Y Huang, JG Cyster and SR Coughlin (2007), *Science*, 316(5822):295-298.

Parada CA and RG Roeder (1996), Enhanced Processivity of RNA Polymerase II Triggered by Tat-Induced Phosphorylation of its Carboxy-Terminal Domain, *Nature*, 384(6607):375-378.

Park IW, CK Ullrich, E Schoenberger, RK Ganju and JE Groopman (2001), HIV-1 Tat Induces Microvascular Endothelial Apoptosis Through Caspase Activation, *Journal of Immunology*, 167(5):2766-2771.

Parks AL, KM Klueg, JR Stout and MA Muskavitch (2000), Ligand Endocytosis Drives Receptor Dissociation and Activation in the Notch Pathway, *Development*, 127:1373-1385.

Paxton WA, T Dragic, RA Koup and JP Moore (1996), The Beta-Chemokines, HIV Type 1 Receptors, and Exposed Uninfected Persons, *AIDS Research and Human Retroviruses*, 12(13):1203-1207.

Pear WS, JC Aster, ML Scott, RP Hasserjian, B Soffer, J Sklar and D Baltimore (1996), Exclusive Development of T cell Neoplasms in Mice Transplanted with Bone Marrow Expressing Activated Notch Alleles, *Journal of Experimental Medicine*, 183(5):2283-2291.

Peduto L, S Dulauroy, M Lochner, GF Spath, MA Morales, A Cumano and G Eberl (2009), Inflammation Recepitulates the Ontogeny of Lymphoid Stromal Cells, *Journal of Immunology*, 182(9):5789-5799.

Perl A (2003), Role of Endogenous Retroviruses in Autoimmune Diseases, *Rheumatic Disease Clinics of North America*, 29:123-143.

Perreau M, AL Savoye, E De Crignis, JM Corpataux, R Cubas, EK Haddad, L De Leval, C Graziosi and G Pantaleo (2013), Follicular Helper T Cells Serve as the Major CD4 T Cell Compartment for HIV-1 Infection, Replication, and Production, *Journal of Experimental Medicine*, 120(1):143-156.

Pfeiffer F, V Kumar, S Butz, D Vestweber, BA Imhof, JV Stein and B Engelhardt (2008), Distinct Molecular Composition of Blood and Lymphatic Vascular Endothelial Cell Junctions Establishes Specific Functional Barriers Within the Peripheral Lymph Node, *European Journal of Immunology*, 38:2142-2155.

Phan TG, D Paus, TD Chan, ML Turner, SL Nutt, A Basten and R Brink (2006), High Affinity Germinal Center B Cells Are Actively Selected into the Plasma Cell Compartment, *Journal of Experimental Medicine*, 203(11):2419-2424.

Piguet V, O Schwartz, S Le Gall and D Trono (1999), The Downregulation of CD4 and MHC-I by Primate Lentiviruses: A Paradigm for the Modulation of Cell Surface Receptors, *Immunology Reviews*, 168:51:63.

Piguet V, L Wan, C Borel, A Mangasarian, N Demaurex G Thomas and D Trono (2000), HIV-1 Nef Protein Binds to the Cellular Protein PACS-1 to

Downregulate Class I Major Histocompatibility Complexes, *Nature Cell Biology*, 2(3):163-167.

Podgrabinska S, P Braun, P Velasco, B Kloos, MS Pepper, DG Jackson and M Skobe (2002), Molecular Characterization of Lymphatic Endothelial Cells, *PNAS*, 99(25)16069-16074.

Pöhlmann S, EJ Soilleux, F Baribaud, GJ Leslie, LS Morris, J Twosdale, B Lee, N Coleman and RW Doms (2001), DC-SIGNR, a DC-SIGN Homologue Expressed in Endothelial Cells, Binds to Human and Simian Immunodeficiency Viruses and Activates Infection in Trans, *Proceedings of the National Academy of Sciences USA*, 98(5):2670-2675.

Pollakis G and WA Paxton (2012), Use of (Alternative) Coreceptors for HIV Entry, *Current Opinion on HIV and AIDS*, 7(5):440-449.

