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"Signal transduction and extracellular vesicles release induced by HIV-1 Nef protein on plasmacytoid dendritic cells"

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“The fun of the scientific research is also always finding other frontiers to overcome, building more powerful means of investigation, more complex theories, always trying to progress knowing that you will probably get closer to understanding the reality, without ever completely understanding it”.

Margherita Hack

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IV. RESULTS

ABBREVIATIONS

AIDS = Acquired Immunodeficiency Syndrome

APC = Antigen Presenting Cells

DCs = Dendritic cells

Env = Envelope

ESCRT = Endosomal Sorting Complex Required for Transport

EVs = Extracellular vesicles

FC = Flow Cytometry

HEV = endothelial venules

HIV = Human Immunodeficiency Virus

IDO = indoleamine-pyrrole 2,3-dioxygenase

IFN = interferon

ILVs = Intraluminal vesicles

IPC = Interferon Producing Cells

IRF = Interferon Regulatory Factor

ISGs = Interferon stimulated genes

MAPK = Mitogen-Activated Protein Kinase

mDCs = myeloid dendritic cells

MDMs = Monocytes-Derived Macrophages

MHC = Major Histocompatibility Complex

MVBs = Multivesicular Bodies

Nef = Negative Factor

NFκB = Nuclear Factor κB

NK = Natural killer

PBLs = Peripheral Blood Lymphocytes

PBMCs = Peripheral Blood Mononuclear Cells

pDCs = plasmacytoid dendritic cells

PI3K = Phosphatidylinositol-3 Kinase

SIV = Simian Immunodeficiency Virus

STAT = Signal Transducers and Activators of Transcription

TLR = Toll-Like Receptor

Treg = Regulatory T cells

“Trasduzione del segnale e rilascio di vescicole extracellulari indotti dalla proteina Nef dell’HIV-1 in cellule dendritiche plasmacitoidi”

Le cellule dendritiche plasmacitoidi (pDCs) costituiscono una particolare sottopopolazione di cellule dendritiche specializzata nella produzione di interferon di tipo I (IFN). Il loro ruolo nell'infezione da HIV-1 e nella sua patogenesi è complesso e non ancora ben definito. Sebbene questa popolazione non rappresenti uno dei principali *reservoir* dell’HIV, è noto che le pDCs possono essere infettate dal virus contribuendo dicotomicamente sia all'attivazione immunitaria cronica che all'immunosoppressione [Aiello et al., 2018]. Ad oggi, la maggior parte degli studi riportati è incentrata sull'analisi della risposta delle pDCs infettate dal virus. Tuttavia, negli ultimi anni molteplici studi hanno sottolineato la capacità della proteina Nef di essere trasferita mediante contatto cellula-cellula e vescicole extracellulari [Campbell et al., 2008; Xu et al., 2009; Muratori et al., 2009; Lenassi et al., 2010], esercitando in questo modo effetti sia in cellule infette che non infette. Inoltre, nel siero di soggetti con infezione da HIV è stata rilevata la presenza della proteina Nef e di anticorpi anti-Nef [Fujii et al., 1996; Ameisen et al., 1989] che supportano l’idea del possibile *uptake in vivo* della proteina localizzata nello spazio extracellulare da parte di cellule non infette. Alla luce di quanto sopra riportato, in questo lavoro sono stati caratterizzati gli effetti indotti dalla sola proteina Nef del clone virale SF2 in pDCs non infettate da HIV.

Studi precedentemente condotti su colture di macrofagi primari umani (*Monocytes-Derived Macrophages*, MDMs) hanno dimostrato che il trattamento esogeno con la proteina Nef ricombinante miristoilata ($myrNef_{SF2}$) induce la rapida attivazione di vie di segnalazione intracellulari che coinvolgono IKK/NFκB, alcune MAPKs (*Mitogen-Activated Protein Kinase*) e il fattore IRF-3 (*Interferon Regulatory Factor 3*), inducendo la produzione e il rilascio di citochine/chemochine, tra cui l’IFN-β, in grado, a loro volta, di attivare in modo autocrino e/o paracrino alcuni trasduttori del segnale e attivatori della trascrizione della famiglia STAT (*Signal Transducers and Activators of Transcription*), in particolare STAT-1,-2 e -3 [Olivetta et al., 2003; Mangino et al., 2007; Federico et al., 2001; Percario et al., 2003]. Perciò, come prima cosa sono state analizzate le possibili alterazioni nel *signalling* intracellulare indotte da Nef nelle cellule dendritiche plasmacitoidi. I risultati preliminari ottenuti nelle pDCs primarie hanno rivelato che il trattamento esogeno con la proteina Nef incrementa l'espressione di *mxa*, un gene indotto da IFN di tipo I e III. Inoltre, immagini di microscopia confocale hanno evidenziato che Nef induce l'incremento e la parziale traslocazione nucleare del fattore IRF-7 (*Interferon Regulator factor 7*), evento che potrebbe indurre la produzione di IFN di tipo I. Poiché le cellule dendritiche plasmacitoidi rappresentano una frazione minoritaria dei leucociti del sangue periferico, al fine di

facilitare le analisi biochimiche e avere un sistema più stabile e riproducibile ulteriori analisi sono state effettuate utilizzando una linea di cellule dendritiche plasmacitoidi umane, GEN2.2, acquistata attraverso un *Material Transfer Agreement* con il CNCM (*Collection Nationale de Cultures de Microorganismes*), Istituto Pasteur di Parigi. Analisi di western blot eseguite sulle GEN2.2 hanno rivelato che il trattamento con la proteina Nef induce la fosforilazione in tirosina di entrambe le proteine STAT1 e STAT2 a partire da 3 ore. In particolare, attraverso l'attivazione delle STAT Nef influenza in maniera significativa anche l'espressione genica, come indicato dall'induzione tardiva di IRF-1, STAT1 e ISG15. Al contrario, il trattamento con il mutante Nef 4EA, mutato nel dominio acido della proteina, non è in grado di indurre la fosforilazione di queste proteine né la conseguente modulazione dell'espressione genica dal momento che IRF-1, STAT1 e ISG15 non sono risultate incrementate. Questi risultati evidenziano l'importanza del dominio acido nella via di segnalazione indotta da Nef e aggiungono ulteriore rilevanza ai risultati ottenuti precedentemente nei macrofagi primari [Mangino et al., 2007 e 2011].

Per quanto riguarda l'espressione di molecole co-stimolatorie come CD40, CD80 e CD86, che sono note accompagnare l'attivazione e/o maturazione delle pDCs rendendole efficienti cellule presentanti l'antigene (APCs), è stato osservato che la proteina Nef non ne altera l'espressione suggerendo in questo modo che la proteina virale possa agire sulle pDCs favorendo l'acquisizione del fenotipo di cellule producenti interferon (IPCs) piuttosto che di APCs.

L'analisi del secretoma ha rivelato che la proteina Nef induce nelle GEN2.2 la produzione di citochine regolatorie (IL-2), fattori di crescita (FGF basico e G-CSF), e fattori chemiotattici e/o pro-infiammatori (MCP-1, IL-8, IFN-γ, IP-10, MIP-1α, MIP-1β e TNF-α). Altri mediatori come IL-4, IL-5, IL-17, Eotaxin e RANTES sono risultati solo debolmente prodotti in risposta a Nef. Poiché i macrofagi sono ampiamente riconosciuti come uno dei principali *reservoir* dell'infezione, il secretoma delle GEN2.2 indotto da Nef è stato comparato con quello delle THP-1, che sono state differenziate con PMA in modo da acquisire un fenotipo simil-macrofagico. Diversamente dalle GEN2.2, nelle THP-1/PMA Nef induce debolmente o per nulla l'espressione di IL-8, G-CSF e MCP-1, mentre promuove la secrezione di alcune citochine che sono scarsamente indotte o non prodotte nelle GEN2.2 come PDGF, IL-1β, IL-5, IL-15, IL-17, RANTES e VEGF. Altri mediatori come IL-1ra, IL-4, IL-12 (p70), IFN-γ ed Eotaxin vengono solo debolmente modulati nelle THP-1/PMA. Fattori solubili come FGF basico, IL-2, IP-10 e TNF-α vengono secreti da entrambi i tipi cellulari seppure in misura diversa. In particolare, il TNF-α è maggiormente indotto nelle THP-1/PMA. In conclusione, questi dati evidenziano la capacità della proteina Nef di indurre un diverso *pattern* di citochine/chemochine a seconda del tipo cellulare contribuendo probabilmente ad alimentare in maniera diversa l'intensa "tempesta di citochine" che caratterizza l'infezione da HIV

[Wang et al., 2017]. Inoltre, dal momento che durante l'infezione le pDCs sono esposte al microambiente locale che è influenzato dalle molecole immunostimolatorie rilasciate dalle cellule infette, è stata verificata la risposta delle GEN2.2 al *pattern* di citochine/chemochine rilasciato dalle stesse GEN2.2 in risposta allo stimolo con Nef. A tale proposito, è stato osservato che la fosforilazione in tirosina della STAT1 si verifica più rapidamente (già dopo 30 minuti) rispetto al trattamento con Nef (3 ore), dimostrando che le cellule rispondono prontamente all'ambiente extracellulare circostante. Inoltre, è interessante notare che i supernatanti depleti delle vescicole extracellulari (EVs) mantengono la capacità di attivare precocemente la STAT1 già dopo 30 minuti, suggerendo che le citochine/chemochine responsabili di questo fenomeno siano secrete per lo più in forma libera.

Successivamente, considerata l'importanza che le vescicole extracellulari sembrano avere per il loro ruolo nella comunicazione intercellulare in condizioni sia fisiologiche che patologiche, inclusa l'infezione da HIV [Dias et al., 2018], sono state caratterizzate e quantificate le vescicole extracellulari (esosomi e microvescicole) rilasciate dalle GEN2.2 in risposta al trattamento con Nef. A tale scopo, le cellule sono state marcate con un acido grasso, Bodipy FL C_{16}, disponibile in commercio e le EVs rilasciate, essendo fluorescenti, sono state esaminate e quantificate come riportato da Sargiacomo e colleghi [Coscia et al., 2016]. È interessante notare che, a differenza di altri tipi cellulari, nelle GEN2.2 Nef riduce del 40% la quantità di esosomi rilasciati. La proteina viene specificatamente incorporata negli esosomi ma non nelle microvescicole, suggerendo l'esistenza di uno specifico meccanismo che indirizzerebbe Nef negli esosomi. Inoltre, è stato osservato un aumento del livello di espressione dei marker esosomiali CD81, Tsg101 e Flotillin-1 nella frazione di esosomi secreti dalle GEN2.2 trattate con Nef, probabilmente dovuto ad una diversità nelle vescicole rilasciate in risposta al trattamento con la proteina rispetto a quelle secrete da cellule non trattate. Infine, considerata la particolare attitudine delle pDCs a secernere IFNs e la loro continua esposizione a questo tipo di citochina durante l'infezione da HIV, è stato valutato come il numero di vescicole extracellulari secrete dalle pDCs potesse essere influenzato dal trattamento con gli IFNs. A tale proposito, è stato osservato che né l'IFN di tipo I, II o III alterano significativamente il rilascio di vescicole, ma sembrano influenzare qualitativamente il tipo di popolazione di esosomi rilasciata considerata la ridotta espressione dei marker esosomiali CD81, Tsg101 e Flotillin-1 osservata.

Complessivamente, i risultati di questo lavoro gettano nuova luce sugli effetti esercitati dalla sola proteina Nef sulle pDCs non infettate da HIV, contribuendo a fornire un quadro più completo per una comprensione approfondita del ruolo di queste cellule nell'infezione che potrebbe aiutare a definire le funzioni delle pDCs e a sviluppare strategie terapeutiche.

ABSTRACT

Plasmacytoid dendritic cells (pDCs) are a unique dendritic cell subset specialized in type I interferon (IFN) production and whose role in HIV-1 infection and pathogenesis is complex and not yet well defined. Although they do not represent the main reservoir of HIV, it is reported that pDCs can be infected by the virus contributing dichotomously to both chronic immune activation and immunosuppression [Aiello et al., 2018]. To date, most of the reported studies have been focused on the analysis of pDC response following HIV infection. However, emerging evidences point out the ability of the viral protein Nef to be transferred through cell-to-cell contact and extracellular vesicles (EVs) [Campbell et al., 2008; Xu et al., 2009; Muratori et al., 2009; Lenassi et al., 2010; Pužar Dominkuš et al., 2017], thus exerting specific effects on both infected and uninfected cells. Moreover, both Nef and anti-Nef antibodies were detected in the serum of HIV-infected individuals [Fujii et al., 1996; Ameisen et al., 1989] supporting the possible *in vivo* detection of extracellular Nef by uninfected cells. In light of what reported above, in this work the effects induced by the pathogenic accessory protein Nef alone of HIV-1 SF2 strain on HIV-not infected pDCs were characterized.

Previous results obtained in our laboratory demonstrated that the recombinant myristoylated Nef protein ($myrNef_{SF2}$) was rapidly internalized in primary monocyte-derived macrophages (MDMs) and triggered NF-κB, MAPKs (Mitogen-Activated Protein Kinase) and IRF-3 (Interferon Regulatory Factor 3) activation inducing the production and release of a set of cytokines/chemokines including IFN-β [Mangino et al., 2007 and 2011]. The latter, in turn, activated some signal transducers and activators of transcription (STAT) molecules in an autocrine and/or paracrine manner, in particular STAT-1, -2 and -3 [Olivetta et al., 2003; Mangino et al., 2007; Federico et al., 2001; Percario et al., 2003]. Therefore, we started to investigate the possible alterations in intracellular signalling induced by Nef. The preliminary results obtained in primary pDCs revealed that the exogenous treatment with Nef protein up-regulated the expression of *mxa*, an IFN-inducible gene, whose protein is usually used as surrogate marker for type I and III IFN production. Moreover, confocal images showed that Nef induced the increase and a partial nuclear translocation of IRF-7 that could cause type I IFN production. Since plasmacytoid dendritic cells represent a minor fraction of peripheral blood leukocytes, in order to facilitate biochemical analyses and have a more stable and reproducible system we decided to carry out further analyses using a human plasmacytoid dendritic cell line, GEN2.2. The latter was provided by Dr. Laurence Chaperot through a Material Transfer Agreement with the CNCM (Collection Nationale de Cultures de Microorganismes), Pasteur Institute of Paris.

Western blot analyses performed on GEN2.2 revealed that Nef induced the tyrosine phosphorylation of both STAT1 and STAT2 proteins starting from 3 hours. Notably, Nef substantially influenced also the gene expression program via STAT activation, as indicated by the late induction of IRF-1, STAT1 and ISG15. In contrast, the treatment with the Nef mutant 4EA, mutated in the acidic domain of the protein, was not able to induce the phosphorylation of STAT1 and STAT2 proteins and did not even modulate the gene expression since neither IRF-1, STAT1 nor ISG15 were increased. These results highlight the importance of the acidic domain in the signalling pathway induced by Nef and add relevance to the previous findings obtained in primary macrophages [Mangino et al., 2007 and 2011].

Concerning the expression of co-stimulatory molecules such as CD40, CD80 and CD86, which usually accompany the activation or maturation of plasmacytoid dendritic cells making them efficient antigen presenting cells (APCs), it was observed that Nef protein did not alter them thus suggesting that Nef could act on pDCs by favouring the acquisition of an interferon producing cells (IPC) phenotype rather than an APC one.

The secretome analysis performed on the supernatants after Nef treatment revealed that the protein induced in GEN2.2 the production of regulatory cytokines (IL-2), growth factors (FGF basic and G-CSF), and chemotactic and/or pro-inflammatory mediators (MCP-1, IL-8, IP-10, MIP-1α, MIP-1β, IFN-γ and TNF-α). Other mediators, such as IL-4, IL-5, IL-17, Eotaxin and RANTES resulted to be only weakly secreted in response to Nef treatment. Since macrophages are widely recognized as one of the main reservoir of HIV infection, we compared the pattern of cytokines/chemokines induced by Nef in GEN2.2 with that of THP-1, a monocytic cell line that was differentiated adding PMA in order to acquire a macrophage-like phenotype. Notably, unlike GEN2.2, in THP-1/PMA Nef did not affect or only weakly the expression of IL-8, G-CSF and MCP-1, whereas promoted the secretion of some cytokines weakly induced or not produced in GEN2.2 such as PDGF, IL-1β, IL-5, IL-15, IL-17, RANTES and VEGF. Other mediators such as IL-1ra, IL-4, IL-12 (p70), IFN-γ and Eotaxin were only weakly modulated in THP-1/PMA. Soluble factors such as FGF basic, IL-2, IP-10 and TNF-α were secreted by both cell types, although at different extent. Indeed, TNF-α was much more induced in THP-1/PMA. In conclusion, these results highlight the ability of Nef protein to induce the release of a different pattern of cytokines/chemokines according to the cell type probably contributing to fuel in different ways the intense “cytokine storm” that characterizes HIV infection [Wang et al., 2017].

Furthermore, since during HIV infection pDCs are exposed to the local microenvironment influenced by the immunostimulatory molecules secreted by infected cells into the extracellular space, we verified the response of GEN2.2 to the cytokine/chemokine milieu released by GEN2.2

in response to Nef stimulus. It was observed that STAT1 tyrosine phosphorylation occurred more rapidly (already after 30 minutes) than following Nef treatment (3 hours) showing that pDCs are promptly responsive to the surrounding extracellular milieu. Interestingly, supernatants depleted of extracellular vesicles (EVs) maintained the capacity to early activate STAT1 already after 30 minutes indicating that the cytokines/chemokines responsible for this phenomenon should be mostly secreted in free form and not associated with EVs.

Subsequently, considering the emerging importance of the EVs for their role in the intercellular communication in both physiological and pathological conditions, including HIV infection [Dias et al., 2018], we characterized and quantified the EVs (exosomes and microvesicles) released by GEN2.2 in response to Nef treatment. To fulfil our purpose, we used the commercially available Bodipy FL C_{16} fatty acid to label the cells. The fluorescent EVs released from the cells were examined and quantified as reported by Sargiacomo and colleagues [Coscia et al., 2016]. Interestingly, unlike what reported in literature regarding other cell types, we found that Nef did not increase the production of exosomes in GEN2.2, but it induced a 40% reduction. Moreover, Nef was preferentially incorporated into the exosomal pellet after cell treatment, but not in microvesicles, suggesting the presence of a specific mechanism that would address the protein to be released into exosomes. In addition, we observed an increased expression of the exosomal markers CD81, Tsg101, and Flotillin-1 in exosomes secreted from GEN2.2 treated with Nef possibly reflecting the diversity of the vesicles released in response to Nef treatment compared to those secreted by untreated cells.

Finally, considered the particular attitude of pDCs to secrete IFNs and their continuous exposure to these types of cytokines during HIV infection, we analysed how they could influence the number of EVs secreted by pDCs. In this regard, we observed that neither type I, II or III IFN alter significantly the exosome or microvesicle release. However, they seem to affect from a qualitative point of view the type of vesicles released because in response to all IFN types a down-modulated expression of the exosomal markers CD81, Tsg101, and Flotillin-1 was observed.

Altogether, the results of this work shed new light on the effects exerted by Nef protein alone on uninfected pDCs by contributing to provide a more comprehensive picture for a thorough understanding of pDCs roles in HIV infection that may help to define pDCs functions and develop therapeutic strategies.

I. INTRODUCTION

CHAPTER 1

Plasmacytoid dendritic cells and their role in HIV infection

1.1 Introduction to plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are one of the two principal subsets of human dendritic cells (DCs). The discovery and identification of pDCs was the result of converging studies that date back to 1950s. The term "plasmacytoid" refers to their plasma cell-like morphology, due to an abundant cytoplasm with a well-developed endoplasmic reticulum. pDCs differ in morphology, phenotype and function from myeloid DCs (mDCs, also referred to as conventional or classical DCs) and they express specific surface markers such as human blood dendritic cell antigen (BDCA)-2 and BDCA-4, immunoglobulin-like transcript 7 (ILT7) and IL3Rα (CD123).

Originally described in humans, pDCs have also been characterized in other mammalian species including mice, rats and monkeys. The characterization of pDCs in mice was of particular importance to garner data regarding developmental origin and transcriptional control of the pDCs lineage (reviewed in ref. [Reizis, 2010]). Ever since the identification of pDCs as a distinct cell type, their origin and lineage affiliation has been controversial, in part because these cells show features of both lymphocytes and dendritic cells. However, the current view is that these cells can be derived from either myeloid or lymphoid precursors.

Initial studies showed that pDCs migration is quite different from that of mDCs. The latter, following their development, leave the bone marrow to give rise to resident and migratory DCs. Instead, pDCs are mostly confined to primary and secondary lymphoid organs (lymph nodes, spleen), and they are found in rare numbers in peripheral tissues under homeostatic conditions. Moreover, unlike mDCs that reach lymph nodes (LNs) via afferent lymphatic vessels, pDCs circulate through the body via the bloodstream and enter lymphoid tissues directly via high endothelial venules (HEV) [Segura et al., 2012]. In inflammatory conditions, pDCs leave the bloodstream and accumulate at the site of infection, where they can secrete IFN-α, take up antigens and migrate to draining LNs for antigen presentation [O'Brien et al., 2013]. pDCs can also accumulate in inflammatory sites, as in the case of systemic lupus erythematosus (SLE) or psoriasis, and infiltrate primary and malignant melanoma, ovarian and breast carcinoma [Swiecki & Colonna, 2015]. The recruitment into these sites suggests that pDCs may contribute to the ongoing inflammatory response through the release of cytokines and chemokines or, alternatively,

to the induction of tolerogenic responses. The factors that has been reported to influence pDC migration are CD62L (L-selectin), PSGL1, β1 and β2 integrins and multiple chemokines receptors, such as CXCR4, CCR7, CXCR3, CCR5, CCR2 and CCR6 [Sozzani et al., 2010; Seth et al., 2011], which promote recruitment in steady-state and during inflammation. The migration of pDCs to lymphoid tissue is promoted by expression of L-selectin (non-inflamed states) or E-selectin (inflamed states) in HEV [Yoneyama et al., 2004], while pDCs egress from the bone marrow into the blood is dependent on CCR5 and CCR2. The high expression of CCR7 at the surface of pDCs promotes the migration toward increased concentration gradient of its ligand CCL9 and CCL21 abundantly secreted by LNs, thus contributing to pDCs homing in LNs [Seth et al., 2011]. Moreover, pDCs employ both CCR7 and CXCR4 as critical chemokine receptors to migrate into the splenic white pulp under steady-state conditions. CXCR4 also promotes pDCs recruitment to tumors that produce CXCL12 [O'Brien et al., 2013]. In addition to chemotactic chemokines, pDCs can be recruited also in response to signals associated with inflammation and tissue damage, such as IL-18, thanks to the engagement of receptors for chemerin (ChemR23), adenosine (A1-R) as well as the anaphylatoxins C3a and C5a [O'Brien et al., 2013].

1.2 pDCs as effectors at the interface of innate and adaptive immunity

pDCs are key players in the early antiviral response thanks to the substantial production of type I and III IFN in response to viral RNA or DNA through activation of Toll like receptor (TLR)-7 and -9. In addition to their role in antiviral immunity, recent studies suggest that pDCs also play an important role in antifungal immunity (for a comprehensive review see ref. [Maldonado et al., 2017]). Furthermore, pDCs can act as antigen-presenting cells (APCs), a process typically referred to as "priming". Although pDCs are generally thought to be less efficient compared to mDCs, they can efficiently induce memory $CD4^+$ and $CD8^+$ responses when activated, and in some instances, can prime naïve T cells [O'Brien et al., 2013]. These two specialized roles exist at distinct functional stages characterized by different morphologies. Therefore, when pDCs are stimulated, their functional response to pathogens is flexible and it is influenced by specific signals according to which they can differentiate into Interferon producing cells (IPCs) or APCs.

An additional role for pDCs as cytolytic effector cells was reported by Tel and colleagues, who termed these cells "killer pDCs" [Tel et al., 2012]. Moreover, the ability of pDCs to migrate from the blood to LNs, where they can interact with T cells, as well as to sites of inflammation also places these cells ideally at the interface between innate and adaptive immunity.

Studies of the last years have revealed that pDCs exhibit a functional dichotomy: they can display both pro-inflammatory and immunosuppressive tolerogenic properties. The local

microenvironment and the extrinsic stimuli influence pDCs phenotype and hence could control the phenotypic switch toward inflammation or tolerance.

1.2.1 Pro-inflammatory properties

The primary function of pDCs is the recognition of pathogen-associated molecular patterns (PAMPs), such as viral single-strand RNA or bacterial CpG nucleotide DNA sequences through TLR7 and TLR9 respectively, and the production of large amounts of type I IFN in response to infection [Rogers et al., 2013]. Although constituting only 0.2-0.8% of human blood cells, pDCs are responsible for over 95% of type I IFN produced by peripheral blood mononuclear cells (PBMCs) in response to many viruses. Within 6 hours of activation, human pDCs dedicate 60% of the induced transcriptome to type I IFN genes, producing 200 to 1000 times more type I IFN than any other cell type (3-10 pg of IFNα/cell), firmly establishing their key role as professional IPCs. Multiple subtypes of type I IFN are secreted by pDCs, including IFN-α, β, κ, ω and τ [Ito et al., 2006]. Additionally, pDCs have the ability to produce a significant amount of type III IFN (IFN-λ1, –λ2 and little if any IFN-λ3) [Yin et al., 2012]. The extraordinary ability of pDCs to mount a rapid and massive IFN response is linked to several unique features of these cells. First, pDCs selectively and abundantly express TLR7 and TLR9, two innate endosomal sensors, which make them superbly sensitive to internalized nucleic acid agonists [Gilliet et al., 2008]. Second, while interferon regulatory factor 7 (IRF7), one master transcriptional regulator of IFN-α production, is expressed at low levels in most cells and is only upregulated upon microbial infections, pDCs constitutively express high levels of IRF7 as well as the related IRF4 and IRF8 possibly due to the low expression of the negative translational repressors 4EBPs [Ito et al., 2006]. The third reason for their capability of producing high levels of type I IFN is related to their unique ability to retain TLR-activating ligands in early endosomes for extended periods of time, which allows a sustained activation of IRF7 [Honda et al., 2005; Guiducci et al., 2008].

Type I IFNs represent the first line of defence against viral infections, as they are capable of mediating immunoregulatory, growth-inhibitory and antiviral activities. All type I IFNs bind to the same IFNα/β receptor (IFNAR), which is widely expressed by most nucleated cells in the body [Schreiber et al., 2017]. The resulting signalling cascade induces a multitude of interferon-stimulated genes (ISGs) that encode for antiviral proteins, which in turn make cells resistant to viral infections and promote apoptosis of virally infected cells [Swiecki & Colonna, 2010]. Although viral interference is the first described function of IFN, its role is also to provide an interface between innate immune effectors and other cells of innate and adaptive responses (reviewed in ref. [Fitzgerald-Bocarsly et al., 2008]). For example, type I IFN induces the

differentiation, maturation and activation of mDCs, which in turn promote antiviral T cell immunity [Fonteneau et al., 2004]. Moreover, pDCs-derived type I IFN stimulates activation of NK cells, biases the immune system toward a Th1 response, primes $CD8^+$ T-cells and induces memory $CD8^+$ T cells, promotes the development of regulatory T (T_{regs}) cells and differentiation of B cells into antibody-secreting plasma cells [Aiello et al., 2018]. Type I IFN also possesses strong inflammatory properties through the activation of NLRP3 inflammasome contributing to the inflammasome-dependent caspase-1 activation that leads to the production of IL-1β and IL-18 and finally to pro-inflammatory pyroptotic cell death [Aiello et al., 2018]. In addition to IFN, pDCs have also the ability to produce a number of inflammatory cytokines and chemokines including IL-6, TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), and CXCL10 (IP-10). Another mechanism by which pDCs contribute to inflammation is by performing granzyme B- and caspase-dependent cytotoxicity against target cells [Bratke et al., 2010].

Different studies indicate that pDCs are implicated in advanced inflammatory response in several autoimmune diseases that are characterized by a type I IFN signature. Indeed, chronic activation of pDCs and sustained over production of type I IFN appear to be contributing factors in initiating and/or promoting psoriasis, SLE and type 1 diabetes (T1D). Therefore, the blockade of type I IFN or antibody-mediated depletion of pDCs could represent a potential therapy in these diseases.

1.2.2 Tolerogenic properties

There is increasing evidence that unstimulated or alternatively stimulated pDCs can act as tolerogenic cells, while pDCs activated through TLR7 and TLR9 act as immunogenic cells. Indeed, when compared to mDCs, pDCs exhibit poor immune-stimulatory ability and their interaction with T cells often favours the generation of T_{regs}, potent suppressors of T cells and DC activation [Matta et al., 2010]. The mechanisms by which pDCs induce tolerance have been actively investigated. The poor capacity to stimulate T cells may be a function of persistent membrane-associated MHC class II ubiquitin E3 ligase RING-CH1 (MARCH1) expression despite maturation, a feature that mDCs lose when activated. Consequently, there is the formation of continuously ubiquitinated, internalized and unstable antigen-MHC class II complexes on their surface [Matta et al., 2010]. Since low antigen levels were found to promote development of T_{regs} [Turner et al., 2009], it is feasible that the rapid turnover of antigen-MHC class II complexes by pDCs could translate into presentation of low levels of Ag (*i.e.* weaker TCR stimulation) which promotes T_{regs} induction and function.

Another pDCs-mediated immunosuppressive mechanism involves the indoleamine-pyrrole 2,3-dioxygenase (IDO), an enzyme catabolizing L-tryptophan to N-formyl kynurenine that possesses

immunosuppressive properties by depleting the tryptophan pool that T cells require to generate effective responses. In pDCs, IDO expression could be induced through: i) non-canonical NF-κB signalling by CD200-Ig-dependent stimulation of CD200 receptor (CD200R) with the involvement of the IFN-α/β receptor signalling [Fallarino et al., 2004], ii) IFN-γ mediated feedback (by inducing the IDO enzymatic function) and iii) a TGF-β-dependent manner (by inducing the IDO regulatory function) [Fallarino et al., 2012]. IDO promotes the differentiation of naïve $CD4^{+}$T cells into T_{regs} by stimulating CD40/CD40L-mediated signalling through cytotoxic T-lymphocyte antigen 4 (CTLA4)/CD80, CD40/CD40L and glucocorticoid-induced tumor necrosis factor receptor (GITR)/GITR ligand mechanisms [Fallarino et al., 2005]. The maintenance of IDO-dependent tolerogenic properties of pDCs requires a positive feedback loop mediated by kynurenine through the aryl-hydrocarbon receptor (AhR) on the surface of pDCs together with stimulatory signals from T_{regs} [Harden et al., 2012]. In addition to IDO, the expression of inducible costimulatory ligand (ICOS-L) on activated human pDCs can promote the generation of IL-10 producing T_{regs} from naïve T cells [Ito et al., 2007]. The upregulated expression of the immunosuppressive molecule PD-L1 (programmed death-ligand 1) on the surface of pDCs is another mechanism to mediate tolerance. The interaction between PD-1 and its ligand PD-L1 promotes tolerance by blocking the TCR-induced stop signal in the target T cell [Fife et al., 2009]. Finally, IL-3 could activate expression of granzyme B in pDCs, and its release suppresses proliferation of effector T cells [Jahrsdörfer et al., 2009].

The mechanisms stated above allow pDCs to promote immunosuppression and tolerance to tumor cells and in graft-versus-host disease (GVHD), and in addition renders antigens harmless [Rogers et al., 2013]. In addition to the peripheral tolerance, pDCs found in the cortical and medullar layers of the thymus play a role in inducing and maintaining central tolerance. Indeed, recirculating pDCs might present self-Ags in the thymus and contribute to the inactivation, or deletion, of autoreactive T cells.

1.3 Mechanisms of pDCs activation

The activation of pDCs is mainly due to the induction of the cellular network regulated by the endosomal sensors TLR7 and TLR9 [Webster et al., 2016]. TLR binding signals employ Myd88 (myeloid differentiation primary response 88), an adaptor protein that forms a scaffold with IRAK-4, TRAF-6 and Btk and induces the formation of the TRAF-3//IRAK-1/IKK-α/OPN/PI3K complex. Downstream IFN signalling occurs in response to activation of IFN genes through the phosphorylation and nuclear translocation of IRF-7. Instead, downstream signalling of NF-κB and MAPK pathways lead to the secretion of inflammatory cytokines and chemokines (IL-6 and TNF-

α), and the upregulation of MHC and costimulatory molecules such as CD80 and CD86 necessary for maturation into APCs [Lande et al., 2010]. As posited by the spatiotemporal model of pDCs sensing, differential pDCs activation is likely related to the subcellular location where the TLR sense the pathogen [Gilliet et al., 2008]. The engagement of TLR7/9 in the early endosomes of pDCs preferentially triggers the IRF-7 signal cascade, leading to the acquisition of an IPC phenotype, whereas the engagement of TLR7/9 in lysosomes is associated with the trigger of the NF-κB signal cascade that results in an APC phenotype [Honda et al., 2005; Guiducci et al., 2006]. In the context of viral infections, it has been observed that pDCs activation can occur through different ways (see Fig. I.1); in particular, it seems that their activation can be dependent or not on the exosomal transfer [Assil et al., 2015].

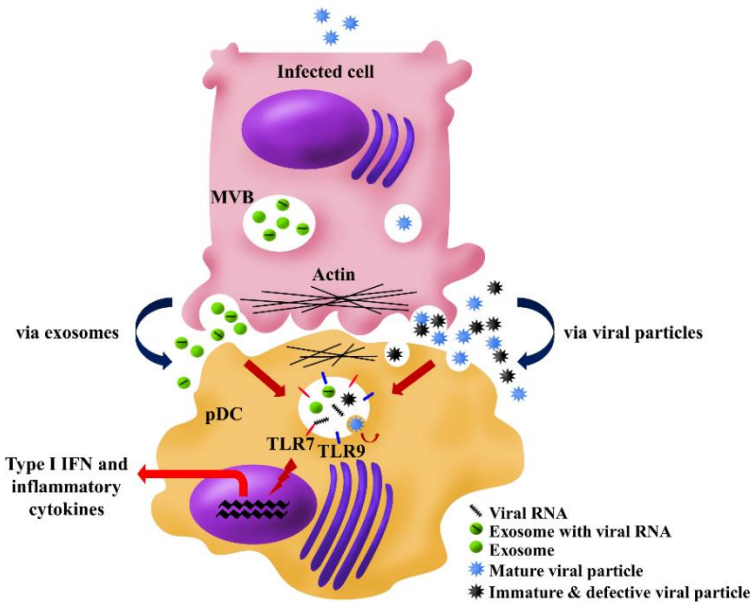


Figure I.1 pDC activation by cell-to-cell contact with infected cell independently of productive infection. Viral RNAs can be transmitted via various carriers including exosomes, which are small membrane bound vesicles budding in multivesicular bodies (MVB), and viral particles to activate pDCs. In some infections, pDCs preferentially respond to viral RNA transferred by exosomes rather than conventional viral particles. Viral RNA packaged in exosomes is then recognized by the endosomal TLR7 in pDCs leading to robust production of type I IFN and other cytokines. In the case of Dengue virus, mature wild type viral particles poorly activate pDCs, likely because they could escape from the recognition by TLR7. In contrast, mature defective and immature viral particles retained in the endo-lysosomal compartment could release viral RNA, hereby resulting in a robust production of type I IFN by pDCs [Aiello et al., Cytokine Growth Factor Rev, 2018].

The exosomes are small membrane bound vesicles (40-100 nm) that bud in multivesicular bodies (MVBs) and are released in the extracellular space. They are an important intercellular carrier not only for many proteins and lipids but also for small segments of nucleic acids [Raposo & Stoorvogel, 2013]. pDCs are sensitive to the exosome stimulation as demonstrated by recent *in vitro* studies in which exosomes produced by infected cells play a key function in the activation of the immune response mediated by pDCs and are involved in the type I IFN production [Dreux et

al., 2012]. This activation mechanism is not limited to a single viral family but seems to be preserved evolutionarily. Moreover, it seems that pDCs preferentially respond to viral RNA transferred by exosomes rather than conventional viral particles. Indeed, it was reported for HCV that while HCV exosomes induce a strong response of pDCs and a high production of IFN, the infectious HCV particles block the pathway induced by TLR7 activation and trigger a negative regulation of the pDCs IFN response [Dreux et al., 2012, Florentin et al., 2012]. This kind of process was reported not only for HCV but also for other viruses as human papillomavirus and for the hepatitis B virus (HBV) [Hasan et al., 2007; Xu et al., 2009]. It seems clear that exosomes carrying viral elements as proteins or RNAs to recipient cells and canonical infectious viral particles use separate modalities for the transmission. However, it remains unclear how exosomes are recognized, internalized and processed to play their roles inside the recipient cells such as pDCs [Assil et al., 2015].

There are some similarities in the mechanism of pDCs activation triggered either by exosomal or non-exosomal pathways. In particular, pDCs activation seems to require the establishment of cell-to-cell contacts with infected cells [Assil et al., 2015]. Cell to cell contact favours the concentration of immunostimulatory molecules in the intercellular space making the activation more efficient [Dreux et al., 2012; Décembre et al., 2014]. Furthermore, analysing the interface of the contact between pDCs and infected cells, the presence of structural viral components has been observed besides the accumulation of the actin cytoskeleton. Starting from this observation it seems that the actin network acts as a structural platform for the transmission of activating signals to pDCs [Décembre et al., 2014]. Notably, since TLR7 is localized in endo-lysosomal compartment, the immunostimulatory signal must be internalised to promote the activation. Therefore, a productive viral infection in pDCs is not required for the activation since the signalling pathway can be stimulated by the recognition of incoming viral RNA during the internalization. Indeed, pDCs activated without detectable viral genome amplification and/or viral protein expression have been identified during infection with different viral families and the activation of pDCs after the treatment with UV-inactivated virions also supports this hypothesis [Assil et al., 2015]. Likely, it is the rapid production of IFN, typical of pDCs, that makes them refractory to many viral infections. However, it is still unclear how the viral genome is transmitted and exposed to the immune-sensor. It is conceivable that the intracellular proteases and lipases, which are present in the endo-lysosomal compartment, favour the digestion and the exposition of the nucleic acids in particular of viral particles that are defective in penetration. In this respect, it is important to remind that RNA viruses produce a huge amount of defective particles during their replication. Future studies should also investigate the possible involvement of cellular surface factors in the

remodelling and polarization of pDCs during the establishment of the cell-to-cell contact [Assil et al., 2015].

1.4 The case of HIV infection

Although the development of combined antiretroviral therapy has allowed HIV patients to suppress viral replication and live longer lifespans, HIV evades the immune system and persists, causing chronic immune activation and inflammation. The role of pDCs in HIV-1 infection and pathogenesis is not well defined. It is clear that pDCs can be infected with the virus and/or respond to it with robust IFN secretion. The key unresolved question is whether HIV-induced pDCs activation is beneficial and/or harmful for the host. Indeed, although pDCs play a critical role in antiviral immunity thanks to the secretion of type I IFN that inhibits HIV-1 replication, dysregulation of these cells during HIV-1 infection seems to be involved in immune pathogenesis by contributing dichotomously to both immune activation and immunosuppression (see Fig. I.2).

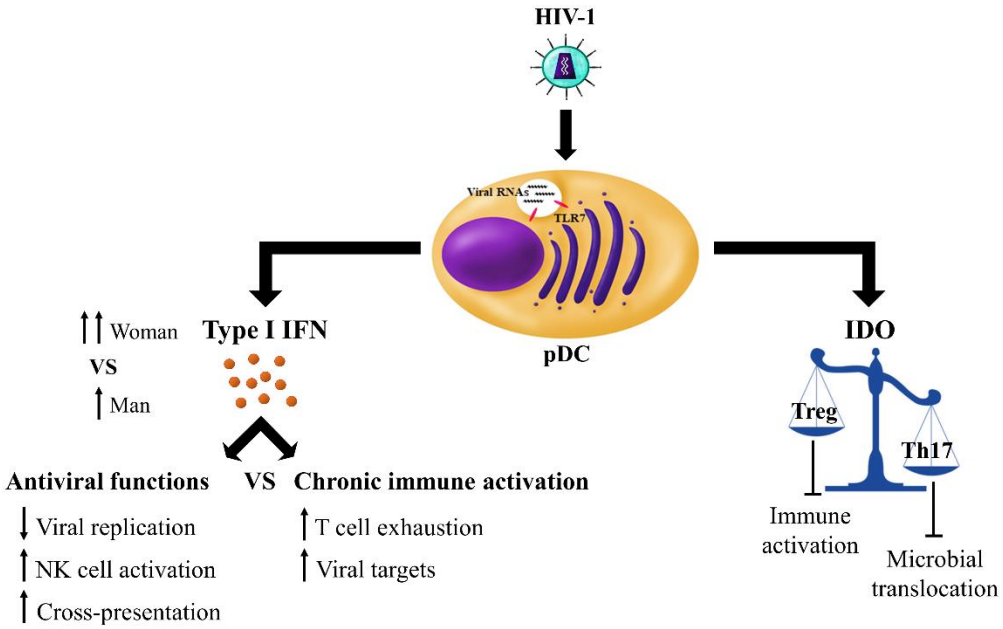


Figure I.2. Opposing roles of pDCs in HIV-1 infection. HIV-1 stimulates pDCs to produce Type I Interferon (IFN-I), which acts as a double-edged sword. On one hand, it exerts antiviral functions; on the other hand, it contributes to chronic immune activation. Moreover, HIV-1 stimulates pDC to secrete indoleamine 2,3 dioxygenase (IDO) that induces the generation of T_{reg} at detriment of Th17 differentiation. Treg are regulatory cells that dampens immune activation, while Th17 inhibit microbial translocation. Th17/Treg deregulation in HIV infected patients leads to an increased microbial translocation and immune activation [Aiello et al., Cytokine Growth Factor Rev, 2018].

