Universitat Autònoma de Barcelona

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Susana Benet Garrabé

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AIDS Research Institute - IrsiCaixa

Universitat Autònoma de Barcelona

Department of Biochemistry and Molecular Biology

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Susana Benet Garrabé

Institut de Recerca de la SIDA – IrsiCaixa

Hospital Universitari Germans Trias i Pujol

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Directors: **Dr. Javier Martínez-Picado**

Dra. Nuria Izquierdo-Useros

Tutor: **Dr. Jaume Farrés Vicén**

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IrsiCaixa
Institut de Recerca de la Sida

El Dr. Javier Martínez-Picado, investigador principal i professor de recerca ICREA a l'Institut de Recerca de la SIDA, IrsiCaixa (Hospital Universitari Germans Trias i Pujol).

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IrsiCaixa
Institut de Recerca de la Sida

La Dra. Nuria Izquierdo-Useros, investigadora principal a l'Institut de Recerca de la SIDA, IrsiCaixa (Hospital Universitari Germans Trias i Pujol).

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Badalona, 23 de desembre de 2020.

Dr. Jaume Farrés Vicén

'He preguntat a les persones que t'han conegut i tots hi estan d'acord, així que pots quedar-te'

Dr. Bonaventura Clotet, 2013

Quan de resident et vaig preguntar si podia quedar-me a fer el doctorat

'Ho aconseguiràs i ho faràs bé, no en tinc cap dubte'

Dr. Javier Martínez-Picado, Novembre 2020

Durant el període de tancament tesil

'A tozuda me ganas'

'Puedes conseguir cualquier cosa que te propongas, sólo tienes que decidir qué es lo que quieres hacer'

Dra. Nuria Izquierdo-Useros, 2013-2020

Tus dos frases que me han acompañado a lo largo de todo el doctorado

'Estic segura que se't quedaran, ets molt bona i tens molt potencial'

Dra. Bea Mothe, whatsapp 05/03/2020

'Estic molt orgullosa de tú'

Mami, whatsapp 14/12/2020

'Tú ponte con la tesis que yo me encargo...'

De los peques, de comprar, de cocinar... y un largo etcétera

Jaime, durante el periodo de encierro tesil

'Mami, avui no treballes?'

Martina

Al meu pare

L'immens amor que sempre em vas transmetre

em segueix donant forces per viure, ara que ja no hi ets.

'Let it be, let it be

Let it be, let it be

Yeah, there will be an answer, let it be'

The Beatles

ABBREVIATIONS

AIDS: acquired immunodeficiency syndrome

APCs: antigen presenting cells

APOBEC3G: apolipoprotein B mRNA editing enzyme catalytic subunit 3G

ART: antiretroviral therapy

BLT: bone marrow, liver and thymus

CCR5: C-C chemokine receptor type 5

CTL: cytotoxic T lymphocyte

CXCR4: C-X-C chemokine receptor type 4

DAMP: damage-associated molecular pattern

DCs: dendritic cells

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3 (ICAM)–grabbing non-integrin

DNA: deoxyribonucleic acid

Env: envelope

ESCRT: Endosomal sorting complex required for transport

EVs: extracellular vesicles

Gag: group-specific antigen

GALT: gut-associated lymphoid tissue

GBS: group B Streptococcus

GFP: green fluorescent protein

HAART: highly active antiretroviral therapy

HIV: human immunodeficiency virus

HIV-1: human immunodeficiency virus type 1

HLA: human leukocyte antigen

HTLV-III: human T-lymphotropic virus type III

IFNα: interferon alpha

IFNγ: interferon gamma

Ig: immunoglobulin

IL: interleukins

ILVs: intraluminal vesicles

INSTIs: integrase strand transfer inhibitors

I-type: immunoglobulin-type

LAV: lymphadenopathy-associated virus

LPS: lipopolysaccharide

LTRs: long terminal repeats

mAb: monoclonal antibody

mDCs: mature dendritic cells

MHC: major histocompatibility complex

MHC-I: major histocompatibility complex class I

MHC-II: major histocompatibility complex class II

MLV: murine leukemia virus

Mtb: *Mycobacterium tuberculosis*

MVBs: multivesicular bodies

MVs: microvesicles

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NNRTIs: non-nucleoside reverse transcriptase inhibitors

NRTIs: nucleoside-analog reverse transcriptase inhibitors

OIs: opportunistic infections

PAMPs: Pathogen-Associated Molecular Patterns

p.i.: post-infection

PIs: protease inhibitors

PIC: pre-integration complex

Pol: polymerase

PRRs: pattern recognition receptors

RAB: Ras-associated binding

RNA: ribonucleic acid

SAMHD1: sterile alpha-motif (SAM) and histidine-aspartate (HD) domain-containing protein 1

SHCS: Swiss HIV-1 Cohort Study

Siglecs: sialic-acid-binding Ig-like lectins

SIV: simian immunodeficiency virus

SNARE: soluble N-ethylmaleimide-sensitive fusion attachment protein receptors

TB: tuberculosis

TCR: T cell receptor

TEM: transmission electron microscopy

TLRs: Toll-like receptors

TNF: tumor necrosis factor

VLPs: virus-like particles

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SUMMARY

Antigen presenting cells of the myeloid lineage have the ability to respond rapidly and effectively to infection by coordinating innate and adaptive immune responses. However, in the case of human immunodeficiency virus type 1 (HIV-1) infection, these cells might contribute to viral pathogenesis through the capture and transmission of infectious viral particles to target cells, a process known as *trans*-infection. This mechanism depends on Siglec-1 (CD169), a myeloid-cell surface receptor that recognizes sialylated gangliosides present on the viral membrane. To dissect the contribution of *trans*-infection in HIV-1 pathogenesis *in vivo*, we searched for *SIGLEC1* null individuals and identified 85 heterozygous and 2 homozygous people with a loss-of-function variant that abrogates Siglec-1 expression. Importantly, cells from these individuals were defective for Siglec-1 activity in HIV-1 capture and transmission. Despite this phenotype, we did not observe prominent differences on HIV-1 susceptibility nor progression to acquired immunodeficiency syndrome (AIDS) in individuals harboring the *SIGLEC1* null variant. Nonetheless, analysis of the effect of Siglec-1 truncation on progression to AIDS was not conclusive due to the limited cohort size, the lack of complete clinical records such as the seroconversion date, the restriction to study only off-therapy periods, and the co-infection with additional pathogens that might influence the observed phenotype in the opposite direction from what was expected.

As a matter of fact, the latest limitation prompted us to investigate the effect of the *SIGLEC1* null variant in HIV-1 co-infections and we found a significant association between this variant and extrapulmonary dissemination of *Mycobacterium tuberculosis (Mtb)* in two clinical cohorts comprising 6,256 individuals. When we analyzed the absence of Siglec-1 in a murine model, local spread of bacteria within the lung was apparent in *Mtb*-infected Siglec-1 knockout mice which, despite having similar bacterial load, developed more extensive lesions compared to wild type mice. Moreover, we demonstrated that Siglec-1 is necessary to induce antigen presentation through extracellular vesicle uptake. We postulate that lack of Siglec-1 delays the onset of protective immunity against *Mtb* by limiting antigen exchange via extracellular vesicles, allowing for an early local spread of mycobacteria that increases the risk for extrapulmonary dissemination. Overall, through this thesis we have explored the concept of antagonistic pleiotropy in co-infected individuals harboring the *SIGLEC1* null variant, where the impaired immune control of *Mtb* in the absence of Siglec-1 could influence the clinical

course of HIV-1 infected individuals, thus masking the expected benefits of this variant on delaying AIDS progression.

Les cèl·lules presentadores d'antigen de llinatge mieloide tenen la capacitat de respondre a una infecció d'una manera ràpida i eficient coordinant respostes immunitàries innates i adaptatives. Malgrat això, en el cas de la infecció pel virus de la immunodeficiència humana de tipus 1 (VIH-1), aquestes cèl·lules poden contribuir a la patogènesi viral a través de la captura i la transmissió de partícules virals a les cèl·lules diana, un procés conegut com *trans*-infecció. Aquest mecanisme depèn de Siglec-1 (CD169), un receptor de membrana de les cèl·lules mieloides que reconeix gangliòsids sialilats presents a la membrana del virus. Per tal d'analitzar *in vivo* la contribució de la *trans*-infecció en la patogènesi del VIH-1, vam buscar individus *SIGLEC1*-deficients i vam identificar 85 individus heterozigots i 2 homozigots per una variant de pèrdua de funció que aboleix l'expressió de Siglec-1. De manera rellevant, les cèl·lules d'aquests individus mancaven de l'activitat de Siglec-1 en relació a la captura i la transmissió del VIH-1. Malgrat aquest fenotip, no vam observar diferències prominents pel què fa a la susceptibilitat a la infecció per VIH-1 ni a la progressió cap a la síndrome d'immunodeficiència adquirida (SIDA) en els individus portadors d'aquesta variant de *SIGLEC1*. Malgrat tot, l'anàlisi de l'efecte del truncament de Siglec-1 en la progressió a SIDA no va ser concloent degut a la mida limitada de la cohort, la manca d'una història clínica complerta amb informació sobre la data de seroconversió, la restricció d'estudiar només períodes sense tractament i la co-infecció amb patògens addicionals que podrien influenciar el fenotip observat en la direcció oposada al que s'esperava.

De fet, aquesta darrera limitació ens va portar a investigar l'efecte de la variant *SIGLEC1*-deficient en les co-infeccions associades al VIH-1 i vam trobar una associació significativa entre aquesta variant i la disseminació extrapulmonar de *Mycobacterium tuberculosis (Mtb)* en dues cohorts clíniques que inclouen 6,256 individus. Quan vam analitzar l'absència de Siglec-1 en un model murí, els ratolins *knockout* per Siglec-1 van presentar una propagació local de bacteris al pulmó i malgrat tenir una càrrega bacil·lar similar, van desenvolupar lesions més extenses en comparació amb els ratolins salvatges. A més a més, vam demostrar que Siglec-1 és necessari per tal d'induir la presentació d'antígens a través de la captura de vesícules extracel·lulars. Proposem un model on l'absència de Siglec-1 endarrereix l'inici d'una immunitat que protegeix enfront el micobacteri limitant l'intercanvi d'antígens mitjançant vesícules extracel·lulars, permetent així una propagació local del micobacteri que incrementa el risc d'una disseminació extrapulmonar. En resum, al llarg d'aquesta tesi hem explorat el

concepte d'antagonisme pleiotròpic en individus co-infectats portadors de la variant *SIGLEC1*-deficient, on l'alteració del control immunitari del micobacteri en absència de Siglec-1 podria influenciar el curs clínic dels individus infectats pel VIH-1, emmascarant així els beneficis esperats d'aquesta variant en retardar la progressió a SIDA.

Chapter 1

INTRODUCTION

Fighting infectious agents that constantly threaten our health is a daily challenge that our immune system has to face. Once a pathogen invades our organism, the immune system triggers a rapid and specific response to efficiently contain these infectious agents. **Antigen presenting cells** (APCs) constitute one of the key fighters of the host defense program. However, some pathogens, such as the **human immunodeficiency virus type 1** (HIV-1), have evolved to alter the host defense program triggered by APCs to promote viral transmission and infectivity. Yet, these APCs are required to effectively mount early responses against other infectious agents such as ***Mycobacterium tuberculosis*** *(Mtb).* In this thesis we have focused in the **host-pathogen interactions** established between APCs of myeloid origin and two major infectious agents such as HIV-1 and *Mtb*. Our search on how these host-pathogen interactions could play a deleterious role favoring the pathogenicity of HIV-1 has led us to discover how, surprisingly, the very same interaction could be beneficial to trigger immunity against *Mtb.* Thus, the "yin and yang" of biological functions involved in pathogen control are discussed throughout this thesis. In this chapter, we provide an overview of several mechanisms by which APCs initiate immune responses to protect the host from invading pathogens, how HIV-1 evades these strategies to promote viral dissemination, and how early immunity against *Mtb* is mounted.

1. THE ROLE OF ANTIGEN PRESENTING CELLS IN HOST DEFENSE

Upon infection, APCs coordinate a rapid but unspecific mechanism of defense that constitutes the **innate immunity**, along with a more potent and specific response that arises later, which is known as **adaptive immunity**[1]. In addition, adaptive responses can be efficiently amplified through the close interaction of APCs and **extracellular vesicles** (EVs) containing antigens released upon infection.

1.1 APCs in innate immunity

Innate immunity constitutes the first line of defense against pathogens through the induction of a rapid response aimed to eliminate or control infectious agents until the specific adaptive immune responses become effective[1,2]. APCs of the myeloid lineage initiate innate immune

responses[3] after the recognition of conserved molecular structures of microbial agents, termed **pathogen-associated molecular patterns** (PAMPs). In addition, these essential structures are shared by large groups of pathogens, allowing a limited number of germline-encoded receptors to recognize a great variety of molecular signatures associated with pathogens[4,5]. That is the case of the bacterial lipopolysaccharide (LPS), which is shared by all gram-negative bacteria. Viral RNA or DNA genomes and mannans constitute other examples of PAMPS[1,6]. The host receptors, present in APCs, which can specifically recognize these PAMPs, are referred as **pattern recognition receptors** (PRRs)[6]. Importantly, to avoid autoimmunity events, PRRs have the ability to discriminate between self-constituents from pathogenic or non-self components[7]. There are several classes of PRRs that orchestrate the initiation and regulation of innate immune responses. Functionally, PRRs can be divided into three classes: secretory, endocytic, and signal triggers[5].

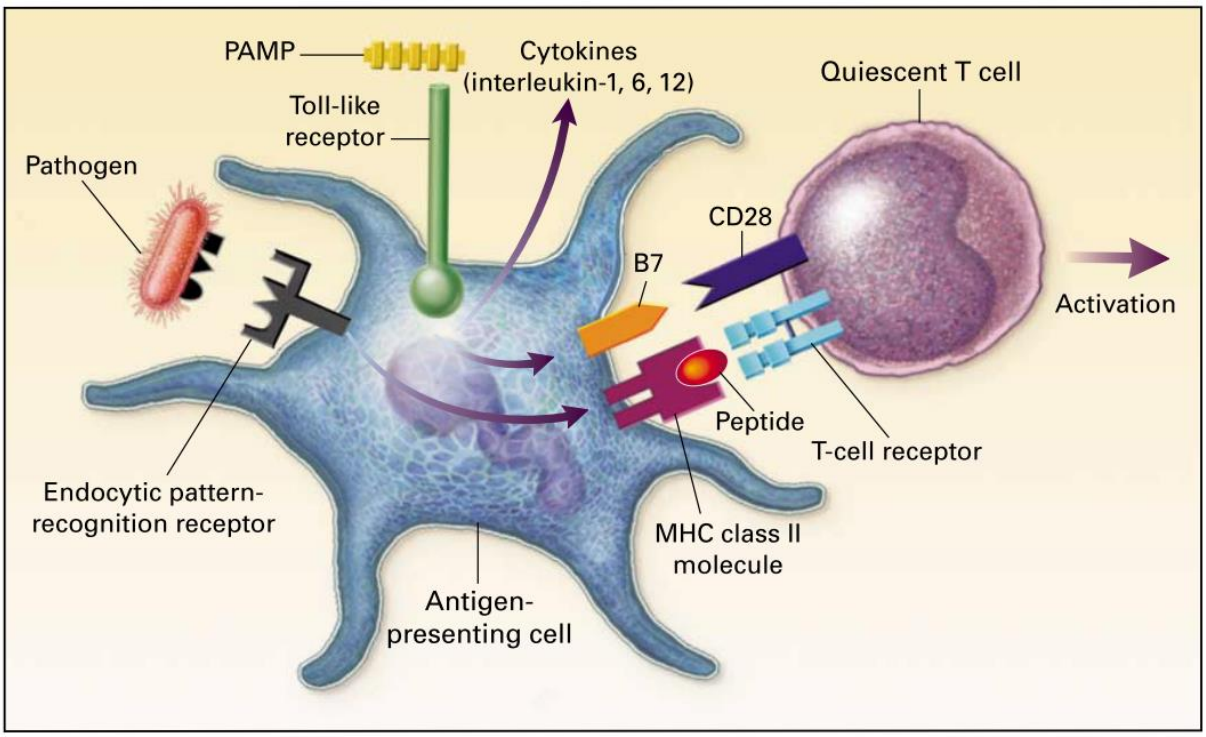

Figure 1.1 APCs orchestrate innate and adaptive immune responses through pathogen sensing via PRRs. Upon infection, APCs have the capacity to initiate innate responses through the recognition of the conserved PAMPs by the PRRS expressed on these cells. Endocytic PRRs expressed on the surface of APCs mediate the uptake and phagocytosis of microorganisms, which will be processed in the lysosome to form a complex with the MHC class II molecule for T-cell receptor recognition. PAMPs can be recognized by other type of PRRs, such as TLRs, leading to the activation of signaling pathways that induce the expression of cytokines,

chemokines, and co-stimulatory molecules, necessary for the initiation of adaptive immune responses. From ref.[5].

Secreted PRRs bind to microbial cell walls and flag them for recognition by the complement system and phagocytes. The best-characterized receptor of this class is the mannan-binding lectin, which recognizes microbial carbohydrates. **Endocytic receptors**, such as the mannose receptor, present on the membrane of APCs, mediate the uptake and delivery of the pathogen into lysosomes, where can be processed and presented by major histocompatibility complex (MHC) molecules (**Figure 1.1**). Other characteristic endocytic receptors are immunoglobulin-type (I-type) lectins, which constitute a group of glycan-binding receptors within the immunoglobulin (Ig) superfamily[8,9]. The sialic-acid-binding Ig-like lectins (Siglecs) are the best characterized members in this group. They are present on the surface of APCs and recognize sialic acid found on several pathogens such as HIV-1 or sialylated bacteria, and also in host cells[8,9,10,11,12,13,14]. Finally, **signaling receptors**, such as the Toll-like receptors (TLRs), activate signal-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines, chemokines and co-stimulatory molecules (**Figure 1.1**). At least 10 human TLR have been identified to date, each with a particular ligand specificity[15-16]. The first human TLR characterized was TLR4, which recognizes LPS, leading to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Another example is TLR2, which is involved in the induction of tumor necrosis factor (TNF) in APCs, being essential to generate a protective immune response against several microorganisms, including *Mtb*[17,18]. These signaling pathways mediated by PRR activation, induce the expression of a variety of cytokines and co-stimulatory molecules that are crucial to initiate adaptive immune responses[19].

APCs have therefore the capacity to trigger rapid innate immune responses through pathogen sensing via PRR interaction. This process leads to the activation of signaling pathways that induce the expression of cytokines that are essential for the development of protective inflammatory responses and co-stimulatory molecules required for the activation of T cells, thus leading to the initiation of adaptive immunity.

1.2 APCs in adaptive immunity

In addition to their role in innate immunity, APCs play a critical role in adaptive immunity. Dendritic cells (DCs) are considered the most potent APCs[20,21] with a unique ability to initiate, coordinate, and regulate adaptive immune responses. **Immature DCs** residing in peripheral tissues have the capacity to efficiently capture pathogens, which are degraded into peptides in endocytic compartments. Later, these peptides will be loaded into MHC molecules to function as immunogenic peptides once DCs mature.[22,23]. Thus, immature DCs are specialists in surveying the periphery, acquiring and storing antigens, but have a limited capacity for direct T-cell activation[24]. After detecting microbial products or proinflammatory cytokines these DCs experiment a cytoplasmic reorganization characterized by a redistribution of MHC from intracellular compartments to the plasma membrane and these activated DCs migrate to the secondary lymphoid tissues[25], where fully **mature DCs** (mDCs) present these antigens to naïve T cells, promoting their activation[26]. In addition, maturation enhances the expression of co-stimulatory molecules such as CD80, CD86 and CD40 and the transport of peptide-MHC complexes to the cell surface for antigen presentation and priming of T cell responses[27,28,29]. The interaction of the peptide-loaded MHC molecules exposed on the surface of APCs with antigen-specific T cell receptors (TCRs) occurs in a complex and dynamic structure known as the **immunological synapse**[30,31]. Once activated, T cells bearing antigen-specific TCRs are selected for clonal expansion and differentiation into effector and memory T cells aimed at eliminating the invading microorganism[32].

All DCs have functional MHC class I (MHC-I) and MHC class II (MHC-II) presentation pathways. The use of one route or another partially relies on the endogenous or exogenous origin of the antigens. **Endogenous antigens** are produced by the APC itself. That is the case of viral antigens, which are degraded mainly in the cytosol by the proteasome after APC infection. Once processed, these peptides are loaded onto MHC-I molecules forming the MHC-I:peptide complexes that are presented to $CD8^+$ T cells triggering cytotoxic responses[33,32] (**Figure 1.2,** left panel). Alternatively, **exogenous antigens** follow a different pathway and can be endocytosed by pinocytosis, phagocytosis or receptor-mediated endocytosis[34]. Once internalized, antigens become accessible to endosomal proteases and the derived peptides can be presented by MHC-II molecules[35] (**Figure 1.2,** central panel). MHC-II:peptide complexes

are recognized by CD4⁺ T cells, which differentiate into several effector cell subtypes[36]. Importantly, the endogenous and exogenous antigen presenting pathways are not mutually exclusive and mDCs can also present exogenous antigens to CD8⁺ T cells through the MHC-I molecules. This mechanism is known as **cross-presentation** and allows the priming of cytotoxic T lymphocyte (CTL) responses against intracellular microbial infections, such as *Mtb*[37,38,39] and also when DCs are not productively infected, as it happens in HIV-1 infection[40,41,42] (**Figure 1.2,** right panel). In a similar way, endogenous antigens can also be presented to CD4⁺ T cells via MHC-II (**Figure 1.2**, dotted line). This non-classical presentation pathway is described for viruses such as influenza A virus[43,44] and HIV-1[45].

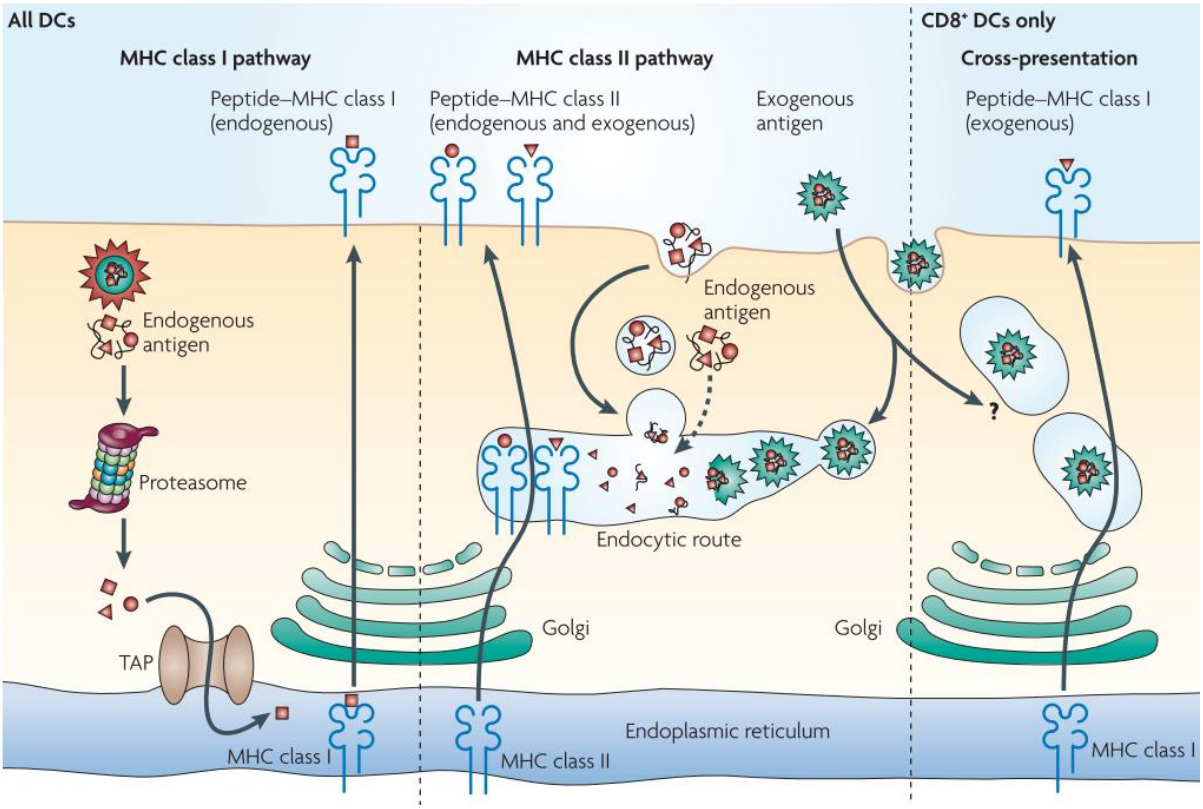

Figure 1.2 Antigen presentation pathways in DCs. Endogenous antigens can follow two routes of presentation: the classical one by which antigens are processed by the proteasome and then loaded onto MHC-I molecules (left panel), or the non-classical pathway that consists of their access to endocytic route for MHC-II presentation (central panel, dotted line). Exogenous antigens can either be internalized and digested in endocytic compartments following antigen loading onto MHC-II (central panel) or be presented through the cross-presentation pathway via MHC-I (right panel). From ref.[46].

Immature DCs act as sentinels of the immune system patrolling peripheral tissues. After encountering an invading pathogen, activated DCs process captured antigens and migrate to the secondary lymphoid tissue, where they establish a close interaction with T cells and present antigens. Peptides derived from invading pathogens can follow multiple antigen presentation pathways leading to the activation and proliferation of T-cell responses, which are necessary to mount a highly specific and long-lasting adaptive immunity aimed to eliminate the infectious agent.

However, DCs migrating from the periphery may not be the only ones exclusively presenting the captured antigens to T cells in the lymphoid tissues. These cells may transfer their antigens to other DCs which may have not been in direct contact with the pathogen. This antigen transfer could occur either by phagocytosis of the antigen-loaded DCs[47] or by the release of antigen-bearing **EVs** generated by DCs[24,48]. This antigen exchange between immune cells through EVs can increase the number of APCs with MHC:peptide complexes thus leading to the **amplification of adaptive immune responses**[49,50].

1.3 EV regulation of immune responses

EVs are a heterogeneous group of small membrane vesicles that are secreted by most cell types and mediate intercellular communication processes. EVs were originally identified in 1946 by Chargaff and West as procoagulant platelet-derived particles in normal plasma[51]. In 1983 EVs were described by Johnstone and Stahl groups while studying the redistribution and externalization of the transferrin receptor during *in vitro* maturation of sheep reticulocytes[52,53]. The two groups simultaneously observed small vesicles with an average diameter of 30-100 nm accumulating in multivesicular bodies (MVBs), where the transferrin receptor was endocytosed (**Figure 1.3, A**). They showed beautifully by scanning and transmission electron microscopy (TEM) how these MVBs fused with the plasma membrane, and released their content by exocytosis[54,52,53] (**Figure 1.3, B**).

Recently after the discovery of EVs, it was believed that the formation of these vesicles was part of a mechanism for removing unnecessary proteins and other molecules from producer cells, functioning only as cellular garbage disposals[55,56]. It was not until the mid-1990s when EVs were shown to have an immunological relevance, as B cell-derived EVs express functional

MHC-I and MHC-II molecules[57,58]. Furthermore, DC-derived EVs carry co-stimulatory molecules that can generate specific T-cell responses[58]. In recent years, the knowledge on the functional role of EVs has increased exponentially.