Poudrier J, X Weng, DG Kay, G Paré, EL Calvo, Z Hanna, MH Kosco-Vilbois and P Jolicoeur (2001), The AIDS Disease of CD4C/HIV Transgenic Mice Shows Impaired Germinal Centers and Autoantibodies and Develops in the Absence of IFN-γ or IL-6, *Immunity*, 15:173-185.

Poudrier J, X Weng, DG Kay, Z Hanna and P Jolicoeur (2003), The AIDS-Like Disease of CD4C/Human Immunodeficiency Virus Transgenic Mice Is Associated With Accumulation of Immunature $CD11b^{Hi}$ Dendritic Cells, *Journal of Virology*, 77(21):11733-11744.

Powell BC, EA Passmore, A NEsci and SM Dunn (1998), The Notch Signalling Pathway in Hair Growth, *Mechanisms of Development*, 78(1-2):189-192.

Preusser A, L Briese, AS Baur and D Willbold (2001), Direct In Vitro Binding of Full-Length Human Immunodeficiency Virus Type 1 Nef Protein to CD4 Cytoplasmic Tail, *Journal of Virology*, 75:3960-3964.

Prodger JL, R Gray, G Kigozi, F Nalugoda, R Galliwango, T Hirbod, M Wawer, SO Hofer, N Sewankambo, D Serwadda and R Kaul (2012), Foreskin T-Cell Subsets Differ Substantially From Blood With Respect to HIV Co-Receprot Expression, Inflammatory Profile, and Memory Status, *Mucosal Immunology*, 5(2):121-128.

Proweller A, L Tu, JJ Lepore, L Cheng, MM Lu, J Seykora, SE Millar, WS Pear and MS Parmacek (2006), Impaired Notch Signaling Promotes De Novo Squamous Cell Carcinoma Formation, *Cancer Research*, 66:7438-7444.

Prowse D, L Bolgan, A Molnár and GP Dotto (1997), Involvement of the Sp3 Transcription Factor in Induction of p21Cip/WAF1 in Keratinocyte Differentiation, *Journal of Biochemistry*, 272 :1308-1314.

Purcel DF and MA Martin (1993), Alternative Splicing of Human Immunodeficiency Virus Type 1 mRNA Modulates Viral Protein Expression, Replication, and Infectivity, *Journal of Virology*, 67:6365-6378.

Racz P, K Tenner-Racz, F van Vloten, H Schmidt, M Dietrich, JC Gluckman, NL Letvin and G Janossy (1990), Lymphatic Tissue Changes In AIDS And Other Retrovirus Infections: Tools and Insights, *Lymphhology*, 23(2)85-91.

Radtke F, A Wilson, G Stark, M Bauer, J van Meerwijk, HR MacDonald and M Aguet (1999), Deficient T Cell Fate Specification in Mice with an Induced Inactivation of Notch1, *Immunity*, 10(5):547-558.

Rahim MA, P Chrobak, C Hu, Z Hanna and P Jolicoeur (2009), Adult AIDS-Like Disease In A Novel Inducible Human Immunodeficiency Virus Type 1 Nef Transgenic Mouse Model: CD4+ T Cell Activation Is Nef Dependent And Can Occur In The Absence Of Lymphophenia, *Journal of Virology*, 83(22): 11830-11846.

Rangarajan A, C Talora, R Okuyama, M Nicolas, C Mammucari, H Oh, JC Aster, S Krishna, D Metzger, P Chambon, L Miele, M Aguet, F Radtke and GF Dotto (2001), Notch Signaling is a Direct Determinant of Keratinocyte Growth Arrest and Entry into Differentiation, *The EMBO Journal*, 20(13):3427-3436.

Reid W, M Sadowska, F Denaro, S Rao, J Foulke Jr, N Hayes, O Jones, D Doodnauth, H Davis, A Sill, P O'Driscoll, D Huso, T Fouts, G Lewis, M Hill, R Kamin-Lewis, Wei C, P Ray, RC Gallo, M Reitz and J Bryant (2001), An HIV-1 Transgenic Rat that Develops HIV-Related Pathology and Immunologic Dysfunction, *Proceedings of the National Academy of Sciences USA*, 98:9271-9276.