1.4.1 Entry and activation of pDCs by HIV-1

Although pDCs do not represent the reservoir of HIV, their interaction with HIV virions is possible because pDCs express the surface molecules that are targeted by the virus: CD4 receptor and the co-receptors CXCR4 and CCR5. It is accepted that HIV-1 virions enter pDCs mainly through CD4

receptor- and dynamin-dependent endocytosis for endosomal degradation [Haupt et al., 2008; Beignon et al., 2005; Pritschet et al., 2012], by inducing cellular activation. HIV-1 detection by pDCs results in abundant IFN production, but low NF-κB-dependent production of TNF-α and minimal upregulation of costimulatory molecules, suggesting that HIV-1 preferentially promotes pDCs to become IPCs rather than APCs [McKenna et al., 2005; O'Brien et al., 2011]. On the contrary, HIV-2 stimulation favours the development of an APC phenotype in pDCs despite similar upregulation of ISGs and viral restrictions factors. Consistent with these observations, Royle and colleagues propose that the pDCs differentiation profile contributes to the differences in pathogenicity between HIV-1- and HIV-2-induced diseases [Royle et al., 2014].

It should be noted that cell-free virions of most HIV-1 isolates are relatively weak stimulants of pDCs and initiation of IFN-α production requires high concentration of HIV-1 particles when compared to other viruses [Beignon et al., 2005; Lo et al., 2012]. In this regard, it was shown that pDCs are highly resistant to HIV infection due to the expression of various host restrictions factors, such as SAMHD1, resulting in low levels of viral replication [Bloch et al., 2014]. On the other hand, HIV-1 infected $CD4^+$ lymphocytes were shown to be more efficient at stimulating pDCs [Schmidt et al., 2005], but precise mechanisms underlying differences between cell-free and cell to cell pDCs activation are not clearly defined and require further studies.

Cellular mechanisms underlying HIV-stimulated IFN production by pDCs have been studied in detail. It was previously shown that IFN is produced by pDCs upon HIV stimulation through endosomal recognition of viral RNA by TLR7 and activation of IRF-7 signalling, since TLR7 oligonucleotide inhibitors are much more potent than TRL9 inhibitors in blocking IFN production by HIV-exposed pDCs [Pritschet et al., 2012]. The upstream events that determine this response, and in particular HIV virion trafficking in pDCs, are currently only partially understood. Prior studies demonstrated that HIV endocytosis and endosomal acidification, but not fusion or viral replication are required [Beignon et al., 2005]. Moreover, in a recent report Veenhuis and colleagues observed that alternative methods of viral uptake can also activate the IFN pathway in pDCs when endocytosis is inhibited without blocking the acidification process critical to TLR signalling [Veenhuis et al., 2017].

The presence of HIV envelope glycoprotein (Env) on viral particles also seems to play a major role, as it was observed that pDCs are not activated by non-virion-associated Env and may actually inhibit their activation [Beignon et al., 2005, Martinelli et al., 2007]. Indeed, HIV entry and pDCs activation require the CD4-gp120 binding on pDCs, as supported by the finding that the affinity of gp120 to CD4 determines the degree of IFN-α production, but not the co-receptors CXCR4 and CCR5 that result to be only dispensable for IFN-α production [Haupt et al., 2008]. Moreover,

O'Brien and colleagues have recently demonstrated as HIV trafficking and pDCs phenotype is predominantly determined by envelope-CD4 interactions and it is regulated by receptor targeting motifs, such that manipulation of HIV envelope or CD4 intracellular trafficking allows modulation of divergent sensing of HIV. They found that HIV virions pseudotyped with influenza hemagglutinin (HA) envelope were rapidly routed to late endosomes/lysosomes and trigger activation of NF-κB similar to influenza, contrary to HIV with its native envelope [O'Brien et al., 2016]. Regarding the role of CD4, it contains a dileucine motif in its intracytoplasmic domain, which is essential for CD4 endocytosis and, in addition, two adjacent serines, Ser^{408} and Ser^{415}, which enhance CD4 endocytosis and redirect CD4 to lysosomal compartments when phosphorylated. HIV-activation of pDCs does not seem to alter CD4 internalization, therefore HIV traffics by default to recycling endosomes due to the CD4 dileucine motif [O'Brien et al., 2016].
In a recent study, Reszka-Blanco and colleagues (2015) have also shown that Env and the multifunctional pathogenic accessory protein Nef cooperatively contribute to HIV-1 induced pDCs activation through their domains involved in CD4 binding and CD4 down-regulation, respectively [Reszka-Blanco et al., 2015]. They hypothesized a model according to which virion-incorporated Nef, by transporting CD4 for endolysosomal degradation and interaction with the AP1 to -3 complex, also interacts with viral RNA and facilitates its presentation to TLR7 in early endosomes to trigger pDCs activation [Reszka-Blanco et al., 2015]. Alternatively, Nef may act indirectly via stimulation of exosome formation in producer cells. Exosomes are usually enriched in viral or cellular structures that may increase activation of pDCs through TLR7-dependent or independent pathways [Lenassi et al., 2010].
Classically, dendritic cells that have been activated by a pathogen become refractory to subsequent activation. This does not happen in pDCs where HIV activates the type I IFN receptor-mediated autocrine feedback loop and persistently activate them to produce IFN-α, probably due to the prolonged localization of HIV in early endosomes. Moreover, pDCs possess specialized large perinuclear intracellular stores of MHC-I molecules with characteristic of recycling endosomes in immature pDCs. These intracellular stores may function as a stable compartment in which HIV accumulates in pDCs and prolonged localization in these early recycling endosomes might ultimately have important consequences also for HIV antigen cross-presentation, which is strongly enhanced when pDCs are activated by maturation stimuli [O'Brien et al., 2016].

1.4.2 Role of pDCs in HIV transmission

Although HIV lentiviral infection is characterized by a protracted clinical course and a long incubation period before the symptoms appear, the initial transmission event that leads to systemic

infection is rapid and explosive. HIV-1 infection is generally acquired via the mucosal surfaces of the genital tract and it is commonly initiated by a single founder virus [Keele et al., 2008]. Studies in the Simian Immunodeficiency Virus (SIV) rhesus macaque NHP model of mucosal transmission showed that virus replication is initially confined to the mucosal infection site. Subsequently, virions cross-mucosal epithelia through transcytosis and/or trans-epithelial emigration of infected DCs and comprise a small homogeneous founder population [Carreno et al., 2002; Stoddard et al., 2009]. The latter then undergoes a local expansion to establish an irreversible, systemic and self-propagating chronic infection.

Of the potential target cells present in the genital tract, DCs, macrophages and $CD4^+$ T cells are the most likely cells infected by HIV. Since $CD4^+$T cells are sparsely distributed at non-inflamed genital mucosae, sentinel DCs, macrophages and epithelial cells have a crucial role in the activation and recruitment of additional target cells through the release of cytokines and chemokines, favouring in this way virus propagation. In a SIV-macaque model, it was observed that endocervical epithelial cells produce macrophage inflammatory protein 3 (MIP-3α or CCL20), which in turn attracts substantial numbers of pDCs. The latter are the first predominant cell type to arrive to infected mucosal sites and are activated by HIV to produce factors including type I IFN and the chemokines MIP1α and β (i.e., CCL3 and CCL4), which recruit $CD4^+$ T cells amplifying the pool of locally available target cells [Li et al., 2009]. Although local immune activation and cellular infiltration facilitate virus replication, HIV-1 must simultaneously avoid being controlled by innate antiviral defences activated at the mucosa, particularly type I IFN. Thus, HIV-1 activates potent secretion of type I IFN and other cytokines/chemokines by pDCs but suppresses type I IFN production and IFN-induced retroviral restriction in infected cells likely to establish a balance between its need to drive inflammation and attract $CD4^+$ T cells to increase replication, and its need to simultaneously minimise local upregulation of antiviral ISGs [Borrow et al., 2011]. Altogether, the innate signalling, including pDCs activation, paradoxically seems to be more a “foe” rather than a “friend” in the context of HIV transmission, as it facilitates rather than restricts viral replication and expansion of infection.

DCs can indirectly facilitate infection of other targets cells by producing cytokines that recruit or activate the target cells so they are easier to become infected, but they can also facilitate HIV transmission by being infected by HIV directly, and then transferring the virus to $CD4^+$ T cells. During HIV-1 replication, there are two major mechanisms of viral transmission between cells (reviewed in ref [Wu & KewalRamani, 2006]). First, HIV-1 can infect target cells, and productively replicate and produce progeny virions that are released to infect new target cells; this is *cis*-infection. Second, the virus is retained at or near the cell surface of a donor cell and

transmitted to a different type of target cell via the close contact and formation of a virological synapse (VS) or via the exosome secretion pathway; this is *trans*-infection [Coleman et al., 2013]. HIV-1 *trans*-infection is that most prominently associated with DCs, as productive HIV-1 replication is relatively inefficient in all DC subtypes (including pDCs) compared to more permissive types, such as macrophages and $CD4^+$ T cells [Dong et al., 2007]. Additionally, internalized HIV-1 is rerouted and polarized at the cell surface to escape canonical degradation routes via the lysosome or proteasome pathways, allowing efficient transmission to $CD4^+$ T cells [Yu et al., 2008]. The migratory capacity of pDCs allows them to contact many T cells also in the lymphoid tissues likely enhancing the effect of such *trans*-infection. Moreover, mDCs or pDCs were observed to be particularly efficient to transfer HIV-1 upon cognate interaction with antigen-specific $CD4^+$ T cells [Loré et al., 2005], possibly resulting in the preferential infection of HIV-specific $CD4^+$ T cells [Douek et al., 2002].

The cellular and viral factors that affect the process of early-stage HIV-1 transmission have been extensively studied. In particular, the Nef protein seems to play a key role in promoting DC-mediated HIV-1 transmission to $CD4^+$ T cells by activating $CD4^+$ T cells [St Gelais et al., 2012] and downregulating the expression of CD4 receptor, which otherwise would inhibit *trans*-infection [Wang et al., 2007]. Further work is necessary to characterize those cellular and viral factors involved in the regulation of DC-mediated HIV-1 transmission to $CD4^+$ T cells that act specifically in different DC subsets.

Prevention of the HIV-1 transmission remains a prominent goal of HIV research. A promising strategy to prevent transmission seems to be the inhibition of innate immune responses and inflammation for example using compounds with anti-inflammatory properties (e.g. glycerol monolaurate), whose efficacy has been demonstrated in animal models [Li et al., 2009]. Moreover, drugs that target initial interactions between DCs and HIV-1 have the potential to be used as topical treatments at the mucosal surfaces to prevent the initial DC-mediated HIV-1 transmission events. In this regard, Gombos and colleagues recently observed a significant reduction in virus transmission using a combination of different neutralizing antibodies that target specific sites on HIV envelope [Gombos et al., 2015].

1.4.3 Role of pDCs in chronic immune activation during HIV infection

HIV-1 infection is marked by aberrant immune activation, which is a better correlate of disease progression to AIDS than viremia. Chronic immune activation persists even under antiretroviral therapy (ART) and contributes to increased risk of infection-associated co-morbidities [Baker et al., 2010; Ho et al., 2010]. It is characterized by increased expression of HLA-DR, CD38 and Ki67

on $CD4^+$ and $CD8^+$ T cells, by T cell exhaustion, upregulation of inhibitory molecules such as CTLA-4 and PD-1 and apoptosis. The pathogenesis of chronic immune activation is complex and incompletely delineated, but stimulation of innate immune cells, directly by HIV and indirectly by products of bacterial translocation, and the persistent IFN-α production may be major contributors. Given that pDCs are the most potent producers of type I IFN, their persistent activation may play a role in HIV disease progression. Indeed, women have pDCs that produce greater amounts of type I IFN and their infection progresses faster to AIDS than in men with similar viral loads [Ziegler & Altfeld, 2017].

During the course of HIV infection, IFN-α appears to be a double-edged sword. Although, it possesses potent antiviral properties because it reduces viral replication and induces apoptosis in HIV-infected cells, both human and animal studies support a role of IFN-α in the pathogenesis of HIV immune activation and inflammation [Herbeuval et al., 2007]. In non-human primates, transcriptional profiling of pathogenic and non-pathogenic infection showed that progressive infection is characterized by a persistent and systemic IFN response signature, in contrary to non-progressive infections, where IFN signature subsides quickly despite high levels of viral replication [Bosinger et al., 2009; Jacquelin et al., 2009]. In HIV-1 patients, a chronic production of IFN-α has been also observed; in particular, the specific subtype namely IFNα2b is preferentially upregulated throughout the course of the disease [Lehmann et al., 2009]. IFN-α contributes to inhibition of T cell differentiation and death of HIV-uninfected bystander cells, favouring T cell exhaustion [Démoulins et al., 2008]. The apoptosis is induced through the expression of the TRAIL factor (TNF-related apoptosis-inducing ligand) and of its DR5 receptor on the $CD4^+$ T lymphocytes present in the blood and LNs, contributing in this way to their depletion, characteristic of the progression to AIDS [Cha et al., 2014]. Moreover, administration of exogenous IFN-α results in increasing $CD8^+$ T cell activation in HIV-infected subjects. Inflammatory cytokines and type I IFN can limit thymic output, enhance bystander T cell proliferation and inhibit telomerase activity in human T cells [Cha et al., 2014].

HIV-1 disease progression is also associated with multi-lineage hematopoietic abnormalities in addition to $CD4^+$ T cell depletion, in which type I IFN produced by pDCs is involved. In this regard, Li and colleagues (2017) showed that HIV-1 infection significantly depleted and functionally impaired human hematopoietic progenitor cells (HPC) in the bone marrow of both HIV-1-infected patients and humanized mice through a pDCs dependent mechanism. Indeed, pDC depletion significantly recovered cell numbers and functions of HPC and their hematopoiesis [Li et al., 2017].

During chronic HIV infection, a substantial depletion of pDCs from the peripheral compartment is observed, which is correlated with high viral load and reduced CD4 counts [Donaghy et al., 2001]. Although ART treatment results in a partial recovery of pDCs numbers, they do not make a full recovery [Chehimi et al., 2007]. The fate of pDCs in the course of HIV-1 infection has been a matter of intense debate and the specific cause of this decline is still under investigation. Initially, their depletion was attributed only to direct virus infection and relocation to lymphoid tissue, but the current data suggest that the decline of peripheral pDCs during the course of HIV infection does not simply reflect a systemic cell loss, but is the result of a combination of pDCs depletion, repopulation and migration [Boichuk et al., 2015]. Lehmann and colleagues reported that circulating pDCs of HIV-1 patients express higher levels of the lymph node homing markers, CCR7 and CD62L; therefore, they relocate to lymphoid tissue where they express high levels of IFN-α before undergoing cell death [Lehmann et al., 2010]. Despite their decline, residual pDCs in peripheral blood express IFN-α at levels markedly higher than pDCs of healthy controls [Lehmann et al., 2008]. Furthermore, pDCs present in LNs of HIV-1 patients display an altered cell surface expression profile of activation/maturation markers with higher CD40, lower BDCA2, a C-type lectin that is a negative regulator of IFN-α production, and stable CD83 and CD86 levels. The lower BDCA2 levels on pDCs are consistent with the notion that HIV promote a maturation as IPCs, therefore these cells have reduced capacity to function as APCs and to stimulate $CD4^+$ T cell proliferation, but retain the ability to express IFN-α [Lehmann et al., 2010]. Since pDCs can be triggered to express IFN-α following exposure to both infectious and non-infectious HIV-1 as well as by viral proteins [Yonezawa et al., 2003; Herbeuval et al., 2005], viral particles and/or proteins trapped in the intercellular space of LNs may be involved in promoting IFN-α expression by pDCs. This suggests that at a certain point during infection, pDCs are chronically activated and are continuously producing IFN-α, contributing to the immune exhaustion of T-cell compartments. This hypothesis is supported by the study of Li and colleagues in which they observed that humanized pDCs-depleted mice infected with HIV show dramatically reduced cell death and immune cell depletion [Li et al., 2014].

Circulating pDCs express also higher levels of CD103 (also known as integrin αE), which in complex with integrin β7 mediates cell redistribution to the intraepithelial sites and *lamina propria* of the gut-associated-lymphoid tissue (GALT) [Lehmann et al., 2014]. Here, they may contribute to immune activation by secreting inflammatory cytokines, leading to the loss of gut tissue integrity and microbial products translocation [Lombardi et al., 2014].

During the course of HIV infection, pDCs show also a different functionality in response to TLR7 and TLR9 stimulation. While pDCs from acute HIV infection are hyperreactive to *ex vivo* stimuli

[Sabado et al., 2010], during chronic infection pDCs that are stimulated *ex vivo* with various TLR7 and TLR9 agonists showed a reduced secretion of IFN-α [Kaushik et al., 2013]. Taken together, these data suggest that some degree of pDCs exhaustion occurs as HIV-1 infection progresses, presumably due to chronic stimulation *in situ* by HIV-1, products of microbial translocation and other immunomodulatory factors, which may lead to a decreased responsiveness to exogenous stimuli.

Although pDCs are considered the main source of IFN in the acute phase, during chronic infection the mechanisms that regulate IFN levels are not completely understood. The recent article of Veenhuis and colleagues shed new light on the possible regulation of IFN production during the late stages of infection [Veenhuis et al., 2017]. Indeed, they demonstrated that both monoclonal antibodies (mAbs) directed against sites outside the CD4-binding sites and Abs generated in people with persistent HIV infection enhance IFN production by pDCs, providing an explanation for high levels of IFN production and immune activation in chronic HIV infection [Veenhuis et al., 2017]. On the other hand, it has been hypothesized that the production of IFNα in plasma and ISG expression may be due also to the contribution of other cell types such as mDCs and macrophages [Lepelley et al., 2011].

1.4.4 Role of pDCs in the immunoregulation during HIV infection

Despite their potential role in chronic immune activation, pDCs may also play a contrary role in immunoregulation during HIV-1 infection through the induction of T_{reg} responses that may have both deleterious and beneficial functions. T_{regs} may limit the formation of HIV-1 specific T cell responses by impairing antigen presentation by mDCs [Manches et al., 2012]. Alternatively, T_{regs} can serve to alleviate chronic immune activation. Which of these roles predominates during the course of HIV-1 infection remains controversial, but it appears that pDCs are important in generation of T_{regs} during the infection. Manches and colleagues described the development of suppressive $Foxp3^{+}CD127^{low}CD25^{+}$ cells from naive $CD4^{+}$ T cells by HIV-exposed pDCs consistent with the phenotype of T_{regs}. Their induction relates to the expression of IDO. pDCs express this enzyme following the endocytosis of HIV-1 through gp120-CD4 binding and the endosomal TLR7 triggering. Interestingly, the induction of T_{reg} cells in this IDO dependent pathway is independent of type I IFN production or of other molecules expressed by HIV-stimulated pDCs including ICOSL [Manches et al., 2008]. In a later study, Manches and colleagues demonstrated that TLR-induced IDO expression in pDCs is dependent upon the non-canonical, but not the canonical NF-κB pathway [Manches et al., 2012].

The generation of T_{regs} following HIV-pDCs interaction may help explaining the strong negative correlation of viral load and allogeneic T cell proliferative capacity of pDCs isolated from HIV patients during acute infection [Sabado et al., 2010]. It is possible that high levels of circulating virus can negatively impact T cell stimulatory capacity of pDCs because of the induction of T_{regs} by HIV-exposed pDCs during acute and chronic infection. Indeed, acute SIV infection is associated with rapid development of T_{regs} and elevated IDO expression [Estes et al., 2006]. Importantly, IDO has been recognized to be an immune response checkpoint that plays an important role in HIV immune dysfunction, even in the context of ART therapy [Routy et al., 2015]. Its activity has been associated with disease progression, as its expression has been shown to prevent conversion of T_{regs} into Th17. Indeed, in HIV-infected donors disease progression is associated with elevated T_{regs} in peripheral blood and gut is related to high levels of IDO and reduced Th17 responses [Favre et al., 2010]. Therefore, IDO acts as a molecular switch to maintain the stability of T_{regs} at detriment of Th17 differentiation in an inflammatory environment [Baban et al., 2009]. Th17 cells are important to host defence against microbes, and in particular are crucial for preservation of the integrity of the gut-associated mucosa and prevention of bacterial translocation. Consequently, Th17/T_{reg} deregulation in blood and mucosal tissues of HIV infected patients leads to the progressive loss of the mucosal epithelial barrier, leading to an increased microbial translocation and immune activation [Aiello et al., 2018]. Mouse models of pDCs depletion showed that pDCs contribute to T_{reg} maintenance in the small intestine, since pDCs depleted mice display increased number of Th17 in the *lamina propria*, confirming the importance of pDCs in regulating Th17/T_{reg} ratios in the gastrointestinal tract [Takagi et al., 2011].

The understanding of the optimal equilibrium of T_{regs} during HIV-1 infection will be vital to understanding the potential beneficial interventions in order to manipulate their function. To date, attempts to block IDO with the inhibitor 1-methyl tryptophan (1mT) have been undertaken in SIV model, but only one of these studies successfully increased the level of circulating tryptophan in animals resulting in an improved viral control in animals receiving ART [Boasso et al., 2009]. Thus, further studies are necessary to optimize IDO blockade and T_{reg} inhibition in order to determine whether therapeutics that target T_{reg} can be useful in improving viral control.

CHAPTER 2

HIV-1 Nef protein and extracellular vesicles

2.1 Introduction

Several viruses manipulate host innate immune response to avoid immune recognition and improve viral replication and spreading. From this point of view, HIV represents a paradigmatic example. It encodes the classical structural and enzymatic factors common to all retrovirus through the *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) genes. In addition, HIV encodes two regulatory proteins, which are essential for viral replication (*i.e.* the transcriptional transactivator Tat and the regulator of virion gene expression Rev), and four "accessory proteins", including the ill-named "Negative effector" Nef (see Fig. I.3). Nowadays, it is known that the so-called accessory proteins are far from being accessory, indeed they carry out several critical functions for both viral replication, immunoevasion and pathogenesis. In particular, HIV-1 Nef protein has proved to be one of the main determinants of HIV pathogenicity, mainly involved in the "hijacking" activity of the immune system. Its pivotal role was confirmed by the fact that the Nef-defective HIV leads to an attenuated clinical phenotype with reduced viral loads in murine models, monkeys and humans, and that Nef transgenic mice develop an AIDS-like disease [Hanna et al., 1998]. Furthermore, macaque infection, with a SIV coding for a prematurely interrupted protein Nef, demonstrated the existence of a strong selective pressure for the expression of a functionally active Nef protein [Arora et al., 2002].

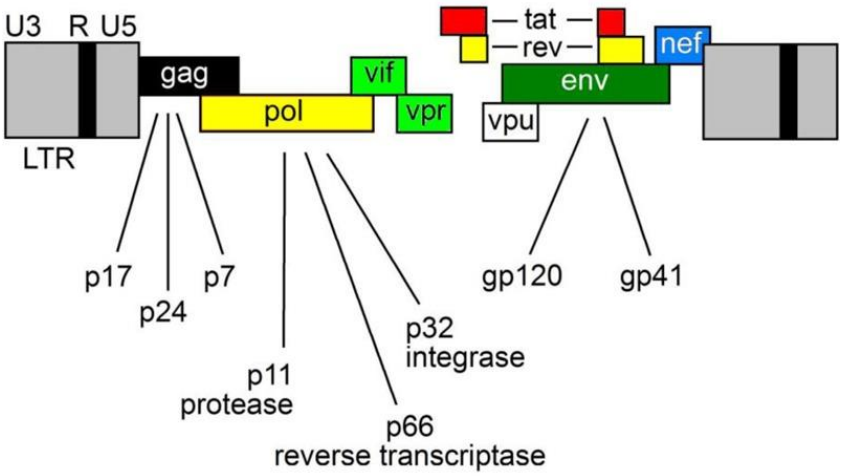


Figure I.3 The HIV-1 genome. HIV-1 encodes three major genes, 5'-gag-pol-env-3' encoding structural, accessory and regulatory proteins [Felli et al., Front Microbiol, 2017].

2.2 The Nef protein

Nef is a molecular multifunctional adaptor of about 200 amino acids (27-34 kDa) and characterized by different alleles that slightly vary in length. It is abundantly expressed already in the earliest stages of infection together with Tat and Rev proteins, but there is evidence of its possible expression also before the integration of the proviral genome [Wu & Marsh, 2001]. Nef is mainly a cytoplasmic protein partially associated with the cell membrane and often concentrated in the perinuclear regions. The protein mediates many and distinct functions that increase the production and infectivity of viral particles and alter specific cellular trafficking and signalling pathways. Since Nef does not possess an enzymatic activity, it acts as molecular adaptor inside the cell exerting its effects through specific protein-protein interaction motifs [Arold & Baur, 2001; Geyer et al., 2001]. It is co-translationally modified by the addition of myristic acid to the N-terminal end (myristoylation) and phosphorylated on specific amino acid residues. Its structure, obtained by nuclear magnetic resonance (NMR) and X-ray crystallography, includes two domains: a flexible N-terminal arm that anchors the protein to the membrane (about 70 residues) and a conserved and folded core domain (about 120 residues), containing a C-terminal flexible loop of 33 residues (152-184) that mediates the interactions involved in the cellular trafficking [Geyer et al., 2001].

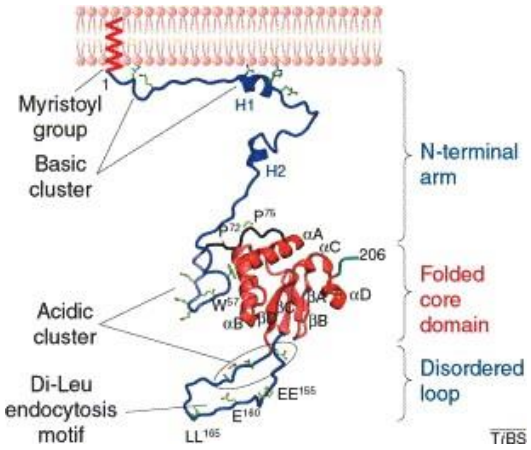


Figure I.4 Nef protein model anchored to the cell membrane. The model was realized based on the NMR structures of the N-terminal myristoylated end of the folded core of Nef [Arold & Baur, Trends Biochem Sci, 2001].

Membrane binding is critical for Nef functions on cell signalling and membrane trafficking and requires both the covalently attached myristic acid moiety and a cluster of N-terminal basic residues, especially the arginine-rich cluster (R17-R22), which ensures a stable binding of Nef thanks to the interaction of the viral protein with the lipid heads of the cell membranes. Instead, the hydrophobic moiety of this region appears to interact with a complex of proteins containing

the tyrosine kinase Lck and with proteins important for the down-regulation of some MHC-I antigens.

The core domain is the only part of Nef that adopts a stable tertiary structure and has been characterized both in free form and linked to SH3 domains (Src-homology domain 3) of cellular proteins which interact with Nef. Mutational analyses have suggested that most of the signalling molecules that bind Nef interact with its core domain, often through a proline-rich sequence, as happens with the Src kinases such as Hck and Lyn, Lck, Fyn and Src. Furthermore, mutational studies have highlighted the importance of residues of the core domain for the interaction with the PAK1/2 kinases (p21-activated kinase 1/2), with the ζ chain of the T cell receptor (TCR), with a human thioesterase and with the CD4 receptor. The core domain mediates also the oligomerization of HIV-1 protein Nef. In this regard, dimers and trimers of the protein have been observed both *in vivo* and *in vitro*, but their role is not yet clear; oligomerization could be functional to the regulatory effect of cellular signalling and endocytosis.

The C-terminal flexible loop is projected outside the core domain and presents three binding motifs, each of which allows Nef to interact with cellular components involved in endocytic pathways. At the centre of the loop is present an internalization motif, containing a pair of leucines (LL^{169} in the Nef SF2 strain), necessary for interaction with the Adaptor Proteins (APs). Near the N-terminal end of the loop, there is a diacid sequence (EE^{159}) necessary for the association of Nef with β-COP (β subunit of the coatomer COPI, Coat Protein 1). Finally, a diacid sequence (ED^{179}), located at the C-terminal end, is required for Nef colocalization with the vATPase (vacuolar proton pump). Since the core loop is variable in the amino acid sequence but has a conserved length, the latter could be maintained to allow exposure of these peptide motifs at a given distance from the core itself.

Arold and Baur proposed a model according to which Nef protein would adopt different structural conformations inside the cell, which allow different localizations and interactions with the different partners, realizing the so-called "Nef cycle" (see Fig. I.5) [Arold & Baur, 2001].

According to the proposed model, Nef, after translation, could adopt a closed conformation in which its binding sites are mainly hidden, because of the interaction of the N-terminal myristoyl moiety with the hydrophobic pocket on the core domain. The closed conformation would explain why the majority of the protein is localized in the cytosol and not associated with membranes when it is analysed its presence in cellular fractions. The subsequent contact with the cell membrane could trigger a conformational change via the interaction of the negative charges of the membrane lipid heads with the positive charges in the N-terminus of Nef (signalling or semi-open conformation), thus reducing the interaction between the N-terminal end and the core. This

conformational change would expose several motifs to bind signal molecules, many of which are present in lipid rafts. However, the association of Nef with the plasma membrane might persist only for a short period due to the subsequent exposure of the core loop (open conformation). The latter, in fact, could bind molecules involved in the endocytic machinery mediating the internalization of Nef together with specific interaction partners such as the CD4 receptor or MHC-I antigens. The protein appears also able to shuttle rapidly from the cytoplasm to the nucleus and *vice versa* inducing the transient translocation to the cell membrane of the Polycomb Group protein Eed, a nuclear transcriptional repressor, thus leading to a potent stimulation of Tat-dependent HIV transcription [Witte et al., 2004].

Overall, the data obtained from functional and structural studies of the protein show how Nef acts as a multifunctional molecular adaptor through the interaction with different partners, which can be divided into two classes: the proteins involved in membrane trafficking or in signalling transduction.

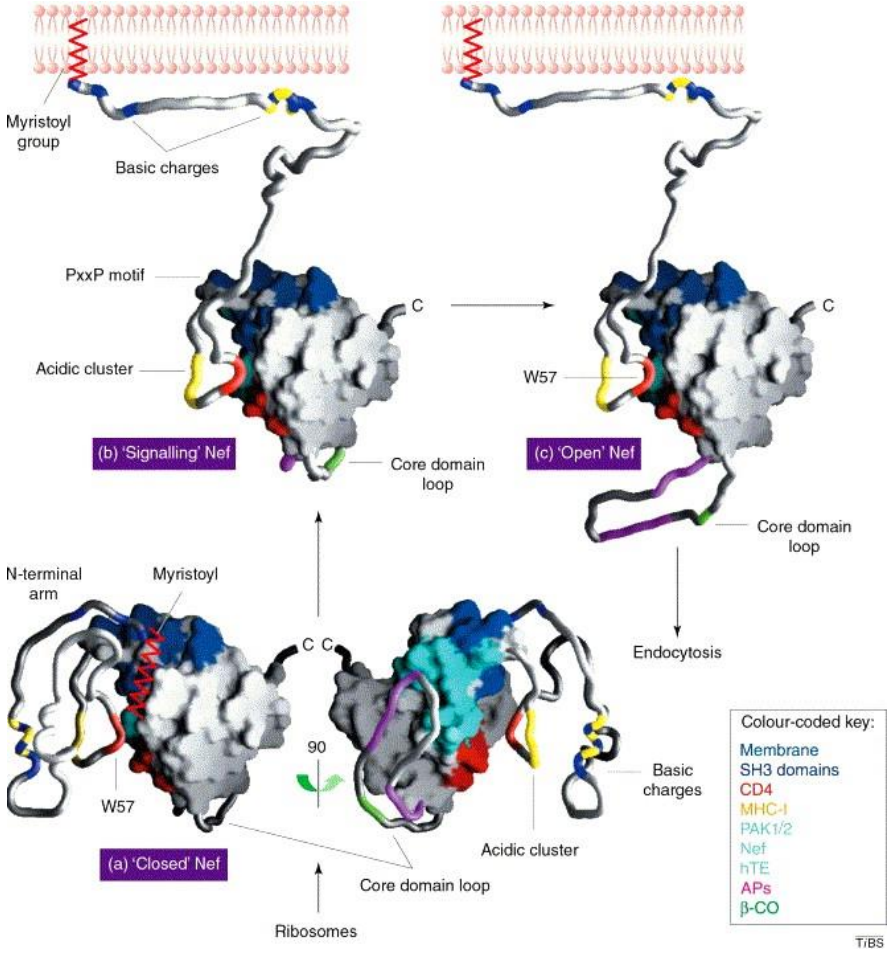


Figure I.5 Structural models of the different stages of the "Nef cycle". (a) "Closed" conformation that Nef would adopt after the translation. (b) "Semi-open" or "signalling" conformation assumed after contact with the membrane, allowing Nef binding to signal molecules in proximity of the TCR. (c) Following the exposure of the core loop, Nef would adopt an "open" conformation [Arold & Baur, Trends Biochem Sci, 2001].

2.3 Nef as a multifunctional viral adaptor

In the last few years, there have been remarkable advances in outlining a defined framework of Nef functions. In particular, Nef results to play a pivotal role in creating an environment suitable for the replication, the persistence and the spreading of the virus. The data obtained from functional and structural studies showed how Nef exerts its functions by acting as a multifunctional molecular adaptor through the interaction with different partners. Depending on its intracellular localization, Nef may exert multiple effects such as interfering with cellular signal transduction pathways or modulating the cell surface expression of many membrane associated-proteins in infected cells [Quaranta et al., 2009]. The most important and best characterized functions of Nef are the following: (a) the modulation of the expression of surface receptors on cellular plasma membrane of T cells, macrophages, dendritic and glial cells [Pawlak & Dikeakos, 2015; Landi et al., 2011]; (b) induction of a pre-activation state in $CD4^{+}$T cells to make them more susceptible to infection and to promote viral gene expression [Simmons et al., 2001]; (c) regulation of apoptosis, by inducing it in bystander uninfected cells meanwhile protecting infected cells by apoptotic stimuli through more than one mechanism [Geleziunas et al., 2001; Wolf et al., 2001; Greenway et al., 2002]; (d) increase of infectivity of produced HIV-1 virions, by preventing the incorporation of two antiviral cellular proteins, SERINC3 and SERINC5 [Rosa et al., 2015; Usami et al., 2015]; (e) regulation of the cytokine network contributing to the chronic inflammation [Swingler et al., 1999 and 2003; Olivetta et al., 2003; Federico et al., 2001; Percario et al., 2003; Mangino et al., 2007; Mangino et al., 2011] and (f) increase of the exosome production from different cellular compartments [Muratori et al., 2009; Lenassi et al., 2010; Baur, 2011].

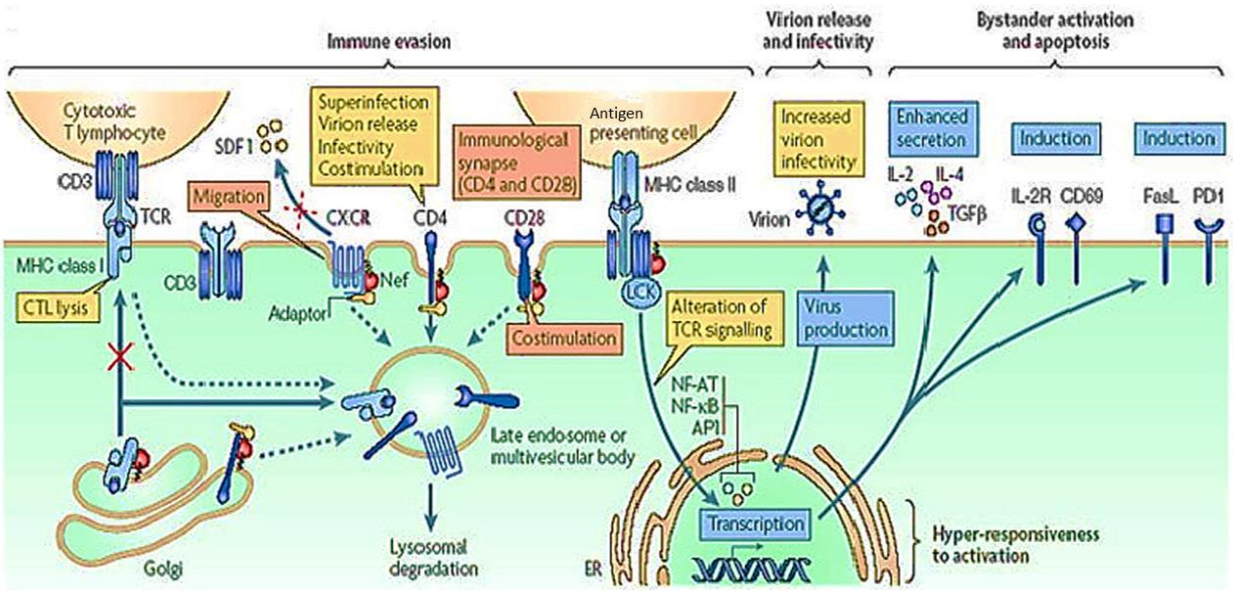


Figure I.6 Schematic representation of some Nef functions [Kirchhoff, Nat Rev Microbiol, 2009].

2.3.1 Nef protein alters the cytokine network

Unlike acute Hepatitis B and C virus infection, during acute HIV-1 infection the increase in plasma viremia was found to be associated with elevated plasma levels of multiple cytokines and chemokines; this phenomenon is known as "early cytokine storm" [Stacey et al., 2009]. Afterwards, during the HIV disease progression, the wearing down of the immune system is accompanied by chronic inflammation, T cell exhaustion and viral immune evasion. Nef regulation of cellular signalling and trafficking pathways in infected and uninfected immune cells strongly suggests that it could influence *per se* the cytokine/chemokine network possibly contributing to chronic inflammation. The first experimental evidences in this direction came from Mario Stevenson laboratory, where it was observed that Nef, when is expressed in macrophages during *in vitro* HIV-1 infection or using adenoviral expression vector, induces the release of a set of paracrine factors including a huge amount of CCL2/MIP-1α and CCL4/MIP-1β, which were able to recruit T cells and make them susceptible to HIV replication [Swingler et al., 1999]. Subsequently, the same group observed that Nef protein also induces the release of two other factors, the intracellular adhesion molecule ICAM-1 (sICAM) and the co-activation molecule CD23 (sCD23), which promote the expression of some receptors on B cells and cooperate with other factors released by macrophages to recall T cells and make them permissive to viral infection (see Fig. I.7). The effects of Nef in macrophages required NFκB pathway activation and mimicked those of CD40 Ligand (CD40L) in activating the CD40 signalling cascades suggesting that Nef may intersect the signal transduction pathway regulated by the CD40 receptor, one of the TNF receptor family members [Swingler et al., 2003].

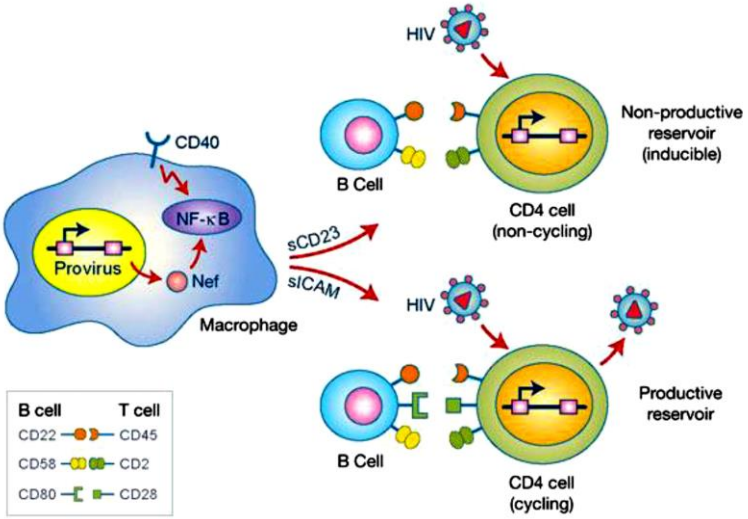


Figure I.7 Nef intersects the CD40 signalling pathway making T cells permissive to infection. The activation of CD40 or the expression of Nef induces the release of sCD23 and sICAM, which promote the expression of co-stimulatory receptors on B-lymphocytes. These, in turn, interact with the corresponding ligands on T lymphocytes making them permissive to infection. The induction of CD22 and CD58 is mediated by sCD23, does not determine the proliferation of T cells and it is sufficient for virus entry and the *de novo* expression of viral proteins, but not for

the release of virions. The induction of CD80 is mediated by sICAM and allows a productive infection of T cells [Swingler et al., Nature, 2003].

Further evidences came from studies conducted in our laboratory that demonstrated how the recombinant myristoylated Nef added in *in vitro* culture of primary human macrophages (MDMs) induces the rapid (15-30 minutes) activation of IKK/ NFκB, MAPKs (i.e. ERK1/2, JNK and p38) and IRF-3, the main transcriptional regulator of the IFNβ gene expression. The prompt transcriptional reprogramming leads in 2 hours to the synthesis and the release of pro-inflammatory cytokines and chemokines including CCL2/MIP- 1α and CCL4/MIP-1β, but also IL-6, TNF-α, IL-1β and IFNβ that, in turn, activate in autocrine/paracrine manner the signal transducers and activators of transcription (STATs), including STAT1, -2, -3 [Federico et al., 2001; Percario et al., 2003; Mangino et al., 2007].

Interestingly, the transient STAT1, -2, -3 tyrosine phosphorylation was also observed early after *in vitro* infection of MDMs with *nef*-expressing Δ*env*, but not Δ*nef*/Δ*env*, HIV-1 pseudotypes [Olivetta et al., 2003], suggesting that the intracellular signalling induced in Nef-treated MDMs could also be activated by Nef after HIV-1 infection or viral reactivation from latency. Further analyses revealed that the conserved Nef domains required for the extracellular Nef effects are the myristoylation site and the conserved N-terminal acidic cluster $E^{66}EEE^{69}$ (numeration according to SF2 HIV-1 Nef allele) involved in MHC-I (HLA-A and -B) down-regulation and also in the interaction with PACS-1, one of the members of a family of molecules involved in endosomal trafficking that controls the TGN localization of the cellular protease Furin also known as PACE (Paired basic Amino acid Cleaving Enzyme) [Dikeakos et al., 2012].