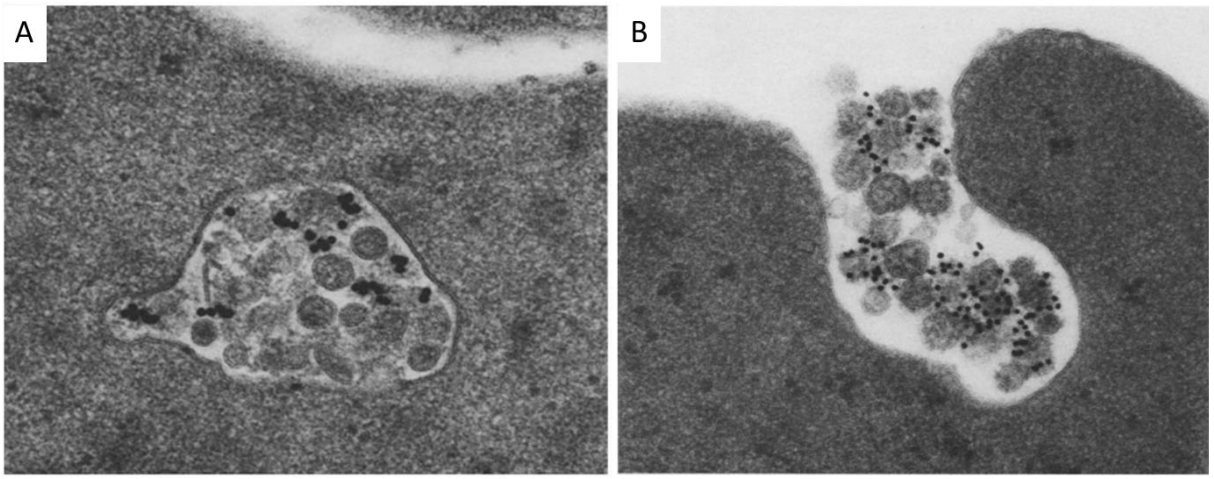

Figure 1.3 TEM visualization of MVBs containing EVs. TEM images of sheep reticulocytes after internalization of colloidal gold-conjugated anti-ferritin antibodies. **A.** MVB containing EVs together with the internalized gold-labelled anti-transferrin receptor antibodies (x85,000 magnification). **B.** Exocytosis of EVs together with the internalized gold-labelled antibodies anti-ferritin after the fusion of MVB with the plasma membrane (x98,625 magnification). From ref.[53].

Based on the current comprehension of their biogenesis, EVs can be broadly divided into two main categories: exosomes and microvesicles (MVs), although apoptotic bodies cannot be excluded from EV preparations. Specifically, the term **exosomes** refers to intraluminal vesicles (ILVs) of 30-100 nm of diameter, formed by the inward budding of the endosomal membrane during maturation of MVBs, which are intermediates within the endosomal system[59,60] (**Figure 1.4**). This process of the exosome biogenesis involves particular machineries, such as the ESCRT (Endosomal Sorting Complex Required for Transport) machinery together with its associated proteins, which drive the membrane shaping and vesicle scission[61]. The ESCRT machinery includes different subunits that act in a stepwise manner. Thus, ESCRT-0 clusters transmembrane proteins in the endosomal membrane in a ubiquitin-dependent manner, ESCRT-I and ESCRT-II induce bud formation, and ESCRT-III finally mediates vesicle scission. However, there are ESCRT-independent pathways involved in the generation of exosomes. Some of these mechanisms require the participation of lipids, such as ceramide[62] or

cholesterol, while others are regulated by proteins, such as tetraspanins[63,64]. Once formed, the secretion of exosomes requires the transport and fusion of MVBs with the plasma membrane, avoiding the lysosomal route of degradation[59,60] (**Figure 1.4**). This process is orchestrated by RAB (Ras-associated binding) proteins that facilitate the MVB interaction with the cytoskeleton allowing its trafficking and docking to the plasma membrane. Then, SNARE proteins (for soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors) mediate the fusion of MVBs with the plasma membrane[65,66].

Several molecules involved in exosome biogenesis have been proposed as exosome markers. That is the case of molecules of early/late endosomes or MVBs like tetraspanins (CD9, CD63 and CD81), proteins associated to the ESCRT machinery, or RAB and SNARE proteins.

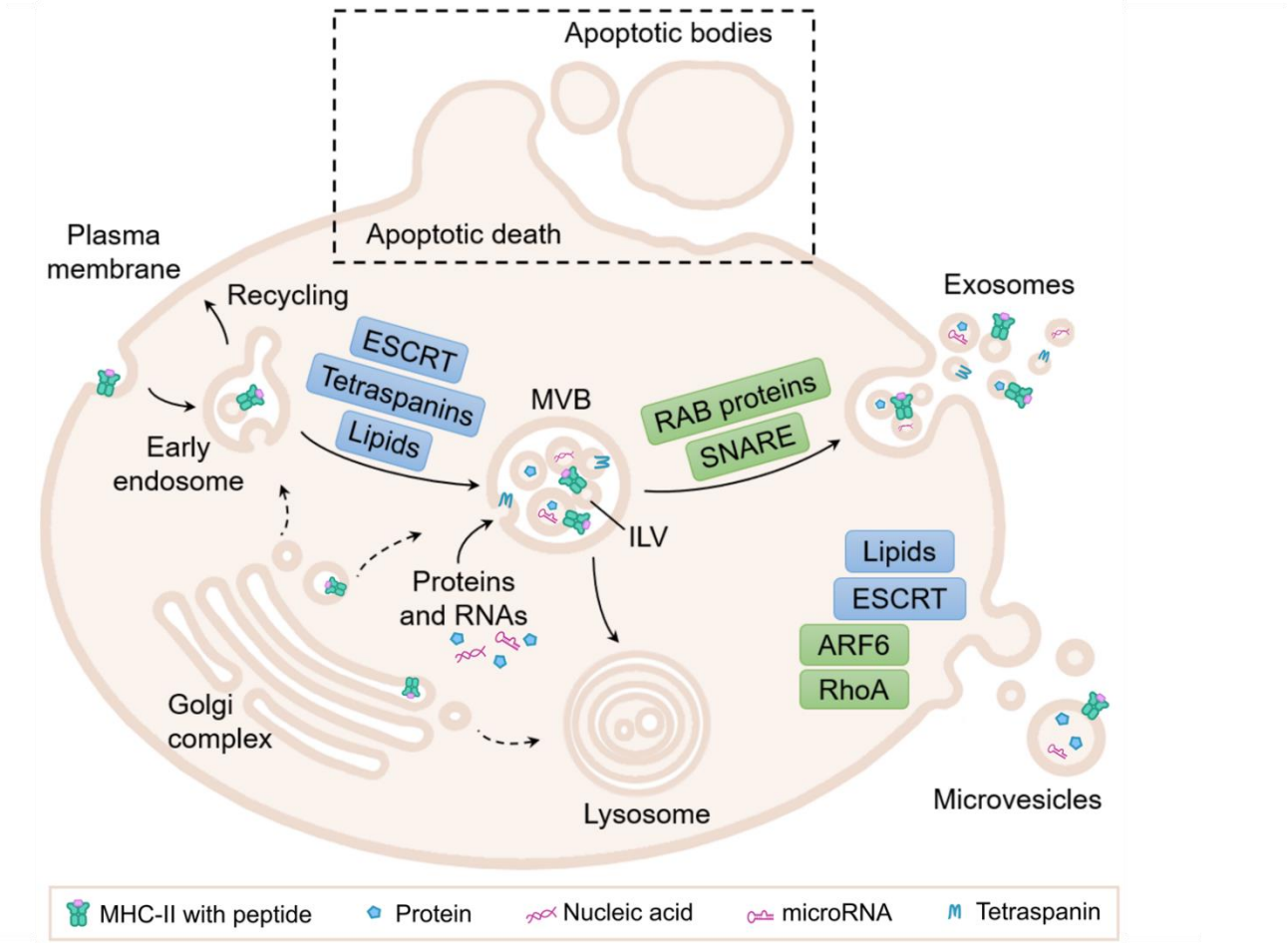

Figure 1.4 Schematic representation of EVs biogenesis and secretion. Several proteins are involved in the formation (shown in blue) and release (shown in green) of EVs. **Exosomes** are generated throughout the endocytic pathway as intraluminal vesicles (ILVs) constituting the multivesicular body (MVB), through ESCRT (Endosomal Sorting Complex Required for

Transport)-dependent or ESCRT-independent (often mediated by tetraspanins or lipids) mechanisms. The release of exosomes is mediated by the Ras-associated binding (RAB) and SNARE (soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors) proteins, which facilitate the trafficking and fusion of MVBs with the plasma membrane allowing the release of exosomes to the extracellular medium. Alternatively, early endosomes can be recycled to the plasma membrane, and MVBs can end up in the lysosome. **Microvesicles**, in contrast, are generated by the budding from the plasma membrane, with the help of molecules from the ESCRT and SNARE machineries, which mediate the budding, and cytoskeletal proteins (ARF6 and RhoA), which are needed for microvesicles release. Through the biogenesis process, exosomes and microvesicles are loaded with different molecules from the originating cell, such as the MHC:peptide complexes, proteins as tetraspanins, often used as an exosome marker, and RNAs (miRNA, mRNA...). Finally, the **apoptotic bodies**, which are generally bigger than exosomes and microvesicles, are released during the apoptotic cell death and carry damage-associated molecular pattern (DAMP) signals like damaged DNA. Adapted from ref.[60].

Microvesicles (MVs) constitute another form of EVs, which are larger than exosomes (ranging in size from 50 nm to 1µm). They originate by an outward budding of the plasma membrane[67,68] (**Figure 1.4**). The biogenesis of MVs requires several molecular rearrangements within the plasma membrane, including changes in lipid and protein composition, and also in calcium levels[69], but the precise mechanism governing MVs formation and shedding is not completely deciphered yet. The main mechanism involved in the release of MVs relies on a rise in intracellular calcium that modulates lipid raft formation, which induces membrane curvature for MV budding. Then, cytoskeleton remodeling through the cleavage of cytoskeletal proteins (ARF6 and RhoA) allows the release of MVs[70]. Other mechanisms proposed for the formation of MVs have implicated the use of the ESCRT and SNARE machineries[71,72] (**Figure 1.4**). Since MVs share the classical exosome markers and have similar size and density, it becomes challenging to distinguish both types of EVs. Finally, the diversity of EVs populations is further increased by the inclusion of additional structures, such as **apoptotic bodies**, which have the peculiarity to be specifically released from cells undergoing apoptosis[73] (**Figure 1.4**). This type of EVs have a diameter of 1 to 5 µm and contain condensed DNA and phosphatydilserine in the outer leaflet of their membrane.

Overall, EVs comprise a heterogeneous population secreted by most cells, including mast cells, dendritic cells, B lymphocytes, neurons, adipocytes, endothelial cells, and epithelial cells[49] and have been found in all types of biofluids[74,75,76]. There is now evidence that the nature and

abundance of EVs cargoes are cell type-specific and are often influenced by the physiological or pathological state of the donor cell, the stimuli that modulates their production and release and the molecular mechanisms that lead to their biogenesis[69]. Thus, each cell type tunes EVs biogenesis and release, which will determine their particular lipid, protein and nucleic acid compositions[66,59]. For example, EVs derived from macrophages infected with *Mtb* contain pathogen-derived antigens[77], and APC-derived EVs are enriched in MHC-I and MHC-II as well as co-stimulatory molecules[78]. Moreover, among the broad spectrum of molecules that can be loaded into the EVs, the presence of glycoconjugates, which might contain sialic acid residues, have become relevant for its emerging role in EV biogenesis, in cellular recognition and in the efficient uptake of EVs by recipient cells through the interaction with lectin receptors, such as one of the members of the Siglec family termed Siglec-1[79,80,81]. In addition, the finding that secreted EVs carry both unprocessed antigenic material and MHC:peptide complexes has generated interest due to their roles in **triggering immune responses**[57,58,82]. Indeed, the exchange of EVs containing MHC:peptide complexes between immune cells is a potential mechanism to amplify the initiation of immune responses[49] (**Figure 1.5**). This mechanism relies on the recognition of incoming pathogens by immature DCs located in the peripheral tissue, which leads to the generation of MHC:peptide complexes that can be secreted on EVs. These trafficking EVs can be internalized by other DCs that have not been in direct contact with the pathogen, but can now present the antigen contained in the EV to amplify adaptive immune responses. Thus, both immature DCs capturing the antigen and becoming activated through the classical pathway, along with bystander activated DCs trapping antigen-bearing EVs could migrate to the secondary lymphoid tissues and activate T-cell responses. As a result, EV production would increase the number of DCs bearing the relevant MHC:peptide complexes, and consequently, amplify the magnitude of the immune response[49] (**Figure 1.5**).

Once EVs have been captured by DCs, they can be used for antigen cross-presentation to $CD8^+$ T cells or to stimulate $CD4^+$ T cell responses *in vivo*[49,83]. To mediate $CD4^+$ T-cell stimulation, DCs can process the antigens contained in the captured EVs, or alternatively, these cells can directly present the antigens coming from EVs, which have been previously processed and loaded into MHC-II molecules[49,83]. Direct presentation of antigens derived from EVs captured by DCs was demonstrated using MHC-II deficient DCs that were still able to activate $CD4^+$ T

cells with MHC:peptide complexes acquired from EVs[83]. Importantly, EVs could not induce naïve T-cell proliferation *in vitro,* unless mDCs were also present, due to the fact that co-stimulatory molecules expressed on DCs surface are also required to trigger immunity[83].

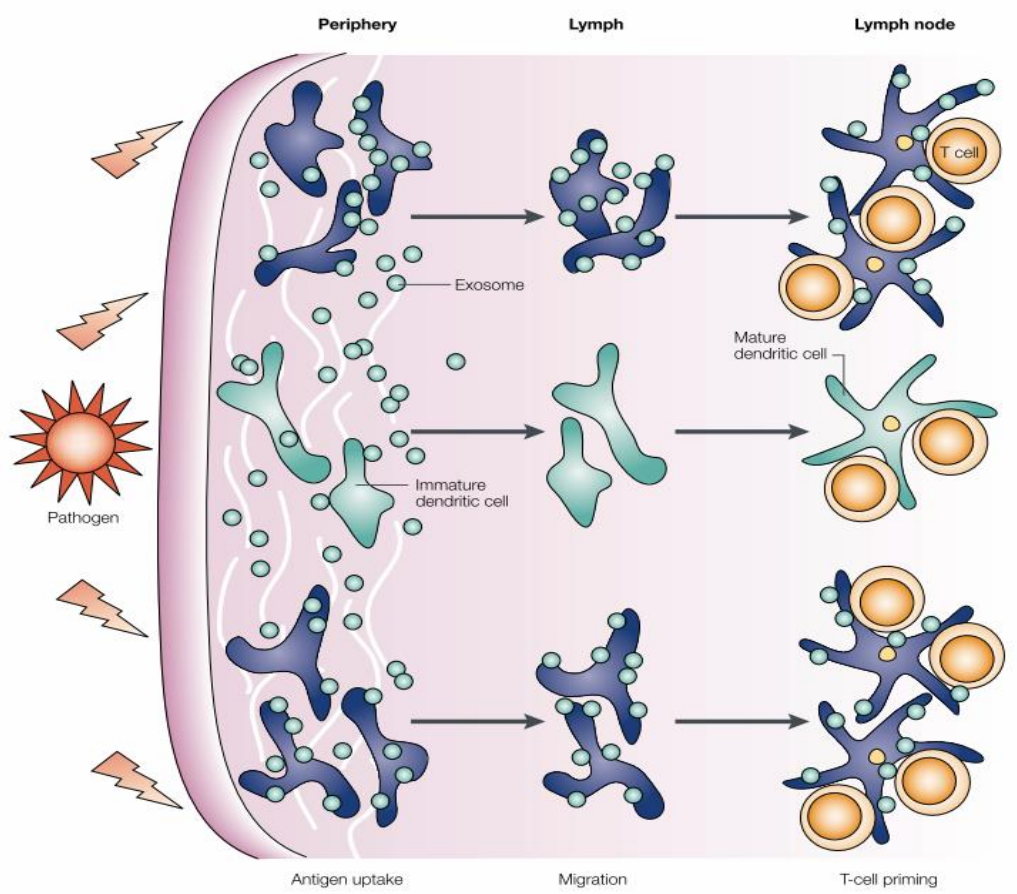

Figure 1.5 Working model for the role of EVs in amplification of immune responses. Immature DCs patrolling the peripheral tissues mediate the uptake of invading pathogens. As a result, DCs generate MHC:peptide complexes, which are incorporated into the EVs released by these cells. These EVs could sensitize other DCs that have not been in direct contact with the pathogen, thus increasing the number of APCs harboring MHC:peptide complexes, which upon migration towards the secondary lymphoid tissues might induce the activation of T-cell responses. From ref.[49].

The role of EVs in antigen presentation was first described in a study using exosomes secreted by a human B cell line[57]. Since then, the immune modulatory properties of EVs derived from other cells have been widely demonstrated[50]. In the context of infectious diseases, it has been observed that EVs released from cells infected with intracellular pathogens such as *Mtb*, *Salmonella typhimurium* and *Toxoplasma gondii* are also implicated in the induction of

immune responses aimed at containing these pathogens[84,85]. In particular, EVs derived from *Mtb*-infected cells contain antigenic bacterial proteins[86,87], which modulate antigen presentation during *Mtb* infection[84,88,89,90,91,92]. It was recently shown in a mouse model infected with *Mtb* that EVs derived from *Mtb* infected macrophages were able to activate antigen-specific $CD4^+$ and $CD8^+$ T cells *in vivo*[90]. Moreover, the potential role of EVs as cell-free vaccines against tumoral or parasitic diseases has been previously described. The first evidence reported *in vivo* of using EVs for cancer immunotherapy was demonstrated in a murine model using tumor peptide-pulsed DC-derived EVs, which were able to prime specific cytotoxic T-cell lymphocytes that effectively suppressed tumor growth[58]. Since then, further studies involving the application of EVs to elicit an immune response against established tumors were explored in human clinical trials against late-stage cancer patients[93]. Also, EVs from convalescent plasma have been used as an antigen source and successful clinical vaccine platform for the veterinary Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)[94]. With regard to parasitic diseases, their value as novel antigenic sources has been shown for *Toxoplasma gondii*[85], *Leishmania major*[95] and *Plasmodium yoelii*[96], where vaccines formulated with EVs from antigen-loaded DCs or from infected cells protect animal models from infection[97]. Thus, the increasing knowledge in the biological functions of EVs has positioned them as potent regulators of immune responses, aimed at eliminating different types of pathogens and tumoral cells.

Despite antigen exchange via EV trafficking between immune cells constitute an efficient mechanism to activate and amplify T-cell responses, retroviruses may exploit these pathways to infect new target cells[98]. In the case of HIV-1, the virus uses the mechanism by which APCs capture and secrete EVs, and may exploit these cells as “Trojan horses” to migrate towards secondary lymphoid tissues, where the virus can find and infect $CD4^+$ T cells, which are their main cellular targets[99,100,101,102]. This mechanism of viral escape known as *trans*-infection is facilitated by the receptor Siglec-1 expressed on APCs, which recognizes sialic acid-containing gangliosides, present both on the viral membrane and also on the surface of EVs[103]. Indeed, in this thesis we explore how Siglec-1-expressing APCs facilitate HIV-1 dissemination *in vivo*, while the capture of EVs by this receptor can be crucial to initiate immune responses against *Mtb*.

2. HIV-1 AND CO-INFECTIONS

HIV is the causative agent of AIDS. In the absence of treatment, the devastating deterioration of the immune system that characterizes AIDS is often associated with the development of opportunistic infections that complicate the prognosis of HIV-1 infected individuals.

2.1 History and epidemiology of HIV

On June 5th, 1981, the report of four cases of pneumocystis pneumonia in previously healthy men who had sex with men from Los Angeles, along with a severe immunodeficiency status[104], captivated the attention of the scientific and medical community worldwide. That was the official date of the beginning of the global AIDS epidemic. In 1983, a group of scientists headed by Luc Montagnier and Françoise Barré-Sinoussi at the Pasteur Institute in Paris first isolated a novel retrovirus from the lymph node of an infected individual with multiple lymphadenopathies, which was first dubbed lymphadenopathy-associated virus (LAV)[105]. In 1984, a team led by Robert Gallo from the National Cancer Institute in Bethesda also claimed the discovery of the virus naming it human T-lymphotropic virus type III (HTLV-III)[106]. Molecular cloning of the virus[107,108,109] led to its full nucleotide sequencing in 1985[110,111,112]. A year later, the etiological agent of AIDS received the final nomenclature of HIV. By that time, a T-cell tropic virus was isolated in monkeys, and it was shown to be closely related to HIV[113]. Since then, several studies have focused on the analysis of similarities and differences between simian immunodeficiency virus (SIV) and HIV, finding strong evidence for cross-species transmission of SIV from simian species to humans[114,115,116]. In 2008, the Nobel Prize in Medicine was awarded to Montagnier and Françoise Barré-Sinoussi for their discovery of HIV. Almost four decades after the first descriptions of HIV cases, 75 million people have been infected with HIV, 32 million people have died from AIDS-related illnesses globally and 38 million people are currently living with HIV, of which around 25 million people live in Africa[117,118].

2.2 Classification and viral structure

HIV belongs to the *Retroviridae* family, *Orthoretrovirinae* subfamily and *Lentivirus* genus[119]. HIV is classified in two main types: HIV-1 and HIV-2. HIV-1 is the major cause of the AIDS

pandemic and it is spread globally, while HIV-2 is endemic of some regions of Western and Central Africa and is less virulent compared to HIV-1[120]. In this thesis we have mainly focused on HIV-1 infection, so we will describe its structure in greater detail. Further HIV-1 classification subdivides the virus in the groups M, N, O and P, being the group M the most prevalent, with a worldwide distribution. As a result of its spread and evolution in humans, HIV-1 group M has been subdivided into subtypes A–K, including subtypes E and I, which form part of circulating recombinant forms[121,122].

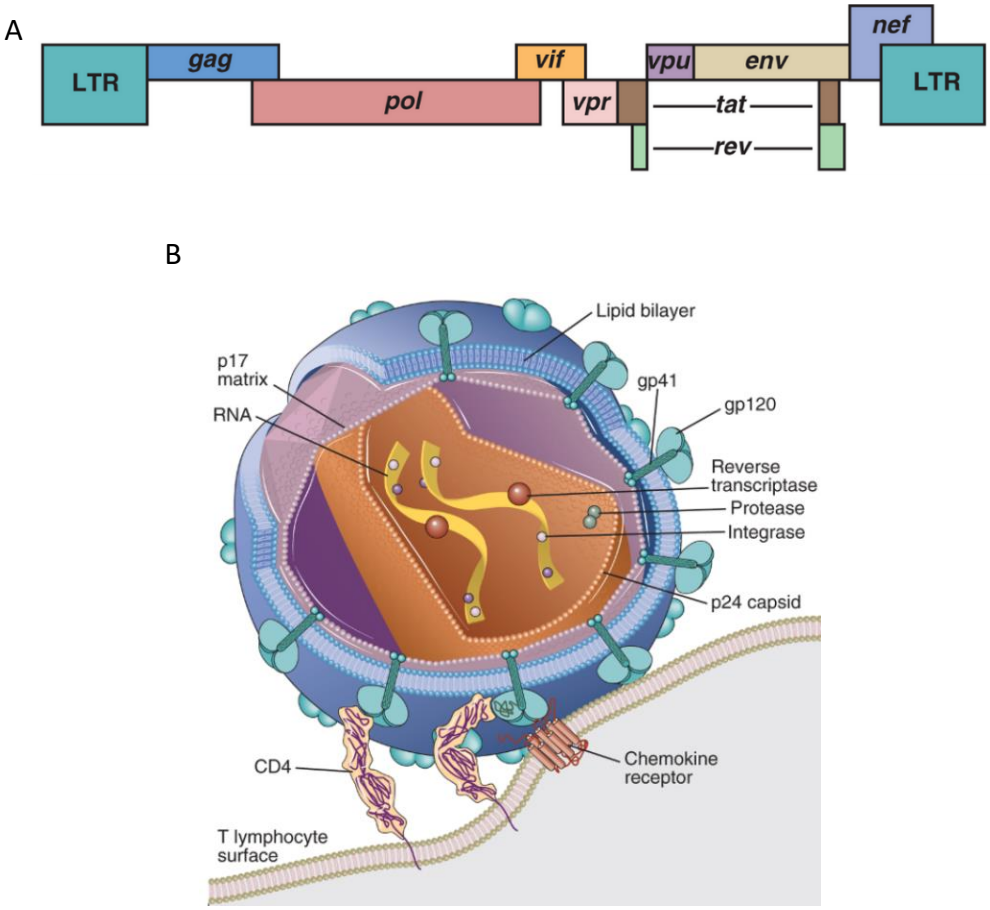

Figure 1.6 HIV-1 genome and viral structure. A. The HIV-1 genome is flanked by two LTRs that delimit three major genes (*GAG, POL* and *ENV*) that encode for major structural and functional proteins, together with other genes that encode for accessory viral proteins. **B.** HIV-1 virions are spherical particles containing two ssRNA molecules and enzymes (reverse transcriptase, protease and integrase), covered by the capsid (p24) and the matrix (p17). They are enveloped by a lipid bilayer containing the glycoproteins gp120 and gp41, which form trimer spikes that allow the recognition of cell surface receptors. From ref.[123].

HIV-1 genome is composed of two copies of single-stranded positive-sense RNA molecules of approximately 9,2 kb in lenght[110,111,112]. The viral genome comprises two long terminal repeats (LTRs) located on each end of the genome, which allows the virus to insert its genetic material intro the host genome, and encodes for three major genes: group-specific antigen *(GAG)*, polymerase *(POL)* and envelope *(ENV)* that encode for major structural and functional proteins, together with other genes that encode for accessory viral proteins: Vif, Vpr, Tat, Rev, Vpu and Nef[124] (**Figure 1.6, A**). The *GAG* gene encodes the p55 polyprotein, precursor of the structural protein of the capsid p24, nucleocapsid p7 and matrix protein p17. The *POL* gene encodes for enzymes crucial for viral replication, which are the reverse transcriptase that converts viral RNA into DNA, the integrase that incorporates the viral DNA into the host chromosomal DNA and the protease that cleaves large gag and pol protein precursors into their components. The *ENV* gene encodes the precursor glycoprotein gp160 that contains gp120 and gp41 subunits, which form trimer spikes that are exposed on the virion membrane allowing for the recognition of cell surface receptors[125].

At **structural** level, the HIV-1 is a spherical particle of 80-150 nm of diameter containing two strains of RNA and viral enzymes necessary for viral replication, covered by a conic capsid formed by the viral protein p24 and the matrix, formed by p17 (**Figure 1.6, B**). They are enveloped by a phospholipid bilayer membrane derived from the host cell, which displays the viral envelope glycoproteins.

2.3 Viral replication cycle

The steps of the HIV-1 replication cycle are orchestrated by the different proteins encoded by the HIV-1 genome, described in Figure 1.6, and include: 1) viral binding and entry; 2) reverse transcription; 3) provirus integration; 4) transcription and translation of viral proteins and 5) viral assembly, budding and maturation (**Figure 1.7**).

HIV-1 entry begins with the adhesion of the virus to the host cell, which leads to the fusion between the viral and cell membrane and the subsequent delivery of the viral capsid into the cytoplasm. The gp120 of the viral envelope glycoprotein first binds to the host $CD4^+$ T cell receptor. This causes conformational changes in the envelope, allowing co-receptor binding, which is mediated in part by the V3 loop of the viral envelope. This initiates the membrane

fusion process as the fusion peptide of gp41 inserts into the target membrane, followed by six-helix bundle formation and complete membrane fusion[126]. There are two possible co-receptors: the chemokine receptor CCR5 or CXCR4. Based on the co-receptor used for infection, HIV-1 strains are classified as R5-tropic, X4-tropic or dual-tropic strains[127] (**Figure 1.7**).