Reid W, S Abdelwahab, M Sadowska, D Huso, A Neal, A Ahearn, J Bryant, RC Gallo, GK Lewis and M Reitz (2004), HIV-1 Transgenic Rats Develop T Cell Abnormalities, *Virology*, 321:111-119.

Resh MD (1999), Fatty Acylation of Proteins: New Insights Into Membrane Targeting of Myristoylated and Palmotoylated Proteins, *Biochimica and Biophysica Acta*, 1451(1)1-16.

Reynolds TC, SD Smith, and J Sklar (1987), Analysis of DNA Surrounding the Breakpoints of Chromosomal Translocations Involving the Beta T Cell Receptor Gene in Human Lymphoblastic Neoplasms, *Cell*, 50:107-117.

Rieder P, B Joos, AU Scherrer, H Kuster, D Braun, C Grube, B Niederöst, C Leemann, S Gianella, KJ Metzner, J Böni, R Weber and HF Günthard

(2011), Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Diversity and Tropism in 145 Patients with Primary HIV-1 Infection, *Clinical Infectious* Diseases, 53(12):1271-1279.

Roozendal R, TR Mempel, LA Pitcher, SF Gonzalez, A Verschoor, RE Mebius, UH von Adrian and MC Caroll (2008), Conduits Mediate Transport of Low-Molecular-Weight Antigen to Lymph Node Follicles, *Immunity*, 30(2):264-276.

Rusnati M and M Presta (2002), HIV-1 Tat Protein and Endothelium: From Protein/Cell Interaction to AIDS-associated Pathologies, *Angiogenesis*, 5:141-151.

Safrit JT, CA Andrews, T Zhu, DD Ho and RA Koup (1994), Characterization of Human Immunodeficiency Virus Type 1-Specific Cytotoxic T Lymphocyte Clones Isolated During Acute Seroconversion: Recognition of Autologous Virus Sequences Within A Conserved Immunodominant Epitope, *Journal of Experimental Medicine*, 179:463-472.

Sakuma T, T Noda, S Urata, Y Kawaoka and J Yasuda (2009), Inhibition of Lhassa and Marburg Virus Production by Tetherin, *Journal of Virology*, 83:2382-2385.

Santini MP, C Talora, T Seki, L Bolgan and GP Dotto (2001), Cross Talk Among Calcineurin, Sp1/Sp3, and NFAT in Control of p21(WAF1/CIP1) Expression in Keratinocyte Differentiation, *Proceedings of the National Academy of Sciences USA*, 98:9575-9580.

Sawaya BE, K Khalili, J Gordon, R Taube and S Amini (2000), Cooperative Interaction Between HIV-1 Regulatory Proteins Tat and Vpr Modulates Transcription of the Viral Genome, *Journal of Biological Chemistry*, 275:35209-35214.

Schacker TW, C Reilly, GJ Beilman, S Wolinsky, M Larson, C Reilly and AT Haase (2002), Collagen Deposition in HIV-1 Infected Lymphatic Tissues and T Cell Homeostasis, *Journal of Clinical Investigation*, 110:1133-1139.

Schacker TW, JM Brenchley, GJ Beilman, C Reilly, SE Pambuccian, J Taylor, D Skarda, M Larson, DC Douek, AT Haase (2006), Lymphatic Tissue Fibrosis Is Associated with Reduced Numbers of Naïve CD4+ T Cells in Human Immunodeficiency Virus Type 1 Infection, *Clinical and Vaccine Immunolog*, 13(5):556-560.

Schacker T (2008), The Role of Secondary Lymphatic Tissue in Immune Deficiency of HIV Infection, *AIDS*, 22 (suppl 3):S13-S18.