In our laboratory have been also performed experiments regarding the possible role of intracellular protein adaptors belonging to the TRAF family (TNF receptor-associated factors) in Nef-mediated activation of NF-κB and MAP kinases. The involvement of TRAFs has been hypothesized given their role in the signal transduction pathway mediated by the CD40/CD40L interaction, which Nef appears to intersect [Swingler et al., 2003]. Modelling analyses allowed the identification of a consensus binding sequence for TRAF2, also compatible with the TRAF6 binding, localized at the N-terminal end of the protein and including the conserved acidic cluster consisting of four glutamates ($EEEE^{69}$ in the SF2 allele). Furthermore, silencing experiments conducted using TRAF2 and TRAF6 specific siRNAs in the human monocyte THP-1 cell line demonstrated the involvement of both these molecular adaptors in the activation of STAT1 and STAT2 by cell treatment with Nef, while pull down experiments highlighted the formation of a Nef-TRAF2 complex [Mangino et al., 2011]. Later on, another research group reported the interaction of the Nef protein with TRAF2, TRAF6 and with TRAF5 [Khan et al., 2013].

It should be noted that other viral proteins, such as EBV LMP-1, HCV NS5A and HHV-8 K15, have evolved the ability to interfere and/or interact with TRAFs. Therefore, it has been proposed that Nef belongs to a family of "viral hijackers" capable of inducing an inflammatory response in MDMs by subduing the signalling pathways mediated by the TRAF intracellular adaptors, which are involved in the signalling pathways activated by the binding of different ligands with their specific receptors (Fig. I.8) [Mangino et al., 2011]. Interestingly, Nef treatment significantly alters the gene expression program of monocytes/macrophages by mimicking a typical inflammatory response.

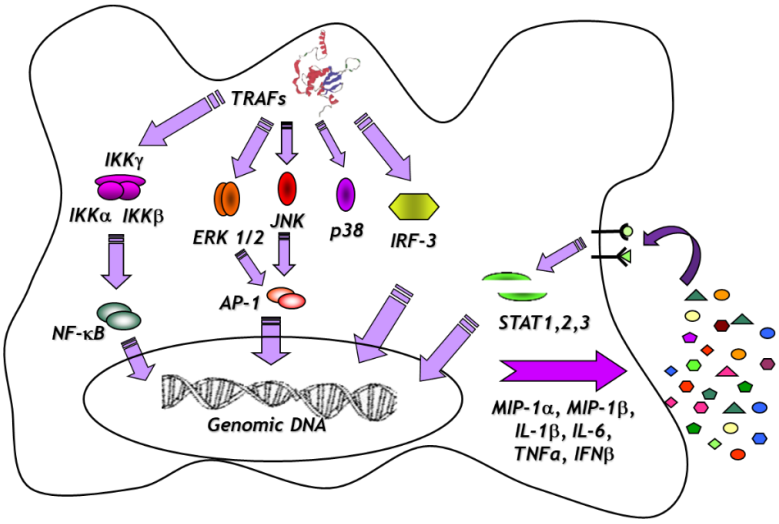


Figure I.8. A representative schematic model of the Nef-induced signalling events in treated human monocytes-derived macrophages (MDMs) [Mangino et al., Plos One, 2011].

Further studies have identified some of the mechanisms that allowed Nef to alter the cytokine network. Lee and colleagues showed that Nef together with the Eed protein forms a complex with paxillin, an integrin-adaptive protein, to recruit and activate the TACE enzyme (*i.e.* ADAM17), a metal-proteinase that converts pro-TNF-α into its active form. Indeed, TNF-α is initially synthesized as a 25 kDa pro-protein bound to membranes and, only later, is converted into the soluble form (17 kDa) by the TACE enzyme. It should be noted that Furin, the previously mentioned protease recruited by Nef through PACS-1 association that require the four acidic conserved element (EEEE[69]) of the viral protein, is involved in the maturation of TACE from its inactive precursor membrane form. Both the transmembrane and the soluble forms of TNF-α have signalling properties interacting with their receptors that can induce either protective or pathological effects in various diseases. Interestingly, the high plasma levels of TNF-α observed during all stages of HIV-1 infection has been associated with an increase in viral replication. Therefore, TNF-α inhibitors have been proposed to modulate the chronic inflammation observed in HIV infection [Kumar et al., 2013].

2.4 Nef transfer from infected to uninfected cells

Both Nef and anti-Nef antibodies have been detected in the serum of HIV-infected individuals [Fujii et al., 1996; Ameisen et al., 1989] supporting the *in vivo* detection of extracellular Nef by uninfected cells. Indeed, HIV-1 Nef was found in considerable numbers of peripheral blood mononuclear cells (PBMCs) from viremic HIV-infected patients not on antiretroviral therapy (ART) and also in patients receiving virologically suppressive ART, though to a smaller degree. Interestingly, these Nef-positive PBMCs constitute predominantly uninfected bystander cells [Wang et al., 2015]. Studies conducted in the last years have identified several possible mechanisms responsible for Nef transfer from infected cells to neighbouring not-infected cells (bystander) (see Fig. I.9).

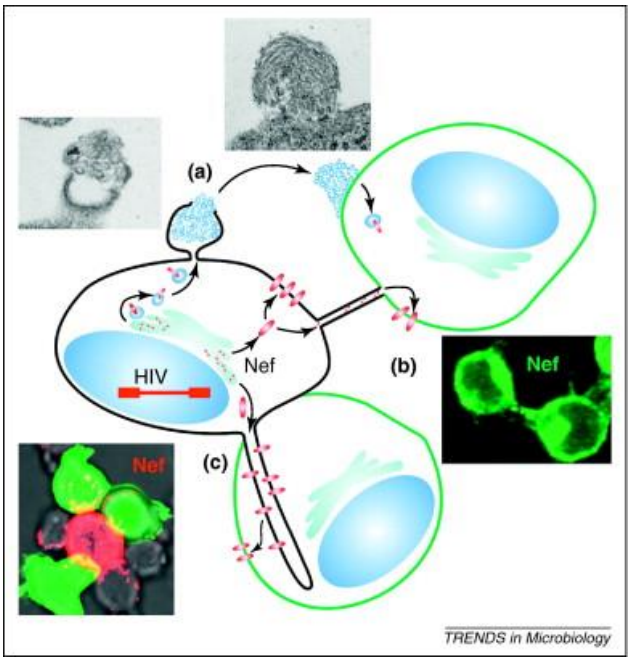


Figure I.9 Transfer of Nef protein from infected to bystander cells. (a) Clusters of microvesicles containing Nef secreted by infected T cells that rapidly attack bystander cells. (b) Cellular protrusions (nanotubes) that connect infected and uninfected cells allowing the transfer of Nef protein. (c) Transfer of the protein by trogocytosis [Baur, Trends in Microbiology, 2011].

Sowinski and colleagues (2008) documented the cell-cell transfer of Nef by means of cellular protrusions (nanotubes). In 2009, Xu and co-workers reported that HIV-1-infected macrophages, in response to Nef expression, are able to form conduits that connect them to follicular B cells. Through these conduits, macrophages transfer membrane-bound Nef and Nef-containing endosomes by an actin-dependent mechanism, mediated by Vav (GEF factor of the G proteins belonging to the Rho family) and dependent by GTPase [Xu et al., 2009]. The formation of nanotubes is mediated by the EXOC (Exocyst complex) proteins. In this regard, Mukerji and colleagues observed that in Jurkat cells Nef protein is associated with five of the eight components

of the exocyst complex (EXOC1, EXOC2, EXOC3, EXOC4 and EXOC6) [Mukerji et al., 2012] and the silencing of EXOC2 abolishes Nef-mediated enhancement of nanotube formation. This association was disrupted by mutations that abrogate the ability of Nef to associate with and activate Pak2, a kinase that regulates T-cell signalling and actin cytoskeleton dynamics. The cell-to-cell transfer of Nef might also explain some effects observed in bystander cells *in vivo*, such as the inhibition of antibody class switching (IgG2 and IgA) in systemic and intestinal lymphoid follicles [Xu et al., 2009]. Based on these results, it was proposed that HIV-1 could exploit the intercellular pathways as a "Trojan horse" to deliver Nef to B cells, thus evading specific humoral immunity, both systemically and at mucosal sites of entry.

Furthermore, it was reported that Nef transfer between cells could be mediated also by trogocytosis, a process that involves the transfer of plasma membrane fragments mainly between cells of the immune system, and extracellular vesicles (exosomes and microvesicles) release. While microvesicles bud directly from the plasma membrane, exosomes are released in the extracellular milieu upon fusion of multivesicular bodies/endosomes (MVBs) with the plasma membrane. The first report relating Nef to vesicle release dates back to 1990 [Guy et al., 1990]. Many years later, the relationship between vesicle release and HIV Nef, as well as their involvement in the pathogenesis of AIDS, returned to be the object of interest by several groups. Although Nef has been consistently reported to increase EV release and to be itself secreted in them, it remains unclear which type of EV is concerned by these findings. Campbell et al. (2008) described that Nef-transfected HEK293 cells could secrete vesicles containing Nef-GFP into the extracellular medium. Moreover, EVs containing Nef can also fuse with HIV-1 virions and deliver Nef protein to viral particles [Campbell et al., 2008]. Muratori et al. (2009) observed that Nef accelerated endocytosis and exocytosis in Jurkat cells and stimulated the release of “microvesicle clusters” in a budding-like process seemingly different from the classical exosome release mechanism. These clusters were budding structures > 500 nm containing several small vesicles, which remained together after release and could be found attached to the membrane of bystander cells. This phenomenon was also observed in 36-37% of primary $CD4^+$ T cells of individuals infected with HIV. Interestingly, Raymond et al. (2011) observed that almost the totality of secreted Nef in the plasma is associated to $CD45^+$ EVs (CD45 is reported to be associated with both exosomes and microvesicles) and their amount in plasma did not seem to correlate with viral load or CD4 cell counts [Raymond et al., 2011]. Indeed, EVs-associated Nef were detected at relatively high concentrations despite the use of antiretroviral therapy and even when plasma levels of HIV-1 RNA are undetectable [Ferdin et al., 2018].

Lenassi et al. (2010) claimed that Nef was found in multivesicular bodies (MVBs) and secreted in exosomes. In their work, it was possible to observe a clear co-localization of Nef and CD63, an exosomal marker, in HeLa.CIITA cells. However, in lymphoblastic cell lines, such as Jurkat and Sup-T1, confocal microscopy images provided poor evidence of the co-localization of Nef with MVBs [Lenassi et al., 2010]. Likewise, a recent study by Luo et al. (2015) did not find any evidence of exosome-mediated Nef transfer in Jurkat. Instead, Nef-containing vesicles from peripheral blood lymphocytes (PBLs) represent a mixture of secreted vesicles of plasma membrane and late endosomal origin [Luo et al., 2015]. Overall, several groups refer to the EVs involved in Nef release as "exosomes", but there are also evidences for an association of Nef with vesicle release processes occurring at the plasma membrane according to the cell type. Therefore, Nef increases the production of vesicles from several distinct cellular compartments.

To date, the N-terminal 70 amino acids results to be sufficient for the secretion of Nef containing vesicles. In particular, the critical amino acid residues in the N-terminal region are: (a) the basic cluster of four arginine residues, (b) the acidic cluster and a domain spanning amino acid residues 66-70 (VGFPV), which has been named the Secretion Modification Region (SMR) [Ali et al., 2010]. Shelton et al. (2012) found that Nef binding to mortalin (HSPA9) is involved in its incorporation into EVs since small peptides derived from SMR can inhibit Nef release in EVs [Shelton et al., 2012]. Mortalin is a member of the heat shock 70 kDa protein family that associates with lipid rafts in the plasma membrane and regulates the intracellular trafficking of cell surface receptors, such as fibroblast growth factor 1 (FGF-1) [Mizukoshi et al., 1999]. According to the Vesiclepedia database, mortalin is found in both microvesicles and exosomes, therefore it cannot clearly indicate the origin of the Nef-containing vesicles.

Considering the ability of Nef to downregulate cell membrane proteins and induce secretion of Nef-containing vesicles, it appears that Nef-mediated activation of membrane trafficking is bidirectional: the viral protein influences, in fact, both the endocytosis and the exocytosis. According to Andreas Baur (2011), Nef-induced secretion is most probably the relevant function in the pathogenesis of this elusive viral effector. In conclusion, Nef exploits the transport machinery of the host cell to widen its diffusion, thus acquiring the possibility of exerting effects even in uninfected bystander cells.

2.5 Extracellular vesicles (EVs): definition

During the course of evolution, both prokaryotes and eukaryotes have developed cell-to-cell communication strategies. These strategies play a vital role in multicellular organisms by allowing them to function as a system. Classically, intercellular communication can be mediated through

direct cell-cell contact (juxtacrine signalling) and/or by secreting a diverse array of soluble factors such as hormones, growth factors, cytokines and chemokines (secretome-induced signalling). These soluble molecules can act both on the cell itself (autocrine signalling) and on neighbouring (paracrine signalling) and distant cells (endocrine signalling). In the last two decades, intercellular transfer of extracellular vesicles (EVs) has emerged as a third mechanism of intercellular communication. EVs are heterogeneous membrane-enclosed structures released in the extracellular milieu in an evolutionally conserved manner by cells ranging from organisms such as prokaryotes to higher eukaryotes and plants. The first observation of EVs in the extracellular milieu date back to the late 1940s when Chargaff and West observed EVs as pro-coagulant platelet-derived particles in normal plasma [Chargaff & West, 1946].

Since then, EVs have been isolated from most cell types and biological fluids, including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile and semen [reviewed in Yáñez-Mó et al., 2015]. The accumulating data have indicated that these vesicles have different origins, size and composition and, based on their biogenesis, they have been divided in three main subgroups: microvesicles, exosomes and apoptotic bodies. Although the nomenclature is still a matter of debate because to date there is not a standard method of isolation and analysis of the EVs [Colombo et al., 2014; van Neil et al., 2018], the term microvesicle is generally referred to vesicles (150-1000 nm) that buds directly from the plasma membrane. On the other hand, the term exosome refers to smaller vesicles (30-150 nm) that are released to the extracellular environment upon fusion of multivesicular bodies/endosomes (MVBs) with the plasma membrane. Finally, apoptotic bodies are released when plasma membrane blebbing occurs during apoptosis (Fig.I.10).

Feature*	Exosomes	Microvesicles	Apoptotic vesicles
Size	50–100 nm	100–1,000 nm	50–500 nm
Density in sucrose	1.13–1.19 g/ml	ND	1.16–1.28 g/ml
Appearance by electron microscopy‡	Cup shape	Irregular shape and electron-dense	Heterogeneous
Sedimentation	100,000 g	10,000 g	1,200g , 10,000 g or 100,000 g
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidylserine	Expose phosphatidylserine	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, selectins and CD40 ligand	Histones
Intracellular origin	Internal compartments (endosomes)	Plasma membrane	ND

Figure I.10 Physicochemical characteristics of different types of secreted vesicles. *All vesicle preparations are heterogeneous, with different protocols allowing the enrichment of one type over another, and they can be classified

according to the presence of several (but not necessarily all) of the listed features. ‡Appearance by electron microscopy is only an indication of vesicle type and should not be used to define vesicles, as their microscopic appearance can be influenced by the fixation and phase contrast techniques used. CR1, complement component receptor 1; ND, not determined; TNFRI, tumour necrosis factor receptor I; Tsg101, tumour susceptibility gene 101 [modified by Théry et al., Nat Rev Immunol, 2009].

Exosomes are the most well studied class of EVs. The term "exosomes" for the EVs of endosomal origin was first proposed in 1987, when Johnstone and colleagues observed by ultrastructural studies the presence of vesicles released by multi-vesicular bodies (MVBs) fusing with the cell membrane during the differentiation of reticulocytes in red blood cells [Johnstone et al., 1987]. Initially, it was believed that these vesicles were simply removing unnecessary proteins and other molecules from the releasing cells, therefore they were considered the "garbage bins" of the cells. It was not until the mid-1990s that exosomes were shown to have an immunological function as antigen-presenting and as vesicles able to induce T cell responses [Raposo et al., 1996]. Since then, numerous studies have recognized exosomes as potent vehicles of intercellular communication, due to their ability to transfer proteins, lipids and nucleic acids, thereby affecting a variety of physiological and pathological processes in recipient and/or parental cells, such as the modulation of the immune response.

2.6 EVs: from the origin to the target cells

Cell vesiculation can be induced by multiple stimuli, including cell differentiation, activation, senescence, hypoxia, transformation and viral infections. Among the different types of EVs, exosomes are the best characterized. They are generated intracellularly as intraluminal vesicles (ILVs) by inward invagination of endosomes membranes giving rise to multivesicular bodies (MVBs) (see Fig. I.11). These endosomal compartments may fuse with lysosomes, for ILV degradation, or with the plasma membrane releasing these ILVs in the extracellular milieu as exosomes (reviewed by Colombo et al., 2014; van Niel et al., 2018]. The processes leading to generation of ILVs in MVBs and their fusion with the plasma membrane are not completely known. In this regard, two independent pathways have been proposed. The first one is accomplished by components of the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which comprises four multiprotein complexes: ESCRT-0, -I, -II, -III and accessory proteins (i.e. Alix and VPS4). In detail, ESCRT-0, -I and –II recognize and cluster monoubiquitinated transmembrane proteins at the endosomal membrane, whereas ESCRT-I and-III induce, together with additional factors, the invagination of late endosomal membrane. Afterwards, ESCRT-III binds ESCRT-II leading to the deubiquitination of cargo proteins, the vesicle abscission and, ultimately, the generation of ILVs.

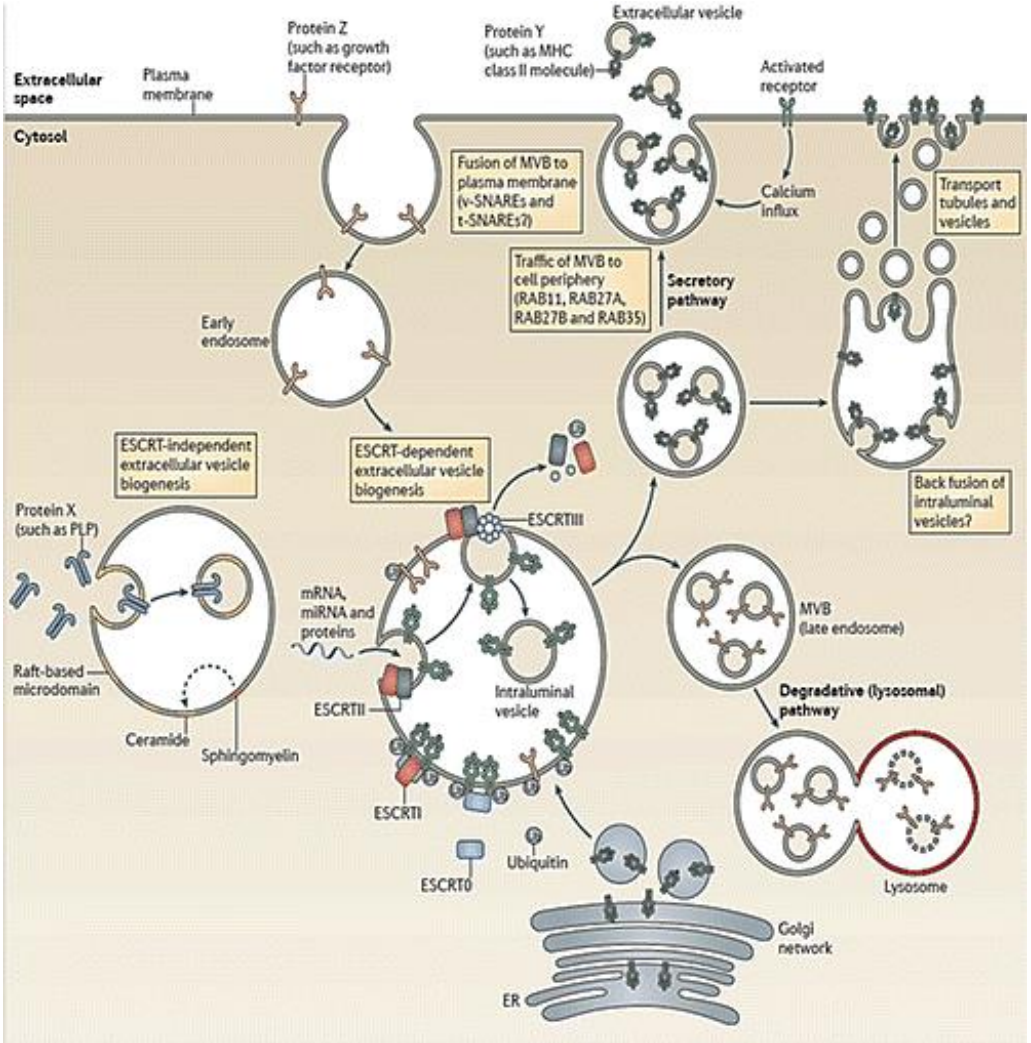


Figure I.11. Exosomal biogenesis in the endosomal network. Exosomes are generated by the formation of intraluminal vesicles (ILVs) in MVBs through an ESCRT-dependent or ESCRT-independent process. These structures can enter both the degrading and secretory pathways. The MVBs destined for the generation of exosomes follow a secretory pathway and, after translocating in the peripheral zone of the membrane, they fuse with the plasma membrane and release their content into the extracellular space. Once secreted, the ILVs are called exosomes. The MVBs that enter the degradative path fuse with lysosomes and their content is degraded [Dreyer & Baur, Methods Mol Biol, 2016].

Recently published evidences have described the existence of ESCRT independent pathways based on lipid composition of the endosomal membranes. Indeed, cells simultaneously depleted of the four ESCRT components continued to form MVBs [Stuffers et al., 2009]. The ESCRT-independent biogenesis of exosomes may involve tetraspanin proteins and lipid molecules, such as ceramide. For instance, oligodendrocytes direct exosome formation via the ceramide pathway, while other cell types rely on oligomerization of tetraspanin complexes [van Niel et al., 2018]. Interestingly, decreased levels of exosomes release were found in oligodendroglial cell line upon inhibition of neutral sphingomyelinase 2 (nSMase2), an enzyme that converts sphingolipids to ceramide, whose accumulation in areas of the endosome membrane containing high concentrations of sphingolipids induces microdomain coalescence thereby triggering ILV budding [Trajkovic et

al. 2008]. Instead, tetraspanins, such as CD83 and CD81, are a superfamily of proteins characterized by four transmembrane domains and they are enriched in MVBs. These proteins form clusters with themselves and other transmembrane and cytosolic proteins that induce inward budding of the microdomains in which they are enriched [Zimmerman et al., 2016].

Once ILVs are released into MVBs, they are either forwarded to degradation through the lysosomal pathway, or transferred to the cell periphery for the secretory pathway. The mechanisms by which secretory MVBs are mobilized to the cell periphery, dock and fuse to the plasma membrane are incompletely understood. Currently, it is known that the trafficking of MVBs to the plasma membrane is mediated by the cytoskeleton, fusion machinery, such as the SNARE proteins and Rab GTPase. While Rab7 mediates the ILV degradation through the fusion of MVBs with lysosomes, several other Rab proteins (i.e. Rab27A, Rab27B, Rab11 and Rab35) are responsible for the intracellular trafficking and secretion together with tetraspanins [reviewed in Colombo et al., 2014]. Endosome-like domains rich in exosomal proteins, lipids and carbohydrates have been found within the plasma membrane of certain cell types [Fang et al., 2007]. These domains are supposed to be involved in either trafficking of cargo from plasma membrane back to MVBs, or in vesicle formation and budding from the plasma membrane [Hurley et al., 2010].

The biogenesis of microvesicles differs considerably from that of exosomes; however, much less is known about the cellular processes leading to their generation. The formation of microvesicles occurs at the plasma membrane. Prior to their shedding, cytoplasmic protrusions are generated by the cell, which undergoes fission events, and finally microvesicles pinch off the cellular membrane [Dreyer & Baur, 2016] (see Fig.I.12). The mechanisms underlying these shedding events are not well elucidated yet; however, microdomain-induced budding processes seem to be involved in their secretion. Despite the fact that microvesicles can be generated by resting cells, stimulation events leading, e.g. to increased intracellular calcium levels, result in cellular membrane remodelling and an enhanced microvesicle secretion [Cocucci et al., 2009].

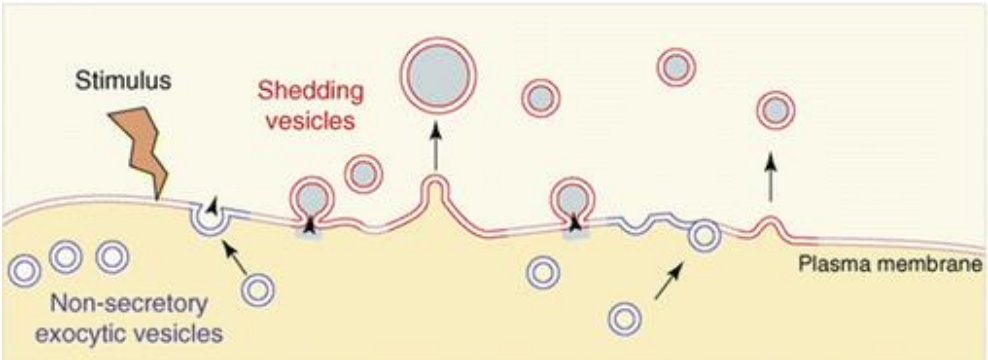


Figure I.12. Biogenesis of microvesicles. They are generated in a constitutive manner or following a stimulation. Non-secretory exocytic (blue) vesicles would release their content on the site of generation of microvesicles

contributing to their biogenesis. Remodelling events of the membrane would be found in specific sites where it occurs biogenesis of microvesicles (in red) [Dreyer & Baur, Methods Mol Biol, 2016].

Experimental evidences indicate that, once released, exosomes can transfer their content into the cytoplasm of target cells. Since exosomes have been isolated from many biological fluids, it is likely that these vesicles can reach very distant recipient cells protecting their cargo from enzymatic degradation during the transit into the extracellular environment. The topology of EVs is similar to that of cells, with extracellular receptors and ligands localized on the outside and cytoplasmic proteins and RNAs on the inside. Therefore, to functionally communicate with cells different types of interactions may be involved for EVs. Concerning the mechanisms underlying exosome internalization in target cells, four process have been proposed: (1) a direct interaction of exosome lipids and/or trans-membrane proteins with receptors on plasma membrane of the target cell, thereby inducing intracellular signalling cascades; (2) fusion events of exosome membrane with plasma membrane, delivering luminal cargo directly into the cytosol; (3) phagocytosis and macropinocytosis of exosomes, with subsequent fusion with other endosomal structures; (4) alternative endocytic internalization processes, including both clathrin-dependent and -independent pathways, the latter involving either caveolin or lipid rafts upon binding with specific receptors (see Fig.I.13) [Arenaccio & Federico, 2017].

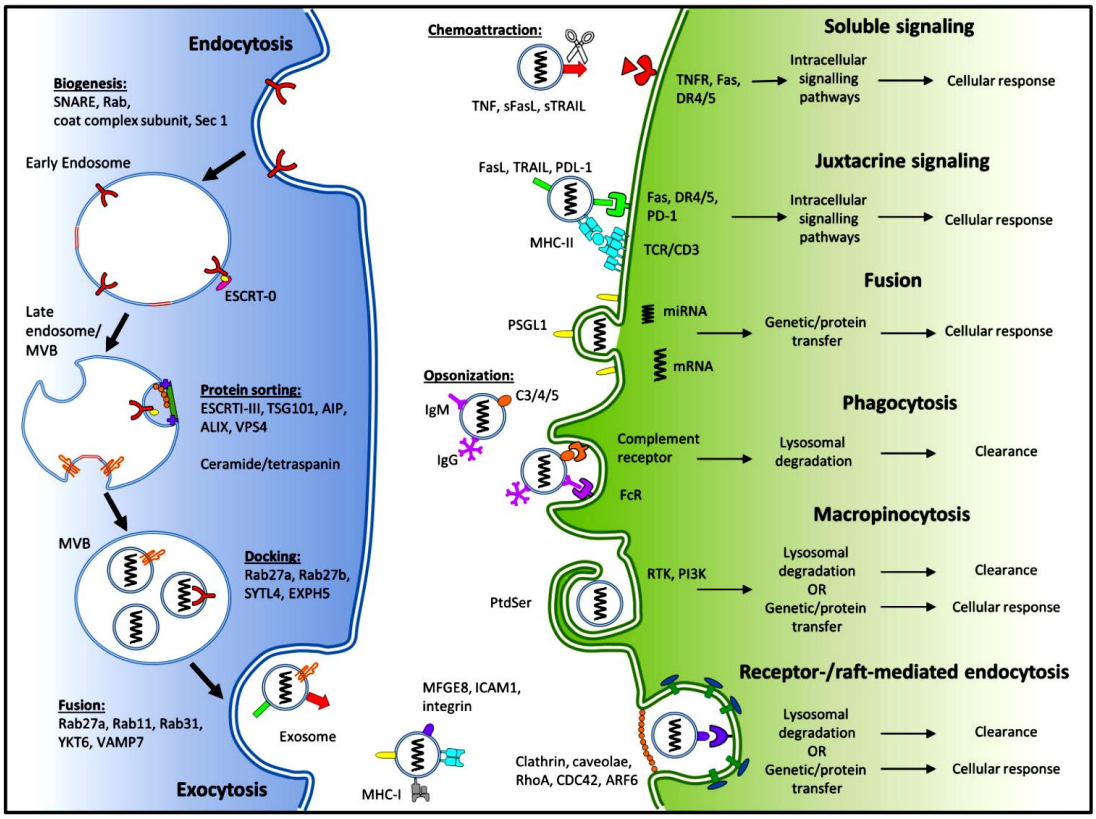


Figure I.13. Schematic representation of exosome biogenesis, internalization and cellular response. The adhesion of exosomes to the recipient cell utilizes the interaction of various exosomal surface proteins and cellular

receptors. Once bound, the exosome may (i) elicit transduction of the signal via intracellular signalling pathways and be released (juxtacrine signalling); (ii) fuse with the cellular membrane transferring protein and genetic contents, into the cytoplasm of the recipient cell (fusion); or (iii) be endocytosed via phagocytosis, macropinocytosis or receptor-mediated endocytosis [McKelvey et al., J Circ Biomark, 2015].

The specific mechanism responsible for the EVs uptake can change according to the recipient cells. In some cases, binding of EVs to recipient cells might be sufficient to induce changes in the physiological state of the recipient cells, for instance during the presentation of MHC-peptide complexes on the surface of antigen-presenting cell-derived EVs to antigen-specific T cells. In other cases, the content of EVs must be transferred inside the recipient cell. Once endocytosed or phagocytosed, EVs can be degraded and their components used by the cells for their own physiology. However, for the content of EVs to gain access to the cytosol of the recipient cell, which is necessary, for instance, for gene silencing or expression induced in the recipient cell by nucleic acids contained inside the EVs, a fusion step with either the plasma membrane or the limiting membrane of endocytic compartments must take place [Colombo et al., 2014]. In tumor cells, fusion was enhanced under an acidic pH [Parolini et al. 2009], which is representative of what occurs in the tumor mass or possibly inside recipient cells in late endosomes or phagosomes.

2.7 Molecular composition of EVs

In recent years, many research groups have focused their efforts on the identification of the content of EVs, in particular exosomes. These works led to the development of two constantly updated databases, i.e., Vesiclepedia (http://microvesicles.org), a compendium where the characteristics of all EVs are summarized, and ExoCarta (http://www.exocarta.org), a manually updated list of proteins, RNAs and lipids identified in exosomes. During their biogenesis and prior to their secretion, various molecules are uploaded into the lumen of EVs (see Fig. I.14).

These molecules can be divided in two main groups. The first one includes molecules relevant for the individual EV biogenesis pathways and for EV secretion. These factors are found in EVs across various cell types and include those involved in MVB formation (e.g., Alix and TSG101), membrane transport and fusion (e.g., annexins, flotillins, GTPase), adhesion (e.g., integrins), tetraspanins (e.g., CD81, CD9, CD63 and CD82) and antigen presentation (MHC class I and II molecules). In addition, heat shock proteins such as HSP70 and HSP90 have been found. The second group involves molecules that are specifically uploaded into vesicles by certain cell types thereby assigning EVs a characteristic cell-type fingerprint. These factors involve cytokines, cell surface receptor, signalling molecules, enzymes and viral proteins. Some proteins are

preferentially uploaded in exosomes, but it still unclear how proteins are targeted specifically to exosomes.

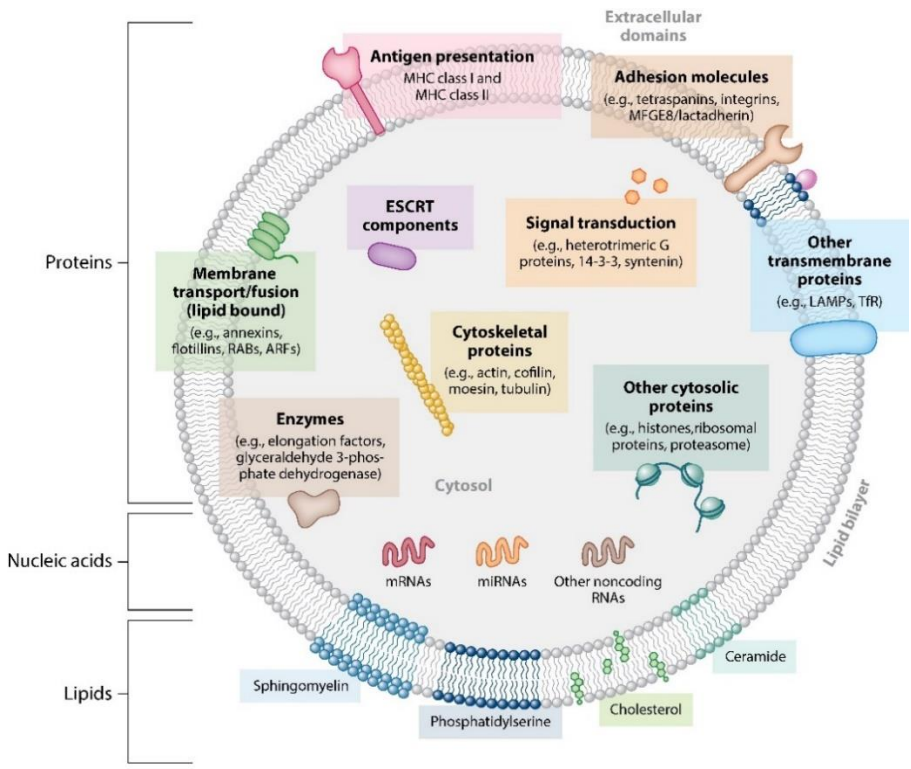


Figure I.14 Overall composition of extracellular vesicles (EVs). Schematic representation of the composition (families of proteins, lipids, and nucleic acids) and membrane orientation of EVs. Examples of tetraspanins commonly found in EVs include CD63, CD81, and CD9. Note that each listed component may in fact be present in some subtypes of EVs and not in others. For instance, histones and proteasome and ribosome components are probably secreted in large plasma membrane–derived EVs and/or apoptotic vesicles rather than exosomes. Abbreviations: ARF, ADP ribosylation factor; ESCRT, endosomal sorting complex required for transport; LAMP, lysosome-associated membrane protein; MHC, major histocompatibility complex; MFGE8, milk fat globule-epidermal growth factor-factor VIII; RAB, Ras-related proteins in brain; TfR, transferrin receptor [Colombo et al., Annu Rev Cell Dev Biol, 2014].

The lipid composition of EVs have been studied and results to be influenced by the nature of the membrane from which the vesicles are generated. In general, exosomes are enriched in lipids such as sphingomyelin, phosphatidylserine, gangliosides and cholesterol, as compared to plasma membrane and other intracellular membranes. Interestingly, exosomes contain mRNAs, microRNAs, whereas limited amounts of DNA or ribosomal RNA were found [Schorey et al., 2015]. When transferred to target cells, mRNAs are translated into proteins, whereas microRNAs can silence target genes. Besides mRNAs and microRNAs, other RNA species have been found within exosomes, such as viral RNAs, fragments of tRNAs, small nuclear RNAs, small nucleolar RNAs, piwi interacting RNAs and long-noncoding RNAs [reviewed in Yáñez-Mó et al., 2015]. However, the mechanisms that regulate the specific loading of RNA species into exosomes are only partly known. Recently, it has been identified a short nucleotide motif regulating the sorting of RNAs into exosomes through binding with the heterogeneous nuclear ribonucleoprotein

(hnRNP)-A2B1, i.e., a ubiquitously expressed RNA-binding protein [Villarroya-Beltri et al., 2013].

2.8 Extracellular vesicles in health and disease

During the past decade, the interest in the role of EVs, particularly exosomes, in both physiological and pathological conditions significantly increased. They are gained recognition as multi-molecular messengers acting in both autocrine and paracrine manners modifying the activity and/or phenotype of recipient cells [Théry et al., 2009]. Recent studies have shown a wide range of pleiotropic functions of these vesicles in several biological processes. In physiological conditions, EVs are involved in antigen presentation, neuronal communication and protection, blood coagulation, wound healing, sperm maturation and regulation of the immune response against the fetus during pregnancy. On the other hand, numerous studies reported the important role played by EVs in the pathogenic processes including cancer, autoimmune disease, inflammation, infections, metabolic and cardiovascular diseases. Exosomes secreted by immune cells can play a role as mediators of immune response. In this regard, it was found that exosomes from DCs carrying MHC-I and II molecules, as well as co-stimulatory molecules such as CD80 and CD86, can induce $CD8^+$ and $CD4^+$ T-lymphocyte activation [Bobrie et al., 2011].

In recent years, the relevance of exosomes in viral infections has been strongly highlighted. In the context of viral infections, EVs play important roles in intercellular communication by incorporating viral proteins and fragments of viral RNAs, therefore by signalling the presence of infectious agents and enabling antiviral responses to neighbour or long distance recipient cells through body fluids [reviewed by Théry et al., 2009]. Exosomes are also involved in a wide range of non-infective human diseases, such as obesity and metabolic syndromes, which induce increased secretion of vesicles [Stepanian, 2013] incorporating specific RNAs and proteins as observed in both rodents and humans [Ferrante et al., 2015]. Many studies demonstrated the role of exosomes in neuronal protection, regeneration and development, as well as synaptic plasticity. Moreover, exosomes have the capacity to cross the blood barrier brain (BBB) making them excellent candidates for therapeutic interventions aimed at regenerating damaged CNS districts.

2.8.1 The ambiguous role of EVs in HIV infection

In the last few years, the potential functions of exosomes during HIV-1 pathogenesis began to emerge. Some studies have pointed to a protective role of EVs against spreading of HIV-1 infection (see Fig. I.15). It has long been known that the non-cytotoxic suppression of HIV-1 replication in $CD4^+$ T cells is an antiviral mechanism mediated by $CD8^+$ T lymphocytes

[Blackbourn et al., 1996]. Subsequent studies aimed to elucidate the effector molecules mediating this antiretroviral activity excluded the involvement of cell-secreted cytokines, chemokines or inflammatory molecules and attributed this activity to soluble factors termed CD8-derived antiviral factor (CAF) [Levy, 2003]. Interestingly, CAF was later linked to EVs secreted by $CD8^+$ T lymphocytes. Specifically, EVs derived from CD8 T cells exerted the HIV-1 replication suppressive activity by inhibiting the transcription of the HIV LTR promoter through an unknown protein moiety localized on the surface of exosomes and also independent of EV internalization [Tumne et al., 2009]. Furthermore, EVs released by $CD4^+$ T cells mediate CD4-dependent inhibition of HIV-1 infection *in vitro*, suggesting a possible interaction of CD4 molecules at EVs surface with HIV envelope proteins that hinder the viral interaction with target cells, hence preventing viral infection [de Carvalho et al., 2014]. Additionally, EVs derived from T cells can contain APOBEC3G (A3G), a cytidine deaminases that have a role on restricting HIV-1 replication [Dias et al., 2018].

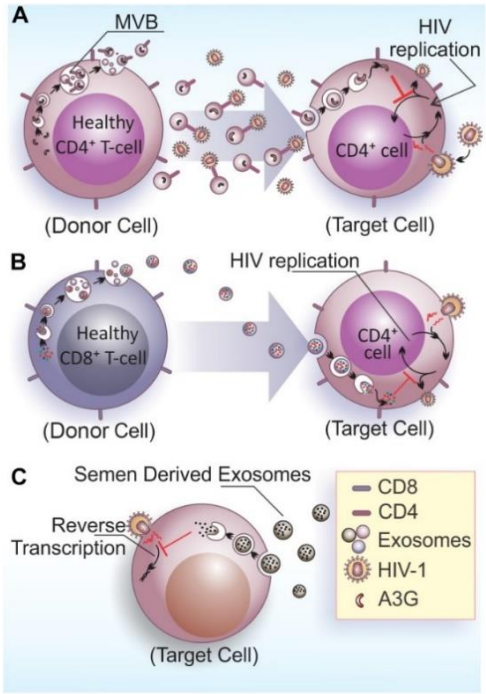


Figure I.15 Anti-HIV-1 effects of EVs. (A) Exosomes released by healthy CD4+ T cells contains CD4 molecules on their surface and hinder HIV-1 infection/dissemination, possibly acting as decoys in the extracellular space. Additionally, exosomes also contain the enzyme A3G that can be internalized by infected cells and inhibit HIV-1 replication in target cells. (B) Exosomes released from CD8+ T cells have non-cytotoxic antiretroviral activity that inhibits HIV-1 transcription in target cells (C) Semen derived exosomes are internalized by target cells (vaginal epithelial cells) and are able to block HIV-1 replication through the impairment of viral RNA reverse transcription [Dias et al., Front Microbiol, 2018].

It is noteworthy that EVs are present in a wide range of human body fluids, and recent reports showed that some of them have an important antiretroviral activity. In this regard, Madison and

colleagues (2014) reported that EVs isolated from semen of healthy men were able to inhibit viral replication of HIV through the impairment of the reverse transcriptase activity [Madison et al., 2014]. Moreover, semen EVs were also capable of blocking HIV-1 transmission from vaginal epithelial cells to monocytic and lymphocytic cell lineages, and to peripheral blood leukocytes [Madison et al., 2015]. Likewise, vaginal fluids also contain EVs that decrease HIV-1 transmission in the Jurkat cells blocking a post-entry step of virus infection [Smith & Daniel, 2016]. Studies performed with EVs isolated from breast milk also provided interesting results on HIV inhibition. In this regard, EVs isolated from breast milk of healthy donors were reported to present a modulatory activity on the immune system [Näslund et al., 2014]. Specifically, they were able to inhibit HIV-1 infection in monocyte-derived dendritic cells (MDDCs) likely due to the binding of EVs to the DC-SIGN receptor, which can compete with the virus and hinder MDDC-mediated viral transfer to $CD4^{+}$T cells. Unlike semen and breast milk, blood-derived EVs did not present antiviral activity [Madison et al., 2014; Näslund et al., 2014]. Therefore, EVs derived from body fluids that naturally display anti-HIV-1 activity are attractive for the development of new antiretroviral therapies.