Once the virus enters the cell, the viral **reverse transcriptase** uses the HIV-1 RNA genome as a template to synthetize a linear dsDNA[128], which together with the viral integrase constitutes the pre-integration complex (PIC)[129]. This PIC is translocated into the cell nucleus and **integrated** within the host genome. **Transcription** of proviral DNA into a messenger RNA (mRNA) begins upon cell activation, and then mRNAs travel into the cytoplasm, where **translation** takes place to synthesize both the structural and functional proteins of new virions [125,129,130] (**Figure 1.7**). In the absence of T-cell activation, however, integrated provirus remain silent and these cells will constitute viral reservoirs that allow HIV persistence even in the presence of specific immune responses and effective antiretroviral therapy (ART)[131].

HIV-1 virion **assembly** occurs at the plasma membrane, within specialized membrane microdomains. The proteins encoded by *POL* and *GAG* genes form the nucleus of the maturing HIV particle, while the gene products encoded by the *ENV* gene form the envelope glycoprotein inserted in the viral membrane[125]. Assembly is mediated by gag and includes membrane binding, making the protein–protein interactions necessary to create spherical particles, recruiting the viral *env*, and packaging the HIV-1 RNA[132]. The assembled viral proteins, together with the two copies of ssRNA and viral enzymes, bud from the infected cell from specialized cholesterol-enriched domains of the plasma membrane, also incorporating host proteins, such as gangliosides, which contain sialic acid[133]. The **budding** event by which the new immature virions are released from the plasma membrane relies on the host ESCRT machinery[129,132]. Finally, upon the budding process, the HIV-1 protease cleaves precursor polyproteins rendering fully mature infectious viral particles[132] (**Figure 1.7**). Of note, it has been shown that the expression of the gag protein alone is enough to direct the assembly and budding of spherical particles from the producing cell[134]. This allows for the generation of virus-like particles (VLPs), which can contain a reporter protein such as green fluorescent

protein (GFP) fused to gag, which can be used as a tool for the study of different virological processes.

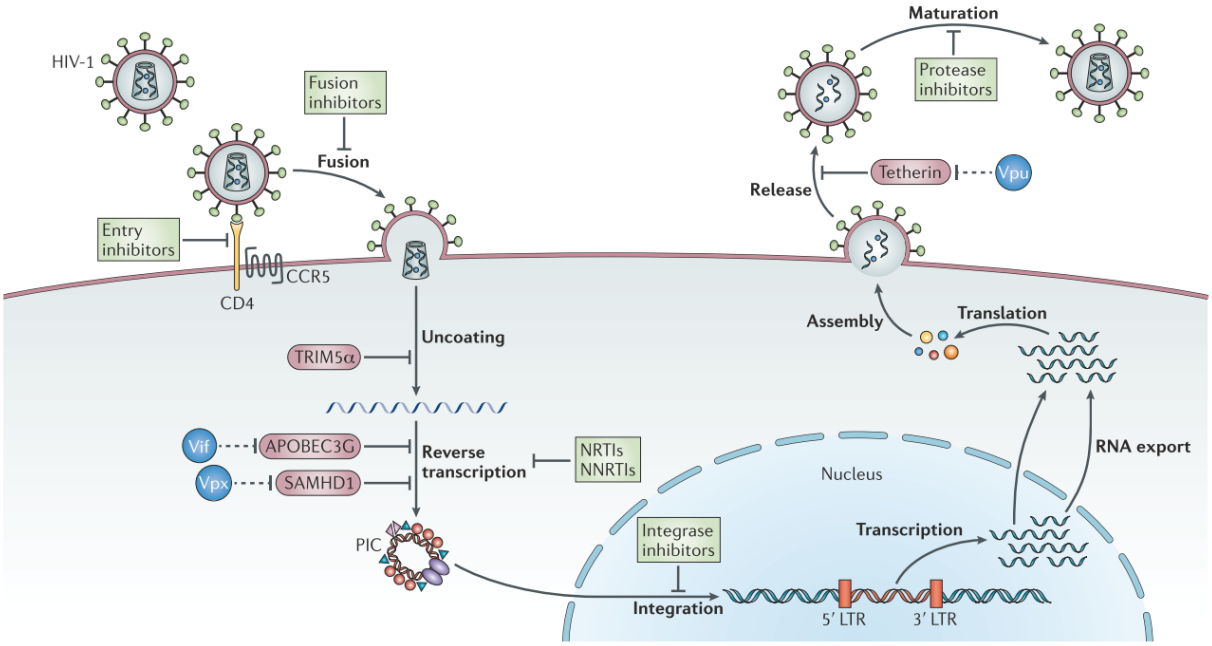

Figure 1.7 Schematic overview of the HIV-1 replication cycle. This figure illustrates the main steps in the viral replication cycle. First, the viral envelope glycoprotein engages the CD4 receptor and the CCR5 (or CXCR4) co-receptor, leading to membrane fusion and entry of the virus particle into the cell. Uncoating of the viral capsid allows the release of the HIV RNA and proteins into the cytoplasm. Viral RNA is reverse transcribed into dsDNA by the viral reverse transcriptase, to form the PIC, which is translocated into the cell nucleus. Once in the nucleus, the viral DNA is integrated into the host DNA by the viral integrase. Upon cell activation, proviral DNA is transcribed and translated to form new viral proteins that assemble in the cell surface into new immature virions. The new viruses bud off and are released. Finally, during maturation, the viral protease cleaves the polyproteins to form mature Gag proteins, resulting in the production of new infectious virions. Each step in the HIV-1 lifecycle is a potential target for antiviral intervention (green). The key viral restriction factors are shown in red and their corresponding viral antagonist are represented in blue. From ref.[135].

2.4 Natural course of HIV-1 infection

HIV-1 is transmitted by sexual contact across mucosal surfaces, by maternal-infant exposure, and by blood contamination[136]. Currently, the most common route of HIV-1 acquisition is **sexual transmission**[117], being the number of copies of HIV-1 RNA per mL of plasma, termed viral load, the most important predictor of the risk of transmission[137]. Following viral

transmission, the natural course of HIV-1 infection includes several phases: eclipse phase, acute or primary infection, chronic infection or clinical latency, and AIDS (**Figure 1.8**).

The **eclipse phase** of HIV-1 infection (which takes place during one or two weeks post-exposure) most often results from the successful transmission and subsequent propagation of a single virus variant, called founder virus[138], which infects permissive cells in the mucosa, such as activated $CD4^+$ T cells, Langerhans cells and macrophages[139]. Shortly after mucosal exposure, the virus can be detected in draining lymph nodes[100,140] and then disseminates systemically. In this viral dissemination route from the viral entry sites to the lymphoid tissue, DCs are thought to play a critical role due to their migratory capacity[100,141,142,143]. In this phase, plasma viremia is still undetectable and neither immune responses nor symptoms of infection are yet apparent[144].

This early phase of HIV-1 infection is followed by the **acute or primary infection** (following three to twelve weeks post-exposure), which is usually characterized by a rapid increase in plasma viremia (**Figure 1.8,** upper panel) and a reduction in the number of $CD4^+$ T cells in blood[144] (**Figure 1.8,** bottom panel, green line). Around the time of peak viremia, the immune response is activated as antibodies against all viral proteins, and as $CD8^+$ T-cell responses against HIV-1 antigens expressed on infected cells[145] (**Figure 1.8,** bottom panel, yellow and blue dotted lines, respectively). At the end of this phase, the level of viremia declines, as a result of both partial control by the immune system and exhaustion of activated target cells, and a limited recover of $CD4^+$ T cells is observed, except for gastrointestinal $CD4^+$ T cells, which are persistently reduced[146]. As a consequence of the destruction of the gut-associated lymphoid tissue (GALT), the intestinal barrier is compromised and bacteria are translocated to the blood[147,148]. This phase is sometimes, but not always, accompanied by the acute retroviral syndrome, which appears usually before seroconversion and is characterized by fever, sore throat, fatigue, weight loss and myalgia and less commonly by headache, retro-orbital pain, enlarged lymph nodes, oral or rectal ulceration and a non-pruritic macular erythematous rash that can last from days to weeks[140,149].

After acute infection it follows a long period of **chronic infection** or **clinical latency** (which varies from one to ten years post-infection) that is characterized by a constant and slow increase of viremia levels, referred to as the viremia “set point”, and steady, near normal or

gradually falling levels of $CD4^+$ T cells in asymptomatic infected individuals[144] (**Figure 1.8,** upper panel). Despite the absence of clinical symptoms during this phase, HIV disease is active in the lymphoid tissue leading to a progressive disruption of the architecture of the lymph node, which causes a progressive involution of the germinal centers that in a later stage will lose the capacity to trap viruses[150].

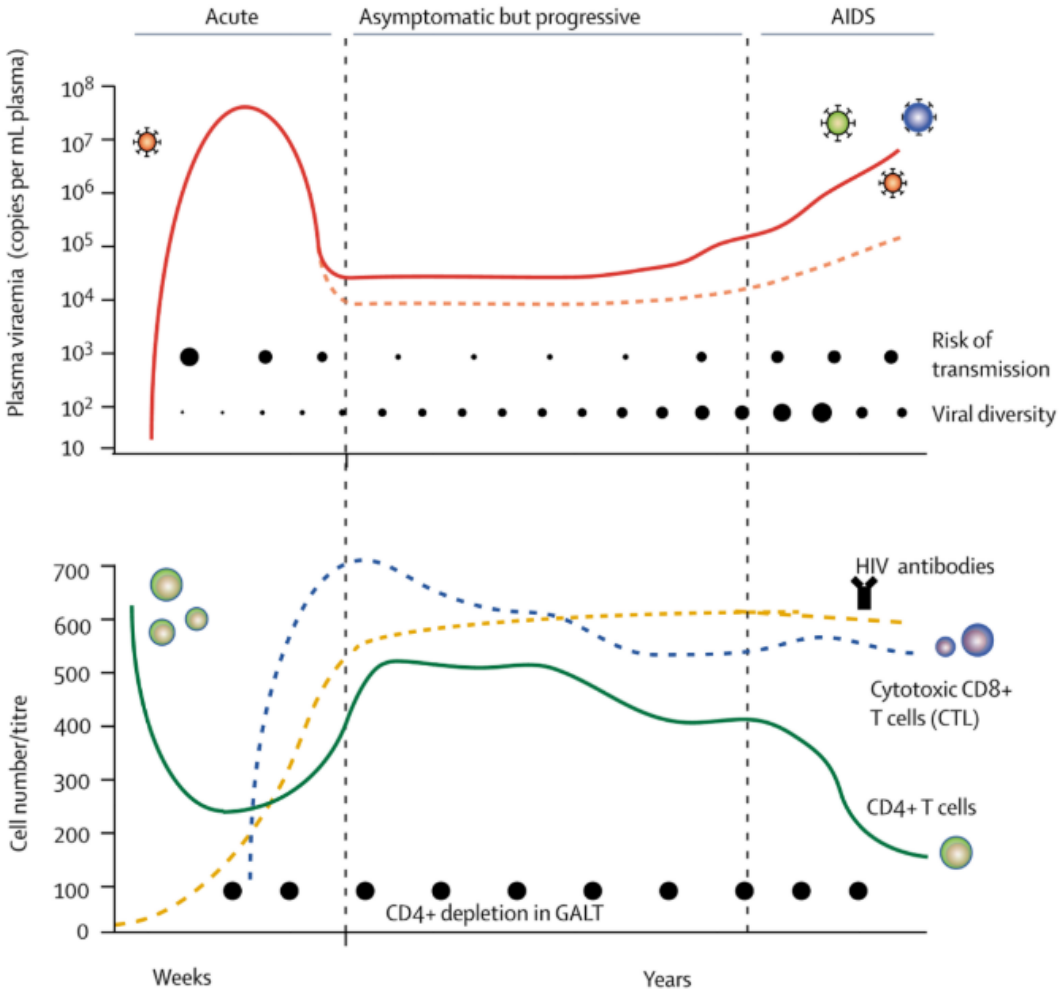

Figure 1.8 Natural course of HIV-1 infection. In the upper panel, evolution of the plasma viremia along the different stages of HIV-1 infection is shown. Orange dotted line represents the variability of viral set-points among infected individuals. The risk of viral transmission is maximum when viral load reaches the peak. Shortly after transmission, HIV-1 evolves generating multiple genetic variants (*quasi*species) that fuel the productive clinical infection and represent the viral diversity shown in the figure. At the bottom panel, evolution of the $CD4^+$ T cell counts (green line), together with the presence of cytotoxic $CD8^+$ T cells (blue line) and specific antibodies (yellow line) are represented through the course of the HIV-1 infection. From ref.[145].

Finally, the **AIDS** phase is characterized by a decline in the number of $CD4^+$ T cells below 200 cells/mm^3, at which immune control of infectious agents can no longer be maintained, and opportunistic infections appear[151] (**Figure 1.8,** bottom panel, green line). The marked immune impairment also results in an increased risk of malignancies. During this later stage of the disease, immune control of HIV-1 replication is also lost, and the level of viremia rises, resulting in the death of the infected individual[144] (**Figure 1.8,** upper panel).

The clinical course and outcome of HIV-1 infection are characterized by a high heterogeneity among untreated infected individuals, with the majority of individuals progressing to AIDS during an average time of 8-10 years. However, a small subset of untreated individuals maintains low to undetectable plasma viremia and high $CD4^+$ T-cell counts over many years (**HIV-1 controllers** and **elite controllers**, respectively)[152,153,154,155]. In contrast, some HIV-1 infected individuals quickly progress to AIDS within the first three years after primary infection (**rapid progressors**). Furthermore, a minority of highly viremic individuals remain asymptomatic with high $CD4^+$ T-cell counts (**viremic non-progressors**). Several factors can explain the high variability in the clinical evolution observed in HIV-1 infected individuals, such as alterations in the virus-specific **immune response**, host genetic differences and the diversity of genetic variants among the **HIV-1 strains**[156,157,158,159,160,161,162]. Regarding **host genetics,** several genome wide association studies have suggested that genetic polymorphisms can explain 15% of the observed variability in HIV-1 plasma set point[163]. Several polymorphisms in the human leukocyte antigen (HLA) region have been associated with slow disease progression (such as HLA-B27, 57, 58) [161,163,164,165,166,167,168,169], supporting the relevance of the CTL response in the control of viral replication. In sharp contrast, a number of HLA alleles have been also associated with accelerated disease progression, such as the HLA-B35/Cw04 haplotype, which is increased in individuals with rapid progression to AIDS[162,170]. In addition to the well-established role of HLA markers in either slowing or increasing disease progression, there are other genetic factors that have been associated with prevention of HIV-1 infection. That is the case of a particular mutation in the gene encoding for CCR5, which is the main co-receptor used by HIV-1 to enter target cells. Particularly, a 32 base pair deletion in the CCR5 gene (CCR5Δ32) causes a truncation of the CCR5 receptor that is not expressed on the cell surface. The homozygous expression of the CCR5Δ32 variant provides *in vivo* resistance to infection by R5-tropic HIV isolates[156,171,172,169].

Aside from the influence of virological, immune or host genetics factors in the clinical evolution of HIV-1 infected individuals, their outcome has changed exponentially since the implementation of ART, which has strongly reduced morbidity and mortality of HIV-1 infected individuals turning a previously fatal condition to a chronic disease with little impact on life expectancy[135,173,174].

2.5 Antiretroviral therapy

The first step in HIV therapy was made in 1987, when a clinical trial showed that azidothymidine (AZT) decreased mortality and opportunistic infections in individuals with AIDS[175]. AZT, which had been originally synthesized as an anticancer treatment, was found to also block the reverse transcription step of the HIV-1 life cycle[176]. However, viral resistance quickly appeared, so new drugs were developed. It was not until the mid-1990s that the treatment was revolutionized by the development of new reverse transcriptase inhibitors and protease inhibitors, and the introduction of drug regimens that combined these agents to enhance the efficacy of the therapy[177].

The **combined regimens** using three drugs directed against at least two distinct molecular targets (two NRTIs along with a third drug of a distinct family), was used to avoid the appearance of drug resistances and markedly reduced morbidity and mortality of HIV-1 infected individuals[173]. Combination ART efficiently reduced the plasma HIV-1 viral load below the limit of detection using the standard clinical assays (<50 RNA copies/ml), reconstituting the immune system[178]. However, despite the efficacy of these combined regimens, NRTI exposure induced mitochondrial toxicity that became evident with the first cases of lipodystrophy, which caused a severe stigma among HIV-1 infected individuals at that time[179]. Thus, ART at the beginning was hampered by the presence of **adverse effects**, **drug-drug interactions** and **high pill burden**, which led to decreased adherence to prescribed regimens and the emergence of HIV-1 drug resistance[180]. The need for less complex regimens that provided a lower pill burden, reducing dosing frequency, generated many clinical studies that demonstrated the efficacy of these new therapeutic interventions[181,182]. Thereby, **single-tablet regimens**, which included three drugs in the form of a single tablet taken once daily, became the preferent initiation regimen in ART-naïve individuals or a simplification strategy in ART-experienced individuals[183]. The development of **new antiretroviral drugs with less**

toxicity such as INSTIs, along with less complex regimens increased the efficacy of these therapies on viral suppression, and had a great epidemiological impact reducing HIV-1 transmission[184]. Given the huge advance of these new therapies and the improved safety profile of the new drugs it was recommended the initiation of ART as soon as seroconversion was documented, regardless the number of $CD4^+$ T cell counts[185].

To date, more than 30 Food and Drug Administration (FDA)-approved drugs are available for the treatment of HIV-1 infection[186]. These **drugs are classified into six distinct classes** based on their molecular mechanism of action within the HIV-1 replication cycle: fusion inhibitors, co-receptor antagonists, nucleoside-analog reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors (INSTIs from integrase strand transfer inhibitors) and protease inhibitors (PIs) (**Figure 1.7**). Although current regimens have a favorable safety profile, **new strategies of treatment** are in development aimed at reducing the number of molecules needed for reaching or sustaining viral suppression. Dual therapies have demonstrated their efficacy in viral suppression as initial therapy in ART-naïve individuals[187] and also as simplification regimens[188]. In addition, new formulations such as long-acting injectable ART are currently being evaluated and are showing promising results when administered every 2 months[189]. These new therapeutic advances have the potential to improve adherence and quality of life of people living with HIV-1.

Overall, if an appropriate drug regimen is selected in the absence of primary resistances along with a good treatment adherence from six to nine months after initiation of ART, most individuals reach undetectable plasma viral loads. However, after treatment interruption, in most individuals, HIV-1 viral load rebounds. This is because ART inhibits viral replication but does not mediate the complete clearance of latently infected cells, whose proviruses are able to produce new mature virions, constituting the HIV-1 reservoir[190,191]. Therefore, current ART does not cure the infection and it needs to be chronically administered to avoid viral rebound and drug resistance emergence. For this reason, alternative therapeutic strategies are being investigated to find a cure for HIV-1. An example of these approaches consists in treating HIV-1 infected individuals with latency-reversing agents in combination with ART aimed at

reactivating the latent proviruses to allow the immune system to recognize and completely eliminate the infected cells[190].

In addition to ART introduction, management of HIV-1 infected individuals includes the prophylaxis and control of **opportunistic infections (OIs)** that can appear during the clinical course of people living with HIV-1. Although the use of ART combined with effective prophylaxis for OIs has reduced their incidence, these infections remain a leading cause of hospitalization and death among HIV-1 infected individuals, especially in those who are not linked to care or have a delayed diagnosis[104].

2.6 HIV-1 co-infections: tuberculosis

Despite the huge advance that ART represents for the prognosis of HIV-1 infection, OIs continue to be a cause of morbidity and mortality in HIV-infected patients[104]. OIs often arise because of severe immunosuppression characterized by a profound depletion of $CD4^+$ T cells[104,192,193] together with a dysregulation of B-cell function[194]. Efficacious regimens for primary and secondary prophylaxis to prevent OIs constituted the first major advance in therapy for HIV-1 infected individuals, significantly decreasing mortality, even before the instauration of ART[195]. ART caused a deep impact in the progression of HIV-1 infection by dramatically reducing mortality and the incidence of OIs[174]. Currently, OIs occur among individuals who are unaware of their seropositive status, when the OI constitutes the first clinical manifestation of AIDS; or among individuals who, despite awareness of their HIV seropositive status, remain unlinked to care or, if receiving care, do not take ART because of psychosocial or economic factors. In addition, OIs also occur among individuals who receive therapy but experiment virologic failure due to poor adherence to therapy or extensive antiretroviral resistance[196]. Finally, they continue to occur among individuals who are not receiving OIs prophylaxis despite having low $CD4^+$ T cell levels.

Among all the OIs associated to AIDS, co-infection with ***Mtb,*** the causative agent of **tuberculosis (TB)**, constitutes a major health issue. Indeed, according to the WHO "Global HIV/AIDS report 2020", TB is the most common illness among people living with HIV-1 and the leading cause of death among them, responsible for nearly one in three HIV-1-associated deaths[197]. HIV-1 prevalence among TB cases is more than 20% in some countries of Africa,

Russia, Ukraine and Turkmenistan[198]. The isolation of the tubercle bacillus causing TB, was first reported in 1882 by Dr Koch, who maintained several weeks a culture plate to observe any signal of bacterial growth[199]. TB is an airborne infectious disease that displays a dynamic clinical spectrum, which ranges from asymptomatic infection to a life-threatening disease[200]. Exposure to *Mtb* can result in the elimination of the pathogen, either because of innate immune responses or acquired T cell immunity. However, if the pathogen is not eliminated, infection with *Mtb* can evolve from containment in the host, in which the bacteria are isolated within granulomas (a clinical conditions known as **latent TB infection**)[201], to a contagious state, in which the patient will show symptoms that can include cough, fever, hemoptysis, fatigue, lack of appetite, night sweats and weight loss (known as **active TB infection**)[200,202]. However, some patients with active disease, whose bacterial cultures are positive, can be asymptomatic and are then referred to as having **subclinical TB**[202].

The route of entry of *Mtb* is via the respiratory tract. Following inhalation, *Mtb* is translocated to the lower respiratory tract, where it encounters alveolar macrophages, which are the predominant cells that *Mtb* infects (**Figure 1.9**)[202]. These cells internalize the bacteria by receptor-mediated phagocytosis. Once internalized, *Mtb* actively blocks phagosome fusion with the lysosome, ensuring its survival[203]. If this first line of defense fails to eliminate the bacteria, *Mtb* invades the lung interstitial tissue, either by the bacteria directly infecting the alveolar epithelium, or by the infected alveolar macrophages that migrate into the lung parenchyma. Subsequently, either dendritic cells or inflammatory monocytes carry *Mtb* to pulmonary lymph nodes for T-cell priming[204,205]. This event leads to the recruitment of immune cells, including T cells and B cells, to the lung parenchyma to form a granuloma (**Figure 1.9**)[202]. Initially, **granulomas** are formed as aggregates of innate immune cells that are recruited to the site of infection. The infected alveolar macrophages release interleukins (IL) 12 and 18, which stimulate T lymphocytes, predominantly $CD4^+$ T helper cells, to release interferon gamma (IFNγ)[206]. This cytokine plays a critical role in the control of *Mtb* infection through different mechanisms.

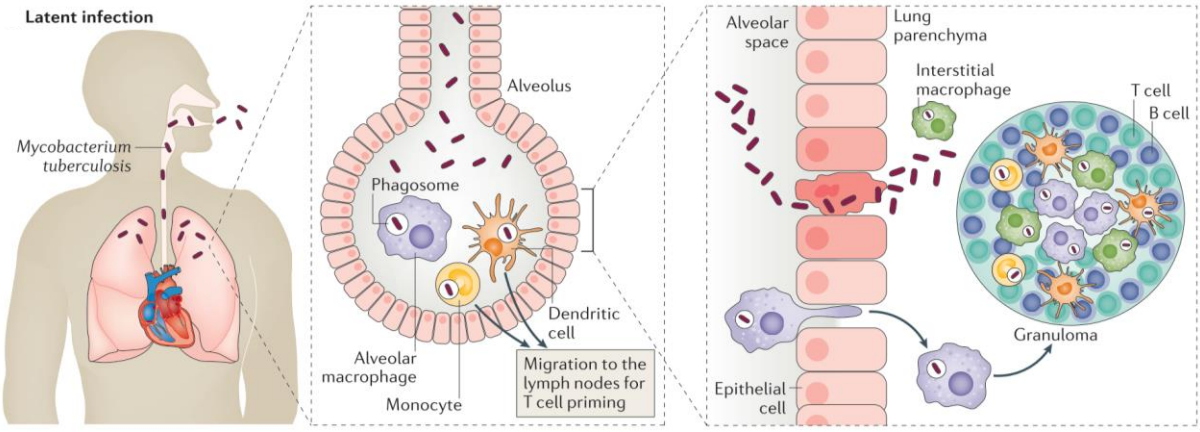

Figure 1.9 Pathogenesis of latent TB infection. Following inhalation, *Mtb* enters the lungs and infects resident alveolar macrophages. If *Mtb* has not been eliminated by the immune system, *Mtb* invades the interstitial tissue by directly infecting epithelial cells or by migrating within alveolar macrophages. Once the primary infection has been established, infected monocytes or DCs migrate to the pulmonary lymph nodes for antigen presentation and T-cell activation, leading to the recruitment of immune cells that will generate a multicellular host response, termed granuloma. From ref.[202].

On one hand, IFNγ stimulates the production of TNF by macrophages, which will be involved in the formation and maintenance of the granuloma, in the induction of cytokine production and also in the control of Mtb spread. Moreover, IFNγ is also involved in the maturation process of the phagosome containing the bacteria stimulating their phagocytosis[207,208]. After the development of adaptive immunity, the granulomas acquire a more intact structure with the macrophage-rich center surrounded by T cells and B cells, resulting in a lymphocytic ring at the periphery of the structures[209,210,211,212,213,214]. Within the growing granuloma the bacteria replicate, but if the bacterial load becomes too high, the granuloma can fail to contain the infection[215], thus favoring the **dissemination of *Mtb*** (**Figure 1.10**). Thereby, the granuloma undergoes complex remodeling events characterized by the accumulation of necrotic material composed of cellular debris that leads to the formation of caseum at the center. The caseum may undergo liquefaction resulting in cavitation, characterized by the destructive fusion of a liquefying granuloma with an adjacent airway, which facilitates bacterial dissemination[216,217,218,219,214]. Therefore, granulomas, while capable of limiting growth of *Mtb,* also provide a survival niche from which the bacteria may disseminate[210].