Schaefer MR, ER Wonderlich, JF Roeth, JA Leonard and KL Collins (2008), HIV-1 Nef Targets MHC-I and CD4 for Degradation via a Final Common beta-COP-Dependent Pathway in T Cells, *PLoS Pathogen*, 4:e1000131.

Schaerli P, K Willimann, AB Lang, M Lipp, P Loetscher and B Moser (2000), CXC Chemokine Receptor 5 Expression Defines Follicular Homing T Cells with B Cell Helper Function, *Journal of Experimental Medicine*, 192(11):1553–1562.

Schubert U and K Strebel (1994), Differential Activities of the Human Immunodeficiency Virus Type 1-Encoded Vpu Protein are Regulated by Phosphorylation and Occur in Different Cellular Compartments, *Journal of Virology*, 68:2260-2271.

Schuurman HJ, WJA Krone, R Broekhuizen and J Goudsmit (1988), Expression of RNA and Antigens of Human Immunodeficiency Virus Type (HIV-1) in Lymph Nodes from HIV-1 Infected Individuals, *American Journal of Pathlology*, 133(3):516-524.

Sharma M, S Callen, D Zhang, PC Shinhal, GB Vanden Heuvel and S Buch (2010), Activation of Notch Signaling Pathway in HIV-Associated Nephropathy, *AIDS*, 24(14):2161-2170.

Sharma M, LK Magenheimer, T Home, KN Tamano, PC Singhal, SP Hyink, PE Klotman, GB Vanden Heuvel and TA Fields (2013), Inhibition of Notch Pathway Attenuates the Progression of Human Immunodeficiency Virus Associated Nephropathy, *American Journal of Renal Physiology*, 304:1127-1136.

Shiow LR, DB Rosen, N Brdichová, Y Xu, J An, LL Lanier, JG Cyster and M Matloubian (2006), CD69 Acts Downstream of Interferon-Alpha/Beta to Inhibit S1P1 and Lymphocyte Egress from Lymphoid Organs, *Nature*, 440(7083):540-544.

Shoham N, L Cohen, A Yaniv and A Gazit (2003), The Tat Protein of the Human Immunodeficiency Virus Type 1 (HIV-1) Interacts with the EGF-Like Repeats of the Notch Proteins and the EGF Precursor, *Virus Research*, 98:57-61.

Sieg SF, B rodriguez, R Asaad, W Jiang, DA Bazdar and MM Lederman (2005), Peripheral S-Phase T Cells in HIV Disease Have a Central Memory Phenotype and Rarely Have Evidence of Recent T Cell Receptor Engagement, *Journal of Infectious Diseases*, 192:62-70.

Siegert S, HY Huang, CY Yang, L Scarpellino, L Carrie, S Essex, PJ Nelson, M Heikenwalder, H Acha-Orbea, CD Buckley, BJ Marsland, D Zehn and SA

Luther (2011), Fibroblastic Reticular Cells from Lymph Nodes Attenuate T Cell Expansion by Producing Nitric Oxide, *PLoS ONE*, 6:e27618.

Siegert S and SA Luther (2012), Positive and Negative Regulation of T Cell Responses by Fibroblastic Reticular Cells Within Paracortical Regions of Lymph Nodes, *Frontiers in Immunology*, 3:1-10.

Simmons A, V Aluvihare and A McMichael (2001), Nef triggers a Transcriptional Program in T Cells Imitating Single-Signal T Cell Activation and Inducing HIV Virulence Mediators, *Immunity*, 14:763-777.

Singhal PC, P Sharma, K Reddy, V Sanwal, J Rawal, N Gibbons and N Franki (1997), HIV-1 gp160 Envelope Protein Modulates Proliferation and Apoptosis in Mesangial Cells, *Nephron*, 76:284-295.

Smith KG, A Light, LA O'Reilly, SM Ang, A Strasser and D Tarlinton (2000), Bcl-2 Transgene Expression Inhibits Apoptosis in the Germinal Center and Reveals Differences in the Selection of Memory B Cells and Bone Marrow Antibody-Forming Cells, *Journal of Experimental Medicine*, 191(3):475-484.