Although EVs derived from uninfected cells may exert a protective role against HIV-1, the virus can take advantage of the endomembrane system not only by enhancing the viral biogenesis itself but also by inducing EVs biogenesis changes. These modifications may involve alterations in cargo composition, frequency of EV release and targeting, which can contribute to immune evasion and increased pathogenesis.

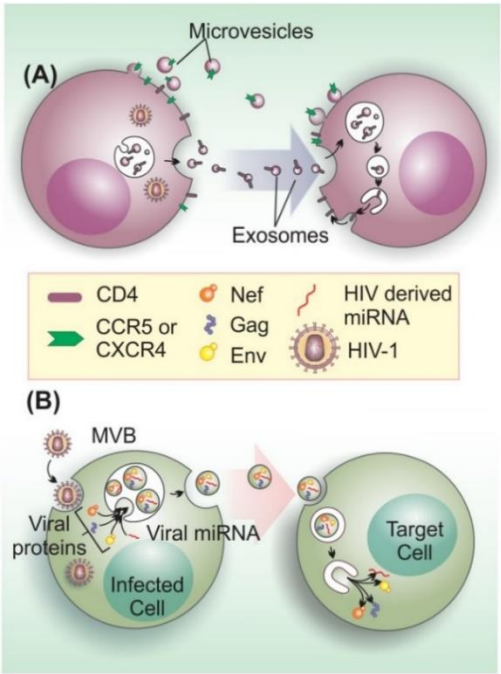


Figure I.16. Extracellular vesicles contribute to the enhancement of HIV-1 infection and pathogenesis. (A) EVs from HIV-1 infected cells transfer HIV-1 receptors (CXCR4, CCR5 and CD4) to null cells to spread infection. (B)

HIV-1 components, such as viral proteins (Gag, Env, and Nef) and microRNAs may be transferred to target cells to enhance infection [Dias et al., Front Microbiol, 2018].

In this context, an important role is played by EVs containing Nef, whose effects on recipient cells have been assessed by several groups. In this regard, exosomes containing HIV-1 Nef protein turned out to have multiple pathogenic effects such as the induction of T-cell apoptosis [Lenassi et al., 2010] and the down-modulation of cell surface molecules (i.e., MHC-I and CD4) for immune evasion [Gray et al., 2011]. Moreover, the expression of HIV-1 Nef induces the release of exosomes incorporating active ADAM17/TACE [Lee et al., 2013], a metalloprotease that promote the maturation of pro-TNFα into its active form. Resting $CD4^+$ T lymphocytes challenged with ADAM17/Nef EVs became competent for HIV-1 expression and replication as a consequence of cell activation induced by TNFα [Arenaccio et al., 2014a and b; Ostalecki et al., 2016]. A similar mechanism was found to be involved in the reactivation of cells latently infected with HIV-1 [Arenaccio et al., 2015]. These mechanisms are likely relevant *in vivo* since the presence of EVs carrying Nef, ADAM17 and several pro-inflammatory factors in the plasma seems to correlate with HIV-1 associated immune pathogenesis in both viremic and non-viremic chronic infection [Lee et al., 2016; Ostalecki et al., 2016]. HIV-1 tends to cause chronic neurologic disease in patients. A few studies have described the role of EVs in neuroimmune pathogenesis. HIV-1 infected microglia releases EVs containing Nef, which, in turn, can disrupt BBB integrity and permeability [Raymond et al., 2016]. In addition, EVs associated Nef induces an increase on Toll-like receptor-induced cytokines and chemokines levels (including IL-12, IL-8, IL-6, RANTES, and IL-17A in microglia) [Raymond et al., 2016]. Another study conducted by Khan et al. (2016) found that the levels of "exosome"-packaged Nef protein and mRNA were higher in the plasma of patients with HIV-1 associated neurocognitive disorders (HANDs). Moreover, these vesicles were capable to deliver Nef mRNA and induce the expression of the viral protein in a neuroblastoma cell line. This expression increased the production and secretion of Beta Amyloid protein possibly contributing to the cognitive impairment observed in HAND [Khan et al., 2016].

Apart from Nef, other viral components are also found in EVs. For instance, a large proportion of EVs released from infected cells contain gp120 HIV-1 envelope (Env) protein and resulted to significantly increase the viral infectivity in human lymphoid tissues [Arakelyan et al., 2017]. Even HIV-1 Gag is transported into EVs, but its effects in non-infected cells are currently unknown. In addition to viral proteins, exosomes from HIV infected cells contain viral RNAs, which stimulate TLR8 signalling to promote TNF-α release and may contribute to chronic immune activation [Bernard et al., 2014]. Furthermore, the HIV-1 trans-activation response (TAR) RNA, a pre-microRNA that produces mature microRNAs, was found in exosomes derived from virus-infected

cells and it was shown that these RNAs could inhibit apoptosis of recipient cells by down-regulating the expression of pro-apoptotic proteins [Narayanan et al., 2013] and modulate the gene expression of pro-inflammatory cytokines, such as IL-6 and TNF-β, in human macrophages [Sampey et al., 2016].

Besides the transport of bioactive HIV-1 derived molecules to bystander cells, EVs can have a role on aiding viral entry. In this regard, different studies showed how EVs can mediate the transfer of host cell surface proteins, such as CCR5 and CXCR4, to tissue that do not express endogenous HIV-1 co-receptors, favouring viral dissemination [Dias et al., 2018]. Moreover, extracellular vesicles can facilitate infection via association with viral progeny, thus camouflaging it from immune system. In conclusion, EVs from different cell sources play different roles in HIV pathogenesis. Whereas the EVs from infected cells may promote viral replication and the dissemination of infection, EVs from uninfected tissues or cells could protect the immune system against the virus. The kind of action depends on the cargo, the type of their cell of origin and the interaction with viral proteins.

2.9 EVs as potential biomarkers and drug delivery tools

There is a growing body of evidence in the literature indicating that EVs play important roles in the intercellular communication, stirring interest on the use of EVs as potential therapeutic tools. In this regard, exosomes are considered excellent biomarker candidates that hold great potential for the detection of many pathological conditions in view of their ability to alter their cargo according to different cell stimuli. Moreover, since exosomes have been shown to be present in many biologic fluids [reviewed in Yáñez-Mó et al., 2015], they result to be easily accessible. In cancer and other disorders, they can be useful to monitor disease progression as well as evaluate therapy responses. In fact, cargo of exosomes released from cancer cells can vary with the development of the disease. For instance, in melanoma patients the proteome of circulating exosomes can be correlated with different clinical tumor stages.

In the case of HIV-1 infection, blood-derived EVs carrying viral components could be considered a promising biomarker regarding the progression of infection and could be used to assess treatment efficacy. As previously mentioned, EVs purified from breast milk [Näslund et al., 2014] and semen [Madison et al., 2014] display intrinsic protective properties that appear to restrain vertical and horizontal viral transmission. However, further studies are necessary to characterize how EVs derived from the various biological fluids correlate with the different pathological states of HIV-1 progression. An interesting recent finding is that the amount of EVs present in the extracellular milieu may also influence HIV production and infectivity. It was found that culturing HIV

producer cells in EVs depleted media leads to increased HIV production and a more infectious viral progeny [Liao et al., 2017]. The authors speculate that cells may respond to EVs scarcity triggering metabolic pathways that could induce viral particle production. Since the ESCRT machinery is hijacked by HIV-1 and is also involved in EVs release, it could be an attractive target for the development of inhibitors. Furthermore, the induction of autophagy during infection could also provide a mean to inhibit the biogenesis of EVs and, therefore, the intercellular transfer of viral molecules mediated by these vesicles. In fact, rapamycin, a specific MTOR inhibitor and inducer of autophagy, inhibits HIV-1 replication [Heredia et al., 2003].

Another aspect that has gained considerable interest in the scientific community is the potential use of EVs, in particular exosomes, as drug delivery vehicles. In fact, exosomes offer distinct advantages as gene therapy delivery vectors as they possess cellular membranes with multiple adhesive proteins on their surface. Furthermore, their small size and flexibility enables them to cross major biological barriers such as blood-brain barrier. Their potential utility in drug delivery is also due to their intrinsic homing capacity. Unlike liposome formulations and lentiviral-based delivery systems, exosomes are naturally secreted by the cells and thus they possess a high biocompatibility, safety and stability in circulation that allow them to overcome many of the limitations of cell-based therapeutics. In this regard, Sun and colleagues (2010) shown that exosomes can deliver the anti-inflammatory agent curcumin which, in this form, was found more stable than free curcumin [Sun et al., 2010].

To date, some studies have used EV-based therapeutics to treat disease by engineering EVs with full-length proteins which were proven effective in inducing specific, unrestricted cytotoxic T cell (CTL) immunity when injected in mice, as in the case of exosomes engineered with antigens from human papillomavirus (HPV) [Di Bonito et al., 2015]. In light of this, exosomes containing antiviral proteins are regarded as possible relevant allies in HIV therapy, since they may be directed to viral reservoirs thus representing a very significant tool in combating the HIV infection.

II. AIMS OF THE WORK

The role of pDCs in HIV-1 infection and pathogenesis is complex and not well defined so far. To date, most of the reported studies have been focused on the analysis of pDCs response following HIV infection. As described in the introduction, the viral protein Nef is transferred through different mechanisms including the transfer via extracellular vesicles (EVs) also in uninfected cells [Campbell et al., 2008; Xu et al., 2009; Muratori et al., 2009; Lenassi et al., 2010; Pužar Dominkuš et al., 2017], thus exerting specific effects on both infected and uninfected cells. Indeed, both Nef and anti-Nef antibodies were detected in the serum of HIV-infected individuals [Fujii et al., 1996; Ameisen et al., 1989]. In light of what is emerging, the aim of this study was to characterize the effects induced by Nef protein on uninfected pDCs.

Since Nef is known to hijack cellular signalling pathways to promote viral replication and spreading and Nef expression in monocytes/macrophages has been correlated with remarkable modifications in the pattern of secreted factors [Percario et al., 2015], we firstly sought to define the possible alterations in intracellular signalling induced by Nef in plasmacytoid dendritic cells. In particular, we focused the attention on the activation of some signal transducers and activators of transcription (STAT) molecules, which are involved in the response of a wide number of cytokines, growth factors and hormones, and subsequently on the analysis of the soluble factors released comparing them with those observed in macrophages.

It is well known that pDCs response to pathogens is variable and it is influenced by specific signals according to which they can acquire two different phenotypes; pDCs can produce large amounts of IFN, hence acting as Interferon producing Cells (IPCs) or differentiate into antigen-presenting cells (APCs) [Aiello et al., 2018]. Hence, we also attempted to identify which kind of phenotype pDCs acquire in response to Nef stimulus analysing the expression of surface molecules, which usually accompany the activation or maturation of pDCs to APCs, and of markers of type I IFN response and production.

In light of the pivotal role of EVs as potent vehicles of intercellular communication in both physiological and pathological conditions including HIV infection [Dias et al., 2018], we sought to characterize and quantify the extracellular vesicles released by plasmacytoid dendritic cells in response to Nef stimulus. Despite the recent expansion of studies conducted on vesicles, nowadays there are few methods for their reliable quantification and characterization. To fulfil our purpose, we used an innovative methodology developed by Sargiacomo and colleagues based on cell treatment with a fluorescent fatty acid analogue, Bodipy FL C_{16}, which labels the cells ultimately

producing fluorescent vesicles that can be examined and quantified by conventional non-customized flow cytometry [Coscia et al., 2016].

Moreover, considered the particular attitude of pDCs to secrete IFNs and their continuous exposure to this cytokine during HIV infection because of its chronic production [Stacey et al., 2009; Malleret et al., 2008], we characterized and quantified the EVs released by plasmacytoid dendritic cells also in response to IFN stimulus.

Altogether, the results of this work contribute to shed light on the effects exerted by the viral protein Nef on uninfected pDCs by providing a more comprehensive picture for a thorough understanding of pDC roles in HIV infection. It may help to define pDC functions and develop therapeutic strategies.

III. MATERIALS AND METHODS

III.1 Cell isolation and culture

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coats obtained from healthy donors at Centro Trasfusionale-Cattedra di Ematologia, Università degli Studi "La Sapienza" Rome. No ethical approval from university La Sapienza or Roma Tre ethics committees nor formal or verbal informed consent from blood donors were necessary to use buffy coats as sources of cells. In particular, PBMCs were isolated with Lympholyte-H (Cedarlane Laboratories Ltd, Ontario, Canada) density gradient centrifugation and maintained in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 2 mM L-Glutamine (Gibco), 100 Units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS), previously inactivated at 56°C for 30 minutes.

Circulating pDCs were isolated from PBMCs by positive selection using an immunomagnetic-based kit (BDCA-4 cell isolation Kit, Miltenyi Biotec, Bologna, Italy) according to the manufacturer's recommendations. The purified pDCs were maintained in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 100 Units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), 25 mM Hepes and 10% heat-inactivated FBS.

Monocytes were isolated by positive selection from total PBMCs using CD14 paramagnetic microbeads (Miltenyi Biotec) and then cultivated for 7 days in RPMI 1640 supplemented with 2 mM L-Glutamine, 1% penicillin/streptomycin, 20% heat-inactivated FBS and 50 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech EC Ltd, London, UK) to promote macrophage differentiation. The eluates corresponding to PBMCs depleted of monocytes (PBLs), PBLs depleted of plasmacytoid dendritic cells (PBLs-pDCs) and PBMCs depleted of pDCs (PBMCs-pDCs) were recovered and the cells were resuspended in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 100 Units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich) and 10% heat-inactivated FBS.

Since primary pDCs are present in very low amount in blood (0.2-0.5% of PBMCs), the experiments were carried out also using GEN2.2, a pDC cell line derived from a leukaemia patient [Chaperot et al., 2006], deposited within the CNCM (Collection Nationale de Cultures de Microorganismes, Pasteur Institute, Paris) on September 24, 2002, under the number I-2938. This cell line was purchased through a signed Material Transfer Agreement (MTA). The proliferation of GEN2.2 is strictly dependent on the presence of a feeder layer made by the murine stromal cell line MS-5 (deposited within the DSMZ [German Collection of Microorganisms and Cell Cultures] under the No. ACC441). Thus, GEN2.2 were cultured in flasks precoated with a sub-confluent

irradiated MS-5 monolayer in RPMI 1640 containing 1% Glutamax (Gibco, cat. 35050-038), 100 Units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), 1 mM sodium pyruvate (Euroclone), 1% nonessential amino acids (Euroclone, cat. ECB3054D) (hereafter referred to as complete medium) and 10% heat-inactivated ultra-low endotoxin FBS (Microgem). Upon arrival, GEN2.2 were expanded, freezed and stored under liquid nitrogen to create a large working cell bank in order to standardize the experiments. GEN2.2 were kept in culture for no more than two months and, for the experiments, only the $CD45^+$ non-adherent fraction (corresponding to GEN2.2 in the supernatant) was used.

THP-1 cells (from ATCC), derived from a human monocytic leukaemia, were grown in RPMI 1640 supplemented with 2 mM L-Glutamine, 100 Units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich) and 10% heat-inactivated FBS. For the experiments, THP-1 were seeded at 100.000 cells/cm^2 and differentiated adding 35 nM of PMA (phorbol 12-myristate 13-acetate) (Sigma-Aldrich) in order to adhere and differentiate acquiring a macrophage-like phenotype, which mimics, in many respects, primary human macrophages [Lund et al., 2016]. In particular, after a PMA treatment of 32 hours, medium was replaced with fresh medium supplemented with 20% FBS. Then, after 1 day of resting, differentiated THP-1 were used for the experiments. All cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

III.2 Recombinant Nef protein preparations and reagents

Wild type recombinant myristoylated Nef protein and a mutant in the acidic cluster $E^{66}EEE^{69}$→AAAA present at N-terminal end (referred to as $myrNef_{SF2}$w.t and $myrNef_{SF2}$4EA respectively) were generated from HIV-1 SF2 allele in the laboratory of Dr. Matthias Geyer (Max-Plank-Institut fur molekulare Physiologie, Dortmund, Germany). These proteins were obtained by co-transformation of an E. coli bacterial strain with two plasmidic expression vectors containing a codon-optimized Nef and a N-myristoyl-transferase coding sequence respectively, both inducible by isopropyl β-D-thiogalactoside (IPTG). Subsequently, they were purified by affinity chromatography as C-terminal hexahistidine-tagged fusion proteins. The myristoylation of recombinant Nef proteins was verified by mass spectrometry at Dr. Geyer's laboratory. All Nef preparations were analysed for the presence of endotoxin as contaminant using the chromogenic Limulus amebocyte lysate assay (LAL-test) (Biowhittaker, Walkersville, MD), and if required, purified using the EndoTrap® red Endotoxin Removal Kit (Cambrex Bio Science, Walkersville Inc). To avoid possible signalling effects due to residual undetectable lipopolysaccharide (LPS) traces in Nef preparations, we performed some experiments in presence of 1 µg/mL of polymyxin

B (Sigma-Aldrich, Milan, Italy), a cationic antibiotic that binds to the lipid A portion of bacterial LPS. In our hands, this polymyxin B treatment blocked the signalling activity of up to 100 endotoxin units (EU)/mL LPS without inducing any differences in the signalling events analysed. For this reason, the experiments described here below were conducted in the absence of polymyxin B.

CpG class A (cat. #ODN2216) was purchased from Miltenyi Biotec (Bologna, Italy) and used as positive control for the innate activation of immune cells, such as human PBMCs and pDCs. Brefeldin A, also known as BFA, ascotoxin, cyanine or decumbin, was obtained from Sigma-Aldrich (Milan, Italy) and used as inhibitor of IFN secretion for confocal microscopic analysis. Lipopolysaccharide (LPS; cat. #L4391) was purchased from Sigma-Aldrich and used as positive control for pDC activation. For the IFN treatments of cells, human recombinant (r) IFN-β (Ares-Serono, Geneva, Switzerland), human rIFN–γ (cat. #300-02; EC Ltd PeproTech, London, UK) and human rIFN-λ1/λ2 (hereafter referred to as IFN-λ), generously gifted by Dr. Eliana Coccia (Department of Infectious Disease, Istituto Superiore di Sanità, Rome, Italy) were used.

III.3 Flow cytometry analysis of cells

The purity of the cells isolated from peripheral blood of healthy donors was assessed by flow cytometry (FC) analysis. For surface staining, cells (10^5) were washed once with 1x phosphate buffered saline (PBS), resuspended in 50 µL of PBS containing 2% FBS and incubated in the dark for 30 minutes at 4°C with the corresponding mixture of antibodies (see Table II.1). As control, we used isotype-matched antibodies labelled with the appropriate fluorochrome. After incubation, cells were washed twice with PBS, fixed with 2% paraformaldehyde (PFA) (Sigma-Aldrich) for 15 minutes on ice and finally left in 1% PFA until the observation with the cytofluorimeter CytoFLEX (Beckman Coulter, USA). Since CD123 and CD14 are respectively the specific cell surface markers for plasmacytoid dendritic cells and monocyte/macrophage cell populations, the purity of these cell types was assessed by means of anti-CD123 and anti-CD14 monoclonal antibodies labelling. The purity of the populations of PBMCs depleted of pDCs and PBMCs depleted of monocytes (PBLs) was measured by evaluating respectively the percentage of CD123 and CD14 positive cells. Cell populations whose purity was below 95% were discarded.

GEN2.2 cell line was assayed for the expression of different markers, in order to verify the purity of the cells recovered from the co-culture with the MS-5 monolayer. GEN2.2 staining was performed as reported above and the markers were analysed using: FITC-conjugated anti-HLADR, APC-conjugated anti-CD44, PE-conjugated anti-CD123, APC-conjugated anti-CD11c, FITC-

conjugated anti-CD29 and FITC-conjugated anti-CD45, FITC-conjugated anti-CD4, FITC-conjugated anti-CD86, FITC-conjugated anti-CD80 (all generously gifted by ImmunoTools GmbH). Furthermore, immunophenotype of GEN2.2 treated with myrNef$_{SF2}$w.t, myrNef$_{SF2}$4EA or LPS was assessed using: FITC-conjugated anti-CD86, APC-conjugated anti-CD40, PE-conjugated anti-CD38 and FITC-conjugated anti-CD80.

Cells	Antibodies	Producers
PBMCs	Lineage-FITC/BDCA2-PE/ HLADR-PerCP/CD123-APC CD3-FITC/CD19-PE/CD14-APC	BD Biosciences; MACS Miltenyi; BD Biosciences; eBioscience; BD Biosciences; BD Biosciences; BD Biosciences.
PBMCs – pDCs	Lineage-FITC/BDCA2-PE/ HLADR-PerCP/CD123-APC	BD Biosciences; MACS Miltenyi; BD Biosciences; eBioscience.
PBMCs – Monocytes (PBLs)	CD3-FITC/CD19-PE/CD14-APC	BD Biosciences; BD Biosciences; BD Biosciences
PBMCs – Monocytes – pDCs (PBLs – pDCs)	CD3-FITC/CD19-PE/CD14-APC	All purchased from BD Biosciences
Monocytes	CD3-FITC/CD19-PE/CD14-APC	All purchased from BD Biosciences
pDCs	CD19-FITC/CD123-PE/CD3-APC	BD Biosciences; eBioscience; BD Biosciences

Table III.1 Antibodies used in the flow cytometer analysis of isolated cells. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, chlorophyll-peridinin-protein complex; APC, allophycocyanin.

III.4 Bodipy FL C_{16} reconstitution and cell labelling

The quantification of extracellular vesicles (EVs) released by GEN2.2 was possible using the labelling protocol developed by Sargiacomo and colleagues [Coscia et al., 2016]. This protocol is based on cell treatment with the commercially available BODIPY FL C_{16} fatty acid (4,4-difluoro-5,7- dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid) (Life Technologies), hereafter indicated as Bodipy C_{16}, a fluorescent lipid that labels the cells ultimately producing fluorescent exosomes and microvesicles. Briefly, the fluorescent lipid was resuspended in methanol at 1 mM final concentration and stored at -20°C in aliquots of 150 µL. Before use, each aliquot was dried under nitrogen gas at room temperature, resuspended with 30 µL of 20 mM KOH to avoid the formation of micelles and promote its solubilisation, heated for 10 minutes at 60°C and finally resuspended in 70 µL of PBS containing 2% of bovine serum albumin (BSA).

For *pulse-chase* studies, $3x10^5$ GEN2.2 were metabolically labelled with Bodipy C_{16} at different times and concentrations as reported in the text. Importantly, to favour the uptake of the fluorescent probe the treatments were performed using complete medium supplemented with only 0.3% FBS.

Afterwards, cells were washed with 1x PBS to remove lipid excess and complete culture medium supplemented with 10% FBS was added. The fluorescence intensity of GEN2.2 was evaluated by flow cytometry analysis and reported in terms of median fluorescence intensity (MFI) and observed by confocal microscopy.

For the isolation of fluorescent extracellular vesicles (EVs), 10^7 GEN2.2 were seeded in 75 cm^2 flasks and incubated for 2 hours at 37°C with 3.5 µM of Bodipy C_{16} in 5 mL of medium supplemented with 0.3% FBS. Then, cells were washed with 1x PBS and resuspended in 12 mL of complete culture medium supplemented with 10% FBS containing or not myrNef_{SF2}w.t or type I, II or III IFN. The FBS added to the medium was previously ultracentrifugated overnight for 18 hours at 100,000 x g in a SW41 Ti rotor (Beckman Coulter, Brea, CA, USA) in order to remove the EVs normally present in serum.

III.5 Extracellular vesicle purification

Extracellular vesicles (EVs) were isolated from identical volumes (12 mL) of cell conditioned and non-conditioned control media, which were harvested after 20h and processed following already described methods for EV purification (see Fig. II.1) [Théry et al., 2018]. In detail, cell cultures or culture medium as control were centrifuged at 290 x g for 7 minutes to remove cells and then at 2000 x g for 20 minutes to eliminate cell debris. Subsequently, supernatants underwent differential centrifugations consisting of a first ultracentrifugation at 15,000 x g for 20 minutes to isolate large/medium EVs (hereafter referred to as microvesicles). Then, to isolate small EVs (referred to as exosomes), supernatants were harvested and ultracentrifugated at 100,000 x g for 3 hours. The pelleted vesicles were left for 20 minutes on ice and then resuspended in 12 mL of 1x PBS and ultracentrifugated again at 100,000 x g for 3 hours. All ultracentrifugation steps were performed at 4°C using a SW41 Ti rotor (Beckman Coulter).

Isolated exosomes and microvesicles were resuspended in 100-200 µL of PBS with protease and phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 µg/mL leupeptin and pepstatin A, 2 µg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and stored at 4°C until counting by flow cytometry and further analyses.

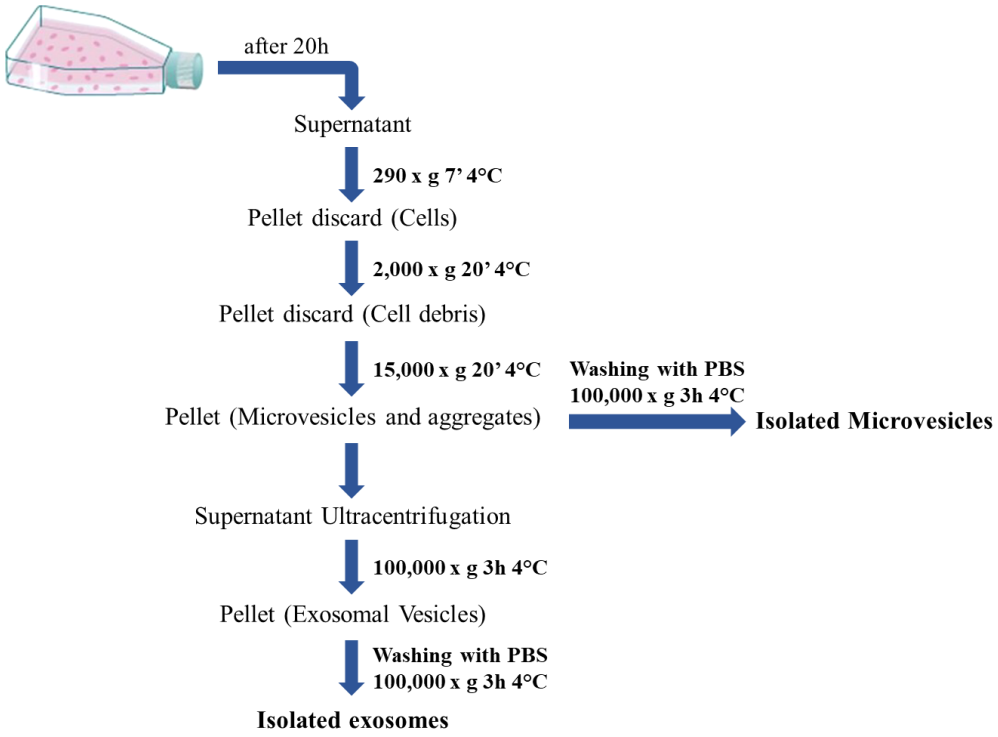


Figure III.1 Experimental workflow of differential centrifugations used for exosomes and microvesicles isolation.

III.6 Quantification of vesicles by flow cytometry

Flow cytometry (FC) of Bodipy labelled EVs was performed on a Gallios Flow Cytometer (Beckman Coulter) at Istituto Superiore di Sanità (Rome, Italy) under the supervision of Dr. Katia Fecchi and Dr. Maria Carollo, using an optimized procedure as previously described (see Fig. II.2 and [Coscia et al., 2016]). Briefly, 5 µL of fluorescent exosomes or microvesicles were mixed with 20 µL of Flow-Count Fluorospheres with size of 100 nm (Beckman Coulter), which were used as internal reference standard, and further diluted with 1x PBS to a final volume of 200 µL. The instrument was set up using control 100-500 nm fluorescent beads in order to identify the right gate corresponding to exosomes (size below 200 nm). FC analysis was performed by plotting fluorescence at 525/40 nm (FL1) versus log scale side scatter (SSarea). The instrument was set at flux high and the analysis was stopped at 2,000 Flow-Count Fluorospheres events. Fluorescent EVs total number was established according the formula: $x = (((y \times a)/b)/c) \times d$ where y = events counted at 2,000 counting beads; a = number of counting beads in the sample; b = number of counting beads registered (2,000); c = volume of sample analysed; and d = total volume of exosome preparation. The total number of exosomes and microvesicles obtained was then normalized against the number of cells counted after 20 hours of treatment. Kaluza Software v. 2.0 (Beckman Coulter) was used for FC analysis.

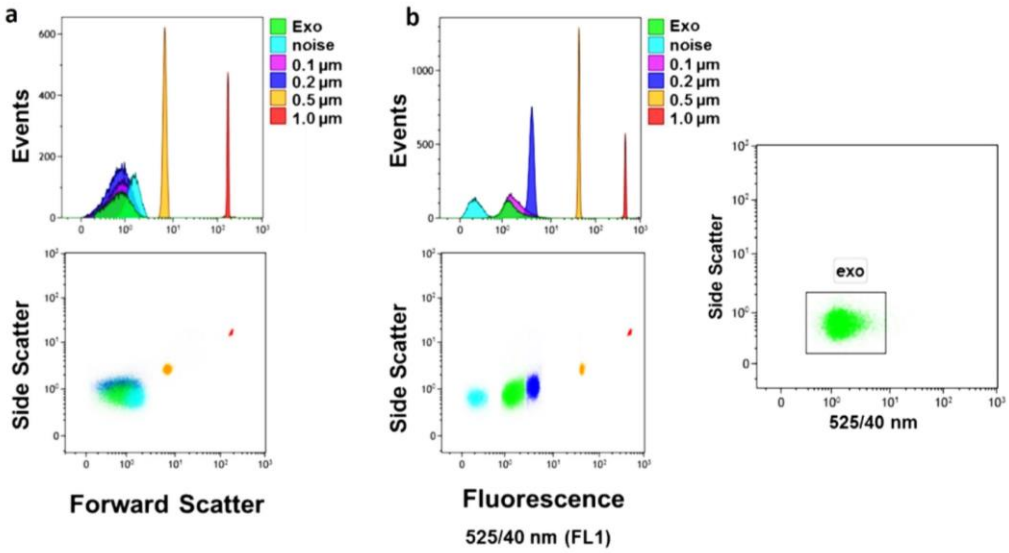


Figure III.2 Setting of the cytofluorimeter for the analysis of Bodipy C_{16}-labelled EVs. FC analysis was performed by plotting fluorescence at 525/40 nm (FL1) versus log scale side scatter (SSarea). Fluorescent beads ranging from 0.1 to 1.0 µm and background noise (noise) were analysed for size (a, forward scatter) and fluorescence (b, 525/40 nm FL1) in order to identify the position corresponding to exosomes. The pair color/sample of the legend matches with sample colors reported in the histograms (upper side) and dot plots (lower side) of (a) and (b). Two thousand Flow-Count Fluorospheres were used to determine the exosomal number. The instrument was set to stop the analysis at 2000 Flow-Count Fluorospheres events. The number of exosomes was registered in the rectangular exosomal region (Exo) [Coscia et al., 2016].

III.7 Western blot assay

Western blot analyses on cell lysates were performed by washing cells twice with ice-cold PBS (pH 7.4) and lysing them for 30 minutes on ice with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0,25% deossicolate sodium, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% non-ionic detergent IGEPAL CA-630 (Sigma-Aldrich), 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 µg/mL leupeptin and pepstatin A, 2 µg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Whole-cell lysates were centrifuged at 6000 x g for 10 minutes at 4°C. The protein concentration of cell extracts was determined by the Lowry protein quantification assay.

Aliquots of cell extracts containing 30 to 50 µg of total proteins were resuspended in loading buffer (Tris 65 mM pH 6.8, 10% glycerol, 5% sodium dodecyl sulfate [SDS], 50 mM dithiothreitol [DTT], 0.05% bromophenol blue) and heated for 5 minutes at 95-97 °C. Then, samples were resolved by 6 to 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting on 0.45 µm pore size nitrocellulose membranes (Amersham™, GE Healthcare Life science) overnight at 35 V using a Bio-Rad Mini Trans-Blot Cell. For Western blot analysis of EVs, they were lysed by repeated freezing and thawing and then analysed as described for cell lysates.

For the immunoassays, membranes were blocked in 3% bovine serum albumin (BSA) fraction V (Biofroxx, Germany) in TTBS/EDTA (10 mM Tris pH 7.4, 100 mM NaCl and 1 mM EDTA, 0.1% Tween 20) for 30 minutes at room temperature (RT). After incubation, membranes were washed twice with TTBS/EDTA for 10 minutes and incubated for 1 hour at RT or overnight at 4°C with specific primary antibodies diluted in 1% BSA/TTBS-EDTA. The antibodies used in immunoblottings were the following: rabbit polyclonal anti-phosphotyrosine (Y701) STAT1 (Cell Signaling, cat. #9171), mouse monoclonal anti-STAT1 (Transduction Laboratories, cat. #G16920), rabbit polyclonal anti-phosphotyrosine (Y689) STAT2 (Upstate Biotech. /Millipore, cat. #07-224), rabbit polyclonal anti-STAT2 (Santa Cruz Biotechnology, cat. #sc-476), mouse monoclonal anti-ISG15 (Santa Cruz Biotechnology, cat. #sc-166755), rabbit polyclonal anti-IRF-1 (Santa Cruz Biotechnology, cat. #sc-497), rabbit polyclonal anti-α-actin (Sigma-Aldrich, cat. #A2066), rabbit polyclonal anti-Lamin A (Abcam, cat. #ab26300), mouse monoclonal anti-TSG101 (Genetex, cat. #GTX70255), rabbit polyclonal anti-Alix (Novus Biologicals, cat. #NBP1-90201), rabbit polyclonal anti-Hsp90 (Santa Cruz Biotechnology, cat. #sc-7947), mouse monoclonal anti-CD81 (Santa Cruz Biotechnology, cat. #sc-166029), mouse monoclonal anti-Flotillin-1 (BD Biosciences, cat. #610821), mouse monoclonal anti-COX4 (Santa Cruz Biotechnology, cat. #sc-376731), mouse anti-Nef ARP3026 (obtained from the NIH AIDS Research and Reference Reagent Program).

Membranes were then washed three times in TTBS/EDTA for 10 minutes at RT. Subsequently, the immune complexes were detected by incubating membranes for 1 hours at RT with horseradish peroxidase-conjugated goat anti-rabbit (Merk Millipore, cat. #AP307P) or goat anti-mouse (Enzo Life Technologies, cat. #ADI-SAB-100-J) antibodies, followed by enhanced chemiluminescence reaction (ECL Fast Pico; Immunological Sciences, Rome, Italy). To reprobe membranes with antibodies having different specificities, nitrocellulose membranes were stripped for 5 minutes at RT with Restore Western Blot Stripping Buffer (Thermo Scientific™ Pierce™ Protein Biology, Rockford, IL), and then extensively washed with TTBS/EDTA.

The ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) instrument and the Image Lab software (Bio-Rad) were used to reveal the chemiluminescence signal. For loading control, α-actin levels were quantified by using a rabbit polyclonal anti-α-actin antibody (Sigma-Aldrich, cat. #A2066).

Densitometric analyses were performed using the freeware Image J software (NIH), by quantifying the band intensity of the protein of interest with respect to the relative loading control band (i.e., actin) intensity. Fold changes of each analysed protein were calculated by dividing the values obtained in treated conditions by those of the corresponding controls and were reported in the histograms as means ± S.D of n independent experiments.

III.8 Nuclear and cytoplasmic extract preparation

GEN2.2 (4 x 10^6 cells) were treated with myrNef$_{SF2}$w.t or IFNs for 20 h, harvested and washed twice in ice-cold PBS buffer by centrifuging at 1200 rpm for 3 minutes at 4°C. Cell pellets were lysed with 200 µL of hypotonic buffer (10 mM Hepes pH 7.8, 10 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM EDTA, 5% glycerol, 1 mM PMSF, 1 µg/mL pepstatin A, 2 µg/mL aprotinin, 1 µg/mL leupeptin, 1 mM Na_3VO_4, 20 mM NaF) and incubated on ice for 15 minutes. Afterwards, 0.58% IGEPAL CA-630 (Sigma-Aldrich) was added, incubated on ice for 2 minutes and then centrifuged at 14,000 rpm for 5 minutes. Supernatants corresponding to the cytoplasmic fraction were harvested, whereas 60 µL of hypertonic buffer (50 mM HEPES, pH 7.8; 400 mM NaCl; 1 mM $MgCl_2$; 1 mM EGTA; 1 mM EDTA; 10% glycerol; 1 mM PMSF; 1 µg/mL pepstatin A; 2 µg/mL aprotinin; 1 µg/mL leupeptin; 1 mM Na_3VO_4; 20 mM NaF) were added to the nuclear pellets, then incubated on ice for 40 minutes and centrifuged at 14,000 rpm for 10 minutes. Supernatants corresponding to the nuclear fraction were harvested. Protein concentrations of both cytoplasmic and nuclear fractions were determined by the Lowry protein quantification assay. All samples were processed and analysed by Western Blot as previously described.

III.9 Confocal microscopy

To detect IFN-α production and to assess IRF-7 increase by Confocal Laser Scanner Microscopy analysis, primary pDCs were seeded at 10^5 cells/200 µL in complete 10% FBS medium in 96-well plates and treated with myrNef$_{SF2}$w.t. (300 ng/mL), myrNef$_{SF2}$4EA (300 ng/mL) or CpG (3 µg/mL). To detect IFN-α production, Brefeldin A was added after 3 hours of treatment to prevent the secretion of IFN-α possibly produced. Cells were harvested at indicated times, washed once in 1x PBS, placed on the microscope slide and left to air dry. Subsequently, they were fixed with 4% PFA for 15 minutes on ice, then washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes on ice. Afterwards, the specimens were incubated for 30 minutes in the dark at RT with 1% BSA in PBS containing far-red fluorescent dye RedDot™2 to stain nuclei (Biotium, Inc. Hayward, CA), washed and then incubated for 1 hours in the dark at RT with a mouse PE-conjugated anti-IFNα antibody (Miltenyi Biotec S.r.l, Bologna, Italy) diluted 1:20 in 0.1% BSA in PBS. To assess IRF-7 increase, primary pDCs were processed as reported above and then incubated in the dark for 1 hours at RT with the following antibodies: rabbit anti-IRF-7 antibody (Santa Cruz Biotechnology, cat. #sc-9083) diluted 1:50 in 0.1% BSA in PBS and AlexaFluor546-conjugated anti-rabbit (Life Technologies cat. #A11010) as secondary antibody diluted 1:200 in 0.1% BSA in PBS. Finally, the specimens were washed four times in PBS and coverslips were mounted using Vectashield antifade mounting medium (Vectashield H-1000;

Vector Laboratories Inc., Burlingame, CA) diluted at 80% in PBS to prepare samples for confocal microscopy observation.

To evaluate the internalization of Nef protein, primary human pDCs (10^5 cells/200 µL), GEN2.2 (0.2 x 10^6 cells/150 µL) and monocyte-derived macrophages (MDMs) (80,000 cells/cm^2) were treated with 300 ng/mL of myrNef$_{SF2}$w.t.-Alexa488, which was labelled using AlexaFluor488 Microscale Protein Labelling Kit (Molecular Probes/Invitrogen, Italy) following the manufacturer's recommendations. After the incubation, cells were washed once in 1x PBS, fixed and mounted as previously described. Plasma membrane counterstaining was performed by treating primary pDCs for 5 minutes with PKH26-GL using PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich) following the manufacturer's recommendations. Nuclei of GEN2.2 were stained with 3 µg/mL DAPI (4', 6'-diamidino-2-phenylindole) (Sigma-Aldrich).

For *pulse-chase* studies, 3x10^5 GEN2.2 were seeded in 48-well plates and metabolically labelled with Bodipy C_{16} according to the concentrations and interval of times reported. Cells were then washed twice with 1x PBS and placed on a microscope slide, fixed with 4% PFA for 15 minutes on ice and washed again for 15 minutes. Finally, samples were mounted with Vectashield antifade mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) for nucleus staining. All the samples were stored protected from the light at -20°C until the observation. Images were acquired with Leica TCS SP5 confocal microscope and processed with LAS AF software (version 1.6.3, Leica Microsystems CMS GmbH). Objective 63.0X. Lasers activated: He/Ne laser at 543 nm to phycoerythrin and Alexa546 excitation, He/Ne laser at 633 nm to dye RedDot^{TM}2's excitation, Argon laser at 488 nm to visualize myrNef$_{SF2}$-Alexa488 (green) and UV laser at 405 nm to observe nuclei stained with DAPI. Images were acquired activating single laser in sequential mode to prevent fluorescence overlay. Several fields were analysed for each condition and representative results are presented.

III.10 RNA extraction and quantitative RT-PCR analysis

RNA extraction and qRT-PCR (quantitative Real Time Polymerase Chain Reaction) were performed at the laboratory of Dr. Eliana Coccia at Istituto Superiore di Sanità, Rome. For RNA extraction, cells were seeded at 10^6 cells/mL and treated for 6 hours with 300 ng/mL of myrNef$_{SF2}$w.t. or with 3 µg/mL of CpG-A as positive control or left untreated. After treatment, cells were washed with ice-cold PBS and centrifuged at 290 x g for 10 minutes. Cell pellets were lysed in RLT lysis buffer containing β-mercaptoethanol (Qiagen Inc, Valencia, CA) and then RNA was isolated using the High Pure RNA Isolation Kit from Qiagen according to the manufacturer's

recommendations. The amount of RNA extracted was measured by means of Nanodrop spectrophotometer (Thermo Scientific, Wilmington DE). The retrotranscription was performed using 0.5-1 µg of mRNA and the Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Life Technologies). According to the protocol, mRNA was incubated for 1,5 hours at 37°C with a mixture containing 1 µM oligo-dT_{12-18}, 1 µM random primers, 0.5 mM deoxynucleotides triphosphates (dNTPs), 10 mM DTT, first Strand Buffer 5X (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 0.04 U/µl of ribonuclease inhibitor RNasiOUT™ and finally 8 U/µl of retrotranscriptase. The obtained cDNA was then purified using the QIAquick PCR Purification Kit (Qiagen Inc.) following the manufacturer's instructions.