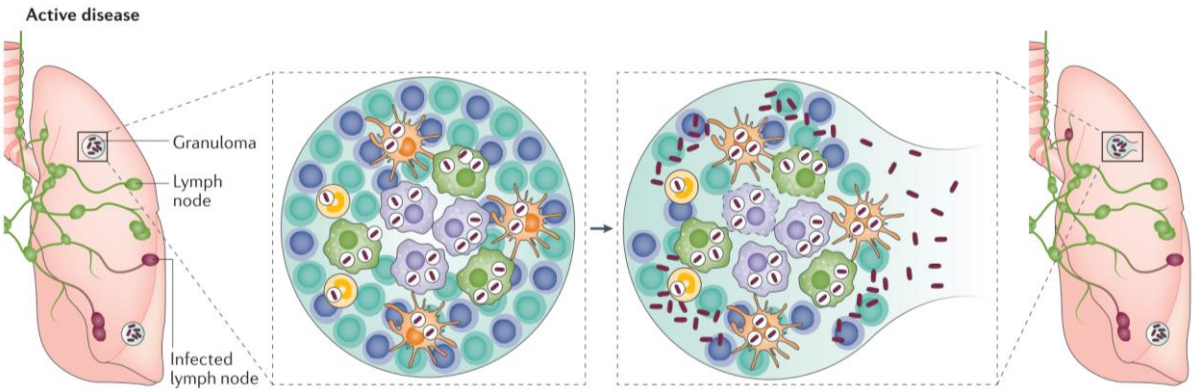

Figure 1.10 Pathogenesis of active TB disease. Once the granuloma has been formed, the immune cells recruited at the site of infection, can either contain the *Mtb* infection (left) or alternatively, allow the infection to progress (right). When *Mtb* replication is too high to be controlled by the immune system, the granuloma undergoes cavitation and finally fuses with an adjacent airway, facilitating bacterial dissemination. Thus, *Mtb* can reach the respiratory tract and be released, and consequently, the host becomes infectious or *Mtb* disseminates systemically to other organs originating an extrapulmonary TB. From ref.[202].

Through the dissemination pathway, *Mtb* can reach again the respiratory tract, from where it can be released, which turns the infected host infectious, or alternatively, *Mtb* can enter the bloodstream to spread systemically to other organs, originating an **extrapulmonary TB**. Extrapulmonary sites of infection commonly include lymph nodes, pleura, and osteoarticular areas, although any organ can be involved[220] (**Figure 1.11**).

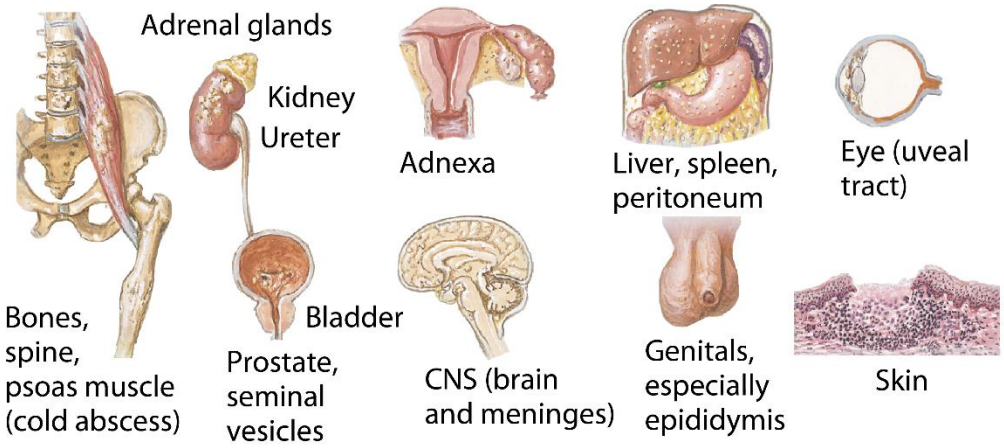

Figure 1.11 Extrapulmonary TB. Once *Mtb* enters the bloodstream, bacteria can spread systemically to other organs originating an extrapulmonary TB. Extrapulmonary sites of infection can include the osteoarticular system, genitourinary tract, reproductive tract, central nervous system, liver, spleen, peritoneum, eyes and skin. From Netter images.

HIV-1 infected individuals, particularly if they have low $CD4^+$ T-cell counts, have a higher risk of extrapulmonary TB, which could result in rapid clinical deterioration and death[202]. Moreover, TB leads to an increase in HIV-1 replication and accelerates the progression of HIV-1 infection, causing high mortality among co-infected individuals[221]. Therefore, there is a synergy between HIV-1 and *Mtb*, which promotes the pathogenesis of both infectious agents. On the one hand, $CD4^+$ T cell decay induced by HIV-1 infection are a leading cause for reactivation of latent TB and progression to active TB disease in individuals with AIDS, and, on the other hand, the course of HIV-1 infection is worsened by active *Mtb* infection[222,223]. Goletti *et al.* first showed that viral load was increased in serum samples from HIV-1 infected individuals at the time of TB diagnosis, compared with serum samples obtained before diagnosis[224]. Activated mononuclear cells expressing HLA-DR, which is a feature of active TB[225], constitute a major source of HIV-1 replication *in vivo*[226]. Increased systemic immune activation in HIV-1/TB coinfection[225] might contribute to increased plasma viremia. Other potential mechanism favoring HIV-1 replication might be explained by the capacity of *Mtb* to activate latent HIV-1 in alveolar macrophages derived from HIV-1 infected individuals[227]. In addition, the local cytokine milieu at sites of *Mtb* infection[228] has been also implicated in the enhanced HIV-1 activity in co-infected individuals. Indeed, during TB infection, an excess of proinflammatory cytokines, such as TNF, which induces viral replication[229], may be critical to increase plasma viremia. Moreover, during *Mtb* infection, activated DCs loaded with HIV-1 might migrate to the draining lymph nodes, where they can contact and infect many $CD4^+$ T cells, which are the main cellular targets of HIV-1[230], as we will explain later in greater detail. Finally, it has been recently shown that the TB-associated microenvironment triggers a tunneling nanotube formation between macrophages, which promotes HIV-1 dissemination during co-infection[222]. Overall, TB infection provides an environment of continuous cellular activation and cytokine production that sustains HIV-1 replication and dissemination[223,222].

2.7 Routes of HIV-1 dissemination

HIV-1 preferentially infects $CD4^+$ T lymphocytes through the interaction of the viral envelope glycoprotein with the CD4 receptor and CCR5 or CXCR4 co-receptors, present on the surface of T cells and other immune cells such as macrophages[231] **(Figure 1.12A)**. Despite the fact that **cell-free virus infection** mediates viral dissemination, cell-to-cell viral transmission is believed

to be a much more effective mechanism *in vivo*. HIV-1 can exploit pre-existing cellular communication mechanisms to enhance viral spread[99,100,101,102]. **Cell-to-cell-mediated viral transmission** mechanism was described three decades ago by the group of Steinman, who discovered DCs and also observed that DCs exposed to HIV-1 *in vitro* could transfer viral particles to $CD4^+$ T cells leading to a higher cytopathic infection compared to that observed in $CD4^+$ T cells by cell-free virions in the absence of DCs[99]. Indeed, infected $CD4^+$ T cells can also contribute to this cell-to-cell viral spread dissemination pathway. This pathway could play a critical role in the pathogenesis of HIV-1 infection, since the infectivity of HIV-1 during cell-to-cell transmission is estimated to be 100 to 1,000 times greater than the infectivity of cell-free virus[232]. In addition, mathematical models have predicted that cell-mediated viral transmission accounts for 90% of new infections in lymphoid tissue, while the remaining 10% is mediated by cell-free virus[233]**(Figure 1.12B-C)**.

This efficient cell-to-cell-mediated viral transmission can be orchestrated by APCs through two well-known mechanisms. One is known as ***cis*-infection** and involves HIV-1 infection of APCs, such as DCs, which transfer newly synthesized viral particles to target $CD4^+$ T cells, through the interaction of the envelope glycoprotein of the virus with the CD4 and co-receptor expressed by DCs[234,235,236] **(Figure 1.12B)**. However, this route of viral dissemination is limited due to the low infectivity rates of DCs[237,238,239,240]. This can be explained, in part, by the ability of DCs to degrade captured virions[241]. In addition, myeloid cells have a restriction factor, the SAM and HD domain- containing protein 1 (SAMHD1), which renders these cells refractory to HIV-1 infection by reducing the intracellular pool of deoxynucleotide triphosphates required for the synthesis of the viral dsDNA[242,243,244]. Another restriction factor present in myeloid cells is the apolipoprotein B mRNA editing enzyme, catalytic subunit 3G (APOBEC3G), which triggers a hypermutation in the nascent retroviral DNA, originating non-infectious viruses[245,246,247]. Thus, quick degradation of incoming viruses along with the activity of host restriction factors renders DCs as a poor HIV-1 replicating cellular target.

The second mechanism that allows an efficient APC-mediated viral transmission to $CD4^+$ T cells in the absence of productive infection is knowns as ***trans*-infection**[99,248]. This process consists in HIV-1 binding to the APC surface, internalization within a virus-containing compartment connected to the extracellular space that, after contact with a T cell, is rapidly polarized

towards the contact zone with the T cell, known as the infectious synapse, allowing viral release and fusion with the target T cell[249,250,251] **(Figure 1.12C)**. Importantly, virus-containing compartments protects viruses from degradation and also from the action of broadly neutralizing antibodies[252], which cannot gain access to the viral particles despite being in a compartment connected to the extracellular milieu[250,251,253]. Thus, through *trans*-infection mechanism, uninfected DCs concentrate and transfer intact viruses at the infectious synapse, facilitating the productive infection of $CD4^+$ T cells.

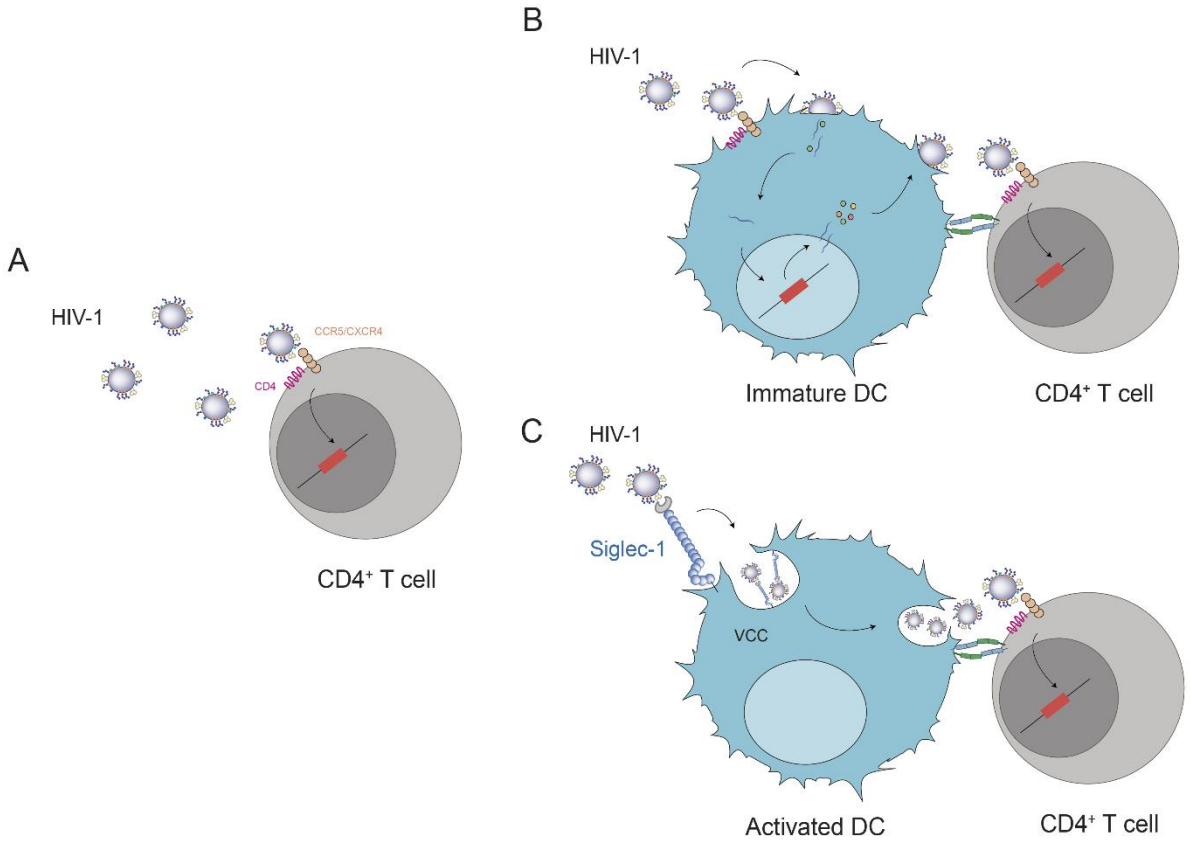

Figure 1.12 Schematic representation of cell-free (A) and cell-to-cell mechanisms of HIV-1 transmission mediated by DCs (B-C). A. Cell-free virus infection of $CD4^+$ T cells. **B.** DCs are susceptible to productive HIV-1 infection or *cis*-infection due to the expression of CD4 receptor and HIV-1 co-receptors. The newly synthesized viral particles are transferred to target $CD4^+$ T cells upon the formation of DC:$CD4^+$ T cell contacts. **C.** Activated DCs mediate viral transmission to $CD4^+$ T cells via *trans*-infection, a mechanism that relies on Siglec-1-mediated capture of HIV-1 particles, which are stored in a virus-containing compartment (VCC) and later released to $CD4^+$ T cells allowing for their productive infection.

HIV-1 *trans*-infection was initially attributed to the capacity of the dendritic cell-specific intercellular adhesion molecule-3 (ICAM)–grabbing non-integrin (DC-SIGN) to bind the virus

through the interaction with the viral envelope glycoprotein[248]. However, later observations revealed that most of the captured virions trapped by DC-SIGN were rapidly degraded and presented via MHC-I and MHC-II to T cells, eliciting adaptive immune responses[241,254]. These results, along with the finding that anti-DC-SIGN antibodies were not able to completely block HIV-1 *trans*-infection[255], indicated that other receptors could be involved in this process[256,257,255,258,259,260]. Indeed, it was reported that DC maturation potently enhanced *trans*-infection in a glycoprotein-independent manner, despite the fact that these cells down-regulated DC-SIGN expression. This new pathway relied on the recognition of sialyllactose-containing membrane gangliosides exposed on the surface of HIV-1 by the myeloid cell surface receptor **Siglec-1**[261,262], which is highly expressed on activated or matured DCs, and has the capacity to interact with sialylated ligands **(Figure 1.12C)**.

Overall, *trans*-infection mediated by Siglec-1 expressing APCs could be a relevant mechanism of viral dissemination. Strikingly, HIV-1 *trans*-infection can be influenced by the presence of *Mtb*, since *Mtb* infection activates macrophages into a particular phenotype that favors viral spread[222]. Transcriptomic analysis of these activated macrophages revealed that Siglec-1 was a potential factor responsible for HIV-1 dissemination upon *Mtb* co-infection[263]. In particular, Siglec-1 was found together with HIV-1 on tunneling nanotubes, which are membranous channels connecting two or more cells, indicating that *Mtb* co-infection induces HIV-1 spread through tunneling nanotubes via Siglec-1[263]. Taken together, these findings suggest an important role for Siglec-1 in HIV-1 spread.

3. HIV-1 DISSEMINATION MEDIATED BY SIGLEC-1-EXPRESSING APCs

Siglec-1, also referred as sialoadhesin (Sn) or CD169, is the key molecule in HIV-1 *trans*-infection mediated by activated DCs[261,262]. At a structural level, Siglec-1 is an **I-type lectin** transmembrane glycoprotein that belongs to the Ig superfamily, composed by a large extracellular region of 17 Ig-like domains, which includes an N-terminal variable domain or V-set that contains the sialic acid binding site and 16 constant Ig-like domains[264,265] (**Figure 1.13**).

Siglec-1 is expressed on the surface of myeloid cells such as DCs, monocytes and macrophages[261,262,266,267,268]. Immune activating signals, such as interferon alpha (IFNα) or

bacterial LPS, induce Siglec-1 expression on myeloid cells[261,262,267]. Both immune activating factors are present during the course of viral infections, such as the ones produced by HIV-1[147] and Ebola[269] viruses, and also in the course of *Mtb* infection[270]. Apart from infectious diseases, an increased expression of Siglec-1 has been observed in the peripheral blood monocytes of patients with autoimmune disorders, such as multiple sclerosis[271], systemic sclerosis[272], systemic lupus erythematosus[273] and rheumatoid arthritis[274].

Siglec-1 specifically **recognizes sialic acid** bound to different sugar moities[268], that can be present in different molecules such as **gangliosides**, which are glycosphingolipids composed by a lipid ceramide and a variable glycan polar head group containing sialic acid[275]. As gangliosides are present in cholesterol-enriched domains in the plasma membrane from which enveloped viruses such as HIV-1 usually bud, they can be incorporated into the viral membrane[276,277,278,279], allowing for the specific recognition by Siglec-1. Aside from HIV-1, other enveloped viruses incorporate sialylated gangliosides during the budding process, such as the porcine reproductive and respiratory syndrome virus, Arenaviruses and Ebola virus, which can be captured by APCs via Siglec-1 interaction[280,269]. In particular, Siglec-1 binding of HIV-1 relies on the recognition of sialyllactose-containing gangliosides, which display sialic acid bound to a lactose, such as GM1 or GM3[261,262,281,282] gangliosides present in the viral membrane (**Figure 1.13**).

Since Siglec-1 interaction with sialic acid has low affinity, high avidity binding requires clustering of both receptors and ligands[13,9,283]. This happens within the HIV-1 particle, where thousands of sialylated gangliosides are packed in a virion exposing the sialylated ligands that can be recognized by Siglec-1 clusters expressed on the cellular membrane of activated DCs. Among the different members of the Siglec family that have been identified in the human cells, Siglec-1 contains the largest number of C2-set Ig-like domains, which separate the sialic acid-binding site far away from the plasma membrane (**Figure 1.13**). This characteristic extended structure facilitates the interaction with viruses, reducing its *cis* interactions with abundant sialic acids on the surface of immune cells, as occurs with other Siglecs[13,264,268,284]. Furthermore, in contrast to other members of the Siglec family, Siglec-1 contains no signaling motif in its cytoplasmic tail, suggesting that Siglec-1 may not play a role in cellular signaling, but could play a predominant role in cell-to-cell interactions[13,284].

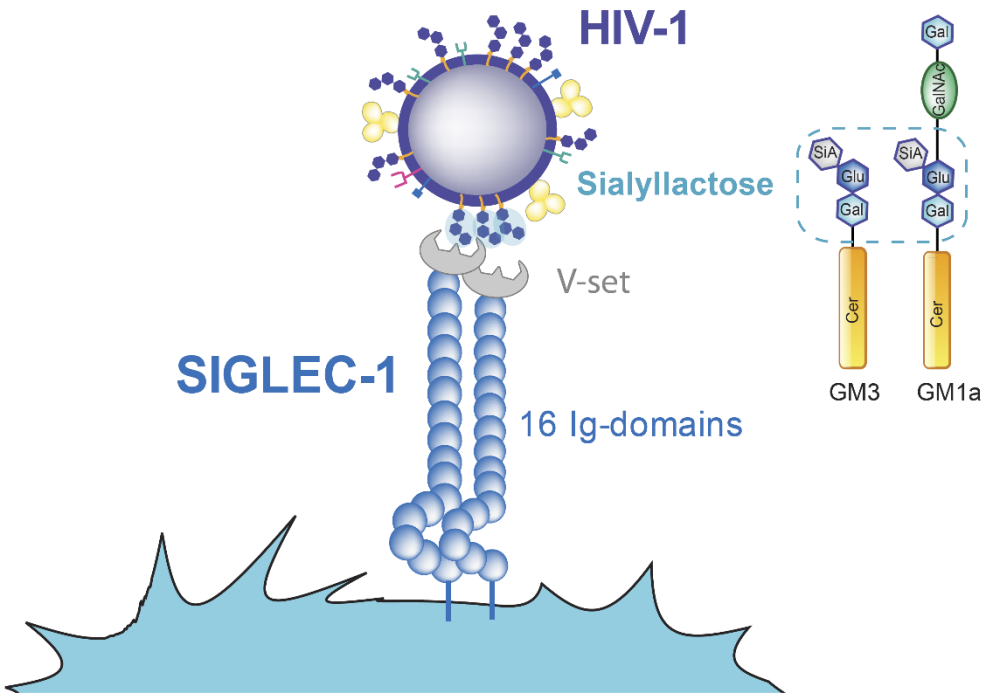

Figure 1.13 Siglec-1 structure and recognition of sialylated ligands present on HIV-1. Siglec-1 is composed of a cytoplasmic tail and 17 Ig-like domains, including the 16 C2-set Ig-like domains that project a V-set domain far from the cell surface. The V-set domain specifically recognizes sialyllactose, present on gangliosides exposed on the HIV-1 membrane, which are acquired during the budding process. Cer: ceramide; Gal: galactose; GalNAc: N-acetylgalactosamine; SiA: sialic acid; Glu: glucose. Adapted from ref.[285].

HIV-1 *trans*-infection mediated by Siglec-1 has been well-described *in vitro* using monocyte-derived APCs[267,261,262] and primary myeloid cells isolated from human tissues[266]. It was demonstrated that myeloid cells activated with either IFNα or LPS up-regulate Siglec-1 expression levels, which enhance viral capture and *trans*-infection[266]. Moreover, it has been reported that the murine leukemia virus (MLV), another retrovirus, disseminates in secondary lymphoid tissues of mice via Siglec-1-mediated *trans*-infection *in vivo* [286]. Taken together, there is a strong evidence suggesting that Siglec-1 mediates HIV-1 *trans*-infection and might increase viral pathogenesis. However, the *in vivo* contribution of this cellular receptor in the progression to AIDS in HIV-1 infected individuals remains to be elucidated.

4. IMMUNE ROLE OF SIGLEC-1 IN APCs

Although Siglec-1 may contribute to viral dissemination, its natural function as an immune receptor is to regulate host defenses via scavenging functions[9]. After mice infection with group B *Streptococcus* (GBS), a bacteria which displays sialic acid ligands on its surface, GBS were taken by marginal zone macrophages expressing Siglec-1 in the spleen, resulting in a limited spread to other organs and consequently in an increased survival compared with knockout mice that lacked Siglec-1[287]. In addition, production of specific anti-GBS IgM antibodies was impaired in Siglec-1 deficient mice after GBS challenge[287,288]. These findings indicate that Siglec-1 is critical for innate recognition of **sialylated pathogens** and can contribute to induce adaptive immune responses against them[288]. Indeed, Siglec-1 can also interact with sialylated ligands present on immune cells, leading to the regulation of host innate and adaptive immune responses[9]. Further support for the role of Siglec-1 in host protection against sialylated bacteria was obtained in studies showing that Siglec-1 functions as a phagocytic receptor promoting the clearance of sialylated pathogens such as *C. jejuni* and *N. meningitidis*[288,289,290,291,292].

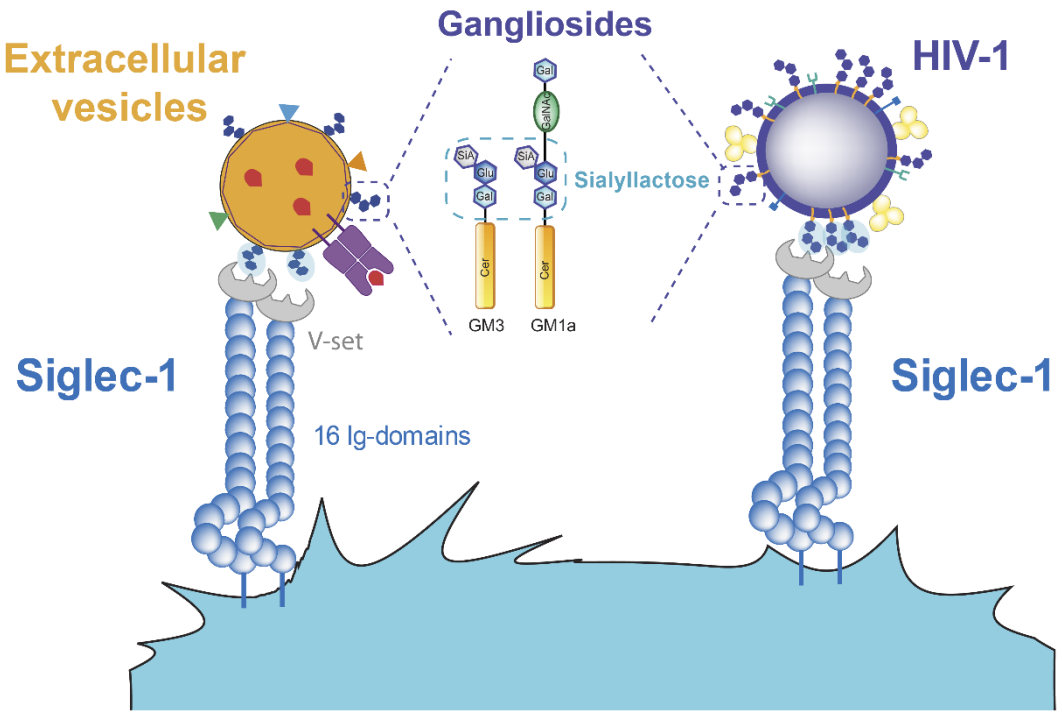

Figure 1.14 Siglec-1 interaction with EVs and HIV-1. Siglec-1 expressed by APCs recognizes specifically the sialyllactose present on gangliosides exposed in the membrane of EVs and HIV-

1. Cer: ceramide; Gal: galactose; GalNAc: N-acetylgalactosamine; SiA: sialic acid; Glu: glucose. Adapted from ref.[285].

Aside from the interaction with sialylated pathogens or immune cells, Siglec-1 is also key to **capture EVs** through the same mechanism exploited by enveloped viruses[103], since these vesicles acquire sialylated gangliosides throughout budding from secreting cellular membranes, as it happens with HIV-1[261,293,294,295] (**Figure 1.14**).