Soilleux EJ (2003), DC-SIGN (Dendritic Cell-Specific ICAM-Grabbing Non-Integrin) and DC-SIGN-Related (DC-SIGNR): Friend or Foe?, *Clinical Science*, 104:437-446.

Sptis H and JP Di Santo (2011), The Expanding Family of Innate Lymphoid Cells: Regulators and Effectors of Immunity and Tissue Remodeling, *Nature Immunology*, 12:21-27.

Sriuranpong V, MW Borges, RK Ravi, DR Arnold, BD Nelkin, SB Baylin, DW Ball (2001), Notch Signaling Induces Cell Cycle Arrest in Small Cell Lung Cancer Cells, *Cancer Research*, 61(7): 3200-3205.

Steinsvoll S' K Helgeland and K Schenck (2004), Mast Cells – A Role in Periodontal Diseases?, *Journal of Clinical Periodontology*, 31(6):413-419.

Stevenson M (2003), HIV-1 Pathogenesis, *Nature Medicine*, 9:853-860.

Sun Z, PW Denton, JD Estes, FA Othieno, BL Wei, AK Wege, MW Melkus, A Padget-Thomas, M Zupancic, AT Haase and JV Garcia (2007), Intrarectal Transmission, Systemic Infection, and CD4+ T Cell Depletion in Humanized Mice Infected with HIV-1, *Journal of Experimental Medicine*, 204:705-714.

Tangemann K, MD Gunn, P Giblin and SD Rosen (1998), A High-Endothelial Cell Derived Chemokine Induces Rapid, Efficient, and Subset-Specific

Arrest of Rolling T Lymphocytes on a Reconstituted Endothelial Substrate, *Journal of Immunology*, 161:6330–6337.

Taylor G, M Lehrer, PJ Jensen, TT Sun and RM Lavker (2000), Involvment of Follicular Stem Cells in Forming not only the Follicle but also the Epidermis, *Cell*, 102:451-461.

Telesnitsky A and SP Goff (1997), Reverse Transcriptase and the Generation of Retroviral DNA. In: Coffin JM, SH Hughes, HE Varmus, editors. *Retroviruses*, Cold Spring Harbor. Cold Spring Harbor Laboratory Press; pp 121-160.

Thélu J, P Rossio and B Favier (2002), Notch Signaling Is Linked to Epidermal Cell Differentiation Level in Basal Cel Carcinoma, Psoriasis, and Wound-Healing, *BMC Dermatology*, 2:7.

Thoulouze MI, N Sol-Foulon, F Blanchet, A Dautry-Varsat, O Schwartz and A Alcover (2006), Human Immunodeficiency Virus Type-1 Infection Impairs the formation of the Immunological Synapse, *Immunity*, 24:547-561.

Tindal B and DA Cooper (1991), Primary HIV Infection: Host Responses and Intervention Strategies, *AIDS*, 5:1-14.

Triant VA, H Lee, C Hadigan, SK Grinspoon (2007), Increased Acute Myocardial Infarction Rates and Cardiovascular Risk Factors Among Patients With Human Immunodeficiency Virus Disease, *Journal of Clinical Endocrinology and Metabolism*, 92(7):2506-2512.

Tschachler E, PR Bergstresser and G Stingl (1996), HIV-Related Skin Diseases, *Lancet*, 348 :659-663.

Turville SG, PU Cameron, A Handley, G Lin, S Pölhmann, RW Doms and AL Cunningham (2002), Diversity of Receptors Binding HIV on Dendritic Cell Subsets, *Nature Immunology*, 3(10):975-983.

Ullman KS, MA Powers and DJ Forbes (1997), Nuclear Export Receptors: From Importin to Exportin, *Cell*, 90(6):967-970.

Ushiki T, O Ohtani and K Abe (1995), Scanning Electron Microscopic Studies of Reticular Framework in the Rat Mesenteric Lymph Node, *The Anatomical* Record, 241(1):113-122.