Quantitative PCR assays to evaluate the expression of *mxA* gene were performed with SYBR Green I technology on the Light Cycler instrument (Roche Diagnostics GmbH). In particular, 2 µL of template cDNA were added in a final volume of 20 µL containing a mix of forward and reverse primers (500 nM each one) specific for the analysed gene (synthesized at Eurofins MWG Operons), the Platinum Taq DNA enzyme Polymerase (Invitrogen Life Technologies) and SYBR Green I (Biowhittaker Molecular Applications, Rockland, ME). In details, primers used were the following: forward, 5'-ATCCTGGGATTTTGGGGCTT-'3 and reverse 5'-CCGCTTGTCGCTGGTGTCG-'3.

The data shown were normalized using the 2-ΔCT formula, where ΔCT represents the difference between the amplification cycles of *mxA* gene and the amplification cycles of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) constitutively expressed in all cell types.

III.11 ELISA multiplex

GEN2.2 were cultured at 10^6 cells/mL in complete 10% FBS medium in 24-well plates and stimulated with 300 ng/mL of $myrNef_{SF2}$w.t or left unstimulated. Supernatants were harvested after 4, 6 and 20 hours, centrifuged at 290 x g for 3 minutes to eliminate the cells and then stored at -80°C for cytokine measurements. In collaboration with professor Roberto Gambari at University of Ferrara, supernatants were then analysed in a Bio-Plex Pro Human Cytokine 27-Plex Immunoassay (Bio-Rad) able to detect the following cytokines: FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF.

Cytokines/Chemokines	Assay Range (pg/mL)	Cytokines/Chemokines	Assay Range (pg/mL)
PDGF-bb	75000 – 4.0	**IL-17**	45000 – 1.5
IL-1β	5000 – 0.05	**Eotaxin**	25000 – 2.0
IL-1ra	180000 – 30	**FGF basic**	12500 – 4.0
IL-2	30000 – 1.0	**G-CSF**	100000 – 5.0
IL-4	4000 – 1.0	**GM-CSF**	6800 – 0.5
IL-5	90000 – 5.0	**IFN-γ**	20000 – 1.1
IL-6	7500 – 1.0	**IP-10**	60000 – 1.4
IL-7	40000 -1.8	**MCP-1 (MCAF)**	6000 – 1.8
IL-8	20000 – 4.0	**MIP-1α**	640 – 1.0
IL-9	45000 – 1.6	**MIP-1β**	6500 – 1.0
IL-10	25000 – 1.25	**RANTES**	15000 – 1.0
IL-12 (p70)	30000 – 1.0	**TNF-α**	54000 – 2.5
IL-13	5500 – 1.0	**VEGF**	200000 - 20
IL-15	165000 – 30		

Table III.2 Sensitivity range of each cytokine/chemokine analysed in the Bio-Plex Pro Human Cytokine 27-Plex Immunoassay.

III.12 Statistical analysis

Differences were statistically evaluated using a two-tailed Student's t test or ANOVA (One-way analysis of variance and Tukey's as post-test). Data were analysed with GraphPad Prism 4 software. p values ≤ 0.05 were considered statistically significant.

IV. RESULTS

IV.1 myrNef$_{SF2}$ induces the tyrosine phosphorylation of STAT1 in human PBLs (*Peripheral Blood Lymphocytes*) but not in PBLs depleted of pDCs.

Previous studies carried out on primary monocyte-derived macrophages (MDMs) showed that myrNef$_{SF2}$ activated indirectly some signal transducers and activators of transcription (STAT) family members in autocrine and/or paracrine manner by inducing the production and secretion of a number of pro-inflammatory factors. The activated STATs were STAT-1, -2 and -3 [Olivetta et al., 2003; Mangino et al., 2007; Federico et al., 2001; Percario et al., 2003]. Hence, the effect of the viral protein was initially assessed by evaluating the tyrosine (Y701) phosphorylation of STAT1 (Signal Transducers and Activators of Transcription 1), a transcriptional factor usually activated in response to a wide range of cytokines, including IFNs. The phosphorylation of this protein in Tyr701 induces its dimerization, nuclear translocation and its binding to specific DNA response elements, ultimately influencing gene expression programs [Ihle et al., 1994]. There are two isoforms of STAT1 protein: α (91 kDa) and β (84 kDa) resulting from alternative splicing. Since primary pDCs are present in very low amount in blood (0.2-0.5% of PBMCs), to facilitate biochemical analyses the experiments were carried out on peripheral blood lymphocytes (PBLs), a population that includes mainly B and T lymphocytes, natural killer cells, dendritic cells and plasmacytoid dendritic cells (pDCs), depleted or not of pDCs. To this purpose, PBLs were isolated from PBMCs by negative selection removing CD14 positive cells (monocytes) and a fraction of PBLs was then further depleted of pDCs in order to evidence the role of this dendritic subset in the response. The efficiency of the cell depletion and the purity of the recovered cells were determined by flow cytometry (FC) analyses (Fig. IV.1A and B).

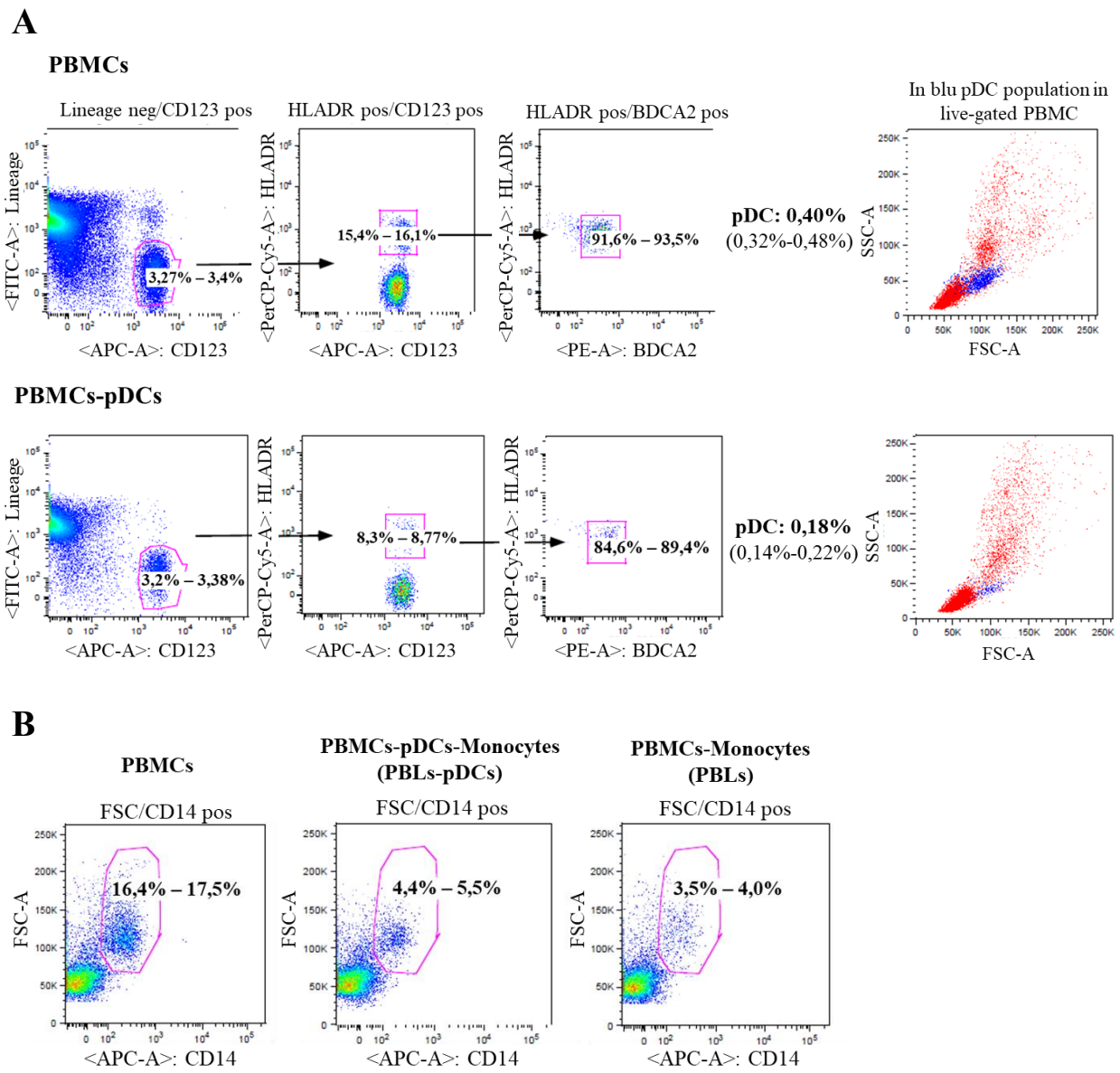


Figure IV.1 Purity of isolated cells. Dot plots show the forward light scatter/SSC profile of the cells. (A) pDC depletion analysis. The frequency of pDCs was determined as cells positive for CD123 and BDCA2 (CD303) within peripheral blood mononuclear cells (PBMCs). (B) Monocyte depletion analysis. The frequency of monocytes was determined within PBLs and PBLs-pDCs as cells positive for CD14.

Usually, specific STAT activation occurs in a very short time after cell treatment with stimulating factors. Afterwards, overcoming of specific inhibitors gradually switches off STAT activation. Thus, to appropriately monitor and characterize possible effects on STAT1 activation, PBLs and PBLs depleted of pDCs (PBLs-pDCs) were treated with myrNef$_{SF2}$w.t at different time intervals (Fig. IV.2). Since IFN-γ is well known to stimulate the phosphorylation of STAT1, cells were treated for 2 hours with 15 IU/mL of IFN-γ as positive control.

As shown, myrNef$_{SF2}$w.t induced in PBLs the tyrosine (Y701) phosphorylation of STAT1 already at 4 hours and the signal persisted also at 6 hours (Fig. IV.2A), whereas PBLs depleted of pDCs

failed to respond (Fig. IV.2B). This preliminary result suggested that pDCs could have a particular importance in the response of PBLs to the viral protein Nef. This data contributed to address our work on this particular dendritic subset, specialized in type I IFN production and involved in the pathogenesis of HIV infection [Aiello et al., 2018].

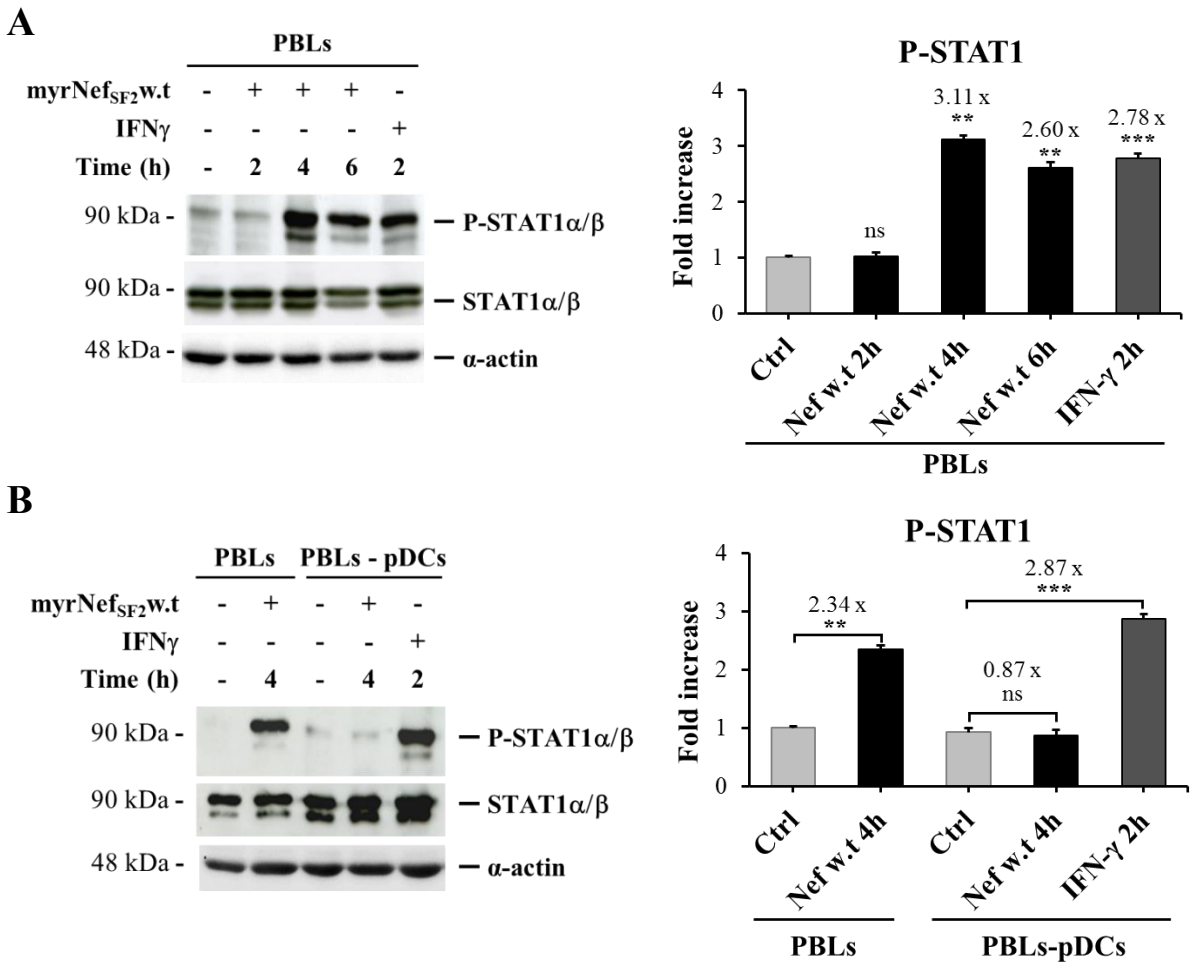


Figure IV.2 myrNef$_{SF2}$w.t induces the tyrosine phosporylation of STAT1 in PBLs, but not in PBLs depleted of pDCs. PBLs were seeded at 4x10^6 cells/each sample in 12 well plate and treated with 100 ng/mL of myrNef$_{SF2}$w.t. for the indicated time points. The treatment with IFN-γ (15 IU/mL) was used as positive control. Cell lysates (50 µg of proteins) were analysed on 9% SDS-PAGE gel and the immunoblotting was performed using a pospho-Tyr(701)-STAT1 specific antibody. P-STAT1 was normalized to actin by densitometric analysis and reported as fold increase compared to ctrl. (A) The results are representative of three independent experiments performed with different donors. (B) The results are representative of replicates of two independent experiments performed with different donors. P-STAT1 was normalized to actin by densitometric analysis and reported as fold increase compared to ctrl. Histograms: mean ± S.D. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ns, not significant vs respective Ctrl (untreated cells).

IV.2 Evaluation of Nef protein internalization in primary pDCs.

Before to get insight into the effects induced by Nef protein on pDCs, we first evaluated the capability of these cells to internalize the recombinant protein. To this aim, primary pDCs were isolated from PBMCs by positive selection using BDCA-4 conjugated microbeads and assayed for their purity by FC analysis (Fig. IV.3A).

Isolated pDCs and monocyte derived-macrophages (MDMs) were treated with 300 ng/mL of myrNef$_{SF2}$-AlexaFluor488 for 24 hours. Confocal microscopy images showed that Nef protein was internalized by primary pDCs but less efficiently than MDMs, probably because of their lesser phagocytic/internalization ability that distinguishes pDCs from macrophages (Fig. IV.3B). The observation of several fields (for a total of about 500 cells) revealed that approximately 30% of pDCs internalized the viral protein. Moreover, the images showed a different aspect of labelled Nef between the two cell types that could be attributed to a different distribution and/or localization of the protein.

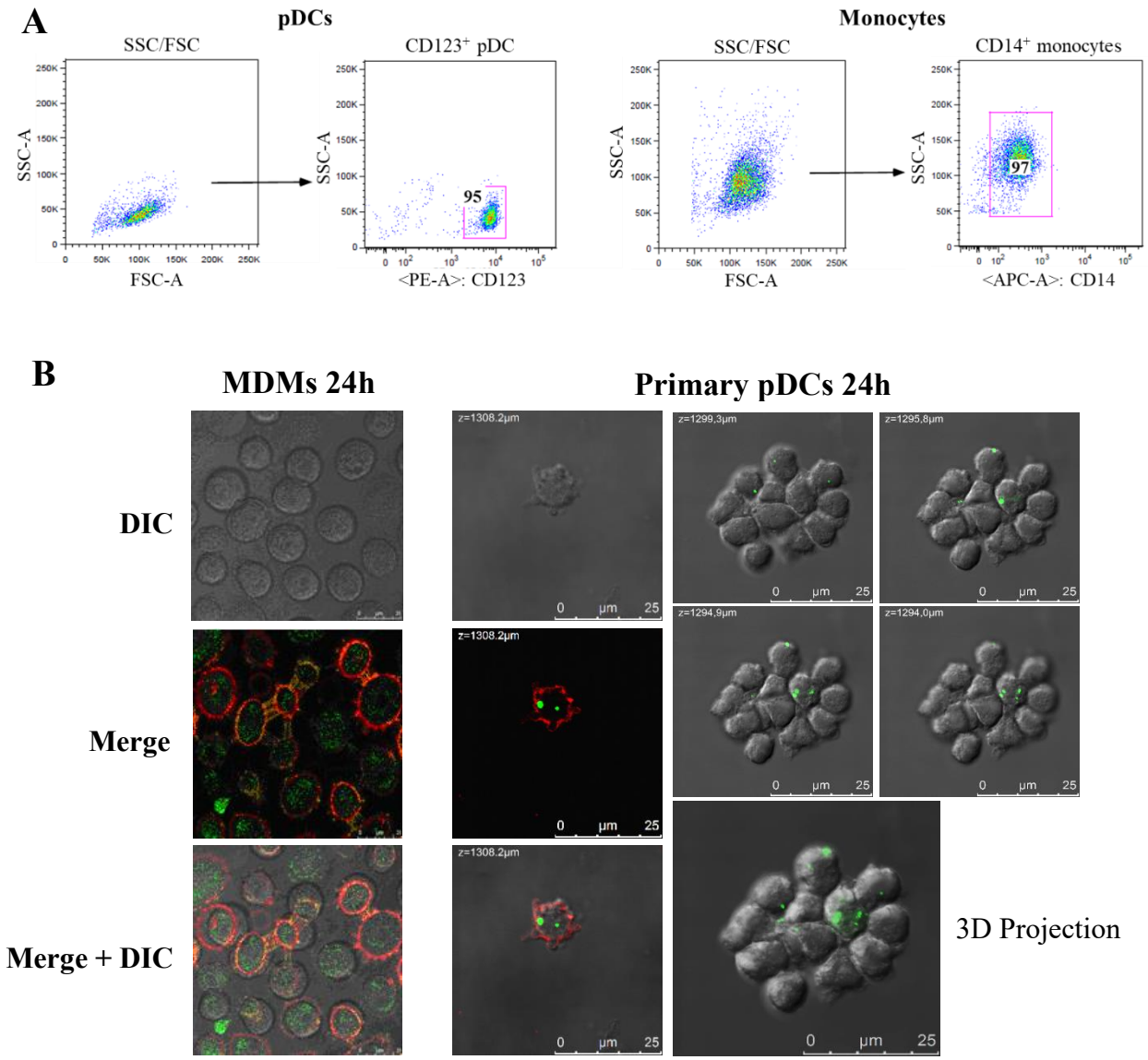


Figure IV.3 Internalization of Nef protein in plasmacytoid dendritic cells and monocyte-derived macrophages. (A) Flow cytometry plots showing the forward light scatter/SSC profile of the cells. Purity of monocytes (left panel) and pDCs (right panel) was determined by staining cells with anti-CD14 and anti-CD123 antibodies respectively. (B) Monocyte-derived macrophages (MDMs) were seeded at 80,000 cells/150 µl in dish coated with poly-d-lysine, specifically designed for confocal observation (MatTek Corporation, Ashland, MA), whereas primary pDCs were seeded at 10^5 cells/200 µL in 96-well plates. Purified MDMs and pDCs were treated with 300 ng/mL of myrNef$_{SF2}$ conjugated with AlexaFluor488 (green) for 24h. Afterwards, cells were fixed as reported in Materials and Methods and analysed by confocal microscope (Leica TCS SP5), software LAS AF version 1.6.3 (Leica Microsystems). Plasma

membrane counterstaining was performed using PKH26-GL (red). Objective 63.0X. DIC: Differential Interference Contrast Images. Scale bars 0-25 µm. Representative images of two independent experiments are shown.

IV.3 Nef up-regulates the expression of *mxa*, induces the increase and nuclear translocation of IRF-7 and stimulates IFN production in primary pDCs.

The chronic production of type I IFN, highlighted through the expression of IFN induced genes in PBMCs, is present in high levels both in individuals infected with HIV-1 and in macaques infected with SIV (Simian Immunodeficiency Virus) [Stacey et al., 2009; Malleret et al., 2008], and is associated with progression to AIDS [O' Brien et al., 2013; Barron et al., 2003; Donaghy et al., 2001, 2003]. Since pDCs are widely recognized as the main producers of type I IFN and play a pivotal role in HIV infection by contributing to chronic immune activation mainly via IFN secretion [Aiello et al., 2018], we asked whether Nef protein induced the expression of the IFN-inducible gene *mxA* (*myxovirus resistance protein A*) in pDCs. The MxA protein was chosen because it is a key mediator of the antiviral response induced by IFNs against a wide variety of viruses. Moreover, its expression is strictly regulated by type I and III IFNs, requires functional activation of STAT1 and is not directly induced by viruses or other stimuli [Haller & Kochs, 2011]. For these reasons, the expression of MxA protein in tissues is used as a surrogate marker of local type I or III IFN production.

The experiments were carried out using total PBMCs and PBMCs depleted of pDCs (PBMCs-pDCs). Both cell types were treated for 6 hours with myrNef$_{SF2}$w.t (100 ng/mL) or with CpG A (1 µM), a TLR9 agonist in response to which pDCs synthesize high levels of IFN-α, as positive control. The results showed that Nef increased *mxA* expression in both PBMCs and PBMCs-pDCs, but a reduction of this increase was observed when PBMCs were depleted of pDCs (Fig. IV.4).

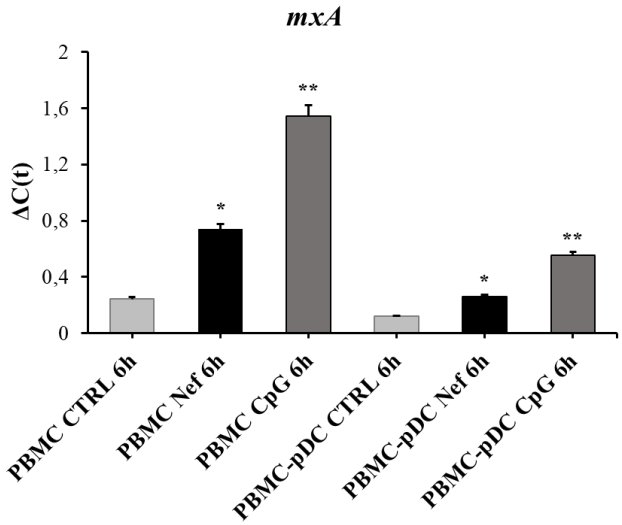


Figure IV.4 Nef protein up-regulates *mxa* expression in pDCs. PBMCs and PBMCs depleted of pDCs (PBMCs-pDCs) were seeded at 2x10^6 /2 mL and treated for 6h with 100 ng/mL of myrNef$_{SF2}$w.t. or 1 µM of CpG A as positive

control. Ctrl: left untreated cells. After treatment, cells were harvested and processed for RNA extraction. *mxa* expression was evaluated by qRT-PCR and the data were normalized using the 2-ΔCT formula, where ΔCT represents the difference between the amplification cycles of *mxA* gene and the amplification cycles of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), constitutively expressed in all cell types. All data were expressed as mean ± S.D. of triplicates of one experiment. One-way ANOVA test; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$ vs respective Ctrl.

Since PBMCs include different cell types, it was not possible to identify a specific population as the only one responsible for *mxa* production. However, this result seemed to suggest that Nef treatment could increase *mxA* in pDCs contributing to the higher response observed in PBMCs. Furthermore, considering that both type I (α/β) and type III (λ) IFN can regulate the expression of this gene, this result was consistent with the idea that pDCs could produce both or one of these types of IFN in response to Nef treatment.

Notably, type III and type I IFN share many immune responses and biological activities, such as antiviral and anti-proliferative activity [Yin et al., 2012]. These functional similarities result from a common signalling pathway (JAK-STAT pathway) that specifically involves the activation of JAK-1, Tyk-2, STAT-1, STAT-2 and the formation of the ISGF3 complex (comprising STAT-1/STAT-2 and IRF9) leading to the expression of type I and III IFN-stimulated genes (ISGs). Even their expression depends on a similar transcription model that requires the previous activation and nuclear translocation of IFN regulatory factors (IRFs), such as IRF-7 and IRF-3 as far as type I IFN production concerns [Zhou et al., 2018].

Given the key role played by IRF-7 for IFN-α production, we evaluated whether Nef treatment induced the activation and nuclear translocation of this factor in pDCs. To this aim, primary pDCs were treated with myrNef$_{SF2}$w.t. (100 ng/mL) for 6 and 20 hours and with CpG A for 20 hours as positive control. Afterwards, cells were harvested and labelled in order to observe IRF-7 by confocal microscopy (Fig. IV.5).

The images revealed that IRF-7 was increased and, although it was mainly localized in the cytoplasm, a partial nuclear translocation was more evident after 20 hours of Nef treatment. Moreover, a basal expression of IRF-7 in untreated cells was observed. This data correlates with what reported in literature, according to which plasmacytoid dendritic cells constitutively express not only IRF-3, but also IRF-7 [McKenna et al., 2005].

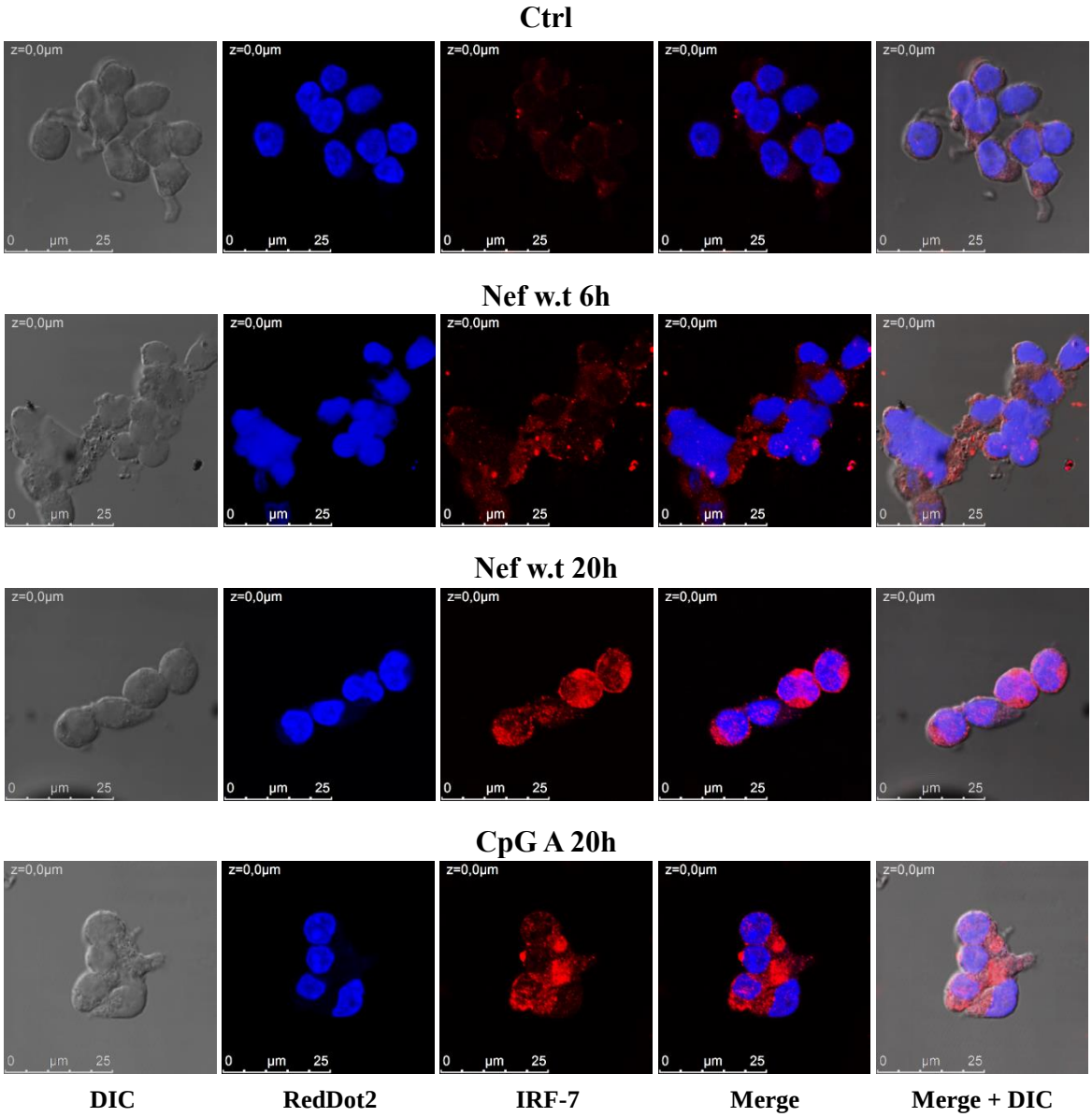


Figure IV.5 IRF-7 is upregulated and translocates to the nucleus after treatment with Nef protein. 0,5 x 10^5 pDCs were treated for 6h and 20h with 100 ng/mL of myrNef$_{SF2}$w.t or for 20h with CpG A (1 µM) as positive control. Ctrl: untreated cells. Cells were afterwards fixed in PFA 4%, permeabilized and incubated with anti-IRF-7 antibody and with a secondary antibody conjugated with AlexaFluor546 (red) as reported in Materials and Methods. Nuclei (blue) were stained using the dye RedDot2. Images were acquired with the confocal microscope Leica TCS SP5 and processed using the software LAS AF version 1.6.3 (Leica Microsystems). Objective 63.0X. DIC: Differential Interference Contrast. Scale bars 0-25 µm. For further details see Materials and Methods section. Representative images of two independent experiments are shown.

However, despite the up-regulated expression of *mxA* gene (Fig. IV.4) and IRF-7 could be related to the production of various types of IFN, our attention was firstly focused on IFN-α because pDCs are defined as "professional IFN-α producing cells". Hence, to evaluate IFN-α production pDCs were isolated by positive selection using anti-BDCA4 paramagnetic microbeads. Purified cells were treated for 6 and 16 hours with 100 ng/mL of the myrNef$_{SF2}$w.t protein, whereas a treatment

of 16 hours with 1 μM of CpG A was used as positive control. After 3 hours of treatment, 10 μg/mL of Brefeldin A were added to cells treated for 6 hours, while only 5 μg/mL were added to the cells treated for 16 hours in order to reduce its toxicity. Brefeldin A is a macrocyclic lactone produced by the fungus *Penicillium brefeldianum* from the palmitate (C16). It is known to inhibit the secretion of proteins in mammals and in other eukaryotic cells by interfering with the function of the Golgi apparatus [Klausner et al., 1992]. Given its role, Brefeldin A was used in order to prevent IFN-α secretion outside the cells thus allowing its observation in cell cytoplasm by confocal microscopy (Fig. IV.6). Confocal images showed as Nef-stimulated pDCs accumulated IFN-α already after 6 hours and the production was detected until 16 hours.

Overall, the data obtained in primary pDCs supported the idea that Nef protein could activate plasmacytoid dendritic cells probably resulting in IFN production.

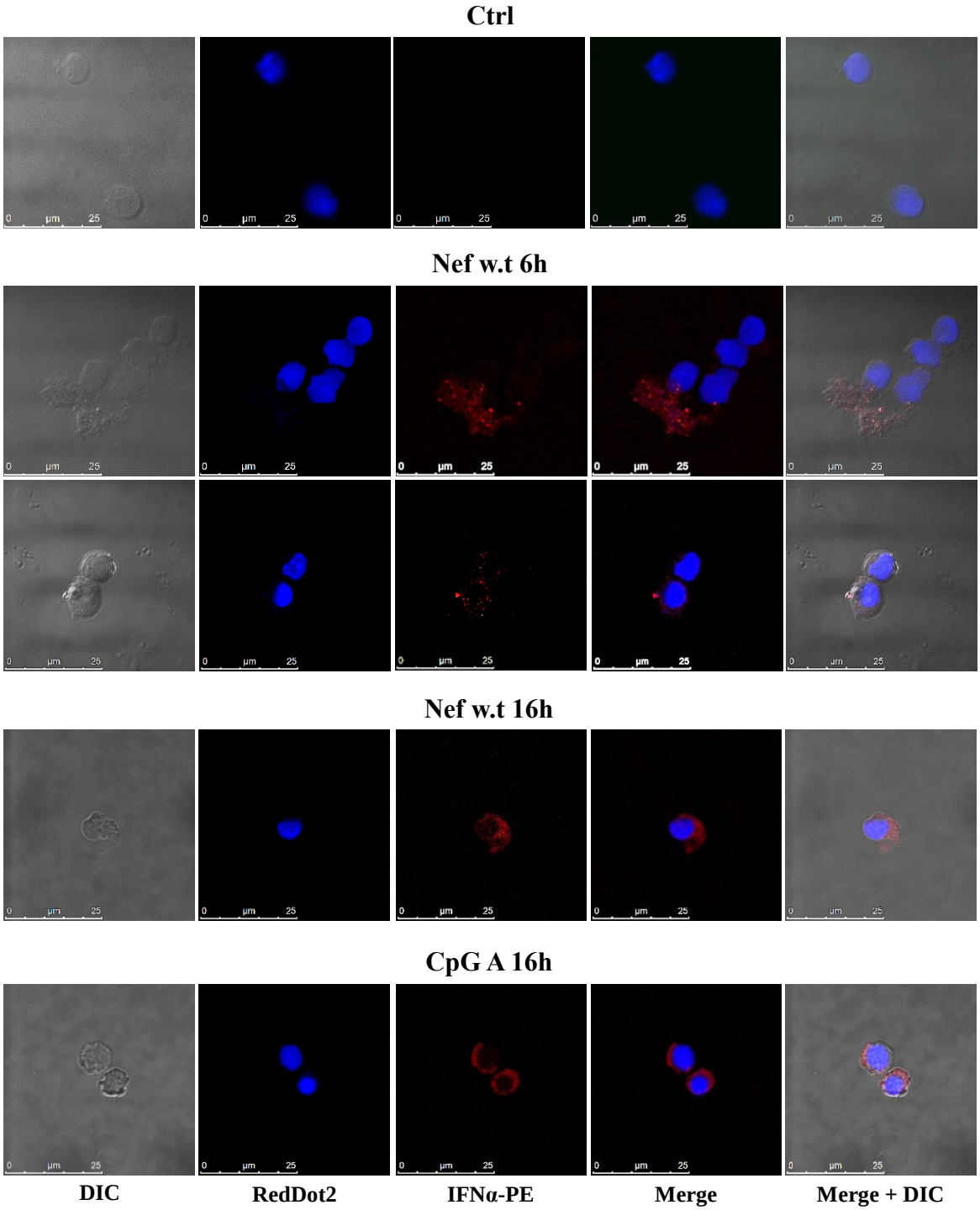


Figure IV.6 Nef treatment induces IFN-α production in primary pDCs. Cells were seeded at 0,1 x10^6 cells/200 µl and treated for 6h and 16h with 100 ng/mL of myrNef$_{SF2}$w.t or for 16h with CpG A (1 µM) as positive control. Ctrl are cell left untreated. After 3h from the start of the treatment, 10 µg/mL of Brefeldin A were added to the cells treated for 6h, while only 5 µg/mL were added to the cells treated for 16h in order to reduce its toxicity. Cells were afterwards fixed in PFA 4%, permeabilized and incubated with anti-IFN-α antibody conjugated with PE (red) as reported in Materials and Methods. To visualize nuclei (blue), cells were incubated with the dye RedDot2. Images were acquired with the confocal microscope Leica TCS SP5 and processed with the software LAS AF version 1.6.3 (Leica Microsystems). Objective 63.0X. DIC: Differential Interference Contrast. Scale bars 0-25 µm. For further details see Materials and Methods section.

IV.4 Characterization and validation of GEN2.2 cell line as good model for the study of the effects induced by HIV-1 Nef on plasmacytoid dendritic cells.

The promising results obtained in primary pDCs led us to better investigate the effects induced by Nef protein on this unique dendritic cell subset. However, to facilitate biochemical analyses of cell signalling, which are difficult to perform in rare and *in vitro* short living human primary pDCs, the following experiments were performed using a human pDC line called GEN2.2, recently established from leukemic pDCs. This cell line shares most of the phenotypic and functional features of primary pDCs [Chaperot et al., 2006], thus it was chosen in order to have a more stable and reproducible system to perform our studies. Moreover, the use of GEN2.2 cell line allowed us to overcome both the problem of isolating and purifying sufficient amounts of cells due to the low frequency of pDCs in human blood, and the variability of the responses due to the different background of the donors.

GEN2.2 proliferate rapidly as a single cell suspension with both no adherent and weakly adherent cells. Since GEN2.2 proliferation is strictly dependent on the presence of a feeder layer made up by the stromal MS-5 cell line, the immunophenotype of GEN2.2 was analysed by flow cytometry for the expression of different markers known to be present on the surface of primary pDCs (see Table IV.1) in order to verify the purity of the cells recovered from the co-culture.

Surface markers	Description	Expression in primary pDCs
HLA-DR	MHC (major histocompatibility complex) class II cell surface receptor.	+
CD123	70 kD transmembrane α chain of the IL-3 receptor usually identify as the specific marker for plasmacytoid dendritic cells.	+
CD44	Cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration.	+
CD11c	α integrin glycoprotein classically employed to define myeloid dendritic cells.	-
CD29	(Integrin beta-1) broadly expressed on a majority of hematopoietic and non-hematopoietic cells.	+
CD45	Known as lymphocyte common antigen, it is a receptor-linked protein tyrosine phosphatase that is expressed on all leucocytes.	+
CD4	Glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells.	+
CD86	Protein expressed on antigen-presenting cells that provides costimulatory signals necessary for T cell activation and survival.	+
CD80	Known as B7, type I membrane protein that belongs to the immunoglobulin superfamily. It is a costimulatory molecule known for its role in T-cell activation and also in regulating normal and malignant B cells activity.	+

Table IV.1 Overview table depicting some of the surface markers present (+) or not (–) on the surface of primary pDCs.

The analysis was conducted at different growing times: soon after the thawing and after two months in order to be sure of the identity of the cells in our hands. Independently from the time

spent in culture, GEN2.2 like primary pDCs were characterized by the expression of CD4, the main cellular receptor mediating HIV binding in pDCs, HLA-DR, CD123, CD44, CD29 and CD45, which is not expressed in MS-5 cells. Instead, GEN2.2 were negative for CD11c, a myeloid dendritic cell marker. Moreover, they expressed high levels of CD86, whereas CD80 was undetectable (Fig. IV.7). For the experiments only the $CD45^{+}$ non-adherent fraction of the culture was used.