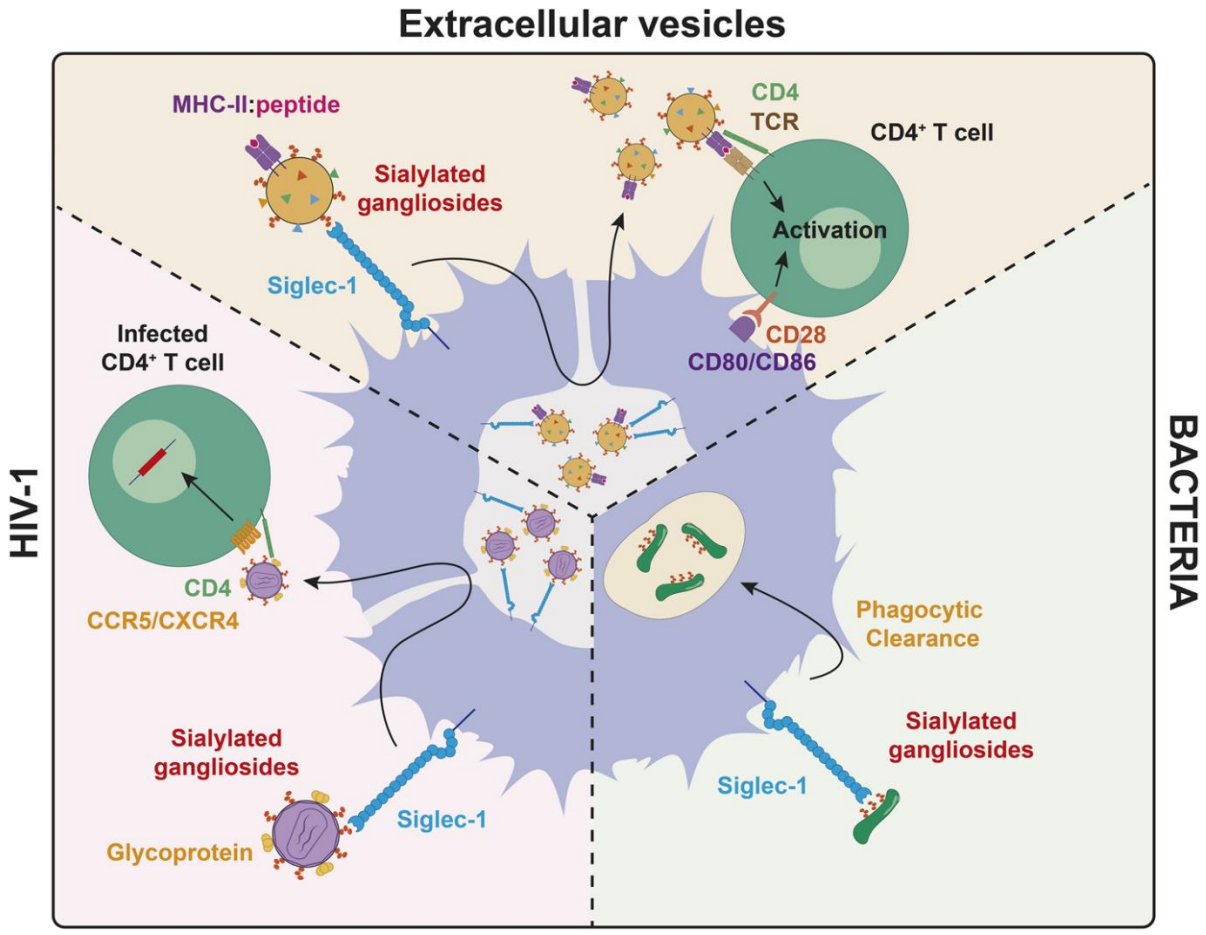

Figure 1.15 Siglec-1 interaction with EVs and bacteria induces immune responses, while in the case of HIV-1 favors HIV-1 dissemination. APCs capture EVs and enveloped viruses such as HIV-1 through the recognition of sialylated ligands by Siglec-1. Then, they are trafficked and stored in a sac-like compartment together with Siglec-1. EVs containing MHC:peptide complexes are released for antigen presentation to T-cells in the presence of co-stimulatory signals provided by APCs leading to the formation of the immunological synapse, while HIV-1 exit leads to the *trans*-infection of CD4+ T cells. In addition, Siglec-1-expressing APCs can capture sialylated bacteria promoting their phagocytic clearance. MHC-II: major histocompatibility complex class II; TCR: T cell receptor. Adapted from ref.[295].

Thus, Siglec-1-expressing APCs have the capacity to capture EVs through the recognition of sialylated gangliosides present on the surface of these EVs[261,293,294]. Once captured, EVs traffic along with Siglec-1 towards the same sac-like compartments where HIV-1 accumulates[103,261] (**Figure 1.15,** top). These EVs can be released for antigen cross-presentation to $CD8^+$ T cells, or stimulate antigen-specific naïve $CD4^+$ T-cell responses[49,83]. $CD4^+$ T-cell activation can occur either by processing the antigens contained in the EVs or by the direct presentation of already processed antigens transported by the EV, which are incorporated in the MHC molecule carried on the vesicle surface[49,83,295] (**Figure 1.15,** top). Therefore, Siglec-1 mediates EV capture, internalization and later release to T cells to initiate immune responses.

Siglec-1 exhibits an important role promoting immune responses against several bacteria, either through the recognition of sialylated ligands present both in EVs released by infected cells (**Figure 1.15,** top) or exposed in the surface of some bacteria, which induces their phagocytic clearance (**Figure 1.15,** bottom right). Strikingly, HIV-1 subverts this system by using this Siglec-1-mediated antigen dissemination pathway or this bacterial clearance mechanism for viral spread (**Figure 1.15,** bottom left). Specifically, HIV-1 interacts with Siglec-1 expressed on APCs and uses these cells as "Trojan horses" to reach $CD4^+$ T cells allowing for their productive infection[99,100,101,102]. However, the *in vivo* relevance of this HIV-1 dissemination mechanism is not known. In this thesis, we focused in understanding the specific contribution of Siglec-1 mediated HIV-1 *trans*-infection to AIDS progression. Since Siglec-1 seems to display opposite "yin and yang" functions by either controlling immune responses against bacteria but also favoring HIV-1 dissemination, in this thesis we also explore the role of this receptor in the context of *Mtb* co-infection.

Chapter 2

HYPOTHESIS AND AIMS

APCs are a heterogeneous group of immune cells that mediate immune responses by processing and presenting antigens for recognition by certain lymphocytes; therefore, their role in initiating immune responses is essential to control infections. Among the several mechanisms of antigen transfer between APCs, secretion of EV-containing antigens may be key to amplify the initiation of immunity. Siglec-1, a myeloid-cell surface I-type lectin receptor, is required to capture these EVs, allowing for the initiation of immune responses against invading pathogens. However, in the case of HIV-1 infection, the virus exploits this pathway by which APCs acquire antigens transported by EVs for viral dissemination. This mechanism relies on Siglec-1 capacity to mediate HIV-1 capture and *trans*-infection to target cells allowing for their productive infection.

Our **hypothesis** was that *in vivo*, Siglec-1-mediated *trans*-infection could fuel HIV-1 transmission in lymphoid tissues, thus accelerating the natural progression to AIDS in HIV-1-infected individuals. To dissect the role of Siglec-1 in the pathogenesis of HIV-1 *in vivo,* we searched for individuals harbouring a loss-of-function variant on the *SIGLEC1* gene. While we were able to identify those individuals and confirm an *ex vivo* loss of HIV-1 *trans*-infection capacity, no significant impact could be associated to HIV-1 progression. The lack of conclusive results derived from our initial study led us to consider different factors that could potentially influence the analysis of the biological function of *SIGLEC1* null genetic variant during HIV-1 infection. That is the reason why we also explored the co-infection with *Mtb*, which is a common scenario in the natural course of HIV-1 infection, and we found how Siglec-1 in this context could promote immunity through EVs. Thus, the “yin and yang” functions of Siglec-1 might help explain how complex host-pathogen interactions shape infectious disease progression.

The aims pursued during this thesis are the following:

Aim 1: To identify and study individuals with a loss-of-function variant in the *SIGLEC1* gene to determine the contribution of *trans*-infection to HIV-1 pathogenesis *in vivo.*

Aim 2: To investigate the effect of the *SIGLEC1* null variant in HIV-1 related co-infections.

Aim 3: To assess which is the mechanism that facilitates the extrapulmonary dissemination of *Mtb* in *SIGLEC1* null humans and the local pulmonary *Mtb* spread in the absence of Siglec-1 in mice.

Chapter 3

RESULTS I

Identification of *SIGLEC1* null individuals infected with HIV-1

The results included in this chapter have been reported in:

Martinez-Picado J*, McLaren P, Erkizia I, Martin M, Benet S, Rotger M, Dalmau J, Ouchi D, Wolinsky S, Penugonda S, Günthard H, Fellay J, Carrington M, Izquierdo-Useros N*, Telenti A*. **Identification of Siglec-1 null individuals infected with HIV-1.** *Nature Communications.* 2016. 7:12412. doi: 10.1038/ncomms12412

*Corresponding authors

Author's contributions: The author of this thesis contributed to the current work by measuring Siglec-1 surface expression by FACS, generating viral stocks and performing VLP capture and HIV-1 trans*-infection assays. The author also contributed to the design of the experiments, analysis and interpretation of the results.*

1. INTRODUCTION

Antigen presenting cells (APCs) of the myeloid lineage, such as monocytes, macrophages and dendritic cells, initiate immune responses and are crucial to control invading viruses. In the case of HIV-1 infection, however, myeloid cells also promote viral pathogenesis through *trans*-infection of $CD4^+$ T cells[99,248]. This mechanism involves HIV-1 capture by sialic acid-binding Ig-like lectin 1 (Siglec-1/CD169), a myeloid-cell receptor that recognizes viral membrane gangliosides[281,282,261,262]. Viral capture facilitates the release of trapped viruses at a cell-to-cell contact zone promoting the *trans*-infection of $CD4^+$ T cells[249]. Immune activating signals, such as IFNα or bacterial LPS, are present throughout the course of HIV-1 infection[147] and induce Siglec-1 expression on myeloid cells[261,262,267]. However, under these inflammatory conditions, DC- SIGN and other C-type lectin receptors previously implicated in HIV-1 *trans*-infection[248,296] play a minor role in viral transmission[261,262,297]. Thus, Siglec-1 is an important inducible receptor that could accelerate HIV-1 transmission in lymphoid tissues, where many T-cells are in contact with myeloid cells. Studies performed *in vivo* in mouse models demonstrated that Siglec-1 blockade significantly reduced HIV-1 infection of mouse splenocytes[286]. Siglec-1 *trans*-infection, cell-free virus infection and cell-to-cell viral transfer between infected and non-infected cells are all important routes of viral dissemination. Yet, the relative contribution of *trans*-infection to HIV-1 transmission and AIDS disease progression remains unknown.

There has been considerable interest in using human genetic diversity to dissect the role of various genes in defense against pathogens *in natura*[298]. Protein-truncating variants have been catalogued in the human genome[299,300,301,302]. These are variants that are likely to disrupt the function of the corresponding allele[303,304]. The identification of the CCR5Δ32 variant was pivotal to understanding how human genetic variation contributes to differences in susceptibility to HIV-1 infection[156], equally affecting all routes of viral spread.

Here, we aimed to identify *SIGLEC1* null individuals to dissect the specific contribution of *trans*-infection to HIV-1 pathogenesis *in vivo*. We found individuals with a specific loss-of-function variant in *SIGLEC1* gene that completely abrogates Siglec-1 receptor expression on primary monocytes and their capacity to *trans*-infect HIV-1 *ex vivo*. Despite this *ex vivo* phenotype there was a striking absence of marked differences in HIV-1 acquisition or clinical evolution of

individuals carrying *SIGLEC1* loss-of-function alleles. Nonetheless, analysis of the effect of Siglec-1 truncation on progression to AIDS was not conclusive due to the limited cohort size, the lack of complete clinical records, and the restriction to study only off-therapy periods.

2. MATERIALS AND METHODS

2.1 Patients

The Swiss HIV Cohort Study (SHCS) is an ongoing observational longitudinal study enrolling HIV-1-infected individuals since 1988 in Switzerland. Demographics, route of transmission, AIDS defining illnesses, co-morbidities, and behavioural, clinical and laboratory data are systematically and prospectively collected. Plasma and cell samples are stored longitudinally every 6 -12 months[305]. To date, more than 20,000 persons have been enrolled. At least 50% of all HIV-1-infected, 72% of all AIDS cases in Switzerland are enrolled[305]. The Multicenter AIDS Cohort Study (MACS) is an ongoing prospective study of the natural and treated histories of HIV-1 infection in homosexual and bisexual men conducted by sites located in Baltimore, Chicago, Pittsburgh and Los Angeles[306]. A total of 6,972 men have been enrolled.

2.2 Ethics statement

The institutional review board on biomedical research from Germans Trias i Pujol University Hospital (HUGTiP) approved this study. Participants of the Swiss HIV Cohort Study (SHCS) and the Multicenter AIDS Cohort Study (MACS) consented to the cohort study and genetic analyses, as approved by the corresponding local Ethics Committees.

2.3 Primary cell culture

Frozen peripheral blood mononuclear cells were obtained from HIV-1 patients of the Swiss HIV Cohort Study. Monocyte populations were isolated using CD14 magnetic beads (Miltenyi Biotec) and cultured 24h with 1,000 U/ml of IFNα (Sigma) in RPMI with 10% of heat-inactivated fetal bovine serum (Invitrogen).

2.4 Siglec-1 surface expression analysis by FACS

IFNα-activated monocytes were blocked with 1 mg/ml of human IgG (Baxter, Hyland Immuno) and stained with 1/10 dilution of α-Siglec-1-PE 7–239 monoclonal antibody (mAb) (AbD Serotec) following manufacturer's instructions at 4ºC for 20 min. Samples were analyzed with FACSCalibur (Becton-Dickinson) using CellQuest software to evaluate collected data. The mean number of Siglec-1 Ab binding sites per cell was obtained with a Quantibrite kit (Becton-Dickinson) as previously described[261]. Briefly, a standard linear regression curve was built with four different precalibrated beads conjugated with fixed amounts of PE molecules per bead. Geometrical mean fluorescence-obtained labeling with α-immunoglobulin G1 (IgG1) PE mAb was used as an isotype control (BioLegend), and Abs-per-cell values were subtracted from values of corresponding samples. Rainbow calibration particles (Becton-Dickinson) were used before quantitation to ensure the consistency of fluorescence intensity measurements throughout all the experiments.

2.5 Generation of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ and HIV-$1_{NL4\text{-}3}$ stocks

Viral like particles (VLP) of HIV-1 Gag tagged to eGFP ($VLP_{HIV\text{-}Gag\text{-}eGFP}$) and HIV-$1_{NL4\text{-}3}$ stocks were generated by transfection of HEK-293T cells with the molecular clones pGag-eGFP and pNL4-3, respectively. Both plasmids were obtained from the US National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. HEK-293T cells were transfected in T75 flasks with 30 μg of plasmid DNA using calcium phosphate (CalPhos; Clontech) and incubated during 48h at 37ºC. After 48 h, supernatants containing virus or VLPs were filtered (Millex HV, 0.45 μm; Millipore) and frozen at -80°C until use. The $p24^{Gag}$ content of viral and VLP stocks was determined by enzyme-linked immunosorbent assay ($p24^{Gag}$ ELISA; Perkin-Elmer).

2.6 VLP capture and HIV-1 *trans*-infection assays

IFNα-activated monocytes ($2x10^5$) were pre-incubated at 16°C for 30 min with 10 μg/ml of α-Siglec-1 mAb (7–239, AbSerotec) or IgG1 isotype control mAb (107.3, BD Bioscience). Capture experiments were performed pulsing monocytes with 150 ng of $VLP_{HIV\text{-}Gag\text{-}eGFP}$ Gag for 3h at 37ºC. After extensive washing, monocytes were acquired by FACS. *Trans*-infection assays were performed pulsing monocytes with 300 ng of HIV-$1_{NL4\text{-}3}$ for 4h at 37ºC. After extensive washing, monocytes were co-cultured in duplicate at a ratio of 1:1 with the TZM-bl $CD4^+$ target

cell line. Cells were assayed for luciferase activity 48h later (BrightGlo Luciferase System; Promega) in a Fluoroskan Ascent FL luminometer (Thermo Labsystems).

2.7 *SIGLEC1* genetic analysis

Data from the Exome Aggregation Consortium that is now the Genome Aggregation Database led us the identification of rs150358287, a stop-gain variant in *SIGLEC1.* Genotyping analysis of this polymorphism in participants of the Swiss HIV Cohort Study (SHCS) consisted on the combination of genotype data from exome sequencing (*n*=392), exome chip (*n*=2,212) and direct genotyping (*n*=1,129). For 392 participants from the SHCS, we captured and sequenced all coding exons using the Illumina Truseq 65Mb enrichment kit and the Illumina HiSeq2000. Sequences were aligned to the human reference genome version 19 (GRCh 37) using BWA. Variant calling was performed using the HaplotypeCaller module of the Genome Analysis Toolkit version 3.1–1. Only variants passing the variant quality score recalibration thresholds were maintained for further analysis. Genotype data for 2,212 individuals were obtained using the Illumina Infinium Human Exome BeadChip. Direct genotyping for rs150358287G4T in 1,129 individuals from the SHCS and 425 individuals from the MACS was performed by Taqman allelic discrimination using a custom design assay from Applied Biosystems (custom Assay ID: AHKAY5K, which now corresponds to the commercial Assay ID: C_167368973_10). Exome sequencing results were confirmed by PCR and direct sequencing (forward primer: 5' AGGACGTGCAGGGTGTGAAG 3'; reverse primer: 5' GCTGGAACAGAGGCTGAGAC 3'; annealing temperature: 62°C; expected fragment: 455 bp).

2.8 Statistical analysis

Statistics of VLP functional assays were performed using unpaired and paired *t* test (considered significant at $P \leq 0.05$) or the Spearman correlation with GraphPad Prism v.5 software. Set point viral load was calculated as the average of at least three measurements obtained during the chronic phase of infection -minimum 6 months after infection and before the initiation of antiretroviral therapy (ART)-. A comparison of variance in set point viral load between genotype groups showed no significant differences (F test P=0.18). Association between viral RNA level and genotype was tested using linear regression. Rate of disease progression was measured using all available $CD4^+$ T-cell counts beginning at enrolment or

estimated date of infection (if known) before the initiation of anti-HIV therapy. The impact of genotype on disease progression was tested using the Cox proportional hazards models.

3. RESULTS

3.1 Genetic description of *SIGLEC1* variants

We used data from the Exome Aggregation Consortium (ExAc.broadinstitute.org) to identify naturally occurring knockout mutations in *SIGLEC1*. In that sample of ~63,000 individuals we observed 70 protein truncating variants, that is, stop-gain, frameshift or splice site (**Figure 3.1**). With the exception of rs150358287, a stop-gain variant resulting in an early stop codon at amino acid position 88 (Glu88Ter), truncating variants are of very low allele frequency (<1%). The Glu88Ter variant occurs in the second exon of *SIGLEC1* (C to A transversion at position 3706494 on chromosome 20, GRCh 38 build reference sequence) and is predicted to truncate both major transcripts of *SIGLEC1*. The frequency of this particular single nucleotide null variant of *SIGLEC1* in the general population is described in the genome aggregation database (https://gnomad.broadinstitute.org/variant/20-3687141-C-A). Particularly, it is found at highest allele frequency in individuals of European and South Asian ancestry (~1.3%) and is rare or absent in African and East Asian populations (<0.5%).

To assess the frequency distribution of this polymorphism in an HIV-1-infected population, we combined genotype data from exome sequencing (*n*=392), exome chip (*n*=2,212) and direct genotyping (*n*=1,129) in participants of the Swiss HIV Cohort Study (SHCS). In 3,733 individuals whose clinical characteristics are detailed in **Table 3.1** (95% reported European ancestry), we observed 85 Glu88Ter heterozygotes and 2 homozygotes for the stop-gain variant (allele frequency = 1.2%). Thus, we identified individuals in whom to assess the consequences of Siglec-1 haploinsufficiency and knockout *in vivo* and *ex vivo.*

To confirm that Siglec-1 Glu88Ter homozygous individuals truly lack receptor expression, we performed functional assays with cryopreserved cells collected from individuals with all three possible genotypes. We induced Siglec-1 expression in isolated monocytes using IFNα and determined the absolute number of Siglec-1 antibody binding sites per monocyte (**Figure 3.2A**).

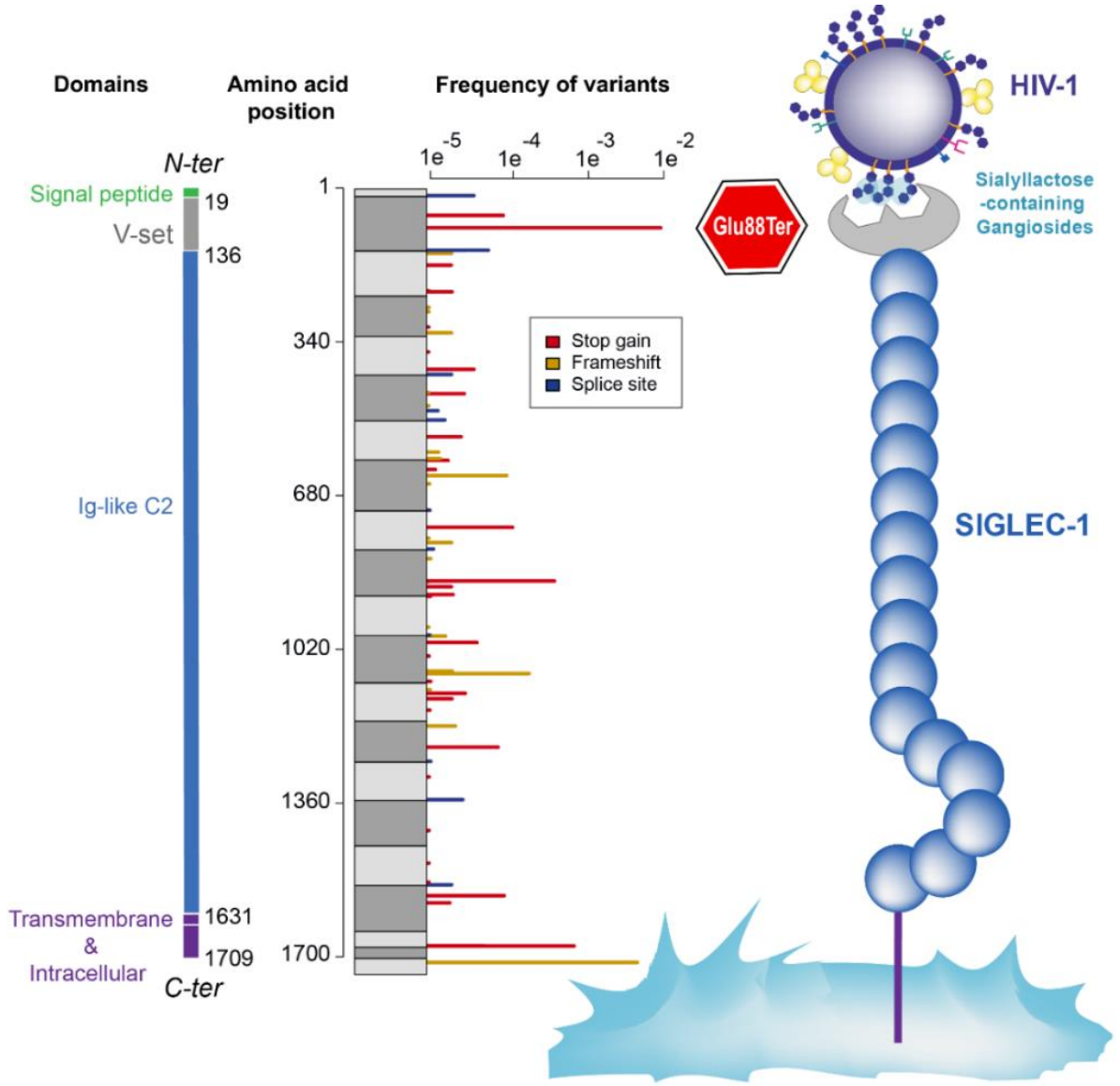

Figure 3.1 Location and frequency of *SIGLEC1* protein truncating variants. Protein domains are represented in different colours. Data from the Exome Aggregation Consortium that is now the Genome Aggregation Database identifies 70 protein truncating variants in *SIGLEC1* including 33 stop gain (red), 24 frameshift (yellow) and 12 splice disrupting (blue) variants. Grey boxes indicate amino acid blocks encoded by each exon. With the exception of Glu88Ter, all protein truncating variants occur at ≤1% frequency. Glu88Ter is located in the V-set domain of Siglec-1, the region that recognizes sialyllactose in HIV-1 membrane gangliosides.

Compared to individuals homozygous for the common allele, heterozygous individuals expressed approximately half of the amount of protein observed, while null individuals showed only background expression levels (**Figure 3.2A**).

Table 3.1 Clinical characteristics of the SHCS cohort		
	Null variant (heterozygous & homozygous)	Common variant (homozygous)
Male *n;* (%)	65 (74.7%)	2835 (77.7%)
Female *n;* (%)	22 (25.3%)	808 (22.3%)
Age median; (IQR)	46 (41-50)	47 (41-53)
Caucasian *n;* (%)	80 (94.1%)	3447 (95.3%)
Peak viremia median; (IQR)	168,423 (42,036; 440,051)	103,094 (31,314; 290,000)
CD4 nadir median; (IQR)	236 (118; 306)	225 (121; 323)
Mode of HIV-1 acquisition *n;* (%)		
Heterosexual	25 (29.4%)	1,116 (30.7%)
Homosexual	39 (45.9%)	1,617 (44.7%)
Intravenous drug user	20 (23.5%)	764 (21.1%)
Other/unknown	1 (1.2%)	120 (3.3%)

IQR, interquartile range.

Table 3.1 Clinical characteristics of the SHCS cohort

Next, we analyzed the ability of monocytes to capture fluorescent HIV-1 virus-like particles (VLPs) displaying specific gangliosides that are efficiently recognized by Siglec-1[281,282]. IFNα-activated monocytes from individuals with the common allele showed the highest viral capture capacity followed by heterozygous and then by null individuals (**Figure 3.2B**), which captured only residual levels of VLPs. To investigate whether this binding was specific for Siglec-1, cells were pre-treated with a mAb against Siglec-1. Treatment led to a significant reduction of VLP uptake in monocytes from common allele and heterozygous individuals (**Figure 3.2B**), while it had no inhibitory effect on *SIGLEC1* null individuals. The VLP uptake of monocytes from distinct *SIGLEC1* genotypes strongly correlated with the mean number of Siglec-1 antibody binding sites per cell (**Figure 3.2C**). To assess the general HIV-1 transfer capacity of Siglec-1 compared to other possible receptors on IFNα-activated monocytes from homozygous individuals, we pulsed cells with equal amounts of infectious HIV-1_{NL4-3} in the presence or absence of blocking mAbs and co-cultured them with a $CD4^+$ reporter cell line (**Figure 3.2D**). Monocytes from individuals with the common allele had higher capacity to *trans*-infect than did *SIGLEC1* null cells (**Figure 3.2D**). *Trans*-infection was inhibited with a mAb against Siglec-1, which had no blocking effect on *SIGLEC1* null monocytes (**Figure 3.2D**). Overall, these results indicated that *SIGLEC1* null individuals lack functional Siglec-1 expression

and HIV-1 *trans*-infection capacity, ruling out genetic compensation mechanisms or a possible stop codon read-through that could alleviate the null status[307].