Uthayakumar S, R Nandwani, T Drinkwater, AT Nayagam and CR Darley (1997), The Prevalence of Skin Disease in HIV Infection and its Relationship to the Degree of Immunosuppression, *British Journal of Dermatology*, 137:595-598.

Van Damme N, D Goff, C Katsura, RL Jorgenson, R Mitchell, MC Johnson, EB Stephens and J Guatelli (2008), The Interferon-Induced Protein BST-2 Restricts HIV-1 Release and is Downregulated from the Cell Surface by the Viral Vpu Protein, Cell Host Microbe, 3:245–252.

Varmus H and R Swanstrom (1991), Replication of Retroviruses. In: Weiss R, N Teich, H Varmus, J Coffin, editors. *RNA tumor viruses. Molecular biology of tumor viruses.* Cold Spring arbor, New York: Cold Spring Harbor Laboratory, pp 369-512.

Vassileva G, H Soto, A Zlotnik, H Nakano, T Kakiuchi, JA Hedrick and SA Lira (1999), The Reduced Expression of 6Ckine in the plt Mouse Results from the Deletion of One of Two 6Ckine Genes, *Journal of Experimental Medicine*, 190(8):1183-1188.

Vázquez M, IM Maldonado, S Almodóvar, C López, M del C Colón, M Hill and E Lorenzo (2010), Short Communication: Human Blood Dendritic Cells Are Infected Separately From Monocytes in HIV Type 1 Patients, *AIDS Research and Human Retroviruses*, 26(4): 471-479.

Vicenzi E and G Poli (2013), Novel Factors Interfering With Human Immunodeficiency Virus-Type 1 Replication *In Vivo* and *In Vitro*, *Tissue Antigens*, 81:61-71.

Vinuesa CG, MA Linterman, CC Goodnow and KL Randall (2010), T Cells and Follicular Dendritic Cells in Germinal Center B-Cell Formation and Selection, *Immunology Reviews*, 237(1):72-89.

Vittecoq D, L Escaut, G Chironi, E Teicher, JJ Monsuez, M Andrejak and A Simon (2003), Coronary Heart Disease in HIV-Infected Patients in the Highly Active Antiretroviral Treatment Era, *AIDS*, Suppl1:S70-76.

Voelkel NF, CD Cool and S Flores (2008), From Viral Infection To Pulmonary Arterial Hypertension: A Role For Viral Proteins?, *AIDS*, 22(suppl 3):S49-S53.

Vogel J, SH Hinrichs, RK Reynolds, PA Luciw and G Jay (1988), The HIV *tat* Gene Induces Dermal Lesions Resembling Kaposi's Sarcoma in Transgenic Mice, *Nature*, 355:606-611.

Vogelzang A, HM McGuire, D Yu, J Prent, CR Mackay and C King (2008), A Fundamental Role for Interleukin-21 in the Generation of T Follicular Helper Cells, *Immunity*, 29(1):127-137.

Von Adrian UH and TR Mempel (2003), Homing and Cellular Traffic in Lymph Nodes, *Nature Reviews Immunology*, 3:867-878.

Vu F, U Dianzani, CF Ware, T Mak and JL Gommerman (2008), ICOS, CD40, and Lymphotoxin Beta Receptors Signal Sequentially and Interdependently to Initiate a Germinal Center Reaction, *Journal of Immunology*, 180(4):2284-2293.

Ware CF (2005), Network Communications: Lymphotoxins, LIGHT, and TNF, *Annual Reviews of Immunology*, 23:787-819.

Warnock RA, S Askari, EC Butcher, von Andrian UH (1998), Molecular Mechanisms of Lymphocyte Homing to Peripheral Lymph Node, *Journal of Experimental Medicine*, 187(2):205-216.

Webster B, EH Ekland, LM Agle, S Chyou, R Ruggieri and TT Lu (2006), Regulation of Lymph Node Vasculature Growth by Dendritic Cells, *Journal of Experimental Medicine*, 203(8):1903-1913.

Wei BL, PW Denton, E O'Neill, T Luo, JL Foster and JV Garcia (2005), Inhibition of Lysosome and Proteasome Function Enhances Human Immunodeficiency Virus Type 1 Infection, *Journal of Virology*, 79:5705-5712.