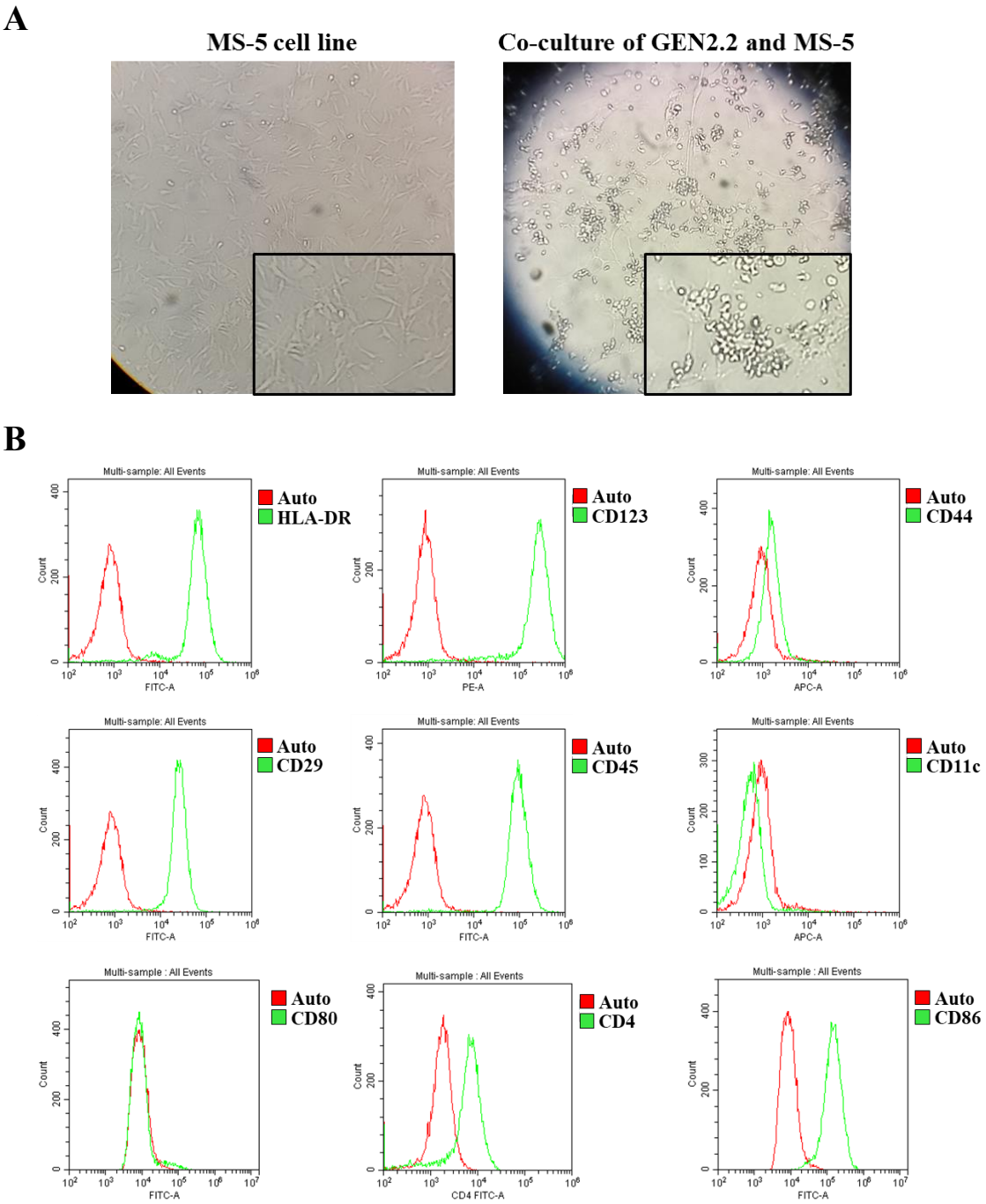
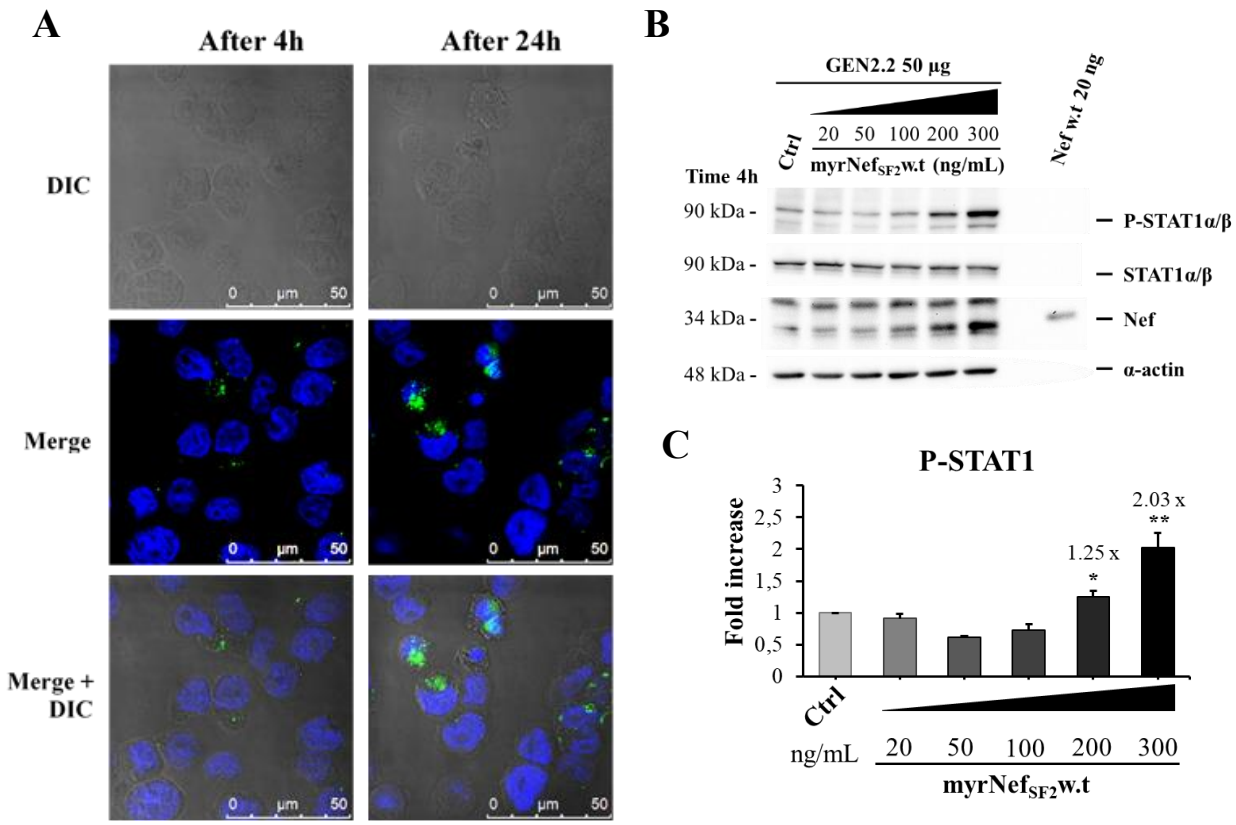


Figure IV.7 Morphology and phenotype of GEN2.2 cell line. (A) Representative images of MS-5 stromal cell line used as feeder layer (left) and of GEN2.2 during the culture on irradiated MS-5 cells (right). At the bottom right of both images, a magnified detail is shown. (B) Phenotypic characteristics of fresh GEN2.2 cells analysed by flow cytometry. For surface staining, 0,3 x 10^6 cells were harvested from the culture and processed as reported in Materials

and Methods section. The autofluorescence of the cells is indicated in red, whereas the expression of the specific markers in green. A representative analysis, out of three independent analyses that yielded similar results, is shown.

As previously performed in primary pDCs, we evaluated the internalization of Nef protein by treating GEN2.2 with myrNef$_{SF2}$ conjugated with AlexaFluor488 and observing them at different time points (Fig. IV.8A). As shown by confocal images, myrNef$_{SF2}$ was taken up by the cells already after 4 hours and its uptake was increased after 20 hours without a significant variation of the percentage of cells that internalized the protein. Importantly, the analysis of several fields (for a total of about 2000 cells) revealed that approximately 50% of GEN2.2 turned out to internalize the protein, but with different efficiency. To further confirm the internalization observed by confocal microscopy, a western blot analysis was performed (Fig. IV.8B). To this end, GEN2.2 were treated with increasing concentrations of myrNef$_{SF2}$w.t for 4 hours, time corresponding to the initial entry of the protein.



IV.8 Internalization of Nef protein in GEN2.2. (A) Confocal microscopy analysis of GEN2.2 seeded at 0.1 x10^6 cells/150 µl and treated for 4h and 24h with 300 ng/mL of myrNef$_{SF2}$w.t conjugated with AlexaFluor488 (green). Afterwards, cells were placed on a microscope slide and fixed in PFA 4%. Samples were mounted with Vectashield antifade mounting medium containing DAPI to visualize nuclei (blue). Images were acquired with the confocal microscope Leica TCS SP5 and processed with the software LAS AF version 1.6.3 (Leica Microsystems). Objective 63.0X. DIC: Differential Interference Contrast. Scale bars 0-50 µm. The images are representative of two independent experiments. (B) Representative western blot is shown. 2x10^6 cells were treated with increasing concentrations of myrNef$_{SF2}$w.t for 4h. Cell lysates (50 µg) were resolved on 11% SDS-PAGE gel and the immunoblotting was performed using a pospho-Tyr(701)-STAT1 and Nef specific antibody. Anti-α-actin was used as internal control of the loaded samples. (C) P-STAT1 was normalized to the actin level by densitometric analysis and expressed as fold

increase with respect to the control. The fold increase was reported as mean ± S.D. of three independent experiments. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs respective Ctrl (untreated cells).

The extent of the protein inside the cellular extract correlated with Nef input. Remarkably, the viral protein was detectable only starting from a concentration of 200 ng/mL. Moreover, we observed that GEN2.2 treated with 300 ng/mL of myrNef$_{SF2}$w.t presented a good amount of the protein inside the cells and responded more strongly. Hence, the following experiments were performed using this concentration of the protein. Considering this result, we can infer that pDCs are less sensitive to Nef treatment comparing to macrophages, which have been reported to be stimulated with lower concentrations of the viral protein [Federico et al., 2001; Mangino et al., 2007]. A possible explanation is that pDCs possess a poor endocytic ability, thus they internalize less protein. However, these data are in agreement with what observed in primary cells and, together with the flow cytometry analysis of surface markers, contribute to validate GEN2.2 cell line as an appropriate model for studying the cell signalling induced by Nef in pDCs.

IV.5 HIV-1 myrNef$_{SF2}$ protein induces the release of STAT-1 and -2 activating factors.

Once validated the system, we attempted to define how GEN2.2 reacted to Nef treatment. Considered the preliminary results obtained in primary pDCs, we focused our attention on the analysis of the STAT pathways involved in the cytokine/chemokine response, including IFNs. In particular, the effect of the viral protein was assessed evaluating the tyrosine phosphorylation of (Y701) STAT1 and (Y689) STAT2 proteins. To this aim, GEN2.2 were treated at different time intervals with 300 ng/mL of myrNef$_{SF2}$w.t or the mutant myrNef$_{SF2}$4EA, whose acidic cluster domain at amino acids (aa) 66 to 69 was inactivated by the substitution with four alanines, in order to define whether this domain was or not important for this signalling as it is in macrophages [Mangino et al., 2011].

Western blot analyses revealed that myrNef$_{SF2}$w.t, but not the mutant, induced the tyrosine phosphorylation of STAT1 (Y701) and STAT2 (Y689) starting from 3 hours and that the signal persisted up to 6 hours (Fig. IV.9A and B). Of note, wild type Nef induced also an increase in the levels of STAT1-α/β proteins, which became clearly detectable 20 hours after the treatment and the increase persisted up to 40 hours (Fig. IV.9B). Moreover, to assess whether Nef-induced STAT1 and STAT2 activation influenced also gene induction, we evaluated the expression of IRF-1 and ISG15.

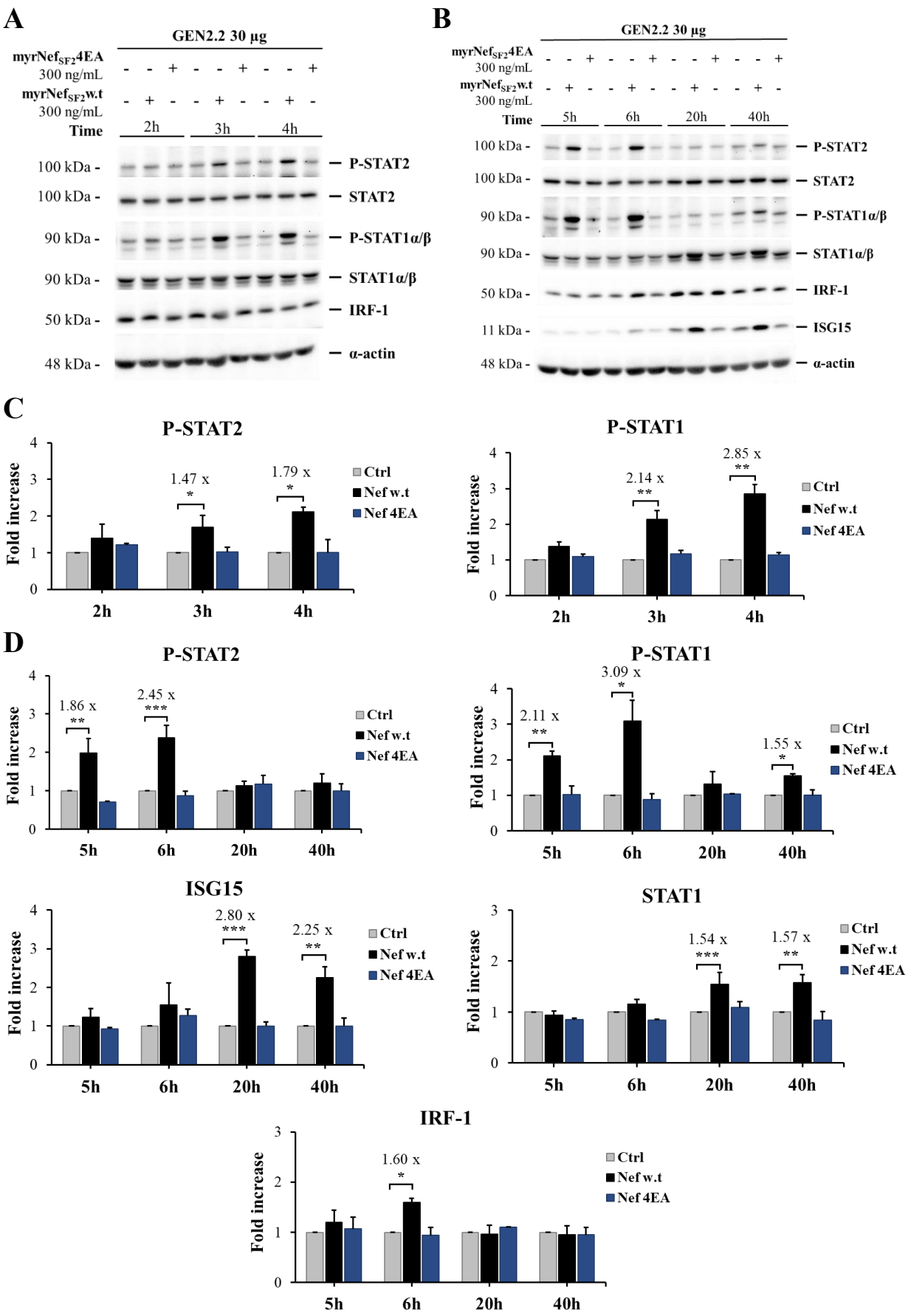


Figure IV.9 HIV-1 myrNef$_{SF2}$w.t protein induces STAT-1 and -2 activation and increased expression of proteins transcriptionally regulated by their activation (*i.e.*, STAT-1, IRF-1 and ISG15). GEN2.2 were seeded at 2x10^6

cells/each sample in 24 well plate and treated with 300 ng/mL of myrNef$_{SF2}$w.t. or myrNef$_{SF2}$4EA or left untreated (Ctrl) for the indicated time points. Cells were lysed and 30 µg of each sample were run on 9%-13.5% SDS-PAGE gel. (A, B) Representative western blots are shown. Anti-α-actin was used as internal control of the loaded samples. (C, D) The expression of P-STAT1, P-STAT2, ISG15 and IRF-1 were normalized to actin by densitometric analysis and expressed as fold increase compared to the respective control. Histograms: fold increase reported as mean ± S.D. of three independent experiments. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs respective Ctrl.

IRF-1 is an interferon regulatory factor encoded by a gene transcriptionally regulated by STAT1 activation that is transiently up-regulated by type I IFN and persistently up-regulated by type II IFN. ISG15 is an ubiquitin-like modifier and, although it was first identified studying type I IFNs-treated cells [Haas et al., 1987], it has been reported to be induced also by type III IFNs, viral and bacterial infections as well as lipopolysaccharide (LPS), indicating that the expression of ISG15 represents a central host response to pathogenic stimuli. It exists as a 17-kDa precursor protein that is rapidly processed into its mature 15-kDa form via protease cleavage to expose a carboxy-terminal motif, which allows the covalent binding of ISG15 to target proteins by a three-step process referred to as ISGylation [Perng and Lenschow, 2018]. Moreover, ISG15 exists as an unconjugated protein that can be released into the extracellular milieu via non-conventional secretion, including exosomes [Sun et al., 2016]. The unconjugated form of ISG15 is the one that was analysed in the figure (Fig. IV.9).

As shown, wild type Nef, but not 4EA mutant, increased IRF-1 amount only after 6 hours, whereas ISG15 production was induced starting between 6 and 20 hours and persisted also at 40 hours.

Interestingly, a further analysis of the nuclear and cytoplasmic fractions revealed that the unconjugated form of ISG15 localized only in the cytoplasmic fraction in cells treated with Nef w.t (Fig. IV.10). Instead, ISG15 was detectable also in the nuclear fraction in cells treated with type I or -III IFN, although in lesser amount with respect to the cytoplasmic fraction. As expected, type II IFN (*i.e.*, IFN-γ) did not induce ISG15.

Since STATs are typically activated in few minutes as a consequence of the JAK phosphorylation that follows the engagement of specific cytokines, chemokines or growth factors to their specific receptors, overall these results suggest that pDCs are stimulated by wild type Nef to release/produce cytokines, including type I or type III IFNs. In fact, these two types of IFN are the only cytokines known to be able to induce tyrosine phosphorylation of STAT2 and ISG15 production. Since myrNef$_{SF2}$4EA is not able to induce the tyrosine phosphorylation of STAT1 and STAT2 proteins and not even IRF-1 or ISG15 production, the acidic domain must have a crucial role in the effect induced by the viral protein here identified, confirming the results previously conducted on primary macrophages in our laboratory [Mangino et al., 2007 and 2011].

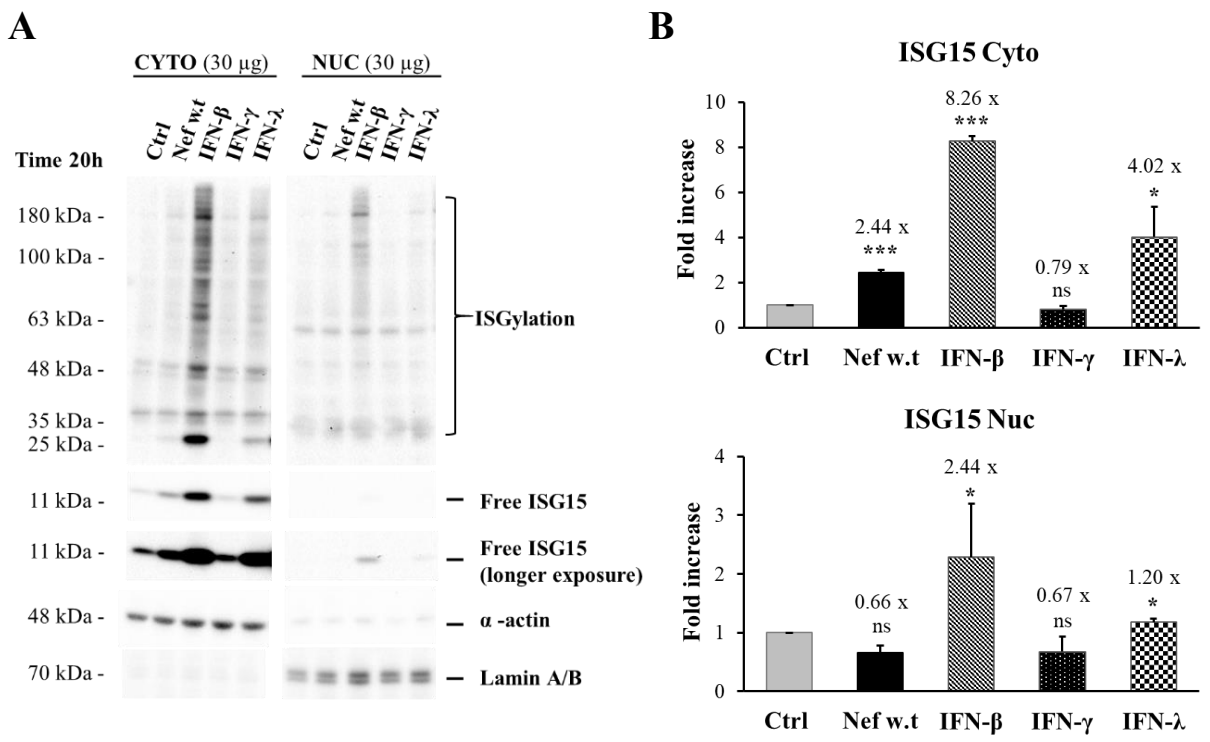


Figure IV.10 HIV-1 myrNef$_{SF2}$w.t protein induces the production of ISG15, which is mainly localized in the cytoplasmic fraction. A total of 4x10^6 GEN2.2 were treated with myrNef$_{SF2}$w.t (300 ng/mL), IFN-β (1000 IU/mL), -γ (100 ng/mL) or –λ1/ λ2 (100 ng/mL) or left untreated (Ctrl) for 20 hours. Cells were lysed and 30 µg of each sample were run on 9%-13.5% SDS-PAGE gel. (A) Representative western blot is shown. (B) The expression of unconjugated ISG15 in the cytoplasmic (Cyto) and nuclear (Nuc) fraction were normalized to actin or lamin A/B respectively by densitometric analysis and expressed as fold increase compared to the respective control. Data shown are representative of three independent experiments. Histograms: mean ± S.D. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs respective Ctrl.

IV.6 HIV-1 Nef protein activates pDCs without modifying the expression of co-stimulatory molecules.

According to the specific signals received, pDCs can acquire two different phenotypes: they can produce large amount of type I IFN, hence acting as IPCs, or they can act as APCs contributing to the T-cell mediated adaptive immune responses [Honda et al., 2005]. Considered that, we wondered whether Nef treatment could modulate the expression of some surface markers, including secondary antigen-presenting-molecules (co-stimulatory molecules) such as CD40, CD80 and CD86, usually accompanying the activation or maturation of plasmacytoid dendritic cells making them APCs as powerful as dendritic cells of myeloid origin for activating T cells [Lande et al., 2010; Schuster et al., 2009]. To this end, GEN2.2 were treated with myrNef$_{SF2}$w.t or its mutant myrNef$_{SF2}$4EA, whereas LPS treatment (100 EU/mL) was used as positive control. After 20 hours, cells were harvested and processed for the staining to analyse the surface phenotype by flow cytometry (Fig. IV.11). As shown, neither Nef w.t nor its mutant modulated the expression

of the analysed molecules. On the contrary, LPS induced a slight increase of CD86 and CD40, whereas CD38 and CD80 remained unchanged. Considering this result, we can speculate that Nef protein promotes pDCs to become preferentially IPCs rather than APCs.

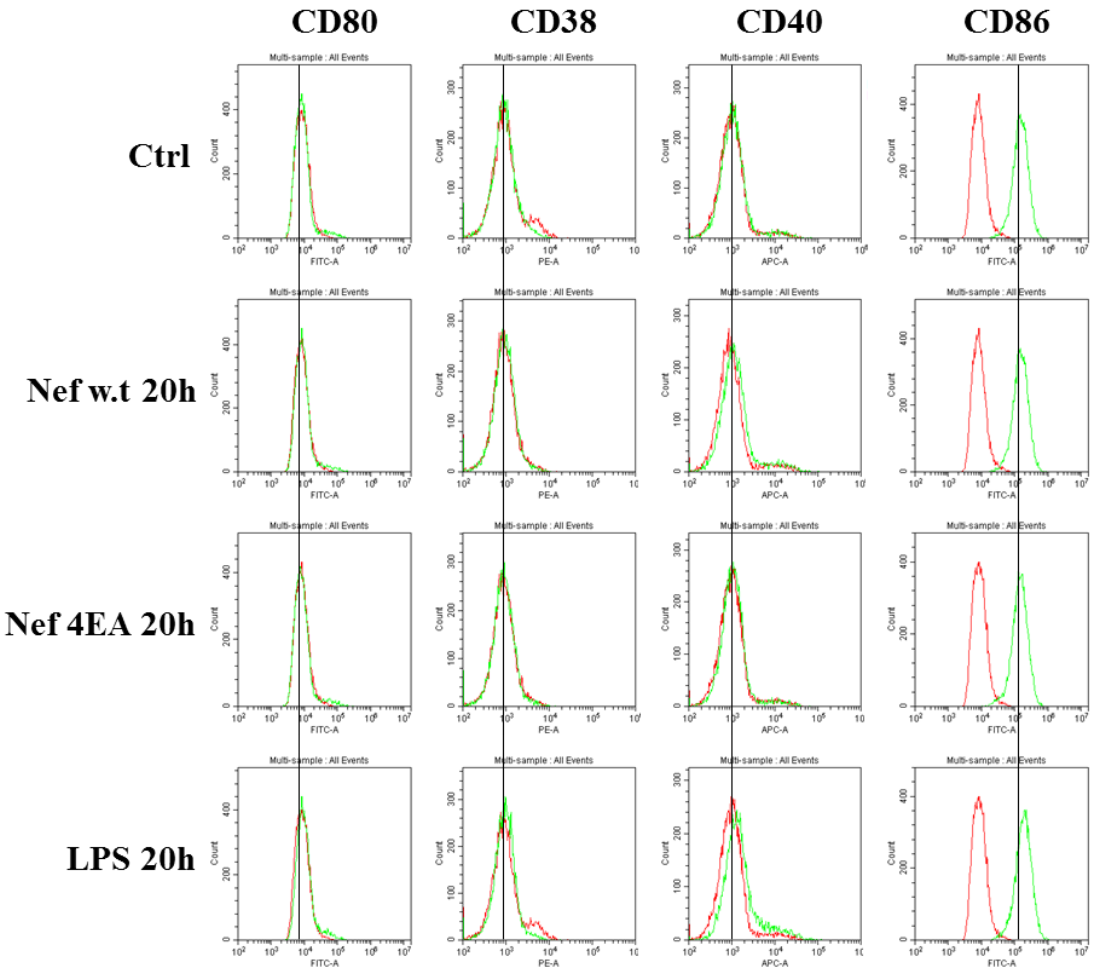


Figure IV.11 HIV-1 myrNef$_{SF2}$ protein and its mutant do not modify the APC immunophenotype of GEN2.2 cell line. A total of 0.3x10^6 GEN2.2 were treated with 300 ng/mL of myrNef$_{SF2}$w.t or myrNef$_{SF2}$4EA for 20 hours. The treatment with LPS (100 EU/mL) was used as positive control. The expression of molecules associated with activation or maturation of plasmacytoid dendritic cells on control and treated cells was analysed by flow cytometry. The autofluorescence of the cells is indicated in red, whereas the expression of the specific markers in green. A representative result, out of three independent experiments that yielded similar results, is shown.

IV.7 Cytokine/chemokine production induced by wild type Nef in GEN2.2 and comparison with the production in differentiated THP-1.

The next step was determining the secreted cytokines/chemokines using the Bio-Plex Pro Human Cytokine 27-Plex Immunoassay able to detect up to 27 cytokines. To this end, GEN2.2 were left untreated or treated with 300 ng/mL of myrNef$_{SF2}$w.t and a time course analysis was performed on supernatants harvested within a 20 hours interval in order to evidence possible time differences in the release of the analysed cytokines/chemokines.

Cytokines/ Chemokines	M.W	GEN2.2									THP-1/PMA		
		4h			6h			20h			20h		
		Ctrl	Nef w.t	Fold increase	Ctrl	Nef w.t	Fold increase	Ctrl	Nef w.t	Fold increase	Ctrl	Nef w.t	Fold increase
PDGF-bb	24300	19,04	19,04	1,00	19,04	144,69	7,60	739,56	732,01	0,99	56,68	364,81	6,44
IL-1β	17300	0,02	0,56	28,00	0,02	0,70	35,00	0,24	0,49	2,04	4,18	76,73	18,36
IL-1ra	16142	4,67	4,67	1,00	4,67	4,67	1,00	4,67	4,67	1,00	8153,02	12614,6	1,55
IL-2	15300	16,96	63,03	3,72	21,93	65,61	2,99	44,11	88,29	2,00	11,13	249,07	22,38
IL-4	17492	0,40	2,29	5,80	0,40	3,81	9,65	0,40	3,52	8,91	5,95	17,74	2,98
IL-5	26500	0,80	0,80	1,00	0,80	6,04	7,60	0,80	8,65	10,88	28,80	62,21	2,16
IL-6	21000	0,12	0,27	2,35	0,12	1,30	11,30	0,12	1,72	14,96	0,29	6,01	20,72
IL-7	24000	2,02	2,02	1,00	2,02	2,02	1,00	2,02	2,02	1,00	2,02	3,11	1,54
IL-8	8400	1,10	189,57	172,34	4,29	247,08	57,59	8,19	226,87	27,70	46374,55	144898	3,12
IL-9	35000	0,41	0,41	1,00	0,41	0,41	1,00	0,41	1,44	3,49	21,86	39,41	1,80
IL-10	20516	0,38	0,38	1,00	0,38	0,38	1,00	0,38	0,38	1,00	0,52	7,59	14,60
IL-12 (p70)	70000	0,07	0,23	3,44	0,07	0,21	3,14	0,07	0,07	1,00	1,18	11,69	9,91
IL-13	17000	0,28	0,28	1,00	0,28	0,28	1,00	0,28	0,28	1,00	0,28	0,42	1,53
IL-15	12900	8,19	8,19	1,00	8,19	8,19	1,00	8,19	8,19	1,00	8,19	92,47	11,29
IL-17	15220	0,37	8,75	23,81	0,37	12,66	34,45	1,61	10,44	6,48	20,95	69,06	3,30
Eotaxin	8400	0,57	2,45	4,30	0,42	2,90	6,90	1,12	2,80	2,50	7,40	21,06	2,85
FGF basic	21000	10,95	38,66	3,53	17,01	45,70	2,69	26,29	37,13	1,41	41,42	96,71	2,33
G-CSF	21000	1,91	192,70	100,89	1,91	350,97	183,75	1,91	125,91	65,92	551,58	520,88	0,94
GM-CSF	24500	0,08	0,08	1,00	0,08	0,08	1,00	0,08	0,08	1,00	1,87	5,34	2,86
IFN-γ	34000	9,28	49,50	5,33	12,08	117,81	9,75	13,39	146,68	10,95	397,39	568,14	1,43
IP-10	8600	10,97	3050,26	278,05	164,65	10882,16	66,09	244,28	27773,64	113,70	11792,30	158825	13,47
MCP-1 (MCAF)	8700	63,36	398,46	6,29	87,20	1069,96	12,27	98,56	1142,57	11,59	2946,35	3451,46	1,17
MIP-1α	7500	0,47	43,74	93,06	0,47	61,47	130,79	0,61	16,52	27,08	1022,40	1346,85	1,32
MIP-1β	7800	0,97	216,00	222,68	1,49	301,08	202,07	2,35	104,68	44,54	773,50	36835,8	47,62
RANTES	7800	0,93	6,27	6,78	0,93	8,43	9,11	4,72	7,45	1,58	6415,60	14757,5	2,30
TNF-α	22000	465,44	2112,57	4,54	607,45	2355,38	3,88	1376,11	2850,31	2,07	123,18	13292,6	107,91
VEGF	42000	179,71	179,71	1,00	179,71	179,71	1,00	9675,55	9903,38	1,02	4494,88	10050,3	2,24

Table IV.2 Cytokines/chemokines induced by Nef protein in GEN2.2 and THP-1/PMA. The reported data were obtained using the Bio-Plex Pro Human Cytokine 27-Plex Immunoassay and are related to the analysis of supernatants harvested after 20 hours from GEN2.2 seeded at the density of $1x10^6$ /ml in 24-well plates (a total of $2x10^6$ cells/well) and from THP-1/PMA seeded at 100.000 cells/cm^2 in 6-well plates (a total of $1x10^6$ cells/well). Both cell types were left untreated or treated with 300 ng/ml of myrNef_{SF2}w.t in a final volume of 2 ml. Values evidenced by intense blue or red indicate the cytokines/chemokines secreted in major amounts by GEN2.2 or THP-1/PMA, respectively in control and treated cells. In the scale gray the fold increase of each cytokine/chemokine is indicated. The reported values are the mean of triplicates of one out of two independent experiments that yielded similar results, and are expressed in pg/mL.

As shown (Table IV.2), Nef induced the production of regulatory cytokines (IL-2), growth factors (FGF basic and G-CSF), chemotactic factors e/o pro-inflammatory mediators (MCP-1, IL-8, IP-10, MIP-1α, MIP-1β, IFN-γ and TNF-α), although at different extent. The low induction of TNF-α detected is in agreement with what reported in literature where HIV-1 detection by primary pDCs results in abundant IFN production, but low NF-κB-dependent production of TNF-α [O'Brien et al., 2011].

Other mediators such as IL-4, IL-5, IL-17, Eotaxin and RANTES resulted to be only poorly secreted in response to Nef treatment. On the other hand, some immune mediators such as PDGF-bb and VEGF were highly secreted by control cells but Nef treatment did not affect their production. Other soluble factors were not detected neither in untreated cells.

Taking into consideration the time course of expression, we noticed that some factors, such as IL-2, IFN-γ, IL-8, MCP-1, IP-10 and TNF-α increased over time and peaked at 20 hours. Conversely, G-CSF, MIP-1α, MIP-1β reached their peak of expression after 4-6 hours, whereas a reduction of their amount was observed 20 hours after treatment (Fig. IV.12).

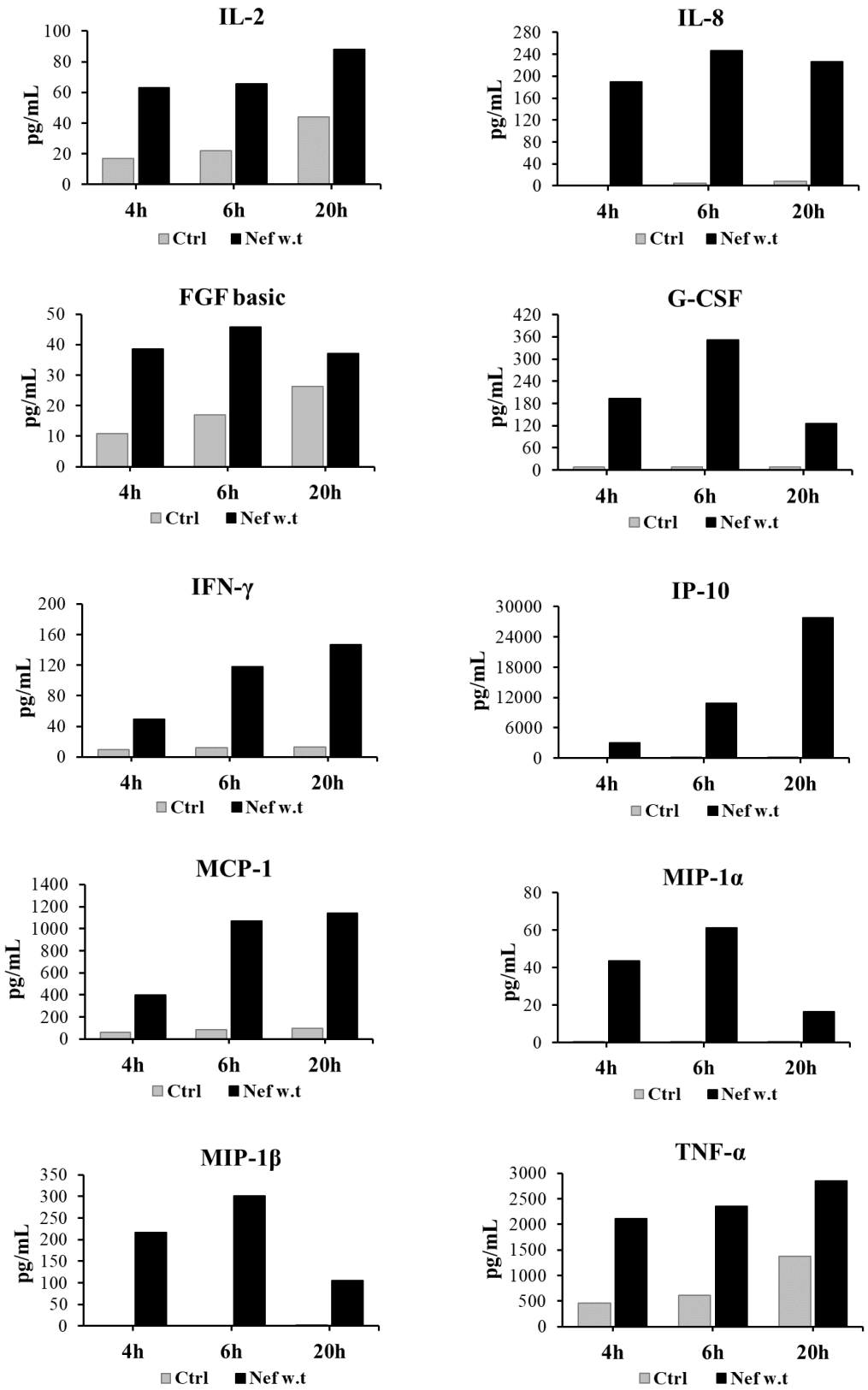


Figure IV.12 Time course production of cytokines/chemokines by GEN2.2 treated with wild type Nef. $2x10^6$ GEN2.2 were seeded in 24-well plates and left untreated (Ctrl) or treated with 300 ng/mL of myrNef_{SF2}w.t in a final volume of 2 ml. Supernatants were collected after 4, 6 and 20h. Data are expressed in pg/mL.

In addition, considering the cytokines/chemokines most relevant in terms of pg produced compared to the total, we noticed that those most secreted changed over time in both untreated and treated cells. Interestingly, some factors such as PDGF, IL-2, FGF basic, IP-10, MCP-1, TNF-α and VEGF were already detectable at medium-high levels in untreated GEN2.2 and increased over time, whereas the values of other cytokines/chemokines were almost undetectable. Importantly, after treatment with myrNef$_{SF2}$w.t, IP-10, known as Interferon gamma induced protein, was the cytokine mostly secreted independently by the time interval (Fig. IV.13).

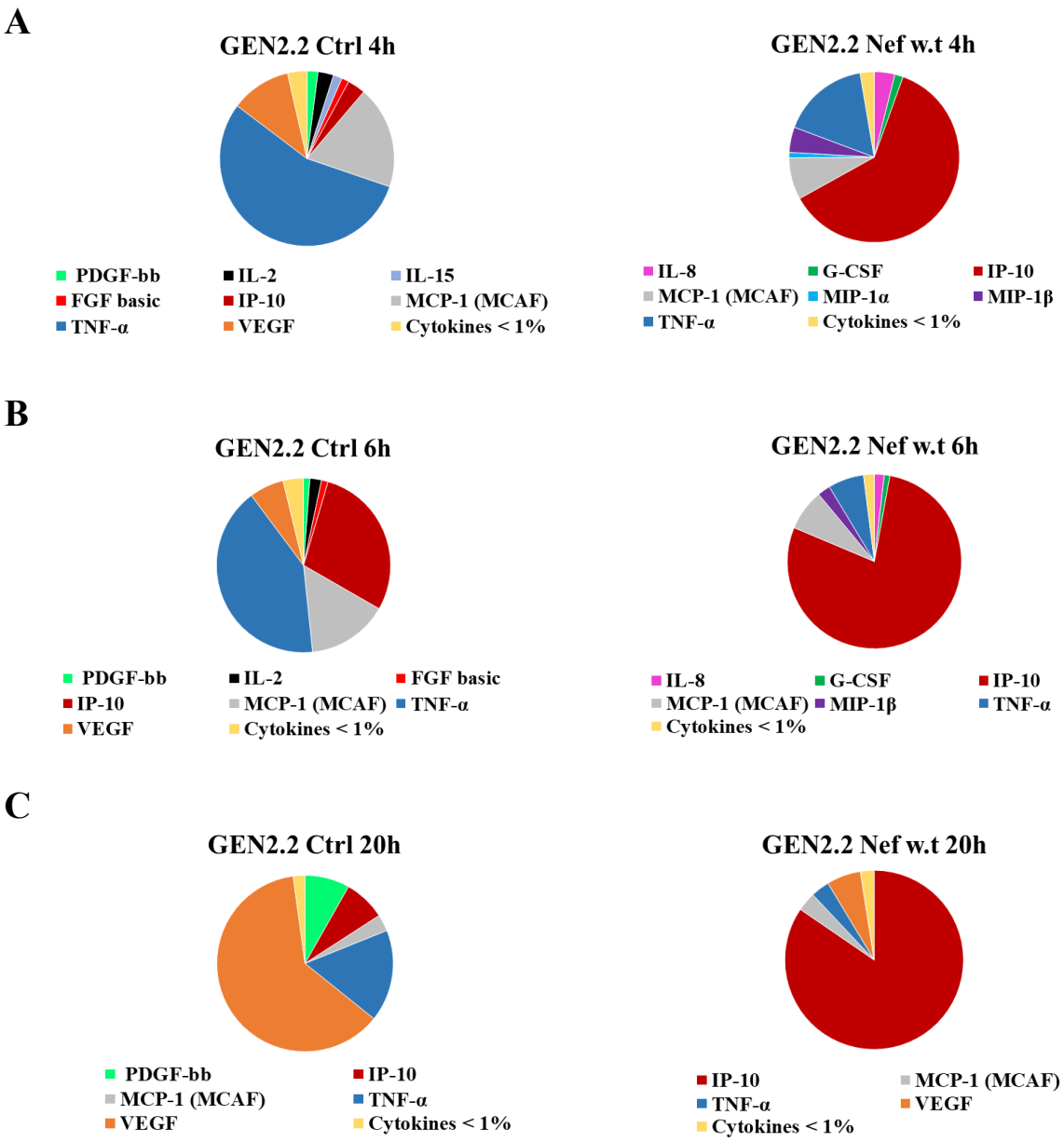


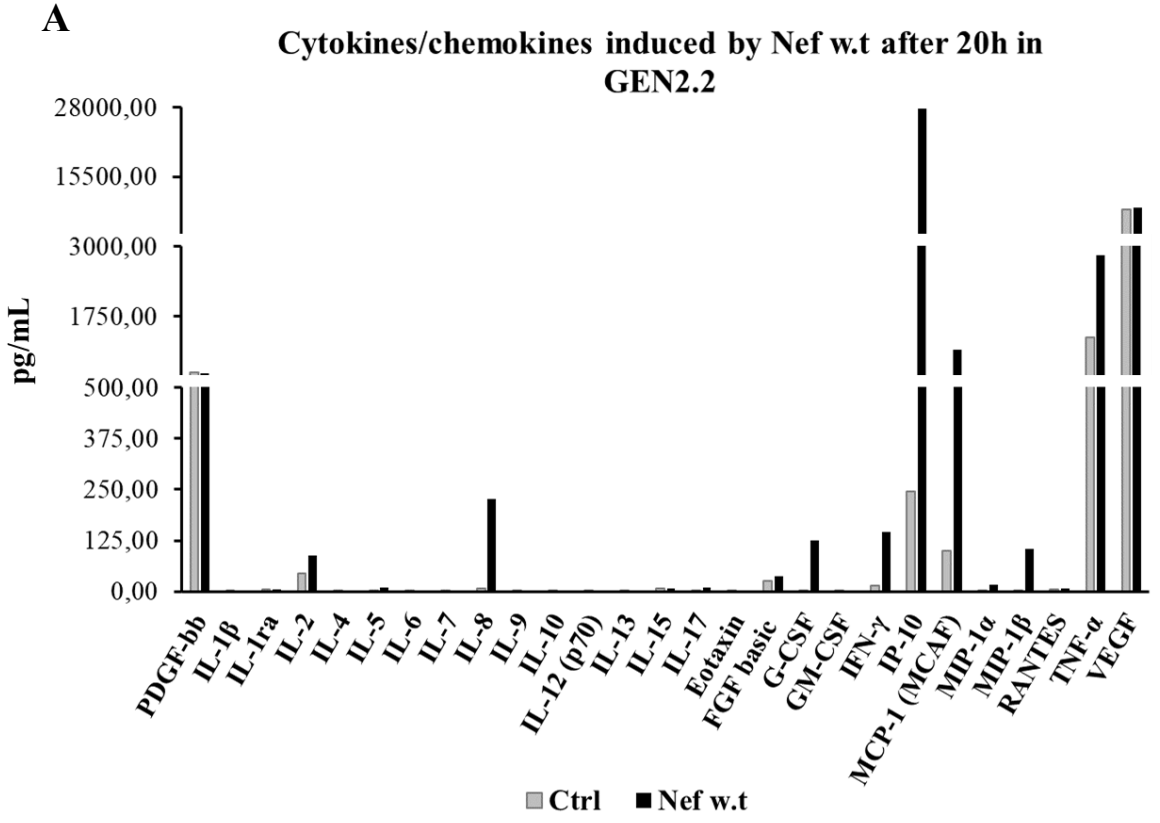
Figure IV.13 The profile of cytokines/chemokines most secreted by GEN2.2 changed over time in both treated and untreated (Ctrl) cells. Pie charts report the cytokines/chemokines more represented in terms of molarity, since their different molecular weights can influence their activity. The analysis was conducted at different time points: 4h (A), 6h (B) and 20h (C). For each experimental condition, only the cytokines/chemokines that reached a percentage

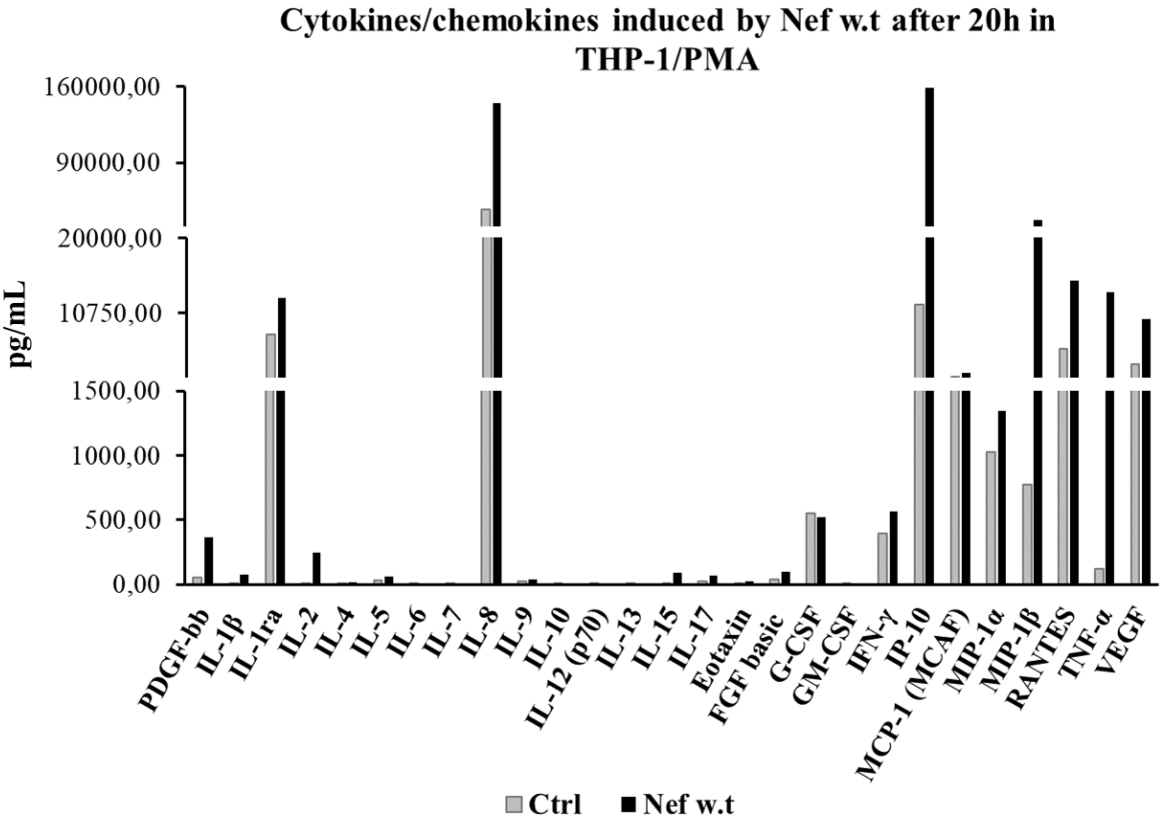
≥1 were indicated. The percentage was obtained dividing the molarity of each cytokine/chemokine, which was calculated according to the molecular weight, by the total molarity.