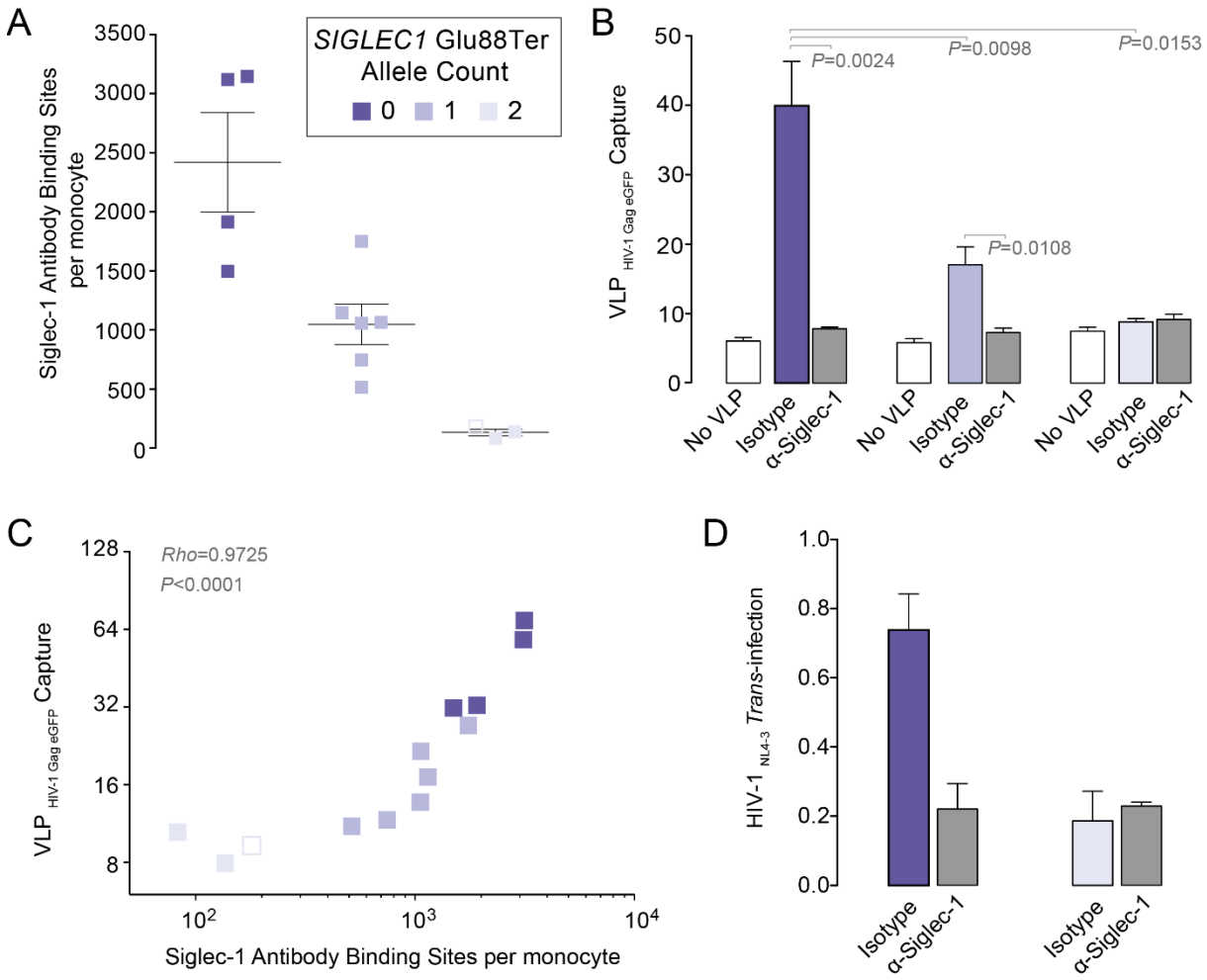

Figure 3.2 Siglec-1 expression and *trans*-infection across distinct *SIGLEC1* genotypes. Monocytes were isolated and cultured 24h in the presence of 1,000 U/ml of IFNα to induce Siglec-1 expression. **A.** Quantification of Siglec-1 expression levels assessed by flow cytometry. Empty box represents a repeat analysis of one Siglec-1 null homozygote. **B.** Capture of fluorescent HIV-1 VLPs by monocytes from distinct genotypes previously exposed to isotype or α-Siglec-1 mAbs. This capture was measured using the geometric mean fluorescence intensity of the cells. Background levels of monocytes not exposed to VLPs are also depicted to show the detection limit of the assay (empty bars). **C.** Correlation between Siglec-1 expression levels and viral capture values of isotype-treated monocytes. **D.** HIV-1 transmission to a reporter CD4⁺ cell line from monocytes of opposing homozygous individuals pre-incubated with isotype or α-Siglec-1 mAbs. HIV-1 infection of reporter cells was determined by induced luciferase activity. Data show mean relative light units and SEM of cells from two homozygous individuals with the common allele and one Siglec-1 null homozygote.

3.3 *SIGLEC1* null variant in HIV-1 acquisition

We next tested for an impact of the Glu88Ter variant on HIV-1 acquisition. Given the population frequency differences at which this variant occurs, we limited these analyses to 3,558 individuals of European ancestry to prevent confounding. The working hypothesis is that if Siglec-1 were essential for infection, homozygous Glu88Ter would not be infected - at least via mucosal exposure. The observation of two HIV-1-infected Glu88Ter homozygotes ruled out a requirement for a functional Siglec-1 protein in HIV-1 acquisition (that is, it does not mirror the CCR5Δ32 effect regarding R5-tropic virus infection). In addition, the observed frequency of the Glu88Ter allele in the HIV-1-infected population (1.2%) is nearly identical to the frequency in Europeans from the ExAc sample (1.3%) and does not differ depending on route of infection (1.15% parenteral, 1.2% sexual, *P*=0.95). Taken together, these results suggest that functional *SIGLEC1* is not required for HIV-1 acquisition regardless of route of exposure.

3.4 *SIGLEC1* null variant in HIV-1 progression

We assessed the potential impact of the *SIGLEC1* null variant on HIV-1 viral load and disease progression. As shown in **Figure 3.3A**, the presence of one or two copies of the Siglec-1 Glu88Ter allele had no impact on plasma set point viral load (n=2,243; **Figure 3.3A**). Similarly, there was no difference in CD4$^+$ T-cell dynamics (n=2,302; **Figure 3.3B**) or in CD4$^+$ T-cell nadir.

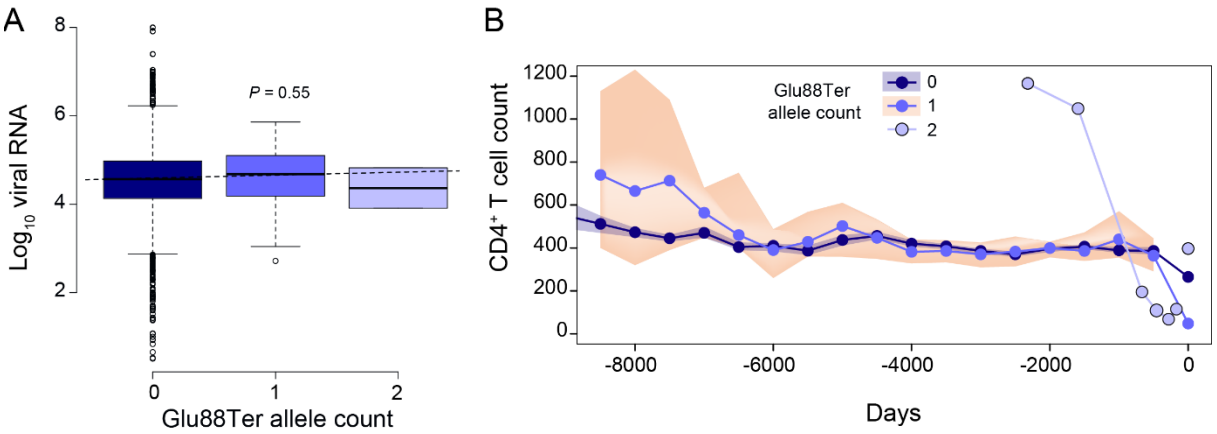

Figure 3.3 Association between different *SIGLEC1* genotypes and HIV-1 clinical parameters. **A.** Set point viral load of individuals from the SHCS with or without the Glu88Ter allele (n=2,243). **B.** CD4$^+$ T-cell count dynamics of individuals from the SHCS with or without the

Glu88Ter allele (n=3,385). CD4⁺ T-cell counts (cells/mm³) were binned using 500 days windows, counting backwards from the date of antiretroviral treatment start or loss of follow-up. Median CD4⁺ T-cell values (lines) and interquartile ranges in each bin (shaded areas) are shown for individuals carrying no copies (n=3,305) or one copy (n=78) of the Siglec-1 Glu88Ter allele. The most updated CD4⁺ T-cell values available at 2016 are shown for the two Siglec-1 Glu88Ter homozygotes.

We also investigated the disease course (viral RNA level and CD4⁺ T-cell counts) for the two homozygous Glu88Ter individuals, which supported viral replication (**Figure 3.4**). The first one represented in the top graph of **Figure 3.4** is a female diagnosed in 1987 that refused treatment and died in 2003 of *Pneumocystis jirovecii* infection. The last CD4⁺ T-cell count was 8 cells/mm³ and the last viremia recorded for this individual was >750,000 cp/ml.

The second Glu88Ter homozygous, whose clinical evolution is shown in the bottom graph of **Figure 3.4**, is a male that was diagnosed HIV-1 in 1987 and registered in the SHCS in 1996, when he initiated antiretroviral treatment while clinically asymptomatic. He has been on antiretroviral treatment for most of the past 20 years (except for a short period in 2003 and 2004), with stable CD4⁺ T-cell count between 400 and 600 cells/mm³. Last clinical values (recorded in October 2015) were a CD4⁺ T-cell count of 602 cells/mm³ (23%) and a viremia <20 cp/ml.

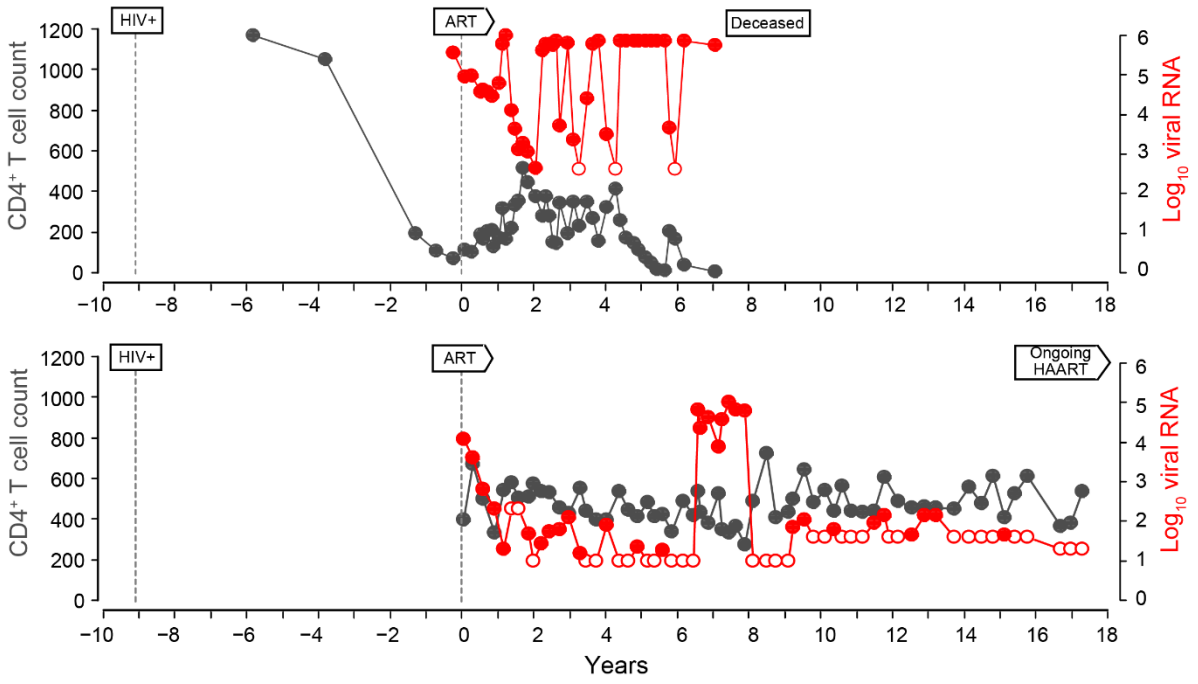

Figure 3.4 (Caption overleaf)

Figure 3.4 Clinical evolution of the two Siglec-1 Glu88Ter homozygous individuals infected with HIV-1 from the SHCS. Plasma viral RNA level (cp/ml) and $CD4^+$ T-cell count (cells/mm^3) dynamics of the two Siglec-1 Glu88Ter homozygotes. The dates of first HIV-1 positive report and of ART initiation are depicted. Open circles indicate negative values bellow the represented detection level.

Finally, we assessed the impact of the Glu88Ter variant on progression to AIDS (as defined in 1987) in the SHCS (**Figure 3.5A**), where we analyzed 52 Glu88Ter heterozygous and 1 homozygous individuals out of 2,511 individuals. We performed the same analysis in an independent cohort (the Multicenter AIDS Cohort Study, MACS) after genotyping 413 Caucasian HIV-1-infected individuals with documented seroconversion date (**Figure 3.5B**). In the MACS cohort, we identified 12 heterozygous individuals, but no homozygous Glu88Ter individuals were found. Although there was a slow progression to AIDS observed in both the SHCS and MACS cohorts for Glu88Ter individuals, it did not reach statistical significance.

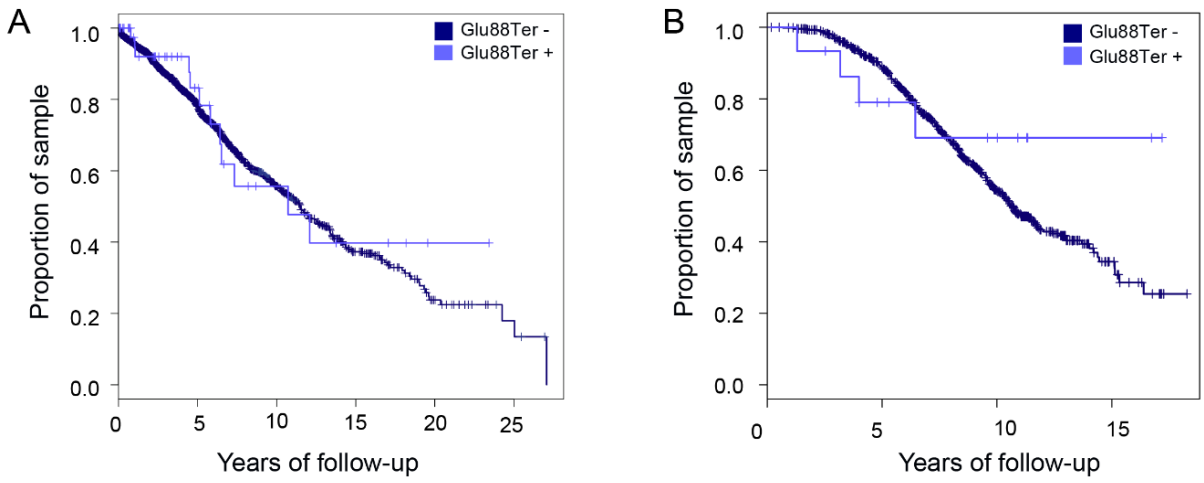

Figure 3.5 Impact of *SIGLEC1* variant on progression to AIDS. A. Time to AIDS measured in the SHCS cohort (n=2,458 common allele Glu88; n=53 Glu88Ter including 52 heterozygous and 1 homozygous individuals) or **B.** the MACS cohort (n=401 common allele Glu88; n=12 Glu88Ter which were all heterozygous).

Taken together, the results in this chapter show HIV-1 infected individuals with a loss-of-function variant in *SIGLEC1* gene that abrogates Siglec-1 expression on primary monocytes and their capacity to *trans*-infect HIV-1 *ex vivo*. Despite this phenotype, our analysis of individuals with this Siglec-1 genetic variant, mainly focused on heterozygotes, which maintain partial receptor expression and function, did not show marked differences in HIV-1 acquisition

or clinical evolution. Given the available sample size of this study and the low Glu88Ter frequency, we can only rule out a large effect of this allele on disease progression. Power simulations indicate that we would need >10,000 samples to detect a relative risk of 5 (similar to the effect of B*57:01 on HIV-1 control) at *P*=0.05 under a recessive model. This sample size far exceeds even the largest genome-wide studies of HIV-1 progression that comprises 6,000 patients[169], which unfortunately does not genotype the Glu88Ter variant and cannot be used to accurately impute the presence of this rare allele. In addition, given that the proposed effect (if any) requires long-term follow-up off therapy (>10 years) it is extremely unlikely that a sufficient sample size could be reached to assess the long-term consequences of this variant on HIV-1 disease. However, the identification of the functional consequences of the Glu88Ter allele might encourage future genetic studies to include this variant and complement our analyses.

In addition, and given the well-known natural history of co-infections during the course of HIV-1 infection, we cannot rule out the possibility that our analysis was influenced by infection with other pathogens. Indeed, the homozygous individual for the rare Siglec-1 allele with worst prognosis had a high CD4+ T cell count that concomitantly dropped when tuberculosis was diagnosed (top graph of **Figure 3.4**). Lack of Siglec-1 could have had a negative impact on the immune control of the mycobacterial infection, masking any putative beneficial effects caused during HIV-1 progression. Previous studies indicate that Siglec-1 expression on myeloid antigen presenting cells (APCs) has a role in combating sialylated bacteria[289,290]. Although sialylation of mycobacteria has not been documented to our knowledge, direct interaction between Siglec-1 and mycobacteria might not be required to impact antibacterial immunity. Alternatively, the lack of Siglec-1 on myeloid APCs could compromise antigen capture via exosome or microvesicle transfer and affect the control of the bacterial infection[84,308,88,89].

Thus, we next aimed to understand if co-infections with additional pathogens such as *Mtb* could worsen the clinical prognosis of HIV-1 infection on individuals bearing the *SIGLEC1* null allele, and mask the potential beneficial effects of Siglec-1 truncation in HIV-1 mono-infected individuals.

Chapter 4

RESULTS II

Increased prevalence of extrapulmonary tuberculosis in individuals harboring the *SIGLEC1* null variant

The results included in this chapter are part of:

Benet S, Gálvez C, Drobniewski F, Kontsevaya I, Arias L, Monguió-Tortajada M, Erkizia I, Urrea V, Ong RY[2], Luquin M, Dupont M, Chojnacki J, Dalmau J, Cardona PJ, Neyrolles O, Lugo-Villarino G, Vérollet C, Julián E, Furrer H, Günthard HF, Crocker PR, Tapia G, Borràs FE, Fellay J, McLaren PJ, Telenti A, Cardona PJ, Clotet B, Vilaplana C*, Martinez-Picado J* and Izquierdo-Useros N*. **Dissemination of *Mycobacterium tuberculosis* is associated to a *SIGLEC1* null variant that limits antigen exchange via trafficking of extracellular vesicles.** *Accepted in J Extracell Vesicles.*

*Senior and corresponding authors

Author's contributions: The author of this thesis contributed to the current work by analyzing the clinical data of the two study cohorts and performing and analyzing the murine experiments, especially the murine histopathological analysis and the cytokine profile. The author also contributed to the design of the experiments, analysis and interpretation of the results, making the figures and writing the manuscript

1. INTRODUCTION

Although Siglec-1 can modulate host immune responses to infection, sialylated viruses like HIV-1 subvert Siglec-1-dependent interactions to hijack antigen presenting cells[9,295]. In the context of HIV-1 infected individuals harbouring the *SIGLEC1* null variant, we hypothesized that co-infections with additional pathogens could worsen the clinical prognosis, masking the potential beneficial effects of Siglec-1 truncation in HIV-1 mono-infected individuals.

The natural function of Siglec-1 is to regulate both innate and adaptive immune responses via interactions with sialylated ligands, present on both immune cells and pathogens[9]. Thus, the primary role of Siglec-1 is to favor immune surveillance and promote pathogen containment. Recognition of sialylated ligands on immune cells modulates Siglec-1 capacity to induce T cell responses[9], via interactions with either antigen presenting cells or specific T-cell populations[309,310]. Moreover, Siglec-1 interacts with sialylated bacteria to promote host defense and pathogen clearance[290,291,289,287]. This has been reported for bacteria containing sialylated lipopolysaccharides such as *Campylobacter jejuni*, group B *Streptococcus* or certain meningococcus[290,291,289,287]. In addition, Siglec-1 also captures sialylated EVs[261,293,294] secreted by antigen-presenting cells interacting with pathogens or by productively infected cells[51]. EVs exchange between antigen presenting cells, which may have not been in direct contact with pathogens can increase the number of cells bearing peptide-MHC complexes leading to the amplification of immune responses[49,50]. Thereby, Siglec-1 can regulate immune responses through the recognition of sialylated ligands exposed on the membrane of immune cells, pathogens or EVs.

Here, we investigated the possible effect of the *SIGLEC1* null variant in HIV-1 related co-infections in two clinical cohorts including 6,256 individuals. We found a significant association between the *SIGLEC1* null variant and extrapulmonary dissemination of *Mycobacterium tuberculosis (Mtb)* in a HIV-1 cohort that was further confirmed when we analyzed an independent clinical cohort of individuals with tuberculosis (TB). In addition, we also studied a murine model lacking Siglec-1 receptor that was infected with *Mtb*. Local spread of bacteria within the lung was apparent in *Mtb*-infected Siglec-1 knockout mice which, despite having similar bacterial load, developed more extensive lesions compared to wild type mice. Thus,

we suggest an antagonistic pleiotropy[311] effect for the *SIGLEC1* null variant in HIV-1 and *Mtb* infected individuals. Since Siglec-1 promotes immunity against several pathogens, co-infection with *Mtb* could influence the observed phenotype in the opposite direction from what is expected, masking any putative beneficial effect of lacking Siglec-1 receptor for avoiding HIV-1 dissemination.

2. MATERIALS AND METHODS

2.1 Ethics statement

The institutional review board on biomedical research from Germans Trias i Pujol University Hospital (HUGTiP) approved this study. Participants of the Swiss HIV Cohort Study (SHCS) and the Russian Cohort gave written consent to the cohort study and genetic analyses, as approved by the corresponding local Ethics Committees.

2.2 Study cohorts

The association of the *SIGLEC1* null variant Glu88Ter with TB was investigated in two different cohorts. First, the Swiss HIV Cohort Study (SHCS), which comprises 3,732 participants that are part of a large cohort study prospectively enrolling HIV-1 infected individuals since 1988 in Switzerland[305], as previously described **(Chapter 3, Materials and methods 2.1).** In the SHCS, TB diagnosis was confirmed by culture, or by combination of response to specific treatment and the presence of acid-fast bacilli in sputum, or by compatible clinical criteria. Selection criteria for pulmonary and extrapulmonary TB are detailed in the SHCS cohort webpage (www.shcs.ch), and are in accordance to the CDC categories. In this study, individuals with concomitant pulmonary and extrapulmonary/disseminated TB were categorized in the extrapulmonary/disseminated TB group.

The second cohort was from Russia, and it includes 2,538 TB patients attending civilian TB dispensaries and TB clinics, along with 2,877 healthy subjects recruited in the blood transfusion services of Samara (Russia). TB patients were initially diagnosed based on information regarding TB contact, medical history, clinical symptoms (cough, hemoptysis, chest pain, fever, weight loss), presence of acid-fast bacilli in sputum smear and characteristic

symptoms and signs of pulmonary TB on chest X-rays, as described previously[312,313,314]. Diagnosis was confirmed by culture of *Mtb* from sputum; otherwise, patients were excluded. Of note, sputum and chest X-ray diagnoses limited the capacity to enroll patients presenting only extrapulmonary/disseminated TB, which are underrepresented in this particular cohort. In the Russian cohort, extrapulmonary/disseminated tuberculosis refers to either lymphatic or hematogenous migration of the bacteria to distant pulmonary areas or different tissues. Individuals with concomitant pulmonary and extrapulmonary/disseminated TB were categorized in the extrapulmonary/disseminated TB group. All individuals lacking information regarding the clinical form of TB were excluded from the analyses, so the final TB cohort included only 2,524 individuals.

2.3 *SIGLEC1* genetic analyses

Genotyping for the HIV-1 Swiss cohort SHCS has been previously described **(Chapter 3, Material and methods 2.7)**. For the Russian cohort, genomic DNA was isolated from frozen whole peripheral blood using the Gentra Puregene Blood Isolation kit (Qiagen) according to the manufacturer's protocol. Samples from individuals with TB diagnosis and control subjects were genotyped for the *SIGLEC1* null variant Glu88Ter (SNP rs150358287C>A) in the *SIGLEC1* gene by TaqMan SNP Genotyping Assay (Assay ID: C_167368973_10; Applied Biosystems). Reactions were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and results were analyzed using StepOne™ Software v2.3 (Applied Biosystems).

2.4 Mice and *Mtb* infection

Six-week-old male C57BL/6 Siglec-1 wild type and knockout mice were shipped from the Division of Cell Signaling and Immunology, School of Life Sciences, University of Dundee, UK. Animal procedures were carried out by the Tuberculosis Experimental Unit and performed according to the protocol DMAH9071, which was reviewed by the Animal Experimentation Ethics Committee of HUGTiP (registered as B9900005) and approved by the *Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural* of the Catalan Regional Government, according to current national and European Union legislation regarding the protection of experimental animals. Mice were supervised daily following a strict monitoring protocol in order to ensure animal welfare, and euthanized, if required, with isoflurane

(inhalation excess). Twelve wild type and Siglec-1 knockout C57BL/6 mice were aerosol challenged with the *Mtb* H37Rv Pasteur strain using an airborne infection apparatus (Glas-col Inc.) delivering around 50 colony forming units (CFU). Three and four weeks after challenge, six wild type and knockout mice were euthanized.

2.5 Murine histopathological analysis

Right upper lung lobe samples were fixed in formaldehyde (Biopsafe), embedded in paraffin and cut in 5-µm sections that were stained with hematoxylin-eosin for microscopic observation (Nikon Instruments Inc). Four distinct sections of each tissue block were used to determine the damaged area as percentage of the total lung area using the NISElements D version 3.0x software package (Nikon Instruments Inc).

2.6 Murine bacillary load measurement

Lung and spleen samples collected from each animal were mechanically homogenized and plated using serial dilutions on nutrient Middlebrook 7H11 agar plates (BD Diagnostics). Visible CFU were counted after incubation for 28 days at 37^{o}C.

2.7 Murine cytokine profiling

Frozen serum samples and snap-frozen lung homogenates from the right lower and middle lung lobes were assessed. Lung samples were thawed, weighted and homogenized on ice with lysis buffer containing 0.05% sodium azide, 0.5% Triton X-100, 1:500 Protease inhibitor cocktail (all from Sigma-Aldrich) in sterile PBS, using 1 ml per 100 mg of tissue. Homogenates were incubated for 1h at 4^{o}C and centrifuged at 3000 × g for 10 min. Supernatants were collected and stored at -80^{o}C until use. Cytokines were measured by Luminex xMAP® technology and analyzed with xPONENT 3.1 software (Luminex Corporation). In serum samples, IFNγ, TNFα, IL-6, LIX and IL-17 were measured using the MCYTOMAG-70K kit. In lung homogenates, IFNγ, TNFα, IL-4, IL-6, LIX, IL-1β, IL-10, IL-12, IP-10, KC, MCP-1 and VEGF were analyzed with the MCYTOMAG-70K kit, and TGFβ with the TGFBMAG-64K kit (EMD Millipore Corporation) following the manufacturer's instructions.

2.8 Statistical analyses

The association between the *SIGLEC1* null allele and the TB phenotypes was assessed by an enrichment analysis using a Chi-squared test, a hypergeometric test, a proportion test or a logistic regression, according to the design of the cohorts. Mean or median changes were analyzed using a paired t-test or a Mann–Whitney U-test, respectively. Test results were considered significant at $P < 0.05$. All analyses and figures were generated with R or GraphPad Prism v.8 software.

3. RESULTS

3.1 Increased frequency of the *SIGLEC1* null allele in individuals with extrapulmonary/disseminated TB from the SHCS HIV-1 Cohort

To assess the possible effect of the *SIGLEC1* null variant in HIV-1 related co-infections, we searched for the co-occurrence of infections in the SHCS. We analyzed the clinical records of 3,732 participants, in which we had previously identified 85 heterozygous and 2 homozygous individuals bearing the *SIGLEC1* Glu88Ter null variant[315]. The characteristics of these patients have been described previously[315] **(Chapter 3, results 3.1)**. It is important to highlight that the SHCS follows the classification of the Center for Disease Control and Prevention (CDC) for AIDS-defining opportunistic infections, and has collected these records since 1988. To evaluate if the *SIGLEC1* null variant was over-represented in any particular infectious disease category, we used a hypergeometric test. This analysis revealed that among the 708 individuals from the SHCS with reported bacterial, fungal, viral or protozoal co-infections, the *SIGLEC1* null variant was significantly associated to *Mtb* infection (*P*-value 0.011; **Table 4.1**).

We next classified TB cases in the SHCS cohort by the site of clinical manifestation of the disease into pulmonary or extrapulmonary/disseminated TB forms, and calculated the proportion of individuals bearing the null variant and the 95% confidence interval (**Figure 4.1**). In these subgroups, the association to the presence of the *SIGLEC1* null allele was only significant for the extrapulmonary/disseminated TB category with an over-representation of 7.15-fold compared to the total SHCS cohort (*P*-value 0.008; hypergeometric test; **Table 4.2**).