Weijzen S, P Rizzo, M Braid, R Vaishnav, SM Jonkheer, A Zlobin, BA Osborne, S Gottipati, JC Aster, WC Hahn, M Rudolf, K Siziopikou,WM Kast and L Miele (2002), Activation of Notch-1 Signaling Maintains the Neoplastic Phenotype in Human Ras-Transformed Cells, *Nature Medicine*, 8(9):979-986.

Weng X, E Priceputu, P Chrobak, J Poudrier, DG Kay, Z Hanna, TW Mak and P Jolicoeur (2004), CD4+ T Cells From CD4C/HIVNef Transgenic Mice Show Enhanced Activation In Vivo With Impaired Proliferation In Vitro But Are Dispensable For The Development Of A Severe AIDS-Like Organ Disease, *Journal of Virology*, 78(10)5244-5257.

Wichukchinda N, Y Kitamura, A Rojanawiwat, EE Nakayama, H Song, P Pathipvanich, W Auwanit, P Sawanpanyalert, A Iwamoto, T Shioda and K Ariyoshi (2007), The Polymorphisms in DC-SIGNR Affect Susceptibility to HIV Type 1 Infection, *AIDS Research and Human Retroviruses*, 23(5):686-692.

Willard-Mack CL (2006), Normal Structure, Function, and Histology of Lymph Nodes, *Toxicologic Pathology*, 34:409-424.

Willey RL, JS Bonifacio, BJ Potts, MA Martin and RD Klausner (1988), Biosynthesis, Cleavage, and Degradation of the Human Immunodeficiency Virus 1 Envelope Glycoprotein gp160, *Proceedings of the National Academy of Sciences USA*, 85(24):9580-9584.

Willey RL, F Maldarelli, MA Martin and K Strebel (1992), Human Immunodeficiency Virus Type 1 Vpu Protein Regulates the Formation of Intracellular gp160-CD4 complexes, *Journal of Virology*, 66:226-234.

Withers DR (2011), Lymphoid Tissue Inducer Cells, *Current Biology*, 21(10):381-382.

Wolf D, V Witte, B Laffert, K Blume, E Stromer, S Trapp, P d'Aloja, A Schümann and AS Baur (2001), HIV-1 Nef Associated PAK and PI3-Kinases Stimulate Akt-Independent Bad-Phosphorylation to Induce Anti-Apoptotic Signals, *Nature Medicine*, 7)11):1217-1224.

Wong DJ and HY Chang (2009), Skin Tissue Engineering, Stembook, ed. The Stem Cell Research Community, Stembook.

Wong JK, M Hezareh, HF Günthard, DV Havlir, CC Ignacio, CA Spina and DD Richman (1997), Recovery of Replication-Competent HIV despite Prolonged Suppression of Plasma Viremia, *Science*, 278:1291–1295.

Xu XN, B Laffert, GR Screaton, M Kraft, D Wolf, W Kolanus, J Mongkolsapay, AJ McMichael and AS Baur (1999), Induction of Fas Ligand Expression by HIV Involves the Interaction of Nef with the T Cell Receptor Zeta Chain, *Journal of Experimental Medicine*, 189(9):1489-1496.

Yarwood JM, DY Leung and PM Schlievert (2000), Evidence for the Involvement of Bacterial Superantigens in Psoriasis, Atopic Dermatitis, and Kawasaki Syndrome, *FEMS Microbiology Letters*, 192:1-7.

Yilmaz BZ, DS Weih, V Sikumar and F Weih (2003), RelB Is Required for Peyer's Patch Development: Differential Regulation of p52-RelB by Lymphotoxin and TNF, *The EMBO Journal*, 22:121-130.

Yoshida H, A Naito, J Inoue, M Satoh, SM Santee-Cooper, CF Ware, A Togawa and S Nishikawa (2002), Different Cytokines Induce Surface Lymphotoxin-αβ on IL-7 Receptor α Cells That Differentially Engender Lymph Nodes and Peyer's Patches, *Immunity*, 17:823-833.