Subsequently, we were interested in evaluating whether and in what extent the cytokines/chemokines released in response to Nef treatment by GEN2.2 differed respect to those secreted by macrophages, a cell population widely known as one of the major reservoir of HIV. Our research group already observed that in primary macrophages Nef induced pro-inflammatory cytokines such as MIP-1β, IL-6, IL-1β, TNF-α and IFN-β. [Olivetta et al., 2003]. However, in this context we analysed the expression of a greater panel of cytokines/chemokines on THP-1 monocytic cell line differentiated with 35nM of PMA for 32 hours in order to acquire a macrophage-like phenotype that mimicked primary human macrophages. After one day of resting, THP-1/PMA were left untreated or treated with myrNef$_{SF2}$w.t and the supernatants were collected after 20 hours. It is noteworthy that the profile of cytokines/chemokines induced by Nef in GEN2.2 was different from that observed in differentiated THP-1 (Fig. IV.14). In particular, Nef did not affect or only weakly the amount of IL-8, G-CSF and MCP-1 in THP-1/PMA compared to GEN2.2. Moreover, Nef promoted the secretion of some cytokines weakly induced or not produced by GEN2.2, such as PDGF, IL-1β, IL-15, IL-17, RANTES and VEGF. As observed in GEN2.2, the viral protein stimulated THP-1/PMA to release a huge amount of IP-10 (Fig. IV.14), although at different extent compared to GEN2.2. Unlike GEN2.2, TNF-α was strongly induced by Nef treatment in THP-1/PMA (Fig. IV.14). Interestingly, not only plasmacytoid dendritic cells and macrophages presented a different panel of cytokines/chemokines induced by Nef, but they differed also for the amount produced by treated and control cells. Indeed, regardless of the type of soluble factor considered, in THP-1/PMA most of the cytokine/chemokine basal levels were much higher than in GEN2.2. In particular, IL-1ra, IL-8, G-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES were the cytokines/chemokines that reached the higher levels. Considering the above, it is possible to hypothesize that the high basal levels of some soluble factors might limit their further increase in response to Nef treatment.

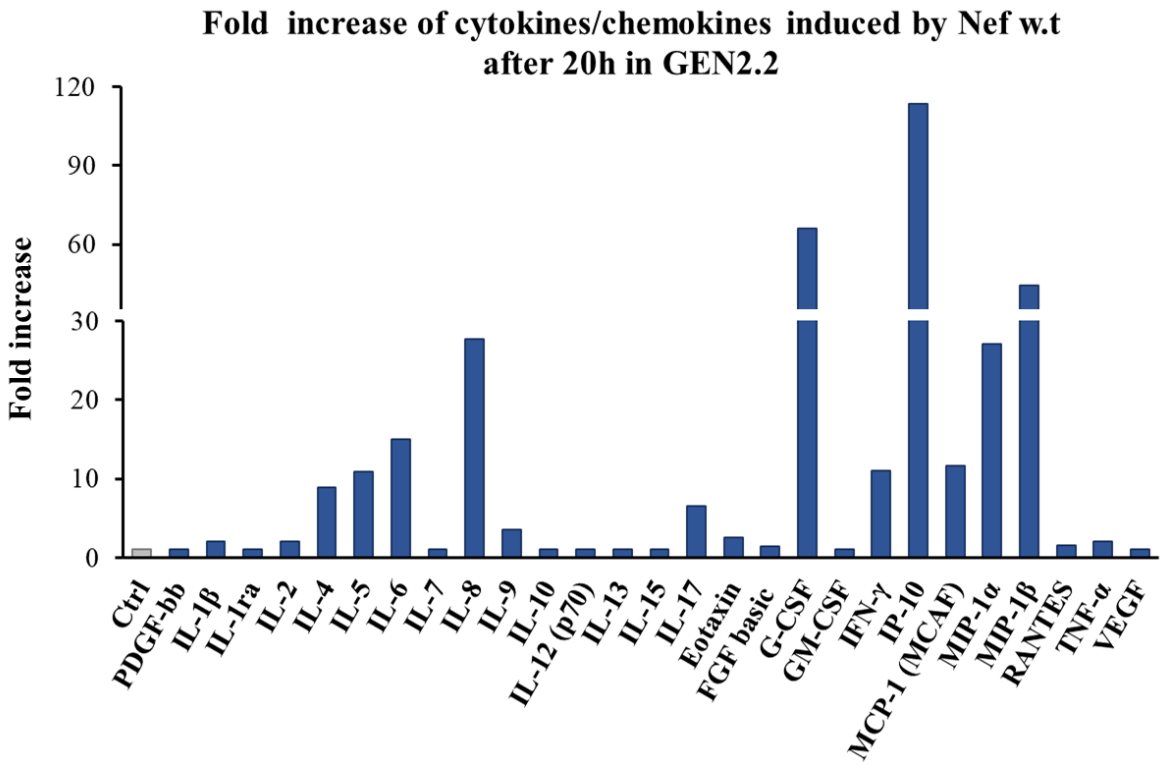
In conclusion, we can state that Nef treatment modifies the secretome of both plasmacytoid dendritic cells and macrophages but in a different manner.

A


Cytokines/chemokines induced by Nef w.t after 20h in GEN2.2
pg/mL
28000,00
15500,00
3000,00
1750,00
500,00
375,00
250,00
125,00
0,00
PDGF-bb
IL-1β
IL-1ra
IL-2
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-12 (p70)
IL-13
IL-15
IL-17
Eotaxin
FGF basic
G-CSF
GM-CSF
IFN-γ
IP-10
MCP-1 (MCAF)
MIP-1α
MIP-1β
RANTES
TNF-α
VEGF
Ctrl
Nef w.t


Cytokines/chemokines induced by Nef w.t after 20h in THP-1/PMA
pg/mL
160000,00
90000,00
20000,00
10750,00
1500,00
1000,00
500,00
0,00
PDGF-bb
IL-1β
IL-1ra
IL-2
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-12 (p70)
IL-13
IL-15
IL-17
Eotaxin
FGF basic
G-CSF
GM-CSF
IFN-γ
IP-10
MCP-1 (MCAF)
MIP-1α
MIP-1β
RANTES
TNF-α
VEGF
Ctrl
Nef w.t

B



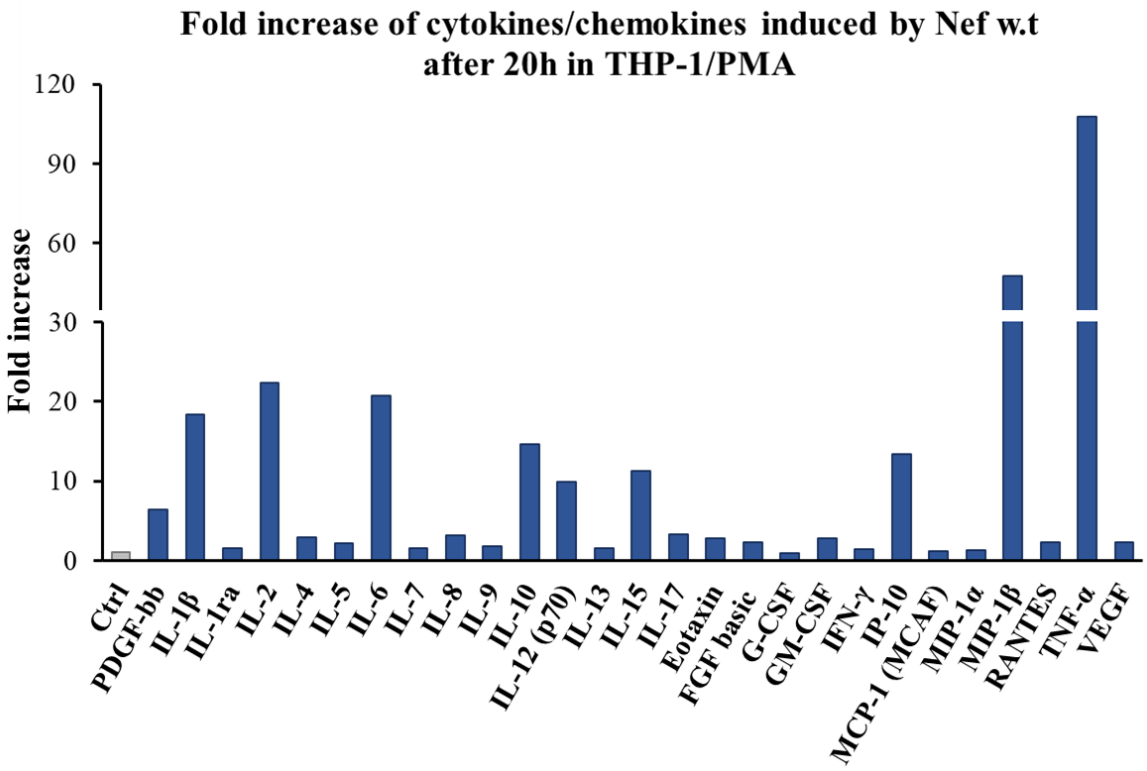


Figure IV.14 Secretome of GEN2.2 and THP-1/PMA in response to Nef treatment. GEN2.2 were seeded at the density of $1x10^6$/mL in 24-well plates, whereas THP-1/PMA were seeded at 100.000 cells/cm^2 in 6-well plates. Both cell types were left untreated or treated with 300 ng/mL of $myrNef_{SF2}$w.t in a final volume of 2 mL. Supernatants were collected after 20h, centrifuged at 290 x g for 3 minutes to remove cells. (A) Data are reported in pg/mL. (B) Data are expressed as fold increase calculated for each cytokine with respect to its own control.

IV.8 Supernatant of GEN2.2 treated with myrNef$_{SF2}$w.t stimulates an early response of other pDCs independently of the extracellular vesicles (EVs) content.

During HIV infection, pDCs are exposed to the local microenvironment that is influenced by infected cells. In this context, their activation is not necessarily caused by the infection with the virus, but it could be the consequence of the interaction with immunostimulatory molecules in the intercellular space. Hence, we wondered whether the cytokine/chemokine milieu released by GEN2.2 in response to Nef was able to stimulate other GEN2.2 not previously treated with the viral protein. To this end, supernatants collected after 20 hours from GEN2.2 cultures treated or not with 300 ng/mL of myrNef$_{SF2}$w.t were used to treat new GEN2.2 at different time intervals. Cell lysates were analysed by western blot to evaluate the phosphorylation of STAT1. As shown, GEN2.2 treated with medium conditioned by Nef-treated GEN2.2 induced an early activation of STAT1, already after 30 minutes (Fig. IV.15A). The fact that the activation occurred more rapidly than following Nef treatment (3 hours) excluded that it could depend on the presence of residual Nef.

Although cytokines are generally thought to exert biologic influence as soluble molecules, several cytokines have been reported to be associated with extracellular vesicles (EVs), such as a membrane bound form of TNF-α, chemokines associated with lipid rafts, or cytokines, such as the IL-1 family, which lack a signal peptide for secretion through the classical pathway [Fitzgerald et al., 2018]. Moreover, EV-associated cytokines resulted to be biologically active upon interacting with sensitive cells, thus representing an important system of cell-cell communication in both health and disease. In this regard, in HIV-infection, it was shown that the amount of EV-associated cytokines was increased [Konadu et al., 2015]. In light of these recent observations, we asked whether GEN2.2 would respond in the same way after treatment with supernatants harvested from treated cells but depleted of EVs.

To this aim, EVs were cleared or not by differential ultracentrifugations from supernatants collected from GEN2.2 cultures treated or not for 20 hours with myrNef$_{SF2}$w.t and used to treat new GEN2.2 cultures (Fig. IV.15C and E). Interestingly, supernatants depleted of EVs maintained the capacity to early activate STAT1, already after 30 minutes, and the signal increased over time reaching a peak after 2 hours. These results suggest that EVs might contribute in some way to modulate the time course of the signalling. Moreover, since GEN2.2 were activated also in response to supernatants depleted of EVs, most of the STAT1 activating factors must be secreted in free form.

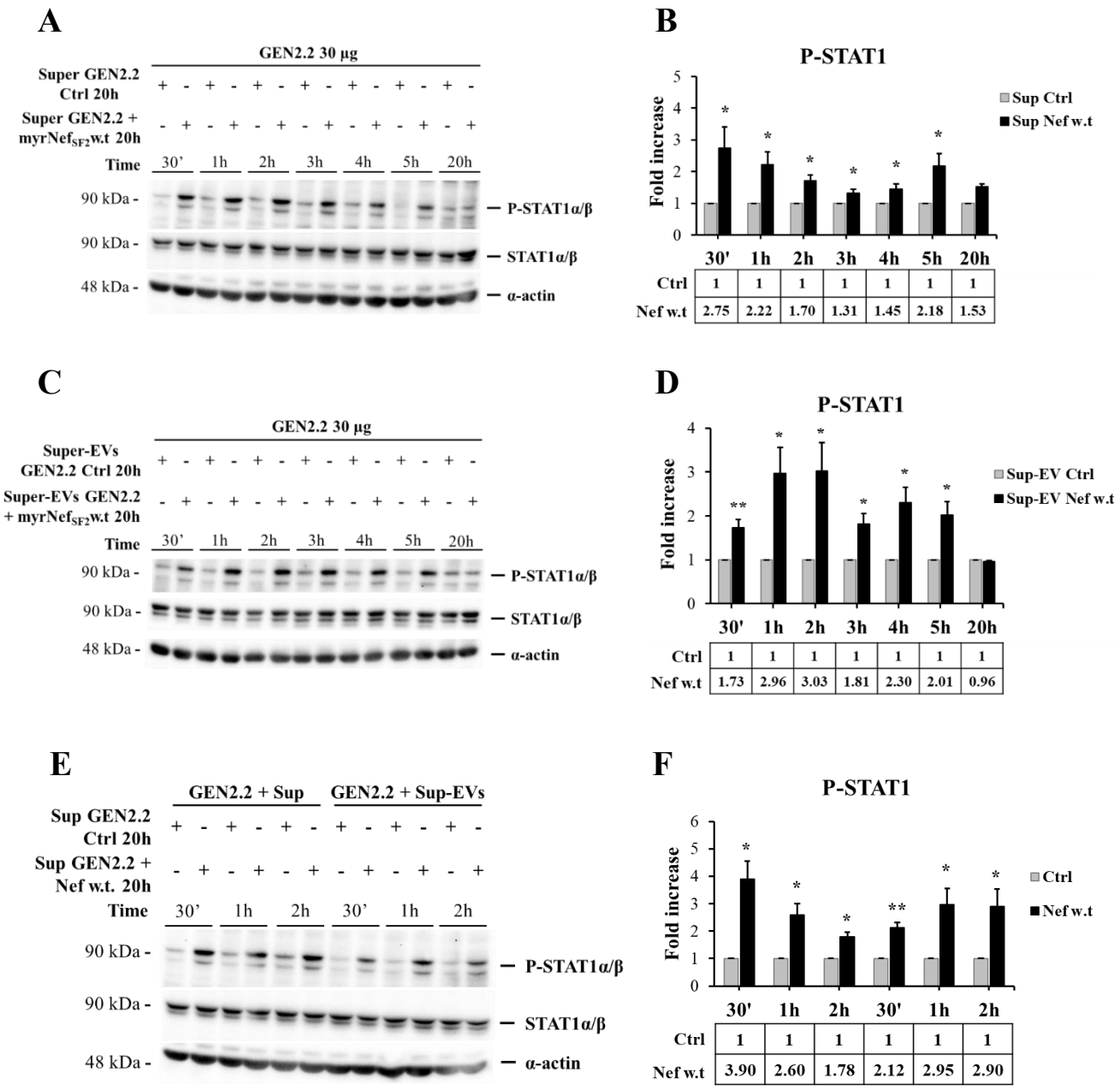


Figure IV.15 Supernatants from Nef-treated GEN2.2 depleted or not of EVs early activate STAT1 tyrosine phosphorylation of GEN2.2. GEN2.2 cells were seeded at $1x10^6$cells/mL in 75 cm^2 flask in 12 mL of final volume and left untreated or treated with 300 ng/mL of myrNef$_{SF2}$w.t. After 20h supernatants from control and treated GEN2.2 were harvested and depleted or not of EVs by ultracentrifugation. Complete supernatants (A) and supernatants depleted of EVs (C) were used to treat fresh GEN2.2 for the indicated time points. (E) Comparison between treatment with supernatant depleted or not of EVs on GEN2.2. Cell lysates (30 µg) were analysed on 9% SDS-PAGE gel and the immunoblottings were performed using a pospho-Tyr(701)-STAT1 specific antibody. (B, D and F) P-STAT1 was normalized to actin by densitometric analysis and expressed as fold increase compared to control. The results are representative of three indipendent experiments. Histograms: mean ± S.D. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs respective Ctrl.

Overall, these data enforce the results previously reported confirming the capacity of Nef to act on pDCs by promoting the release of cytokines/chemokines involved in STAT1 activation and show that pDCs are also promptly responsive to the surrounding extracellular milieu.

IV.9 Set up of the protocol for GEN2.2 cell labelling with Bodipy C_{16}.

The other aspect of plasmacytoid dendritic cells that we wanted to investigate was the production of EVs, which are important for their role in intercellular communication in both physiological and pathological conditions including HIV infection [Yáñez-Mó et al., 2015; Dias et al., 2018]. Considered the relevant amount of cells necessary to isolate a good quantity of EVs and the already known difficulty to isolate sufficient amounts of primary pDCs, the isolation of EVs from primary pDCs would have been a too much great challenge. Therefore, to overcome such a bias, we set up the protocol using the GEN2.2 pDC-like cell line. In particular, we characterized and quantified the exosomes and microvesicles released by GEN2.2 and studied how their release was modulated in response to Nef stimulus and IFNs treatment.

To quantify the EV production, we adopted a methodology developed by Sargiacomo and colleagues [Coscia et al., 2016] based on cell treatment with the commercially available Bodipy C_{16} fatty acid. This latter, upon uptake by the cells, enters the cellular lipid metabolic pathway without affecting the natural lipid metabolism or perturbing the lipid homeostasis inside the cell [Coscia et al., 2016]. As result, labelled cells release exosomes and microvesicles that, because fluorescent, can be examined and quantified with conventional flow cytometry.

To define the optimal conditions for GEN2.2 treatment with the fluorescent lipid, we performed *pulse-chase* experiments. Firstly, cells were pulsed with different concentrations of Bodipy C_{16} for different times and observed by confocal microscopy (Fig. IV.16A). Confocal images showed that the fluorescent probe was taken up by cells very rapidly, just after 15 minutes, and its uptake increased during pulse times. Remarkably, Bodipy C_{16} became more and more concentrated over the time in the perinuclear area, corresponding to the endoplasmic reticulum (ER). To quantify the Bodipy C_{16} uptake, cell fluorescence was measured by flow cytometry (Fig. IV.16B). Independently by the concentration used, we observed that the Bodipy C_{16} uptake reached a plateau between 1-3 hours, thus a time of 2 hours was chosen for cell labelling. However, we did not identify a concentration limit, because independently by the time considered the cells showed a linear uptake, suggesting a capability to continue to internalize the fluorescent lipid even at higher concentrations. The observation of the forward light scatter/SSC profile of Bodipy treated cells by flow cytometry did not evidence any change in the morphological aspect of the cells, thus suggesting the absence of any cellular suffering regardless of the Bodipy concentration. Therefore, for the subsequent analyses we decided to select the two highest concentrations (2.5 and 3.5 µM), whose median fluorescence intensity (MFI) reached high values.

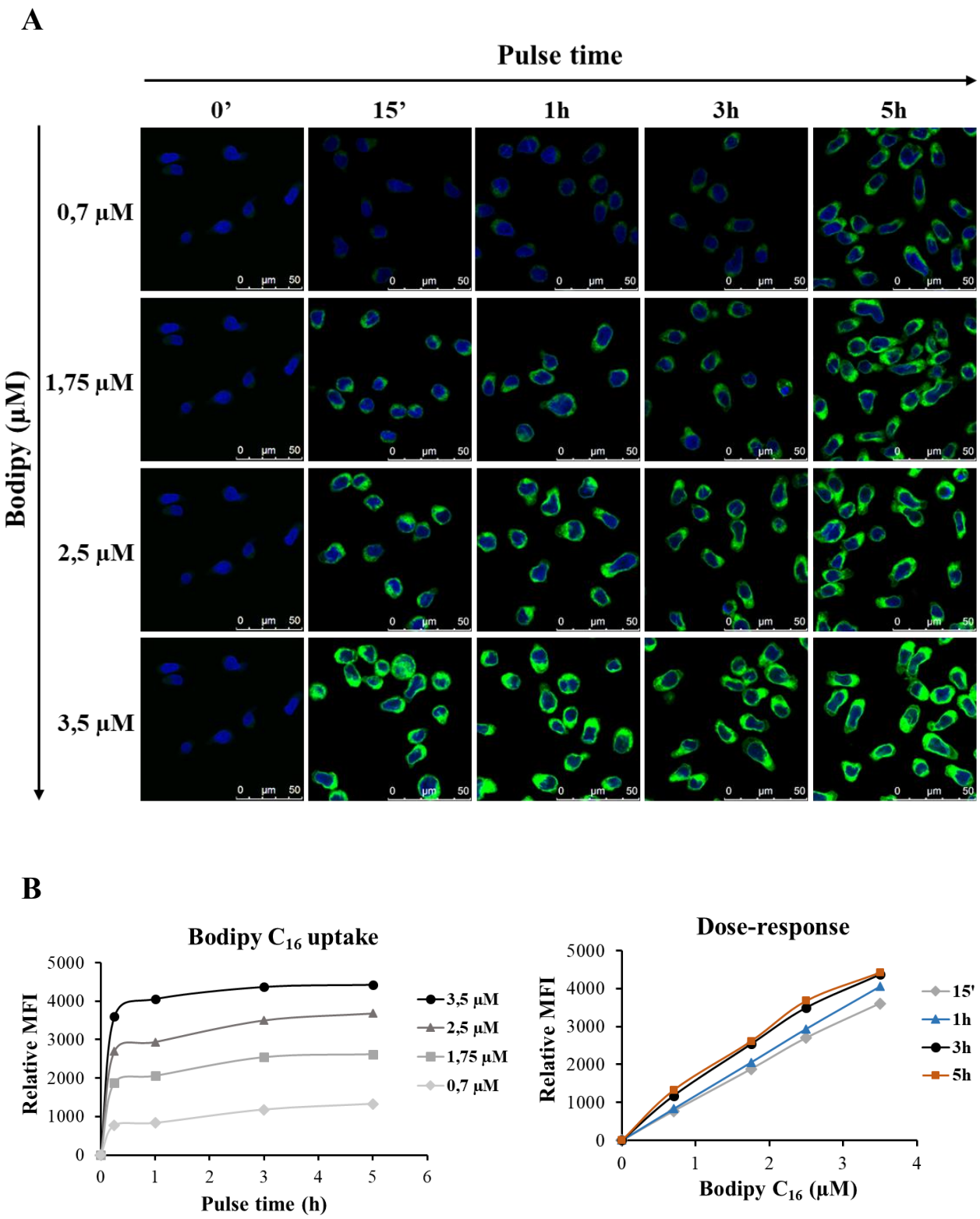


Figure IV.16 Bodipy C_{16} uptake in GEN2.2. A total of 0.3 x 10^6 GEN2.2 were pulsed for different times with different concentrations of Bodipy C_{16} (green) as indicated in the figure. (A) For confocal microscopy analysis, cells were placed on a microscope slide and fixed in PFA 4%. To visualize nuclei (blue) GEN2.2 were stained with DAPI. Images were acquired with the confocal microscope Leica TCS SP5 and processed with the software LAS AF version 1.6.3 (Leica Microsystems). Objective 63.0X. DIC: Differential Interference Contrast. Scale bars 0-50 µm. (B) Cell fluorescence was analysed by FC and expressed as relative MFI (median fluorescence intensity). A representative experiment, out of three independent experiments that yielded similar results, is shown.

Since our interest was collecting exosomes from medium conditioned by Nef-treated GEN2.2 after 20 hours, we had to define how long the fluorescence persisted inside the cells after Bodipy treatment. Thus, GEN2.2 were pulsed with 2.5 or 3.5 µM of Bodipy C_{16} for 2 hours, afterwards cells were washed to eliminate the residual fluorescent probe and fresh medium supplemented with 10% FBS was added. GEN2.2 were then chased for different times up to 24 hours and observed by confocal microscopy, where cell fluorescence appeared more and more diffuse with few spots of fluorescence that were mostly chased out after 24 hours (Fig. IV.17A). Moreover, the cell fluorescence intensity was measured by flow cytometry in order to quantify how fast Bodipy C_{16} was metabolized by GEN2.2 (Fig. IV.17B). During chase times in fresh medium, we observed that GEN2.2 treated with 3.5 µM of Bodipy C_{16} already after 1 hour showed a drastic reduction of cell fluorescence by about 80%, whereas it slowly decreased afterwards. However, the fluorescence was still detectable up to 24 hours ensuring that throughout the period of production the vesicles were able to incorporate the fluorescent lipid. A similar pattern was observed treating cells with 2.5 µM; although after 1 hour cells presented a reduction of the fluorescence of 68% compared to initial values, their fluorescence intensity was slightly lower than cells treated with 3.5 µM. Therefore, a concentration of 3.5 µM was chosen for cell labelling.

The reported data indicate that GEN2.2 internalize Bodipy C_{16} that arises a plateau at 2 hours and, although the fluorescence undergoes a rapid reduction, it does not chase out completely after 24h. The reduction of fluorescence observed is consistent with the idea that the fluorescent lipid, once transported to the endoplasmic reticulum where it is mainly metabolized in phospholipids, is then directed to the endosomal pathway and released into the extracellular milieu as part of the EV membrane.

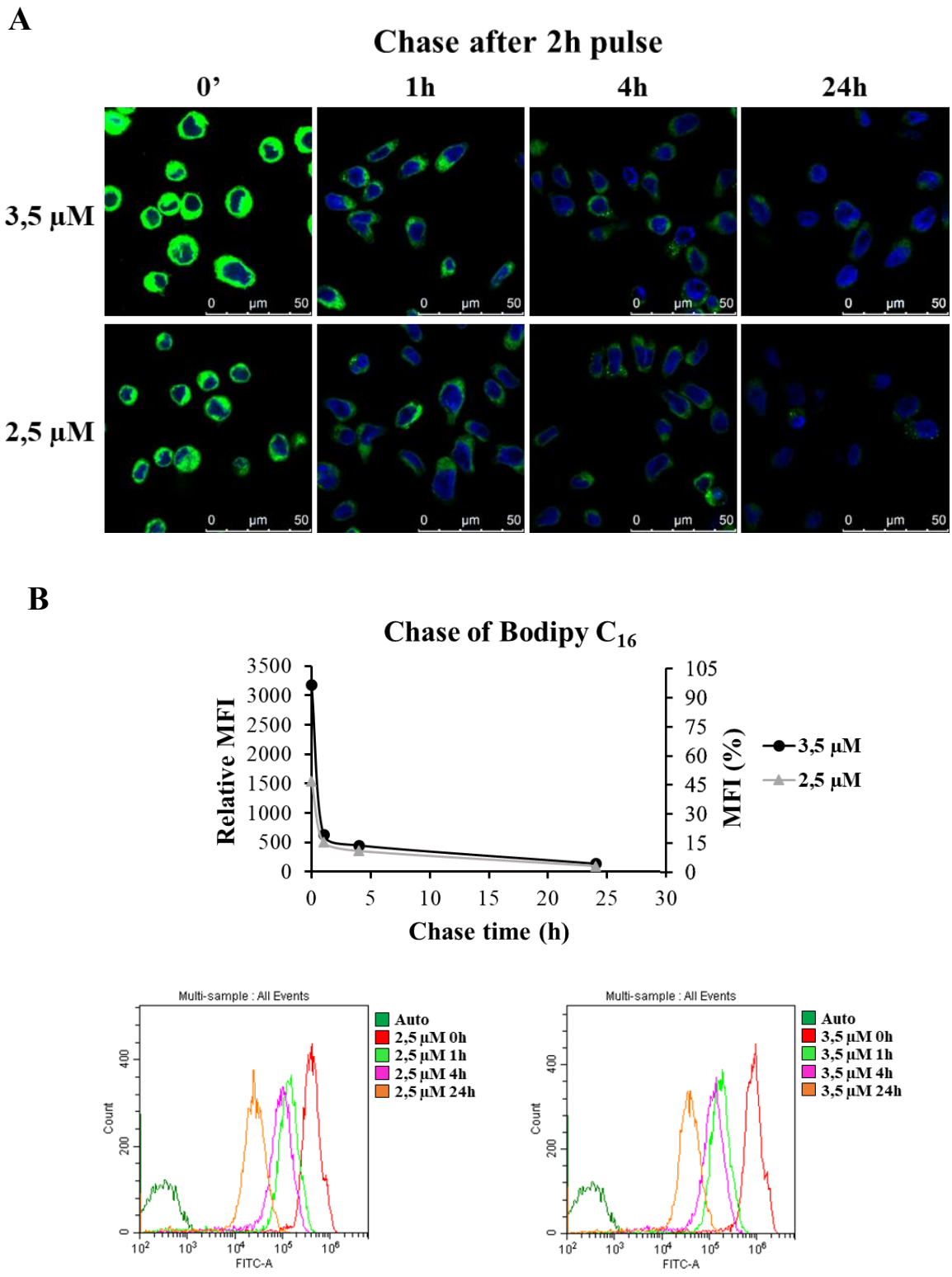


Figure IV.17 Chase of Bodipy C_{16} after 2h pulse. 0.3 x 10^6 GEN2.2 were pulsed for 2 hours with 2.5 µM or 3.5 µM of Bodipy C_{16} in complete medium supplemented with 0.3% FBS. Afterwards, cells were washed and chased in complete medium containing 10% FBS according to the times reported in the figure. (A) For confocal microscopy analysis cells were placed on a microscope slide and fixed in PFA 4%. To visualize nuclei (blue) GEN2.2 were stained with DAPI. Images were acquired with the confocal microscope Leica TCS SP5 and processed with the software LAS AF version 1.6.3 (Leica Microsystems). Objective 63.0X. DIC: Differential Interference Contrast. Scale bars 0-50 µm. (B) Cell fluorescence was analysed by FC and reported as relative MFI and percentage of MFI (upper panel). The corresponding flow cytometry plots were reported in the panel below. A representative experiment, out of three independent experiments that yielded similar results, is shown.

IV.3 Nef reduces the exosome production and affects the type of vesicles released.

The above-described methodology based on cell treatment with Bodipy allows the detection and the count of EVs through conventional flow cytometry, overcoming the problem due to the reduced size of exosomes (below 200 nm). In fact, although it is well known that the detection by flow cytometry based on light-scattering of vesicles or particles smaller than 300 nm is severely hampered by noise events, the novel strategy allows to discriminate fluorescently labelled vesicles from non-fluorescent noise by coupling the fluorescent signal of vesicles with the light-scattering. To study the amount of secreted exosomes and microvesicles in response to Nef treatment, 10^7 GEN2.2 were pre-treated with 3.5 µM of Bodipy for 2 hours in complete medium supplemented with 0.3% FBS. Afterwards, cells were washed to remove residual lipid and fresh medium supplemented with 10% ultracentrifugated FBS and containing 300 ng/mL of myrNef$_{SF2}$w.t was added (Fig. IV.18A). Fluorescent exosomes and microvesicles released into the medium were isolated after 20 hours by differential ultracentrifugations and then processed for FC analysis. Interestingly, comparing Bodipy-exosomes secretion in cells treated with the viral protein with respect to the untreated cells we observed that the production of exosomes was reduced by about 40% in response to Nef stimulus, whereas that of microvesicles did not seem to be influenced (Fig. IV.18B-E).

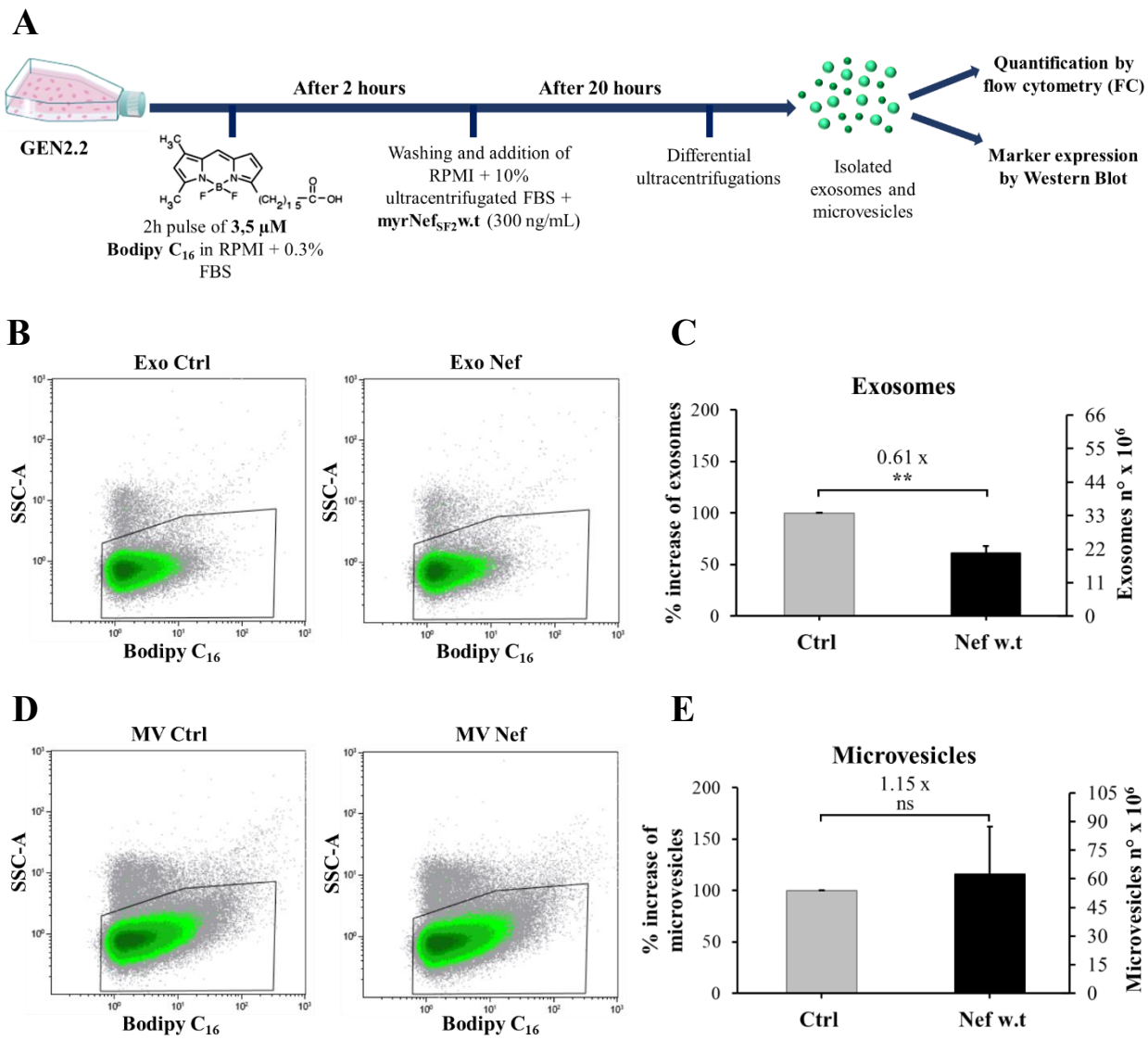


Figure IV.18 HIV-1 myrNef$_{SF2}$ protein affects the exosome but not the microvesicle production of GEN2.2. (A) Workflow of isolation of fluorescent exosomes and microvesicles from GEN2.2 treated with Nef protein. A total of 10^7 GEN2.2 were seeded in 75 cm^2 flask and treated for 2 hours with 3.5 µM of Bodipy C_{16} in 5 ml of complete medium supplemented with 0.3% FBS. Then, cells were washed and 12 mL of fresh medium supplemented with 10% ultracentrifugated FBS and containing or not 300 ng/mL of myrNef$_{SF2}$w.t were added. After 20 hours, conditioned and non-conditioned control medium were harvested and underwent differential ultracentrifugations to isolate exosomes (Exo) and microvesicles (MV). (B and D) Isolated fluorescent vesicles were counted through FC by plotting fluorescence at 525/40 nm (Bodipy C_{16}) versus log scale side scatter (SSC-A). The number of exosomes (B) or microvesicles (D) was registered in the rectangular region corresponding to their specific size as reported in Materials and Methods. (C and E) Histograms show the mean total number of fluorescent exosomes and microvesicles normalized to an equal number of cells and the mean percentage of increase. Values are expressed as mean ± S.D. of triplicates of three independent experiments. Two-tailed t test; **, $p < 0.01$, ns, not significant vs respective Ctrl.

According to what reported by the guideline published in 2018 by Journal of Extracellular Vesicles [Théry et al., 2018], to better characterize the nature of the isolated vesicles we analysed at least one of the transmembrane proteins (CD81) and cytosolic proteins (TSG101, ALIX, HSP90 and Flotillin-1) commonly found in mammalian cell-derived EVs. Furthermore, we evaluated the presence of COXIV, a protein localized in mitochondria, which a *priori* is not enriched in the

smaller EVs (< 200 nm diameter) of plasma membrane or endosomal origin. To this end, isolated exosomes and microvesicles were resolved on 11% SDS-PAGE gel and analysed by western blot (Fig. IV.19).