Table 4.1 *SIGLEC1* null variant and AIDS-defining infectious diseases in the SHCS HIV-1 cohort

AIDS-defining infectious diseases	Null variant (heterozygous & homozygous)	Common variant (homozygous)	*P*-value	Fold Enrichment	Confidence Intervals 95% *Lower bound*	Confidence Intervals 95% *Upper bound*
All bacterial diseases	7	138	**0,051**	**2,071**	**0,976**	**4,393**
Tuberculous mycobacterial diseases	5	54	**0,011**	**3,635**	**1,532**	**8,624**
Non-tuberculous mycobacterial diseases	1	24	0,447	1,716	0,249	11,840
Other bacterial diseases	1	60	0,766	0,703	0,100	4,967
All fungal diseases	10	452	0,650	0,928	0,928	0,928
Pneumocystis pneumonia	3	235	0,924	0,541	0,541	0,541
Other fungal diseases	7	217	0,266	1,341	1,341	1,341
Viral diseases	2	192	0,946	0,946	0,946	0,946
Protozoal diseases	1	57	0,748	0,740	0,740	0,740

Table 4.1 *SIGLEC1* null variant and AIDS-defining infectious diseases in the SHCS HIV-1 cohort

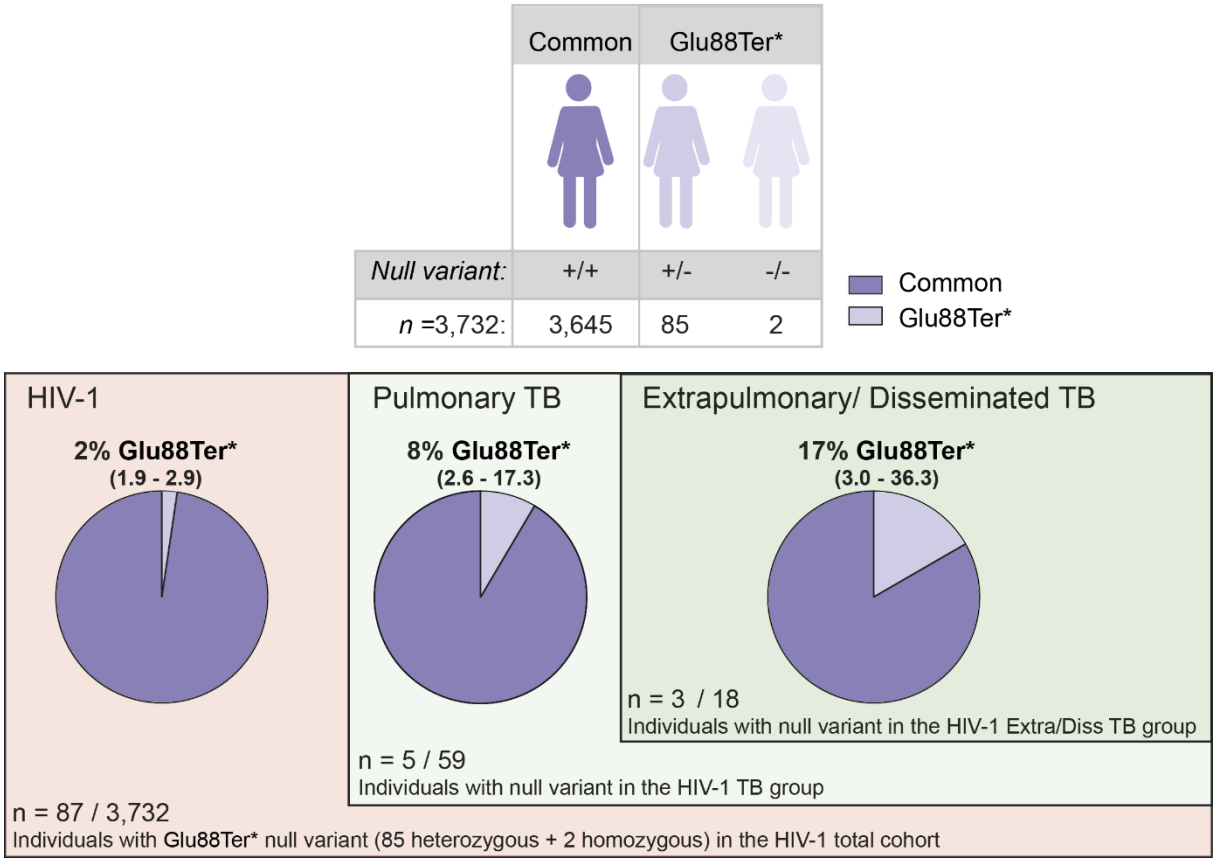

Figure 4.1 Increased frequency of the *SIGLEC1* null allele in individuals with extrapulmonary/disseminated TB from the SHCS Cohort. Representation of the different subgroups analyzed in the SHCS cohort. Estimated proportion and 95% confidence interval of individuals harboring the *SIGLEC1* null allele, including 85 heterozygous and 2 homozygous individuals. Subjects are grouped by TB diagnosis and by the localization of TB into pulmonary or extrapulmonary/disseminated forms.

Table 4.2 *SIGLEC1* null variant and clinical forms of TB disease in the SHCS HIV-1 cohort

Clinical forms of TB disease	Null variant (heterozygous & homozygous)	Common variant (homozygous)	*P*-value	Fold Enrichment	Confidence Intervals 95% *Lower bound*	*Upper bound*
Tuberculous mycobacterial diseases	5	54	**0,011**	**3,635**	**1,532**	**8,624**
Pulmonary TB	2	39	0,248	2,093	0,533	8,215
Extrapulmonary TB	3	15	**0,008**	**7,149**	**2,493**	**20,505**

Table 4.2 *SIGLEC1* null variant and clinical forms of TB disease in the SHCS HIV-1 cohort

In agreement with this association, we observed that one of the homozygous HIV-1 infected Siglec-1 null individuals maintained high $CD4^+$ T cell counts in the absence of antiretroviral treatment for several years (**Figure 4.2**, grey circles). However, this patient concomitantly lost viral control when she was diagnosed with extrapulmonary/disseminated TB along with a pulmonary TB (**Figure 4.2**, green arrow).

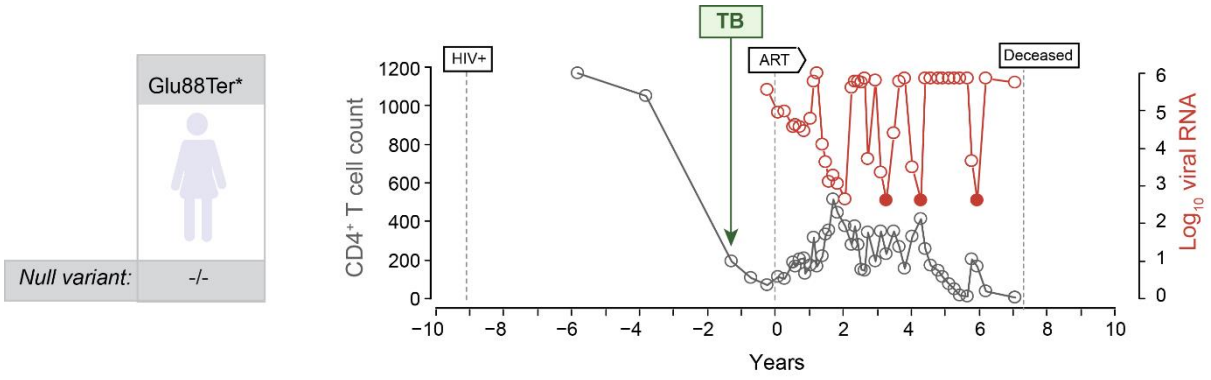

Figure 4.2 Clinical evolution of a Siglec-1 null homozygous individual from the SHCS HIV-1 Cohort diagnosed with TB. Dynamics of the $CD4^+$ T-cell count (cells/mm^3) and plasma viral RNA level (copies/ml) of a Siglec-1 null homozygous individual from the SHCS cohort, who was diagnosed with pulmonary and extrapulmonary TB. The dates of first HIV-1 positive report, of TB diagnosis and of antiretroviral treatment (ART) initiation are depicted. Colored circles indicate values that are below the limit of detection of the viral load. Graph is adapted from **Chapter 3, Figure 3.4**.

The identification of a *Mtb*-infected *SIGLEC1*-null homozygote ruled out a requirement for a functional Siglec-1 for becoming productively infected with *Mtb*. Yet, the lack of Siglec-1 in this particular homozygous individual could have had a negative impact upon *Mtb* infection, complicating HIV-1 progression (**Figure 4.2**). In the SHCS cohort, the significant association found between the *SIGLEC1* null allele and TB appears to be linked to an extrapulmonary dissemination of *Mtb,* what prompted us to confirm this particular association in an independent clinical cohort.

3.2 An increased frequency of the *SIGLEC1* null allele in individuals with extrapulmonary/disseminated TB is also confirmed in the Russian TB cohort

Next, we determined the proportion of the *SIGLEC1* null allele in an independent TB Russian cohort[312] (**Figure 4.3**). DNA samples from 5,401 individuals including 2,877 control subjects and 2,524 individuals with clinical manifestation of TB were genotyped for the *SIGLEC1* null variant previously analyzed in the SHCS HIV-1 cohort. The proportion of individuals bearing the null variant in this Russian cohort is shown in **Figure 4.3**, while the demographic characteristics of the TB group and the control group are shown in **Table 4.3**. In this Russian TB cohort, we observed a significant increase of 6-fold in the frequency of the *SIGLEC1* null allele between the control group and the disseminated TB group (*P*-value 0.007; Confidence intervals 95% =1.5-17.6; Fisher's exact test).

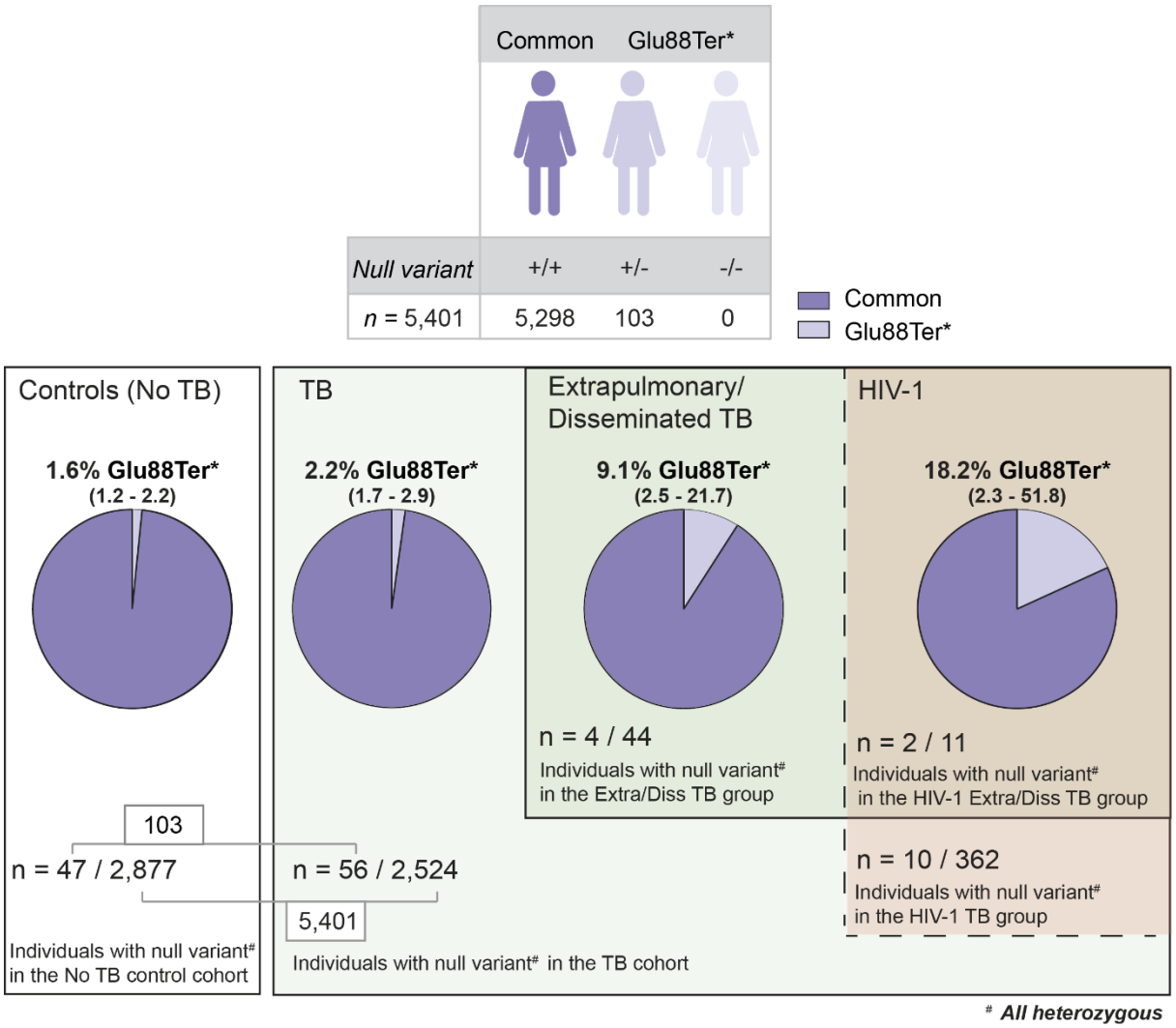

Figure 4.3 (Caption overleaf)

Figure 4.3 An increased frequency of the *SIGLEC1* null allele in individuals with extrapulmonary/disseminated TB in the SHCS HIV-1 cohort is also confirmed in the Russian TB cohort. Representation of the different subgroups analyzed in the Russian TB cohort. Estimated proportion and 95% confidence interval of individuals harboring the *SIGLEC1* null allele, including 103 heterozygous individuals and no homozygous individuals. In the TB group, individuals are categorized by extrapulmonary/disseminated TB and HIV-1 status.

Table 4.3 Demographic characteristics of individuals from the TB cohort

	Controls		TB group	
	Null variant (heterozygous)	Common variant (homozygous)	Null variant (heterozygous)	Common variant (homozygous)
Age median; (IQR)	25.5 (20-35.25)	28 (21-41)	41.5 (32.75-52)	39 (31-50)
Female n; (%)	7 (14.9%)	764 (27%)	12 (21.4%)	592 (24%)
Male n; (%)	40 (85.1%)	2066 (73%)	44 (78.6%)	1876 (76%)
Russian mother n; (%)	44 (93.6%)	2405 (84.9%)	49 (87.5%)	2099 (85%)
Russian father n; (%)	40 (85.1%)	2335 (82.5%)	45 (80.4%)	2024 (82%)

Table 4.3 Demographic characteristics of individuals from the Russian TB cohort

We next focused on the TB group, and since it had a subset of HIV-1 co-infected individuals (**Figure 4.3**), we performed a logistic regression analysis adjusted for HIV-1 to avoid a possible confounding effect, although no significant association between the *SIGLEC1* null allele and HIV-1 infection had been previously found[315]. This analysis revealed only a significant association between the dissemination of *Mtb* and the *SIGLEC1* null allele (*P*-value 0.005; odds ratio of 4.55; Confidence intervals 95%= 1.3-11.9). Overall, data from this independent Russian cohort confirmed the significant association between the disseminated form of TB and the *SIGLEC1* null allele.

3.3 *Mtb* local dissemination in infected *SIGLEC1* knockout mice aggravates pulmonary lesions

To assess the functional association between the *SIGLEC1* null variant and *Mtb* dissemination, we compared wild type and Siglec-1 knockout C57BL/6 mice[316] infected with *Mtb* H37Rv Pasteur strain via aerosol. When the *Mtb*-affected areas of the lungs of these mice were compared to the total lung area by histopathologic analysis (**Figure 4.4A**, images) Siglec-1 knockout mice had slightly smaller affected areas at week 3 post-infection (p.i.), but a much

higher percentage of damaged tissue than wild type mice at week 4 p.i. (**Figure 4.4A**, graphs, *P*-value 0.029; Mann-Whitney test). These results indicate that *SIGLEC1* knockout mice have a delayed generation of pulmonary lesions at week 3 p.i., which are bigger and less structured at week 4 p.i. when compared to wild type mice. Of note, the bacillary load in lungs was similar in both wild type and *SIGLEC1* knockout animals at weeks 3 and 4 p.i. (**Figure 4.4B**), indicating that Siglec-1 is dispensable for the initial infection with *Mtb* in the mouse model as already observed in human cohorts. Equivalent bacillary load was also detected in spleen, denoting that *Mtb* similarly reached lymphoid tissues in both mouse strains at weeks 3 and 4 p.i. (**Figure 4.4B**).

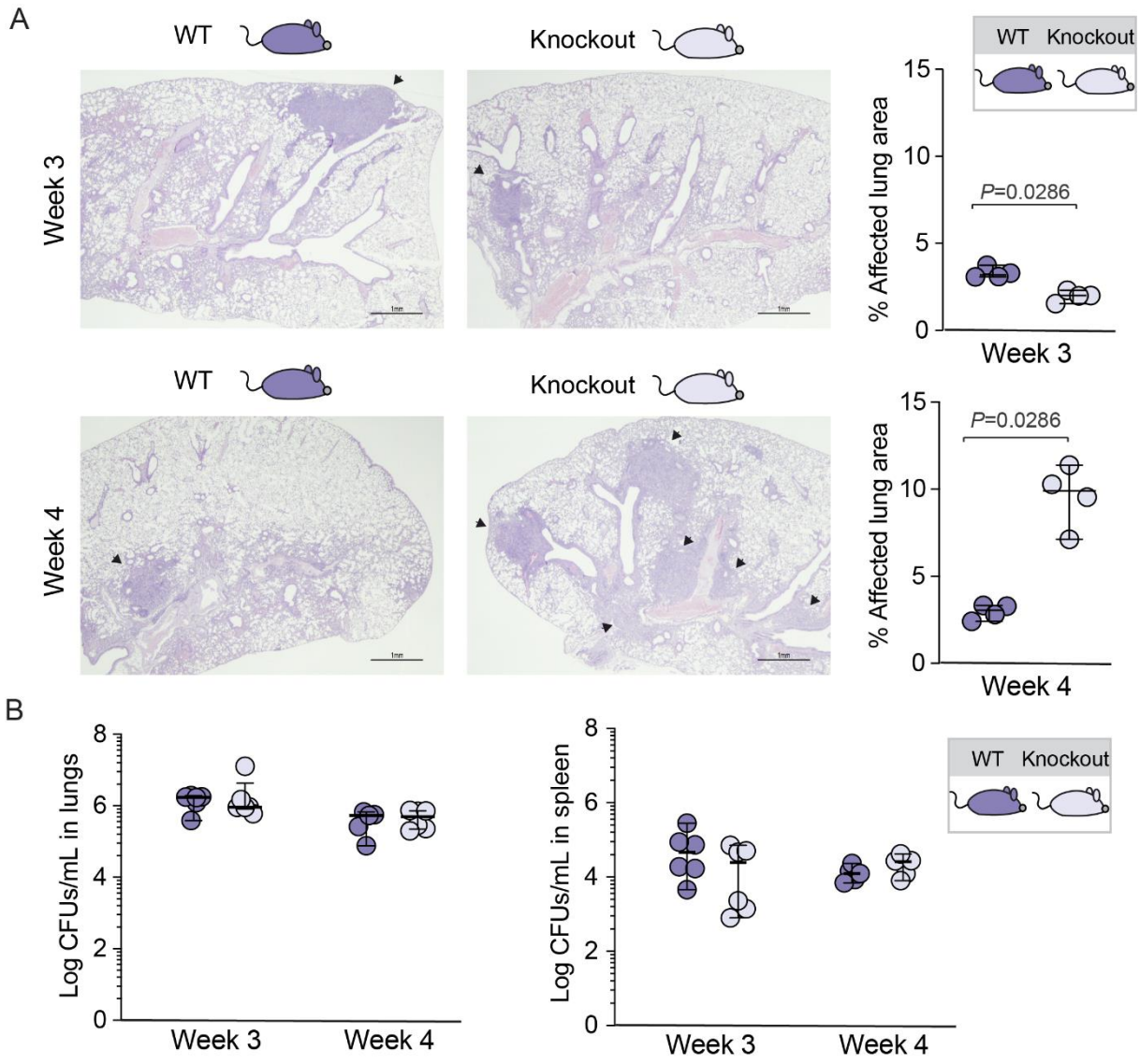

Figure 4.4 *Mtb* local dissemination in infected Siglec-1 knockout mice aggravates pulmonary lesions. A. Images show representative hematoxylin/eosin staining from lungs of wild type (WT) and Siglec-1 knockout C57/BL6 mice challenged via aerosol with *Mtb* H37Rv at 3- or 4-

weeks p.i. Graphs show the corresponding quantification of the damaged area as a percentage of the total lung area analyzed. Median values and range are depicted. Statistical differences were assessed with a Mann-Whitney test. **B.** Growth of *Mtb* H37Rv in the lungs and the spleen of wild type and Siglec-1 knockout C57/BL6 mice infected with *Mtb* for 3 or 4 weeks. Results are presented as the medians and range of Log_{10} of bacterial CFUs per ml.

Accordingly, no significant differences between wild type and *SIGLEC1* knockout mice were observed in cytokine production detected in serum (**Figure 4.5**) or lung homogenates (**Figure 4.6**).

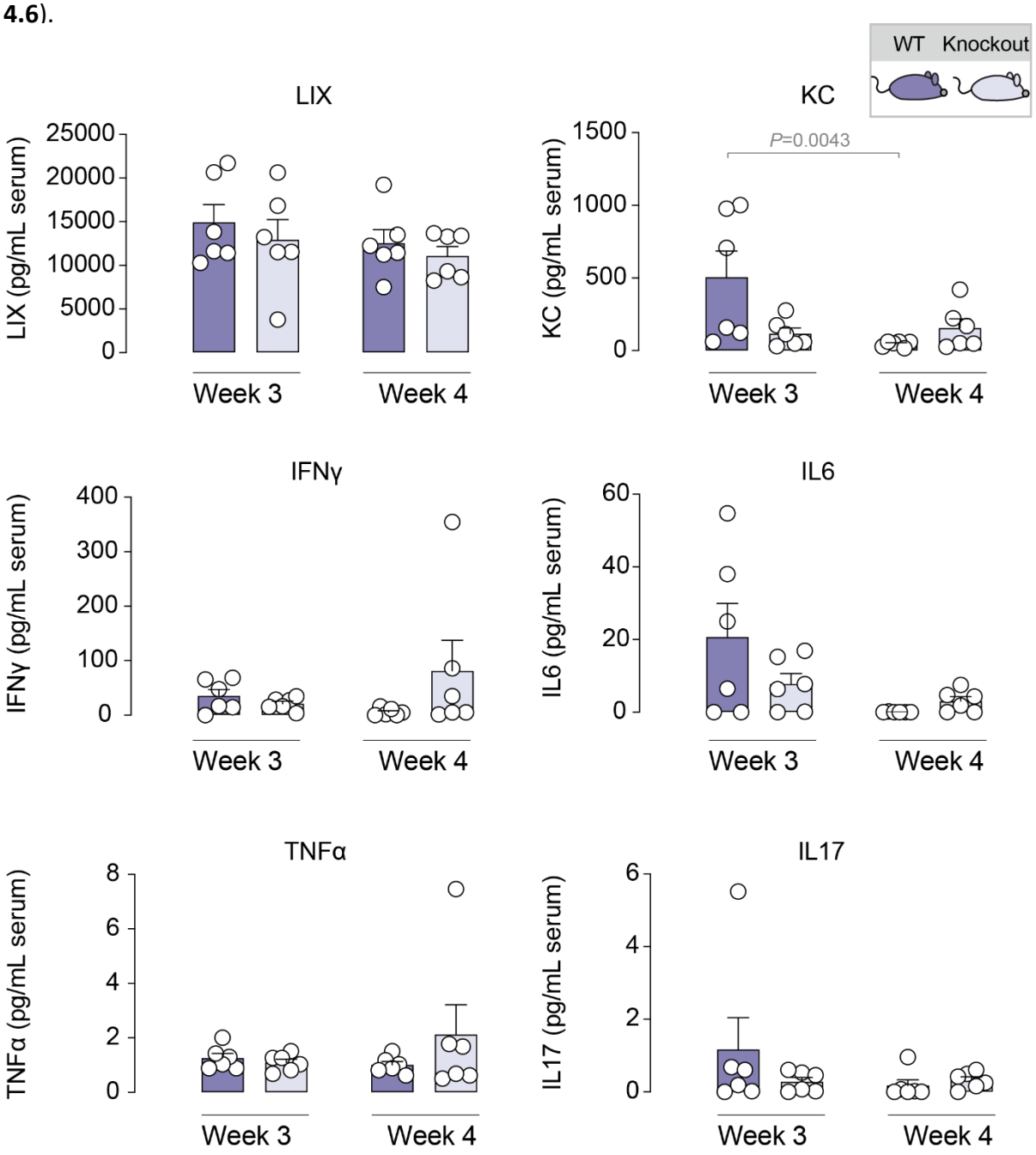

Figure 4.5 (Caption overleaf)

Figure 4.5 Similar cytokine profile was observed in serum samples from wild type and Siglec-1 knockout mice. Comparative cytokine profile measured in serum samples from wild type and Siglec-1 knockout mice at weeks 3 and 4 p.i. Results are expressed as mean and SEM in pg per ml of serum. Statistical differences were assessed with a Mann-Whitney test.