Zack JA, SJ Arrigo, SR Weitsman, AS Go, A Haislip and ISY Chen (1990), HIV-1 Entry in Quiescent Primary Lymphocytes: Molecular Analysis Reveals a Labile, Latent Viral Structure, *Cell*, 61:213-222.

Zagouras P, S Stifani, CM Blaumueller, ML Carcangiu and S Artavanis-Tsakonas (1995), Alterations of Notch Signaling in Neoplastic Lesions of the Human Cervix, *Proceedings of the National Academy of Sciences USA*, 92(14):6414-6418.

Zalia MJ, WP Su and AF Fransway (1992), Dermatologic Manifestations of Human Immunodeficiency Virus Infection, *Mayo Clinical Proceedings*, 67:1089-1108.

Zeng M, AJ Smith, SW Wietgrefe, PJ Southern, TW Shacker, CS Reilly, JD Estes, GF Burton, G Silvestri, JD Lifson, JV Carlis, and AT Haase (2011), Cumulative Mechanisms of Lymphoid Tissue Fibrosis and T Cell Depletion in HIV-1 and SIV Infections, *Journal of Clinical Investigation*, 121(3):998-1008.

Zeng M, M Palardini, JC Engram, GJ Beilman, JG Chipman, TW Schacker, G Silvestri and AT Haase (2012a), Critical Role For CD4 T Cells in Maintaining Lymphoid Tissue Structure For Immune Cell Homeostasis and Reconstitution, *Blood*, 120(9)1856-1867.

Zeng M, AT Haase and TW Schacker (2012b), Lymphoid Tissue Structure and HIV-1 Infection: Life Or Death For T Cells, *Trends in Immunology*, 33(6):306-314.

Zeng M, PJ Southern, CS Reilly, GJ Beilman, JG Chipman, TW Schacker and AT Haase (2012c), Lymphoid Tissue Damage in HIV-1 Infection Depletes Naïve T Cells and Limits T Cell Reconstitution After Antiretroviral Therapy, *PLoS Pathogens*, 8(1):1-13.

Zhang X, J Yu, PM Kuzontkoski, W Zhu, DY Li and JE Groopman (2012), Slit2/Robo4 Signaling Modulates HIV-1 gp120-Induced Lymphatic Hyperpermeability, *PLoS Pathogens*, 8(1):1-13.

Zheng YH, N Lovsin and BM Peterlin (2005), Newly Identified Host Factors Modulate HIV Replication, *Immunology Letters*, 97:225-234.

Zhong Y, B Zhang, SY Eum and M Toborek (2012), HIV-1 Tat Triggers Nuclear Localization of ZO-1 Via Rho Signaling and cAMP Response Element-Binding Protein Activation, *Journal of Neuroscience*, 32(1):143-150.

Zhu AJ and FM Watt (1996), Expression of a Dominant Negative Cadherin Mutant Inhibits Proliferation and Stimulates Terminal Differentiation of Human Epidermal Keratinocytes, *Journal of Cell Science*, 109:3013-3023.

Zhu AJ and FM Watt (1999), β-Catenin Signaling Modulates Proliferative Potential of Human Epidermal Keratinocytes Independently of Intercellular Adhesion, *Development*, 126:2285-2298.

Zotos D and DM Tarlinton (2012), Determining Germinal Center B Cell Fate, *Trends in Immunology*, 33(6):281-288.

Zuo Y, T Matsusaka, J Zhong, J Ma, LJ Ma, Z Hanna, P Jolicoeur, AB Fogo and I Ichikawa (2006), HIV-1 Genes vpr and nef Synergistically Damage Podocytes, Leading to Glomerulosclerosis, *Journal of the American Society of Nephrology*, 17(10):2832-2843.

Zybarth G and C Carter (1995), Domains Upstream of the Protease (PR) in Human Immunodeficiency Virus Type 1 Gag-Pol Influence PR autoprocessing, *Journal of Virology*, 69(6):3878-3884.