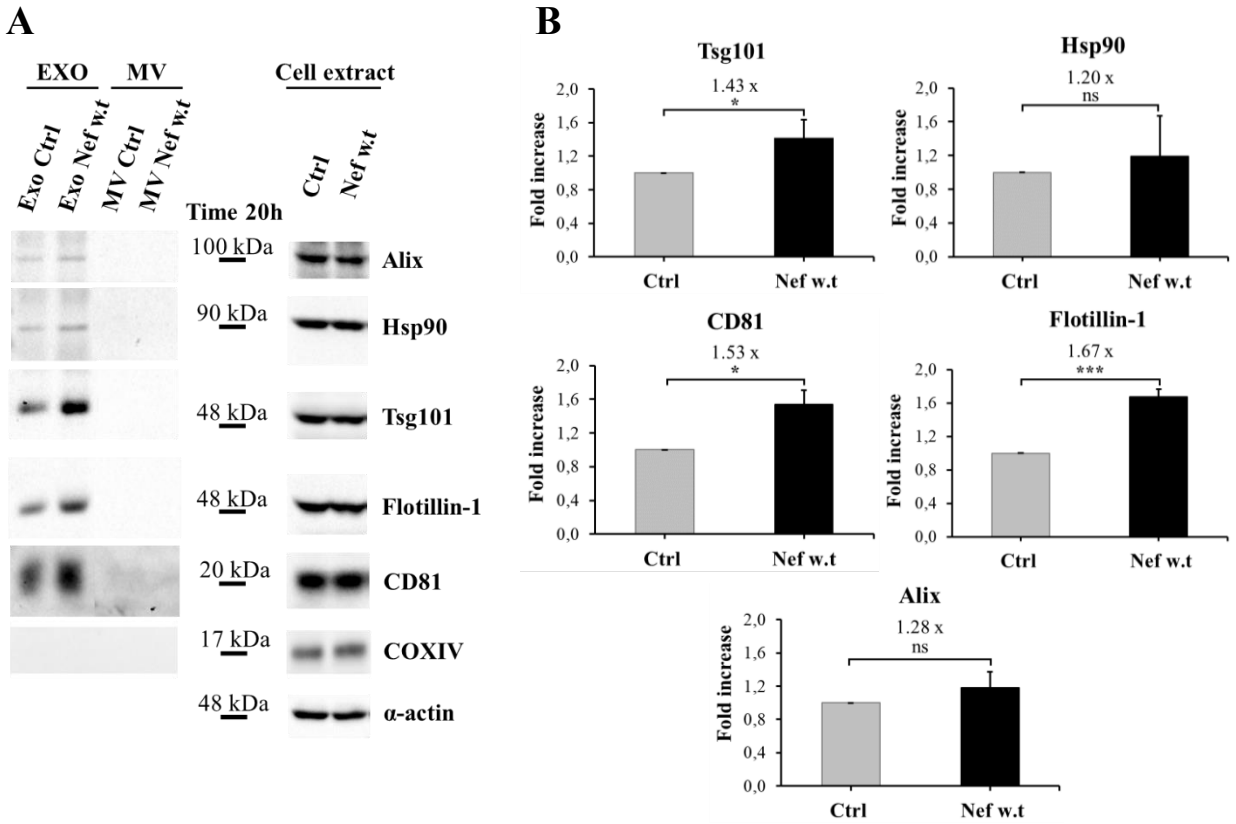


Figure IV.19 Characterization of exosomal markers in exosomes and microvesicles isolated from GEN2.2 treated with Nef protein. (A) Considered the count obtained by FC analysis, an equal number of collected vesicles ($8x10^6$) was loaded on 11% SDS-PAGE gel for Western blot analysis with antibodies specific for Tsg101, Hsp90, Alix, CD81, Flotillin-1 and COXIV. An equal protein content of the cell extracts was loaded and the actin was used as loading control. (B) A densitometric analysis was performed and the results were expressed as fold increase compared to control. Histograms: mean ± S.D. of three independent experiments. Two tailed t test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ns, not significant vs respective Ctrl.

As showed, all specific exosomal markers turned out to be present in the sample corresponding to exosomes but not in the microvesicular one, whereas, as expected, COXIV was detected only in cellular lysates. This analysis formally confirmed the nature and the purity of the isolated vesicles and it allowed to characterize for the first time the marker composition of exosomes isolated from plasmacytoid dendritic cells, revealing their peculiar aspect: the low expression level of tetraspanins such as CD81, whose detection in fact required longer exposure time. The lower expression in the exosomes simply mirrors the low intracellular expression of CD81, which has been recently reported in human primary pDCs [Zuidscherwoude et al., 2017] and that distinguishes pDCs from most of other cell types, including myeloid DCs.

Remarkably, western blot analyses revealed also a different expression rate of Tsg101, CD81 and Flotillin-1 in the exosomes isolated by cells treated with Nef w.t with respect to exosomes derived from untreated cells (Fig. IV.19A). Instead, the expression of the exosomal markers analysed in cellular lysates was not influenced by treatments. In detail, we observed that exosomes from Nef-treated cells showed an up-regulated expression of Tsg101, CD81 and Flotillin-1 (Fig. IV.19B). Since the samples were normalized by loading on the gel an equal number of exosomes and microvesicles, we can infer that the modulation of the exosomal markers observed is certainly not attributable to the presence of a higher or lower amount of vesicles with respect to the control. Therefore, we hypothesize that the different expression level of some exosomal markers reflects the heterogeneity of the vesicles released in response to Nef treatment compared to control.

IV.11 Nef protein is incorporated only into the exosomal fraction, but not into the microvesicular one.

Based on what reported in literature regarding the ability of Nef to be transferred to uninfected cells through extracellular vesicles (EVs), we asked whether the recombinant viral protein followed the same destiny of the viral protein when endogenously expressed in HIV-infected cells [Arenaccio et al., 2014]. Considered the low protein content that can be incorporated inside the vesicles due to their reduced sizes, before verifying the presence of the viral protein different tests were performed to evaluate the sensitivity of the antibody used to ensure that it would be able to detect even low amounts of the protein. As showed, the antibody had a high sensitivity because it was able to detect until 1 ng of Nef (Fig. IV.20A).

Next, to establish whether the viral protein was released into the EVs, GEN2.2 were left untreated or treated with 300 ng/mL of myrNef$_{SF2}$w.t and after 20 hours the conditioned media were harvested and processed for EV isolation. To ensure that the protein in the vesicles could be detected, all pelleted exosomes and microvesicles recovered from the isolation were run on 11% SDS-PAGE gel and analysed on western blot (Fig. IV.20B). We observed that Nef protein was transported through EVs like the protein endogenously expressed during HIV infection. Noteworthy, we demonstrated also that Nef was preferentially incorporated into the exosomal fraction, whereas it was undetectable in the microvesicular one. The specificity of the signal observed was confirmed by the absence of the band corresponding to Nef protein in exosomes isolated from untreated cells. Moreover, as expected, Nef was detected also in the cellular extract, confirming its internalization into GEN2.2 during the treatment. Overall, these result confirm what

already observed in HIV-infected cells, but also suggest the presence of a specific mechanism that would address Nef to be released into exosomes but not into microvesicles.

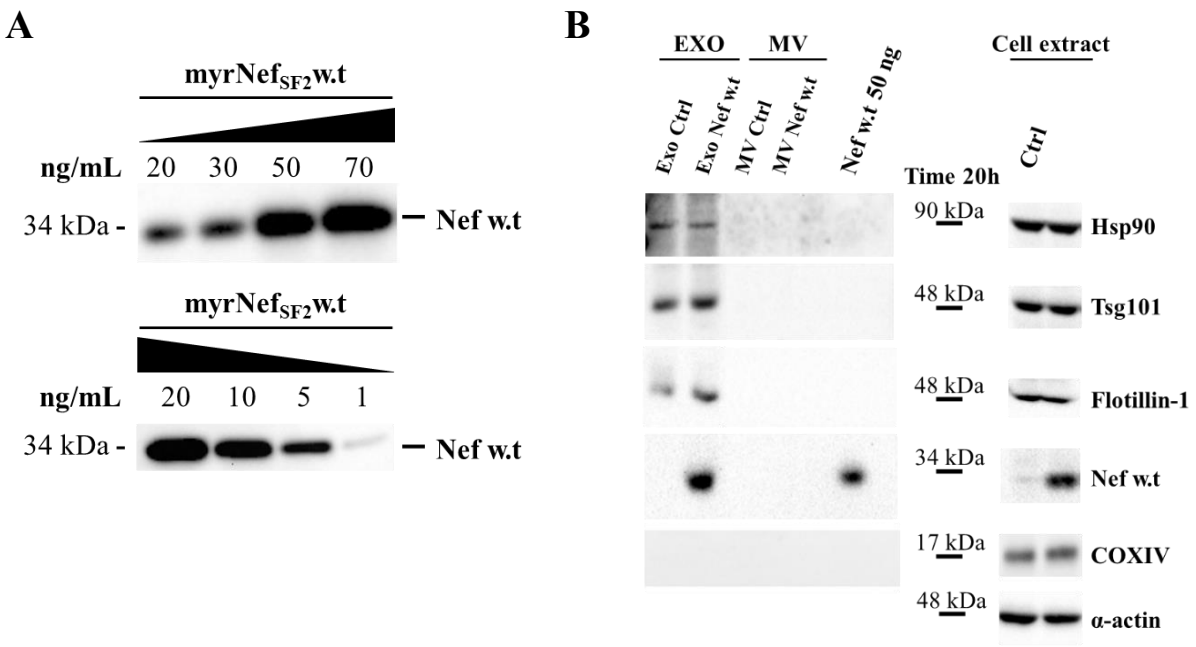


Figure IV.20 Detection of Nef in the exosomal fraction, but not in the microvesicular one. (A) Western blot analyses of different amounts of Nef protein to evaluate the sensitivity of the specific antibody used to detect Nef. (B) An equal number of vesicles ($15x10^6$) purified from supernatants of GEN2.2 treated or not with 300 ng/mL of myrNef$_{SF2}$w.t for 20 hours was loaded on 11% SDS-PAGE gel. An equal protein content of the cell extracts was loaded and the actin was used as loading control. Anti-Nef western blot analysis of both cell extracts and vesicles was shown (EXO: exosomes; MV: microvesicles). To verify the identity of the isolated vesicles, the exosomal marker expression was verified. The results are representative of three independent experiments.

IV.12 IFNs do not alter the amount of EV production, but influence the type of vesicles released.

Considered the particular attitude of pDCs to secrete IFNs and their continuous exposure to this type of cytokines during HIV infection and in response to Nef treatment, we wondered whether the IFN production could influence the secretion of EVs by pDCs. Although HIV-1 infection is marked by a persistent type I IFN production, we tested the effects of all three types of IFN in order to evaluate whether they could differently affect the EV secretion. To this aim, cells were pre-treated with Bodipy as described above, and then it was added fresh medium supplemented with all three types of IFN: IFN-β (1000 IU/mL) ,-γ (100 ng/mL) or –λ1/ λ2 (100 ng/mL). The fluorescent vesicles were analysed by flow cytometry and the results showed that neither type I, II or III IFN significantly affected the yield of exosomes or microvesicles (Fig. IV.21B-E).

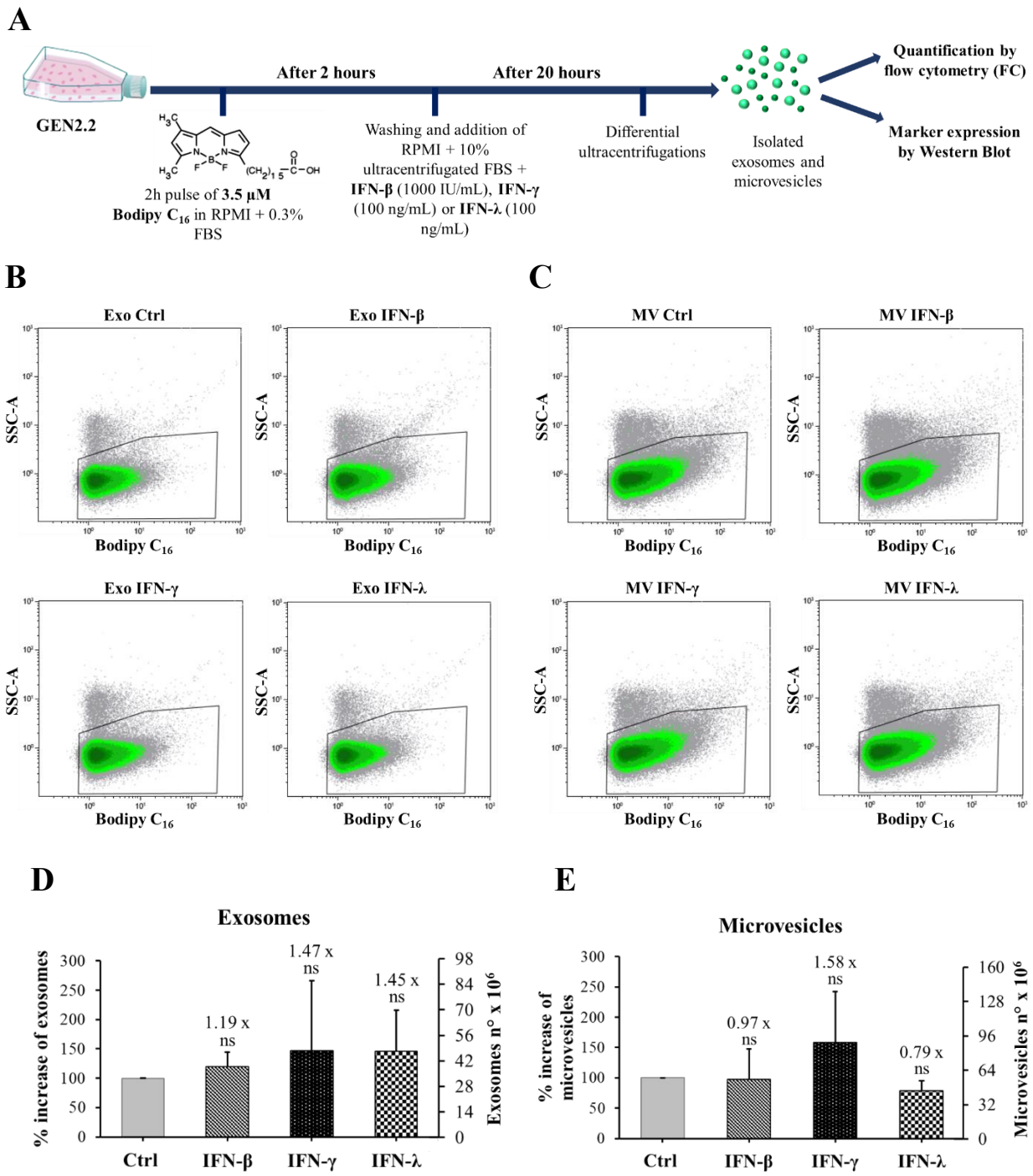


Figure IV.21 IFN treatments do not affect the vesicular production of GEN2.2 from a quantitative point of view. (A) Workflow of isolation of fluorescent exosomes and microvesicles from supernatants harvested from GEN2.2 treated with IFNs. A total of 10^7 GEN2.2 were seeded in flask 75 cm^2 and treated for 2 hours with 3.5 µM of Bodipy C16 in complete medium supplemented with 0.3% FBS. Afterwards, cells were washed and 12 mL of fresh medium supplemented with 10% ultracentrifugated FBS and containing or not IFN-β (1000 IU/mL),-γ (100 ng/mL) or –λ1/λ2 (100 ng/mL) were added. After 20 hours, conditioned and non-conditioned control medium were harvested and underwent differential centrifugations to isolate exosomes (Exo) and microvesicles (MV). (B and C) Isolated fluorescent vesicles were counted through FC by plotting fluorescence at 525/40 nm (Bodipy C_{16}) versus log scale side scatter (SSC-A). The number of exosomes (B) and microvesicles (C) was registered in the rectangular region corresponding to their specific size as reported in Materials and Methods. (D and E) Histograms show the mean total number of fluorescent exosomes and microvesicles normalized to an equal number of cells, and the mean percentage of increase. Values are reported as the means ± S.D. of triplicates of three independent experiments. One-way ANOVA test; ns, not significant.

Remarkably, western blot analysis showed that exosomes released in response to IFN treatment differed with respect to those of control for the expression of some exosomal markers (Fig. IV.22A). In detail, we observed that exosomes released from GEN2.2 treated with all types of IFN showed a down-regulated expression of Tsg101, CD81, Flotillin-1, and the effect of IFN-β seemed to be stronger compared to the others. These data were further confirmed by the densitometric analysis (Fig. IV.22B).

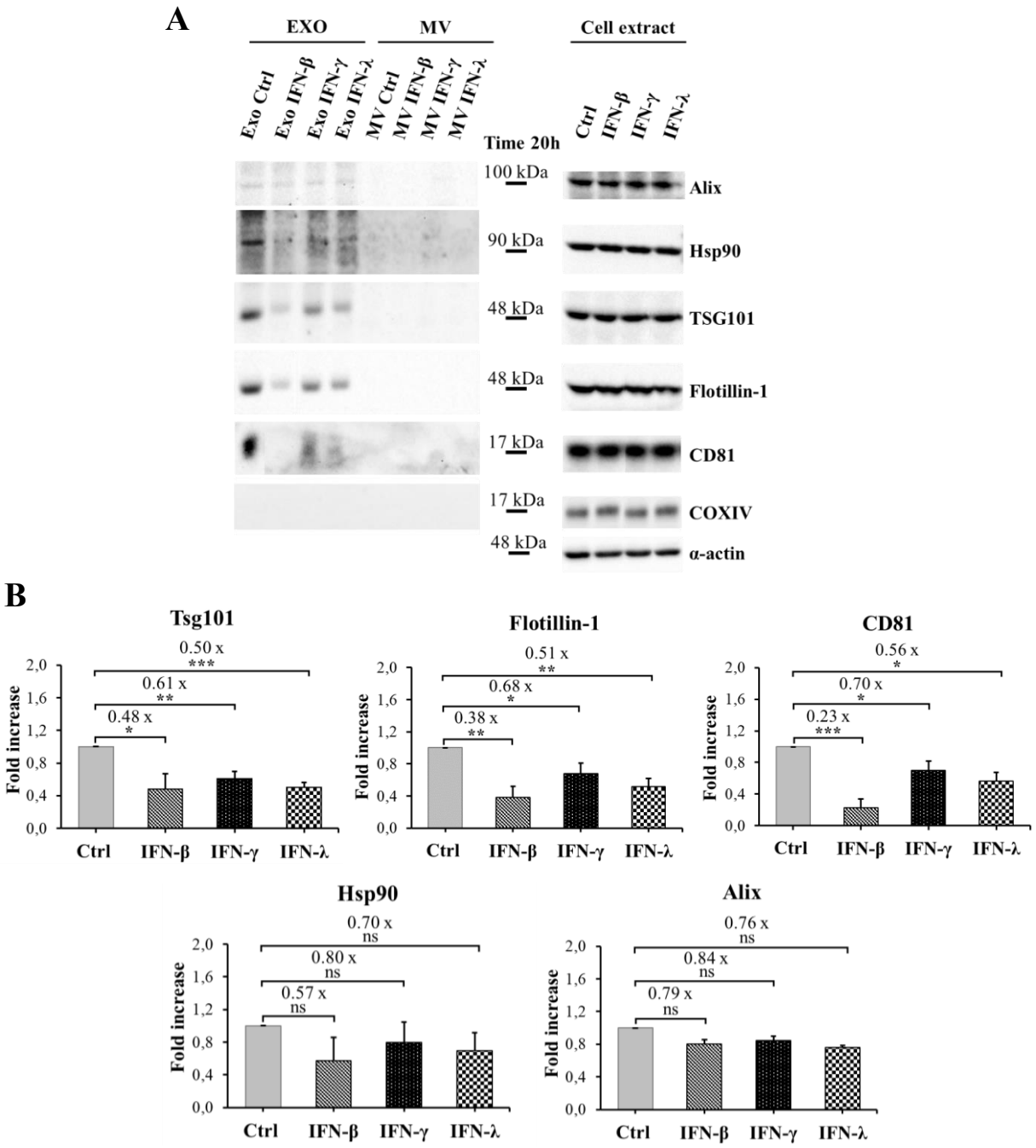


Figure IV.22 IFN treatments induce the release of different exosomes compared to those released by control cells. (A) Vesicles isolated from treated or untreated GEN2.2 were lysed by repeated freezing and thawing. An equal number of collected vesicles ($15x10^6$) and the corresponding whole-cell lysates (80 µg/well) were loaded on 11% SDS-PAGE gel for Western blot analysis with antibodies specific for Tsg101, Hsp90, Alix, CD81, Flotillin-1 and COXIV. An equal protein content of the cell extracts was loaded and the actin was used as loading control. (B) The

expression of exosomal markers in both cells and vesicles is shown. The data were reported as fold increase compared to control. Values are expressed as mean ± S.D. of three independent experiments. One-way ANOVA test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs respective Ctrl.

Taken together, these results confirmed with a novel methodology what recently reported regarding the exosome secretion induced by type I IFN [Villarroya-Beltri et al., 2016]. Furthermore, we demonstrated for the first time that neither type II or III IFN alter the exosome secretion of pDCs from a quantitative point of view and that, like type I IFN, they seem to release a different exosomal population compared to control cells.

V. DISCUSSION

Plasmacytoid dendritic cells (pDCs) are a unique dendritic cell subset specialized in type I IFN production and whose role in HIV-1 infection and pathogenesis is complex and not yet well defined. Although they do not represent the main reservoir of HIV, it has been reported that pDCs can be infected by the virus contributing dichotomously to both chronic immune activation and immunosuppression [Aiello et al., 2018]. Remarkable advances have been made in the past years concerning pDCs dynamics and functions in HIV infection. However, to date, most of the studies have been focused on the analysis of pDC response following HIV infection. Here, instead, we took into consideration another point of view by investigating the effects induced by the multifunctional pathogenic accessory protein Nef alone on HIV-not infected pDCs. Indeed, Nef protein and anti-Nef antibodies have been detected in the serum of HIV-infected individuals [Fujii et al., 1996; Ameisen et al., 1989] supporting the possible *in vivo* detection of extracellular Nef by uninfected cells.

Previous results obtained in our laboratory demonstrated that Nef protein was rapidly and efficiently internalized in primary monocyte-derived macrophages (MDMs) and triggered in few minutes NF-κB, MAPKs and IRF-3 activation inducing the production and release in 2 hours of a set of cytokines and chemokines including IFN-β [Mangino et al., 2007 and 2011]. Since Nef during HIV-1 infection appears to have an important impact on the chemo-cytokine network [Percario et al., 2015] possibly contributing to the chronic inflammation observed during HIV disease progression, we investigated the alterations in the intracellular signalling induced by this viral protein in pDCs. The results obtained in primary pDCs revealed that about 30% of primary pDCs internalized the recombinant myristoylated Nef protein, but less efficiently than MDMs probably because of their lower phagocytic/internalization ability that distinguishes pDCs from macrophages. Moreover, the aspect of the internalized Nef differed in the two cell types, maybe caused by a different distribution of the viral protein or entry mechanism. Regarding the latter, preliminary experiments were performed using different inhibitors of the entry process (data not reported), but the results were not conclusive because none of the tested inhibitors was able to prevent the internalization of Nef. Moreover, the results showed that the exogenous treatment with the recombinant myristoylated Nef protein up-regulated the expression of *mxa*, an IFN-inducible gene, whose protein is usually used as surrogate marker for IFNs production. It was also observed in response to Nef an increase and a partial nuclear translocation of IRF-7 that, according to the confocal images, seems to result in the IFN-α production. It is necessary to underline that the data concerning the IFN-α production provides only a qualitative, but not a quantitative information.

These promising results laid the foundation for further analyses that were carried out using the GEN2.2 cell line in order to facilitate biochemical analyses and to have a more stable and reproducible system. This cell line resulted to be an appropriate model to study cell signalling in plasmacytoid dendritic cells, since it shares most of the phenotypic and functional features of primary pDCs.

Through the GEN2.2 cell line, we demonstrated that Nef acted on pDCs by inducing the tyrosine phosphorylation of both STAT1 and STAT2 proteins starting from 3 hours. Notably, Nef substantially influenced also the gene expression program via STAT activation, as indicated by the late induction of IRF-1, STAT1 and ISG15. In contrast, the treatment with the Nef mutant 4EA, mutated in the acidic domain of the protein, was not able to induce the phosphorylation of STAT1 and STAT2 proteins and did not even modulate the gene expression since neither IRF-1, STAT1 nor ISG15 resulted to be increased. These results highlight the importance of the acidic domain in the signalling pathway induced by Nef and add relevance to the previous findings obtained in primary macrophages by our research group [Mangino et al., 2007 and 2011].

Furthermore, activated pDCs have been described to acquire two different phenotypes according to the specific signals received; they can produce large amounts of type I IFN, hence acting as IPCs, or act as APCs [Honda et al., 2005]. In this regard, we observed that Nef protein did not alter the expression of co-stimulatory molecules such as CD40, CD80 and CD86, which usually accompany the activation or maturation of plasmacytoid dendritic cells making them APCs as powerful as the counterpart of myeloid DCs. These data are in agreement with literature regarding the response of pDCs to HIV-1 detection [O'Brien et al., 2011], thus supporting the idea that Nef alone could mimic some of the effects caused by the virus.

All together, the presented evidences suggest that Nef can act on pDCs by favouring the acquisition of the IPC phenotype rather than the APC one and by promoting the release of soluble factors, since STAT tyrosine phosphorylation is rapidly induced in response of a wide range of cytokines, growth factors and hormones. Our hypothesis was confirmed by the secretome analysis performed on the supernatants harvested 20 hours after Nef treatment. Indeed, we observed that Nef induced the production of regulatory cytokines (IL-2), growth factors (FGF basic and G-CSF), chemotactic factors e/o pro-inflammatory mediators (MCP-1, IL-8, IP-10, MIP-1α, MIP-1β, IFN-γ and TNF-α), although at different extent. Other mediators, such as IL-4, IL-5, IL-17, Eotaxin and RANTES resulted to be only poorly secreted in response to Nef treatment. The other soluble factors were not modulated or detected neither in untreated cells. The positive secretion of some T-cell cytokines, such as IL-2 and IFN-γ, observed in pDCs might seem unusual, but although they are conventional T-cell cytokines they could be released also by other cell types even if in lower

amounts. However, considering the global amount of all the cytokines/chemokines released, these two factors are probably not very relevant and the effects of other soluble mediators, such as IP-10 or IL-8, are definitely the relevant ones.

Overall, these results are consistent with what reported in literature concerning the cytokines/chemokines that can be released by primary pDCs. Indeed, in addition to IFNs, it is known that pDCs can produce a number of inflammatory cytokines and chemokines including TNF-α, MIP-1α, MIP-1β, RANTES, IL-8 and IP-10 [Aiello et al., 2018]. Regarding the specific cytokines/chemokines released in response to HIV-1 infection, most of the studies have been focused on the analysis of IFN production, thus only a few others cytokines/chemokines were analysed. In particular, it was observed that HIV-infected pDCs produced the chemokines MIP-1α and MIP-1β and abundant IFN production, but low NF-κB-dependent production of TNF-α [O'Brien et al., 2011]. These data are in agreement with what observed during our secretome analysis on Nef-treated GEN2.2.

Since macrophages are widely recognized as one of the main reservoir of HIV infection, we compared the pattern of cytokines/chemokines released after 20 hours of Nef treatment of GEN2.2 with that of THP-1, a monocytic cell line that was differentiated adding PMA in order to acquire a macrophage-like phenotype. Unlike GEN2.2, in THP-1/PMA Nef did not affect or only weakly the amount of IL-8, G-CSF, MCP-1, whereas promoted the secretion of some cytokines weakly induced or not produced by GEN2.2 such as PDGF, IL-1β, IL-5, IL-15, IL-17, RANTES and VEGF. Other mediators such as IL-1ra, IL-4, IL-12 (p70), IFN-γ and Eotaxin were only weakly modulated in THP-1/PMA. Among the soluble factors analysed in THP-1/PMA, some of them such as MIP-1β, IL-1β and TNF-α were previously observed to be induced by Nef also in primary macrophages by our research group [Olivetta et al., 2003]. Likewise, MCP-1 it was not increased in response to Nef in primary macrophages and here this data was confirmed.

Although a different profile was observed, macrophages showed a high secretion of IP-10, which reached higher levels than in pDCs culture. This observation is in agreement with what observed *in vivo* infections where macrophages seem effectively responsible for the greatest proportion of IP-10 expression [Lei et al., 2019].

Interestingly, not only plasmacytoid dendritic cells and macrophages presented a different secretome induced by Nef, but they differed also for the amount with which each cytokine/chemokine was produced by control cells. Indeed, some factors such as PDGF, IL-2, FGF basic, IP-10, MCP-1, TNF-α and VEGF were already detectable at medium-high levels in untreated GEN2.2 and increased over time, whereas the values of the other cytokines/chemokines were almost undetectable. In THP-1/PMA, regardless of the type of soluble factor considered,

most of the cytokines/chemokines presented basal levels that are much higher than GEN2.2, except for TNF-α. In particular, IL-1ra, IL-8, G-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES were the cytokines/chemokines that reached the higher levels. Considering the above, it is possible to hypothesize that the high basal levels of some soluble factors in both cell types could limit their further increase in response to Nef treatment.

Altogether, these data highlight the ability of Nef to induce a different pattern of cytokines/chemokines release according to the cell type probably contributing to fuel in different ways the intense "cytokine storm" that characterizes HIV infection [Wang et al., 2017].

Indeed, the types of cytokines/chemokines released in response to Nef in both GEN2.2 and THP-1/PMA seem to play a crucial role during HIV infection.

IL-2 is a potent T cell growth factor that for many years was assumed to amplify lymphocyte responses *in vivo*, but recently it turned out to be critical also for the development and peripheral expansion of $CD4^+CD25^+$ regulatory T cells, which promote self-tolerance by suppressing T cell responses *in vivo* [Nelson et al., 2004]. Considering the above, IL-2 production could suggest a role of pDCs also as tolerogenic cells. Instead, the monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages, which are required for routine immunological surveillance of tissues, as well as in response to inflammation [Deshmane et al., 2009]. Therefore, its release by pDCs in response to Nef would strongly favours monocyte recruitment in HIV infection sites. In the same manner, the release of the chemokines MIP-1α and -1β (i.e., CCL3 and CCL4) and IL-8 would favour the recruitment and activation of $CD4^+$ T cells.

In addition to the regulatory and chemotactic factors, Nef induces the release of pro-inflammatory mediators, among which a particular attention should be paid to the interferon-γ induced protein (IP-10), whose plasma levels have been demonstrated to be abnormally increased after HIV infection and tightly associated with HIV disease progression [Lei et al., 2019]. IP-10 secretion is predominantly driven by IFN-γ but also other cytokines, including IL-2, IFN-α, IFN-β and TNF-α resulted to be involved by acting synergistically with IFN-γ. As chemokine, the most important function of IP-10 is the activation of lymphocytes and the induction of their migration to inflamed regions. In this regard, it has been reported that, after HIV exposure, IP-10 expression levels were increased in human cervical and colonic mucosa tissue epithelia facilitating the transmission process thanks to the increasing IP-10-induced recruitment of HIV target cells to the mucosa surface [Sankapal et al., 2016]. Additionally, numerous studies have demonstrated that high IP-10 levels may impair immune cell functions (T cells and NK cells) and promote HIV replication and latency [Lei et al., 2019]. Regarding the possible mechanism underlying the increase of this

chemokine, it has been thought to be a combination of HIV particles or HIV proteins and TLR7/9 [Lei et al., 2019]. To date, the performed studies reported that HIV itself could contribute to IP-10 induction by infecting monocytes and dendritic cells, which increase the amounts of IFN-α through TLR7/9 dependent mechanism and, in turn, IFN-α further stimulates immune cells to produce IP-10 through the JAK/STAT1 signalling pathway [Simmons et al., 2013]. On the other hand, the HIV accessory protein Tat cooperates with IFN-γ and TNF-α to induce IP-10 expression through NFκB and STAT1 signalling [Williams et al., 2009], whereas the binding of gp120 envelope protein of HIV alone is insufficient to induce IP-10 expression [Wetzel et al., 2002]. Here, we described the ability of the Nef protein alone to induce IP-10 expression in our model of uninfected macrophages (THP-1 cell line) and pDCs (GEN2.2 cell line). Since, Nef stimulates in GEN2.2 the release of both IFN-γ, TNF-α and type I IFN, we can hypothesize that the mechanisms underpinning IP-10 production induced by Nef could involve the cooperation among these cytokines and the activation of JAK/STAT1 and NFκB signalling pathways. Even the late production of ISG15 could contribute to IP-10 expression, since it has been reported that elevated levels of this IFN-induced protein can effectively promote IP-10 expression in macrophages because ISG15 decreases the inhibitory effects exerted by microRNA-21 on IP-10 production [Wu et al., 2017].

Overall, Nef alone can potentially make pDCs able to indirectly amplify and activate the pool of locally available target cells making them susceptible to infection and miming in this way what happens with the virus in proximity of infected mucosal sites, where pDCs migrate in response to inflammatory conditions. Thus, the Nef-induced modification of the pattern of released cytokines/chemokines possibly leads to pathologic effects both in pDCs and in all cells functionally correlated.

Furthermore, since during HIV infection pDCs are exposed to the local microenvironment influenced by infected cells that release immunostimulatory molecules into the extracellular space, we verified the response of GEN2.2 to the cytokine/chemokine milieu released by GEN2.2 in response to Nef stimulus. In this regard, we observed that STAT1 tyrosine phosphorylation occurred more rapidly (already after 30 minutes) than following Nef treatment (3 hours) showing that pDCs are promptly responsive to the surrounding extracellular milieu. Indeed, pDC activation seems to require the establishment of cell-to-cell contacts with infected cells that favours the concentration of immunostimulatory molecules in the intercellular space making the activation more efficient [Dreux et al., 2012; Décembre et al., 2014].

Since several cytokines, including IL-1β and TNF-α, have been recently reported to be associated with extracellular vesicles (EVs) [Fitzgerald et al., 2018] and it has been shown an increase of the

amount of EV-associated cytokines during HIV infection [Konadu et al., 2015], further analyses were performed treating pDCs with supernatants of Nef treated cells depleted of EVs in order to verify whether the cells responded in the same manner. Interestingly, supernatants depleted of EVs maintained the capacity to early activate STAT1 already after 30 minutes but the signal increased over time reaching a peak after 2 hours. Considering these results, EVs could contribute in some way to modulate the time course of signalling in pDCs and, since GEN2.2 were activated also in response to supernatants depleted of EVs, most of cytokines should be secreted in free form.

Emerging evidences also point out the ability of HIV-1 Nef protein to be transferred to neighbouring bystander cells, which can pick the protein up from the extracellular milieu through exosomes/microvesicles or by cell-to-cell transfer [Campbell et al., 2008; Xu et al., 2009; Muratori et al., 2009; Lenassi et al., 2010; Pužar Dominkuš et al., 2017]. Considering the emerging importance of extracellular vesicles for their role in intercellular communication in both physiological and pathological conditions, including HIV infection [Dias et al., 2018], we characterized and quantified the exosomes and microvesicles released by GEN2.2 in response to Nef treatment. Despite the recent expansion of studies conducted on vesicles, nowadays there are few methods for the reliable quantification and characterization of EVs. To fulfil our purpose, we adopted a novel methodology based on cell treatment with the Bodipy C_{16} fatty acid that, upon uptake by the cells, enters the cellular lipid metabolic pathway without affecting the natural lipid metabolism or perturbing the lipid homeostasis inside the cell [Coscia et al., 2016]. As result, labelled cells release exosomes and microvesicles that, because fluorescent, can be examined and quantified with conventional flow cytometry overcoming in this way the problem correlated to the reduced size of exosomes and their detection by means of this instrument. However, the presence of vesicles that may escape the Bodipy labelling cannot be formally ruled out. These vesicles, not being fluorescent, cannot be detected through flow cytometry, thus it is right to point out that the effective number of EVs released by the cells could be underestimated. Interestingly, unlike what reported in literature regarding other cell types, Nef does not increase the production of exosomes in GEN2.2, but it induces a 40% reduction. Indeed, Nef expression has been reported to increase the number of MVBs in other cell types, which could also favour the egress of viral particles in infected cells [Stumptner-Cuvelette et al., 2003; Costa et al., 2006]. In addition, we observed an increased expression level of CD81, Tsg101, and Flotillin-1 in exosomes secreted from GEN2.2 treated with Nef and we hypothesize that it could reflect the diversity of the vesicles released in response to Nef treatment compared to those secreted by untreated cells.

Importantly, here we demonstrated that the exogenous protein is transported into EVs, similarly to the protein endogenously expressed in the cells [Campbell et al., 2008; Xu et al., 2009; Muratori

et al., 2009; Lenassi et al., 2010; Arenaccio et al., 2014; Pužar Dominkuš et al., 2017]. Furthermore, despite the consistently reported association of Nef with EVs, it remains still unclear which type of EVs is involved in this mechanism since according to the cell type Nef was found to be associated to exosomes or microvesicles. Concerning plasmacytoid dendritic cells, we observed that Nef is preferentially incorporated into the exosomal fraction after cell treatment, but not in microvesicles, suggesting the presence of a specific mechanism that would address the protein to be released in exosomes. To date, several groups have explored the cellular mechanisms associated with EVs-mediated Nef secretion; however, they are not yet entirely understood. It has been described the importance of some critical amino acid residues localized in the N-terminal region of the protein, including a motif comprising residues 66-70 (VGFPV) termed the secretion modification region (SMR). This region has been demonstrated to be involved in the binding of the viral protein to the host protein mortalin [Ali et al., 2010] resulting in its release into EVs [Shelton et al., 2012]. Nevertheless, mortalin is a member of the heat shock 70-kDa protein family that associates with lipid rafts in the plasma membrane and regulates the intracellular trafficking of cell surface receptors but, since it is present in both microvesicles and exosomes, its binding to Nef cannot be a determinant factor for its release in exosomes rather than in microvesicles. Considered the above, it is tempting to speculate that the specific internalization of Nef protein in exosomes must be associated to other interactions that could direct the viral protein into the endosomal pathway involved in the biogenesis of exosomes. One possible mechanism could be the direct association of this myristoylated protein with lipid rafts, which results to be enriched in MVBs and may lead to piggybacking of the tethered Nef protein into exosomes [Olivetta et al., 2016]. However, there is still much to be understood.

Considered the particular attitude of pDCs to secrete IFNs and their continuous exposure to these types of cytokines during HIV infection, we analysed how they could influence the number of extracellular vesicles secreted by pDCs. In this regard, we observed that neither type I, II or III IFN altered significantly the exosome or microvesicle release, but all IFN types seem to affect from a qualitative point of view the type of vesicles released since a down-modulated expression of the exosomal markers CD81, Tsg101, and Flotillin-1 was observed. Recently, Villarroya-Beltri et al. (2016) correlated the down-modulation of the exosomal markers observed in response to type I IFN treatment to the ISGylation, an ubiquitin-like modification that affects the exosome secretion. In particular, ISG15 conjugates with Tsg101, a transmembrane protein belonging to the ESCRT complex involved in the exosome biogenesis, promoting its aggregation and degradation. This mechanism does not alter the number of exosome released, but induce the secretion of EVs expressing other markers [Villarroya-Beltri et al., 2016]. Although this explanation can be valid

also for IFN-λ, which induces ISG15 production, it cannot explain the down-modulation of markers observed after IFN-γ treatment. In this case, other mechanisms must be involved.

VI. CONCLUDING REMARKS

The role of pDCs in HIV infection is complex. It is clear that this specialized subset of DCs contributes to HIV infection and pathogenesis through several mechanisms: from initial recruitment of target cells to the site of infection and chronic immune activation with a robust and persistent secretion of IFN to T cell dysregulation and exhaustion. The results here presented highlight the pivotal role that even the Nef protein alone is able to play. Indeed, HIV-1 Nef seems to be involved in AIDS pathogenesis and disease progression by interfering with cellular signal transduction pathways resulting in the production of a wide range of cytokines/chemokines, which possibly lead to pathologic effects both in pDCs and in all cells functionally correlated, including macrophages and $CD4^+$ T cells. Although Nef properties have been associated for long time mainly with its biochemical activities inside infected cells due to its adaptor function, in the last few years the attention has been focused on the effects that the viral protein exert on bystander uninfected cells, where it can be transferred through different mechanisms, including extracellular vesicles.

The discovery of vesicles structures containing Nef has opened a new frontier in the study of the multifaceted role of this viral protein. The latter can exploit the vesicular trafficking machinery of the host as a "Trojan horse" allowing its transfer from one cell to another and escaping the immune system. In this way, Nef may regulate the intercellular communication extending its functions on neighbouring uninfected cells.

Since different forms of Nef protein have been detected in HIV patients, it is possible to envisage that uninfected pDC can pick Nef protein directly from the extracellular milieu as soluble protein, *via* exosomes or following the transfer through cell-to-cell contact. These events could occur in proximity of infected mucosal sites, where pDCs migrate in response to inflamed conditions, or in lymph nodes. However, the effects induced by soluble Nef or Nef containing-exosomes might be different; it is possible to envisage that the exosomes induce a different response because influenced by the entire cargo of the vesicles. Another possibility is that *in vivo* Nef may form immune complexes with anti-Nef antibodies that are produced in infected individuals, and be internalized *via* FcRs in endosomal compartments. Here the acidic environment could favour the release of the viral protein in free form. The latter might be then able to exert its specific effects.

In the case of plasmacytoid dendritic cells, the exosomal transfer of Nef could represent an alternative mechanism of cell activation during HIV infection. Indeed, *in vitro* studies showed that exosomes produced by infected cells play a key function in the activation of the immune response mediated by pDCs and are involved in the type I IFN production [Dreux et al., 2012]. This activation mechanism is not limited to a single viral family but seems to be preserved

evolutionarily. The results here presented open the way to future studies addressed to the dissection of the exosome content and of how this is modulated by Nef in plasmacytoid dendritic cells.

In conclusion, this work provides new insights in order to have a more comprehensive picture for a thorough understanding of pDCs roles in HIV infection that may help to define pDCs functions and develop therapeutic strategies.

VII. REFERENCES

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