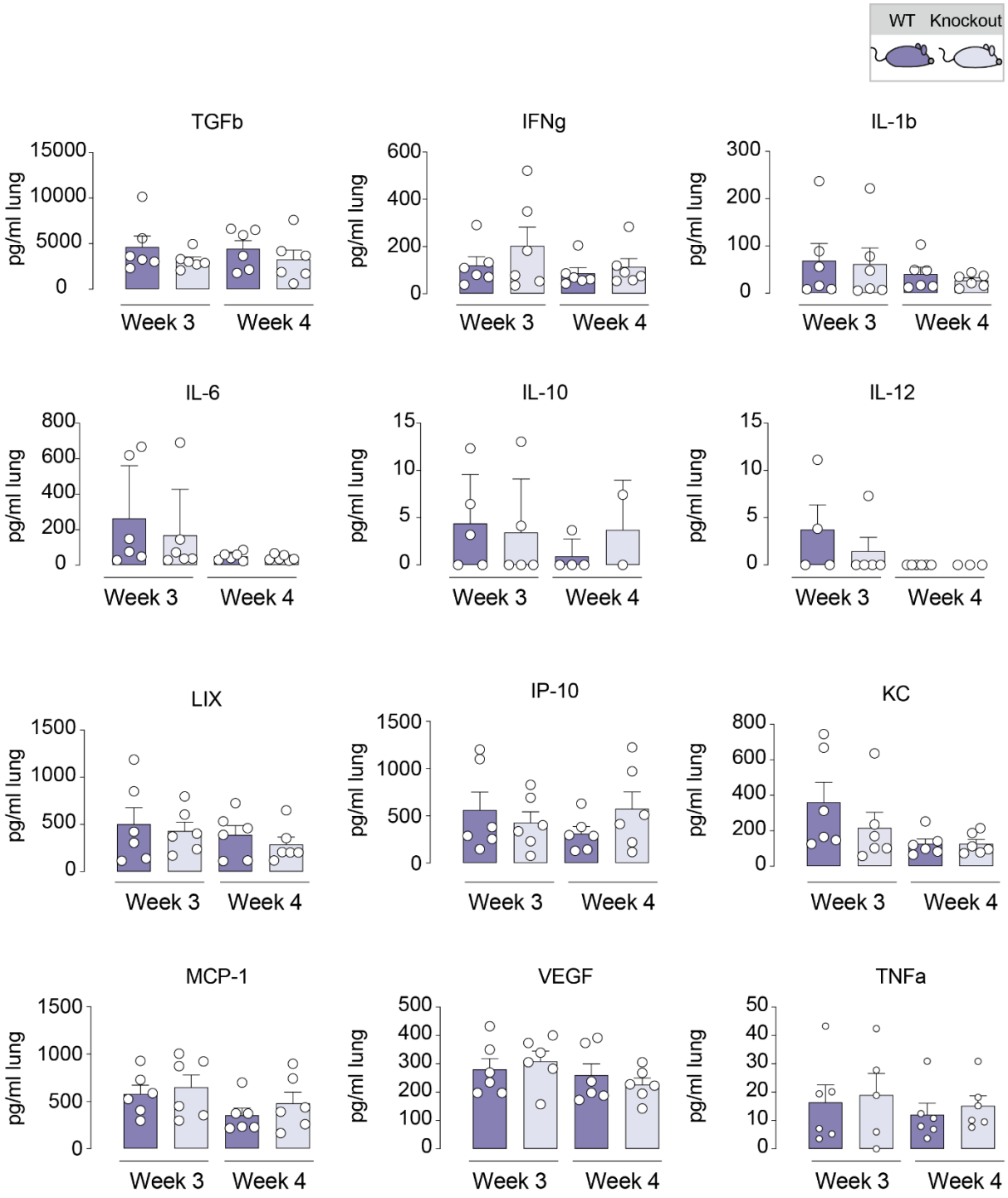

Figure 4.6 Similar cytokine profile was observed in lung homogenates from wild type and Siglec-1 knockout mice. Comparative cytokine profile measured in lung homogenates from wild type and Siglec-1 knockout mice at weeks 3 and 4 p.i. Results are expressed as mean and

SEM in pg per ml of lysate homogenate. Statistical differences were assessed with a Mann-Whitney test.

Results in the mouse model indicate that the absence of Siglec-1 might unfavorably affect bacterial containment within the lung but without detectable changes in bacterial load or cytokine environment.

Here we investigated the effect of a *SIGLEC1* null variant in infectious diseases and found a significant association with an extrapulmonary dissemination of *Mtb* in two independent clinical cohorts. To further gain insights into this association, we studied a murine C57BL/6 model infected with *Mtb*, and compared *SIGLEC1* knockout versus wild type mice. Of note, as opposed to other mouse strains, this particular murine model is highly resistant to TB progression once specific immune responses are mounted 4 weeks p.i. in the lungs[317]. Yet, and despite the limitation of using a murine model that only reflects the human course of TB infection partially, our results showed that in the absence of Siglec-1, the affected area of murine pulmonary lesions assessed by histopathology was larger. This could not be explained by the presence of a higher bacillary load, a lack of bacterial migration towards secondary lymphoid tissues such as the spleen, or reflected in a distinctive cytokine production profile. Thus, in a resistant murine model of TB, the *SIGLEC1* knockout had lower capacity to contain the granulomatous infiltration in the lung.

These results support our initial hypothesis, in which in the absence of Siglec-1, a co-infection with additional pathogens such as *Mtb* could worsen the prognosis of HIV-1 infected individuals. Particularly, *Mtb* co-infected individuals harboring the *SIGLEC1* null variant are prompted to develop an extrapulmonary/disseminated TB. This could lead to a change in the clinical evolution experienced by HIV-1 infected individuals upon *Mtb* infection that could mask the real consequences of the *SIGLEC1* null variant on HIV-1 disease progression. In this scenario, the phenomenon termed antagonistic pleiotropy becomes relevant, as *SIGLEC1* null variant has opposed effects in both infectious diseases.

Altogether, the results from this chapter found both in human and mice suggest that Siglec-1 could protect the host against *Mtb* spread. However, how Siglec-1 regulates this mechanism remains to be elucidated.

Chapter 5

RESULTS III

Dissemination of *Mtb* is associated to a *SIGLEC1* null variant that limits antigen exchange via trafficking of extracellular vesicles

The results included in this chapter are part of:

Benet S, Gálvez C, Drobniewski F, Kontsevaya I, Arias L, Monguió-Tortajada M, Erkizia I, Urrea V, Ong RY[2], Luquin M, Dupont M, Chojnacki J, Dalmau J, Cardona PJ, Neyrolles O, Lugo-Villarino G, Vérollet C, Julián E, Furrer H, Günthard HF, Crocker PR, Tapia G, Borràs FE, Fellay J, McLaren PJ, Telenti A, Cardona PJ, Clotet B, Vilaplana C*, Martinez-Picado J* and Izquierdo-Useros N*. **Dissemination of *Mycobacterium tuberculosis* is associated to a *SIGLEC1* null variant that limits antigen exchange via trafficking of extracellular vesicles.** *Accepted in J Extracell Vesicles.*

*Senior and corresponding authors

Author's contributions: The author of this thesis contributed to the current work by analyzing thin-layer chromatography experiments and gene silencing experiments in human leukocytes and performing and analyzing EV experiments, particularly the uptake assays of fluorescent EVs and the IFN-γ ELISpot assay. The author also contributed to the design of the experiments, analysis and interpretation of the results, making the figures and writing the manuscript.

1. INTRODUCTION

The mechanism that facilitates *Mtb* migration and dissemination in the total or partial absence of Siglec-1 is not known. Siglec-1 has been shown to capture sialylated bacteria, but to the best of our knowledge, *Mtb* is not sialylated. However, Siglec-1 also captures sialylated EVs[261,293,294] secreted by antigen-presenting cells interacting with pathogens or productively infected cells[51]. Particularly, murine studies have confirmed the capacity of Siglec-1 expressed on lymphoid tissues to capture exosomes *in vivo*[294]. Transfer of antigens between immune cells via EVs amplifies the initiation of immunity[49,50], both through direct MHC recycling but also promoting cross-presentation. In turn, this initial boost of antigen transmission mediated by EVs between antigen presenting cells is paramount to mount early and effective responses and contain pathogen invasion. Thus, Siglec-1 can modulate immune responses to infection, not only via interaction with sialylated pathogens, but also trapping EVs.

Thus, we hypothesize that Siglec-1 could either mediate the capture and clearance of *Mtb* or be critical for trapping EVs that are involved in the initiation or amplification of the immune responses against *Mtb*. Here, we did not detect a direct interaction between Siglec-1 and *Mtb*. However, we provide a novel molecular basis to explain how the capacity of Siglec-1 to bind sialylated ligands can initiate immunity against *Mtb* through recognition of EVs that transport antigens. Thus, the lack of Siglec-1 could delay the onset of protective immunity against *Mtb* by limiting antigen spread via EVs, allowing for an early local spread of mycobacteria that increases the risk for extrapulmonary dissemination reported in Chapter 4.

2. MATERIALS AND METHODS

2.1 Siglec-1 siRNA silencing in monocyte-derived macrophages, *Mtb* infection and CFU enumeration

PBMCs were obtained from HIV-1 seronegative donors by Ficoll-Hypaque density gradient centrifugation, and monocyte populations were isolated with $CD14^+$ magnetic beads (Miltenyi Biotec). Silencing in monocytes was performed using reverse transfection protocol as previously described[318]. Monocytes were transfected with 200 nM of ON-TARGETplus

SMARTpool siRNA targeting Siglec-1 or non-targeting siRNA control (Horizon Discovery) using HiPerfect transfection system (Qiagen). Cells were left to adhere to glass coverslips (Dominique Dutscher) in 24-well plates at a density of 0.5×10^6 for 4h prior to the addition of 0.5 ml of RPMI 1640 (Gibco) supplemented with 10% FBS and 20 ng/ml of human M-CSF (Peprotech). Culture media was renewed every three days for seven days.

Monocyte-derived macrophages were then infected with 10^5 *Mtb* H37Rv-DsRed per well (MOI = 0.2) for 4h at 37°C, washed with PBS to remove extracellular *Mtb* and replaced with fresh media. At day 0, 5 or 7 p.i., cells were washed with PBS and lysed with 0.01% Triton X100 (Sigma-Aldrich), serially diluted in PBS and plated onto 7H11- OADC agar medium (Difco) to assess the bacterial intracellular growth. CFU were determined at days 14 and 21 post-plating. Of note, a significant increase in bacterial growth over time was observed for all cellular types tested (P <0.0001; likelihood ratio test for time effect in a fitted linear mixed-effects model). At day 7, part of the cells was assessed for Siglec-1 expression with an APC-anti-Siglec-1 mAb (BioLegend) or the corresponding isotype control by flow cytometry using BD LSRFortessa flow cytometer (BD Biosciences, TRI Genotoul platform) and the associated BD FACSDiva software. Data were then analyzed using the FlowJo_V10 software (FlowJo, LLC).

2.2 Detection of sialylated residues on *Mtb* cell lysates

Mtb H37Rv Pasteur strain was cultured on Middlebrook 7H11 agar plates (BD Diagnostics) at 37°C. After 3 weeks, mycobacterial cells were harvested and extracted with chloroform, methanol and mixtures of chloroform-methanol. The extracts were pooled, dried and partitioned with chloroform/methanol/water (8:4:2 v/v). Aqueous phase and chloroform phase were separated and evaporated to dryness. Presence of free GM3, sialyllactose and sialic acid in these extracts was analyzed by thin layer chromatography developed with chloroform/methanol/water (with 0.2% of $CaCl_2$) (5:4:1 v/v). Thin layer chromatography plates were revealed with resorcinol, a reagent that specifically stains sialic acid or sialic acid-derivatives with a distinctive brown-violet or blue-violet color[319]. Presence of total sialic acid in *Mtb* H37Rv chloroform and aqueous extracts was determined by acid hydrolysis with 1N HCl in methanol at 80°C for 2h and thin layer chromatography analysis as explained above. Purified bovine milk ganglioside GM3, sialyllactose and sialic acid working standards (0.025 mg) were obtained from Sigma-Aldrich.

2.3 *Mtb* infection of THP-1-derived human macrophages

THP-1 were cultured in RPMI with L-Glutamine containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and 100 ng/ml PMA (Sigma-Aldrich) for 48h and a 24h resting period in PMA-free fresh culture medium to induce macrophage differentiation. Then, 2.2×10^6 THP-1-derived macrophages were infected with *Mtb* H37Rv Pasteur strain at a MOI of 0.1 or 1 for 4h or left uninfected. After extensive washing, macrophages were cultured for 72h with EV-depleted culture medium[320]. Briefly, 20% FBS complete medium was ultra-centrifuged in polyallomer ultracentrifugation tubes (Thermo Fisher Scientific) at 100,000 × g for more than 16h (TH641 rotor, adjusted k-Factor = 240.82, Sorvall WX Ultra 100 Series ultracentrifuge, Thermo Fisher Scientific). The supernatant was collected and filtered through a 0.22 µm filter (Sarstedt) to sterilize the medium, which was finally diluted with RPMI medium (1:1) for cell culture.

2.4 EVs isolation and purification

All relevant data have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV190053)[321]. EVs were isolated from supernatants collected from mock or *Mtb*-infected THP-1-derived macrophages after 72h of culture. Supernatants were centrifuged at 400 × g for 5 min and at 2,000 × g for 10 min to exclude cells and cell debris, respectively. Debris-cleared conditioned medium was then concentrated by 100 kDa ultrafiltration using regenerated cellulose Amicon Ultra (Millipore) at 2,000 × g for 35 min, obtaining typically 250 µl concentrated conditioned medium. For tracking purposes, EVs were stained with 5,6-Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE; Invitrogen) by incubating the concentrated conditioned medium with 100 µM CFSE for 2h at 37°C[322]. CFSE positive or unstained EVs were then isolated from the concentrated conditioned medium (and washed away from free CFSE dye) by size-exclusion chromatography (SEC) using a modification of a previous method[320]. Briefly, 12 ml of Sepharose CL-2B (Sigma-Aldrich) were extensively washed with PBS (Oxoid) and packed in a Puriflash dry load empty 12G flash column (Interchim-Cromlab). Concentrated conditioned medium was loaded into the SEC, and 500µl fractions (up to 35) were collected immediately after eluting with PBS. Protein elution was checked by reading absorbance at 280 nm of each fraction using Nanodrop (Thermo Scientific). The presence of EVs in the SEC fractions was determined according to their content

in tetraspanins by bead-based flow cytometry, as previously described[323]. Briefly, EVs were coupled to 4-µm aldehyde/sulphate-latex microspheres (Invitrogen) for 15 min, blocked overnight with BCB buffer (PBS/0.1% BSA/0.01% NaN_3; all from Sigma-Aldrich) and spun down at 2,000 × g for 10 min. EVs-coupled beads were then labelled with the primary antibodies anti-CD9 (Clone VJ1/20) and anti-CD63 (Clone TEA3/18) at 1:100 dilution (kindly provided by Dr. María Yáñez-Mó from UAM; CBM-SO, IIS-IP and Dr. Francisco Sánchez-Madrid from Hospital Universitario de la Princesa, IIS-IP, UAM, CNIC) and secondary antibodies Cy5-conjugated Donkey anti-Mouse or A647-conjugated Goat F(ab')2 Anti-Mouse IgG (Jackson ImmunoResearch), performed at RT for 30 min under mild shaking, washed after each step with BCB buffer and centrifuged at 2,000 × g for 10 min. Data were acquired in a FACSLyric flow cytometer (BD) and analyzed by FlowJo v.10.2 software (BD). EVs-containing fractions were pooled together and adjusted to the desired volume with PBS using 100 kDa-ultrafiltration 2 ml-Amicon Ultra (Millipore). EVs were kept at 4ºC and used within 24h for the *in vitro* experiments or frozen (-1ºC/min) at -80ºC.

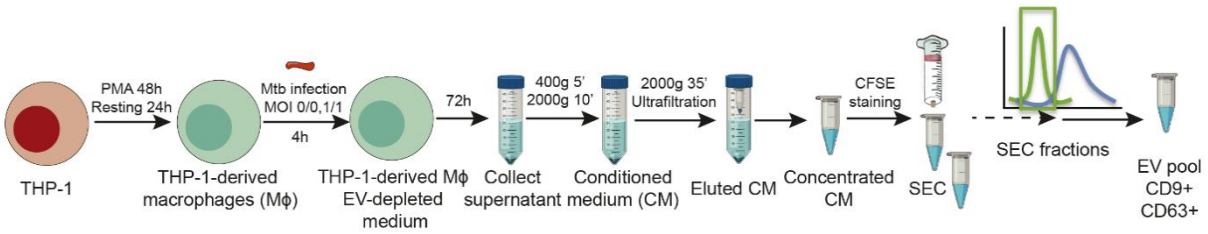

Figure 5.1 Graphical summary of extracellular-vesicle isolation and purification from THP-1-derived macrophages. Supernatants collected from mock or *Mtb*-infected THP-1-derived macrophages after 72h of culture with EV-depleted culture medium were centrifuged to deplete cells and debris. Debris-cleared conditioned medium (CM) is then concentrated by ultrafiltration. This concentrated CM is stained with CFSE and then CFSE positive EVs are isolated by size-exclusion chromatography (SEC). The presence of EVs in the SEC fractions was determined according to their content in tetraspanins (CD9 and CD63) by bead-based flow cytometry.

2.5 Size and morphological analysis of EVs

EVs were examined by cryo-electron microscopy at the Electron Microscopy Service of the Autonomous University of Barcelona. Vitrified specimens were prepared by placing 3 µl of a sample on a Quantifoil® 1.2/1.3 TEM grid, blotted to a thin film and plunged into liquid ethane-

N2(l) in an EM CPC cryoworkstation (Leica). The grids were transferred to a 626 Gatan cryoholder and maintained at -179ºC. Samples were analyzed with a Jeol JEM 2011 transmission electron microscope operating at an accelerating voltage of 200 kV. Images were recorded on a Gatan Ultrascan 2000 cooled charge-coupled device (CCD) camera with the corresponding Digital Micrograph software package.

Size distribution of particles of EV preparations was determined by nanoparticle tracking analysis in a NanoSight LM10-12 (Malvern Instruments Ltd), equipped with a 638 nm laser and CCD camera model F-033. Data was analyzed with software version build 3.1.46, with detection threshold set to 5, and blur, Min track Length and Max Jump Distance set to auto. Samples were diluted in filtered PBS to remove particles in suspension and reach optimal concentration for instrument linearity: 20-120 particles/frame as advised by the manufacturer. Readings were taken on triplicates of 60 s at 30 frames/s, at a camera level set to 16 and with manual monitoring of temperature.

2.6 Uptake assays of fluorescent EVs

DCs were obtained by culturing monocytes obtained with $CD14^+$ magnetic beads (Miltenyi Biotec) in the presence of 1,000 IU/ml GM-CSF and IL-4 (both from R&D) for 7 days and replacing media and cytokines every 2 days. At day 5, DCs were matured with 100 ng/ml lipopolysaccharide (*Escherichia coli* O111:B4; Sigma-Aldrich) to induce Siglec-1 expression. Uptake experiments with CFSE labelled-EVs were performed by pulsing 2.5×10^5 mDCs for 4h at 37ºC.

For immunofluorescence staining, cells were suspended in 500 µL L-15 medium, incubated with 100 nM Abberior STAR RED -1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE, Abberior GmbH) and washed 3 times with L-15. Cells were adhered to poly-L coated coverslips at 37ºC and fixed in 3% PFA plus PBS for 15 min. Fixed samples were permeabilised and blocked using 0.1 % saponin plus 0.5% BSA and 100 µg/mL human serum IgGs (Privigen, Behring CSL). Cells were immunostained with anti-Siglec-1 6H9 mAb[269] directly coupled to Abberior STAR 580 dye (Abberior GmbH). Following immunostaining samples were briefly incubated with NucBlue Live Hoechst 33342 stain (ThermoFisher Scientific) and post-fixed using 3% PFA. Samples were overlaid with SlowFade Diamond mounting medium (ThermoFisher Scientific) and imaged using confocal microscopy. Confocal microscopy

analysis was performed using Zeiss LSM 780 confocal microscope (Jena) equipped with a 63×/1.4 NA oil immersion objective. Image Z-stacks for each channel were acquired sequentially with the following parameters: pinhole size: 1 Airy, XY pixel size: 50 nm, Z pixel size: 250 nm. Following acquisition images were deconvoluted using Huygens Professional software and theoretical PSF parameters corresponding to the system's effective observation spot or point-spread-function. All subsequent image manipulation steps were performed using Fiji (ImageJ distribution) software with the exception of the isosurface generation, which was performed using Imaris.

For flow cytometry analysis, mDCs were left untreated or pre-incubated for 15 min at RT with 10 µg/ml of anti-Siglec-1 mAbs (7-239 or 7D2; both from Abcam) or IgG1 isotype control (BD Biosciences) before pulse with CFSE labelled- EVs. After extensive washing, mDCs cells were acquired with FACS Calibur (BD Biosciences), and the percentage of positive cells was determined using FlowJo v.10.6 software. Forward and side-scatter light gating were used to exclude dead cells and debris from all analyses. Monocytes were also isolated with $CD14^+$ beads from frozen PBMCs obtained from a homozygous HIV-1 null individual and common allele HIV-1 individual with confirmed genotype for the *SIGLEC1* null variant. Monocytes were activated with 1,000 U/ml of Interferon-2α (Sigma-Aldrich) for 24h to induce Siglec-1 expression. 2×10^5 monocytes were pulsed with CFSE labelled-EVs for 4h at 37^{o}C to assess uptake as described for mDCs. Siglec-1 expression on these cells was assessed by flow cytometer, blocking cells with 1 mg/ml of hIgGs and staining them with α-Siglec-1-PE mAb 7–239 or matched isotype-PE control (BioLegend) at 4^{o}C for 30 min. Samples were analyzed with FACSCanto II (BD Biosciences) using FlowJo v.10.6 software.

2.7 IFN-γ ELISpot assay

PBMCs from three HIV-1 positive individuals with TB were thawed and monocytes were isolated and activated with Interferon-2α as described in the previous paragraph. The $CD14^-$ fraction from the same individual was cultured for 24h with 20 U/ml IL-2 (Novartis). Then, monocytes were pre-incubated for 15 min at RT with 10 µg/ml of an anti-Siglec-1 mAb (7D2; Abcam), IgG1 isotype control (BD Biosciences) or left untreated. Monocytes were pulsed for 4h at 37^{o}C with CFSE labelled-EVs isolated from THP-1-derived macrophages infected with

Mtb as previously described. After extensive washes, EV capture was confirmed in a FACS Calibur (BD Biosciences).

After EV exposure, 2.5×10^4 monocytes were cultured with 2.5×10^5 autologous $CD14^-$ cells per well in duplicate for 48h to assess IFNγ production using the Human IFNγ ELISpot^{PLUS} kit (Mabtech) following manufacturers' instructions. Unstimulated cells were used as negative control. PBMCs stimulated with phytohemagglutinin (Sigma-Aldrich) at 15 µg/ml were used as positive control. Of note, the capacity of $CD14^-$ cells to produce IFNγ against *Mtb* peptides was confirmed by detection of spot forming cells after the addition of 10 µg/ml of purified protein derivative or PPD (AJVacines). The number of spots (representing individual IFNγ producing cells) were counted using an automated ELISPOT reader system (ImmunoSpot S6 Versa; CTL), which was manually validated.

2.8 Statistical analyses

Mean changes from 100% of data normalized to percentages were assessed with a one-sample t-test. Longitudinal values of bacterial replication were analyzed using linear models of mixed effects. Mean changes of IFN-γ spot-forming cells (SFC) were assessed with a Wilcoxon test. Test results were considered significant at $P < 0.05$. All analyses and figures were generated with R or GraphPad Prism v.8 software.

3. RESULTS

3.1 Siglec-1 is not involved in *Mtb* capture and clearance

To assess which is the mechanism that facilitates the extrapulmonary dissemination of *Mtb* observed in Siglec-1 null humans and the local pulmonary bacterial spread we found in mice in the absence of Siglec-1, we first tested if it could be related to a lack of direct phagocytic clearance of bacteria via Siglec-1 receptor activity. Given the reported capacity of Siglec-1 to uptake sialylated bacteria and restrict pathogen dissemination through clearance[287], we evaluated whether Siglec-1 could directly interact with *Mtb* to limit local spread and contain the extrapulmonary colonization of bacteria that we found associated to a reduced expression of Siglec-1 in two independent cohorts. We therefore assessed direct *Mtb* uptake on Siglec-1-

expressing human monocyte-derived macrophages, using RNA interference to reduce Siglec-1 expression levels. With this strategy, we were able to reduce the cell-surface receptor expression by 57±14 % (**Figure 5.2A**; *P*-value 0.002; one sample t-test), similar to the cell-surface expression reported for Siglec-1 heterozygous individuals bearing the null allele[315]. These macrophages, along with corresponding non-targeted RNA controls, were infected with *Mtb* H37Rv Pasteur strain *in vitro* and the bacillary load was measured over time. We found no significant differences between the two conditions in bacterial uptake at day 0 or over time (**Figure 5.2B**), regardless of the RNA interference status. These results further confirmed our previous observations in mice, where no differences in bacillary loads were found (**Figure 4.4B**). These findings also suggest that Siglec-1 is not directly involved in *Mtb* clearance.

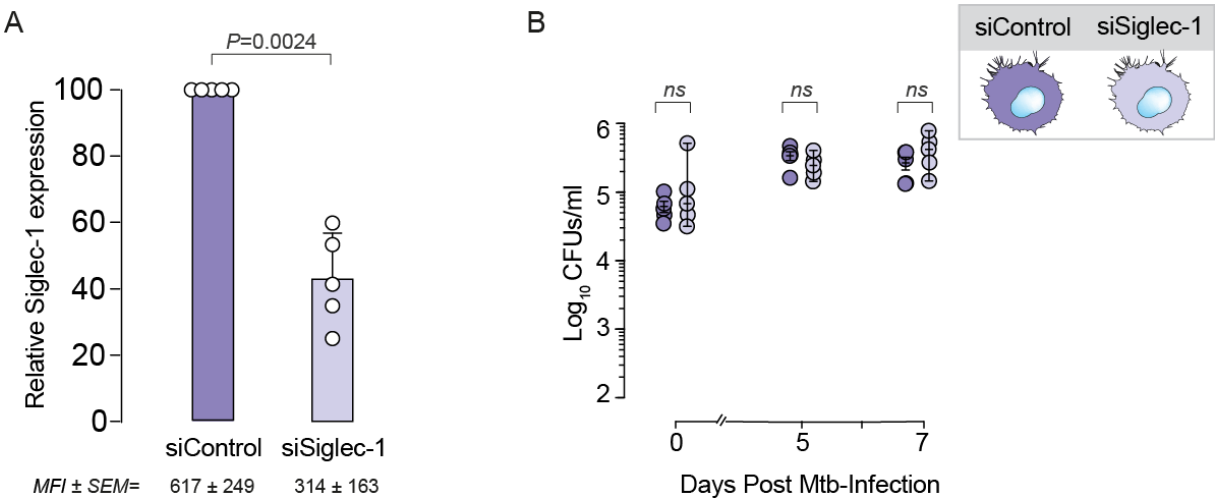

Figure 5.2 Siglec-1 is not involved in *Mtb* capture and clearance. A. Relative Siglec-1 expression on monocytes transfected with a silencing siRNA control (siControl) or with a Siglec-1-targeting siRNA (siSiglec-1). Siglec-1 geometric mean fluorescence intensity (MFI) was assessed by flow cytometry and values were normalized to control monocytes (set at 100%). Numbers indicate mean values and standard error of the means (SEM) obtained from 5 donors tested in triplicates. Statistical differences were assessed with a one sample t-test. **B.** Kinetic analysis of *Mtb* replication (CFU/ml) in monocyte-derived macrophages silenced with a siRNA control (siControl) or with a Siglec-1-targeting siRNA (siSiglec-1). Results are presented as Log_{10} medians of bacterial CFUs and range. Statistical differences were analyzed using linear models of mixed effects. No significant differences were observed between the values of siControl and siSiglec-1 either looking at different time points or focusing on the slope of growth.

As an alternative approach to asses a putative interaction between Siglec-1 and *Mtb*, we next focused on the study of Siglec-1 interacting ligands on the bacteria. We were not able to find prior reports describing the presence of sialylated molecules on *Mtb* that could interact with Siglec-1. Thus, we analyzed their presence in bacterial extracts using thin-layer chromatography. Compared to the positive detection of sialic acid by blue-violet spots on several sialylated standards used (**Figure 5.3A**, lines 1-3; corresponding to GM3-sialyllactose containing lipids, sialyllactose, and sialic acid molecules), no positive blue signals were detected in chloroform or aqueous extracts from *Mtb* H37Rv (**Figure 5.3A**, lines 4-5). To further release any possible sialic acid bound to other bacterial components and increase the likelihood of detection, we next performed an acid hydrolysis of *Mtb* H37Rv extracts. While on the sialic acid standard (**Figure 5.3B**, line 1) this hydrolysis yielded blue-violet spots corresponding to sialylated compounds (**Figure 5.3B**, line 2), no spot with comparable chromatographic appearance was detected on hydrolyzed *Mtb* H37Rv extracts (**Figure 5.3B**, lines 3-4). Thus, thin-layer chromatography analysis did not detect the presence of sialylated ligands associated to *Mtb* that could directly bind to Siglec-1.

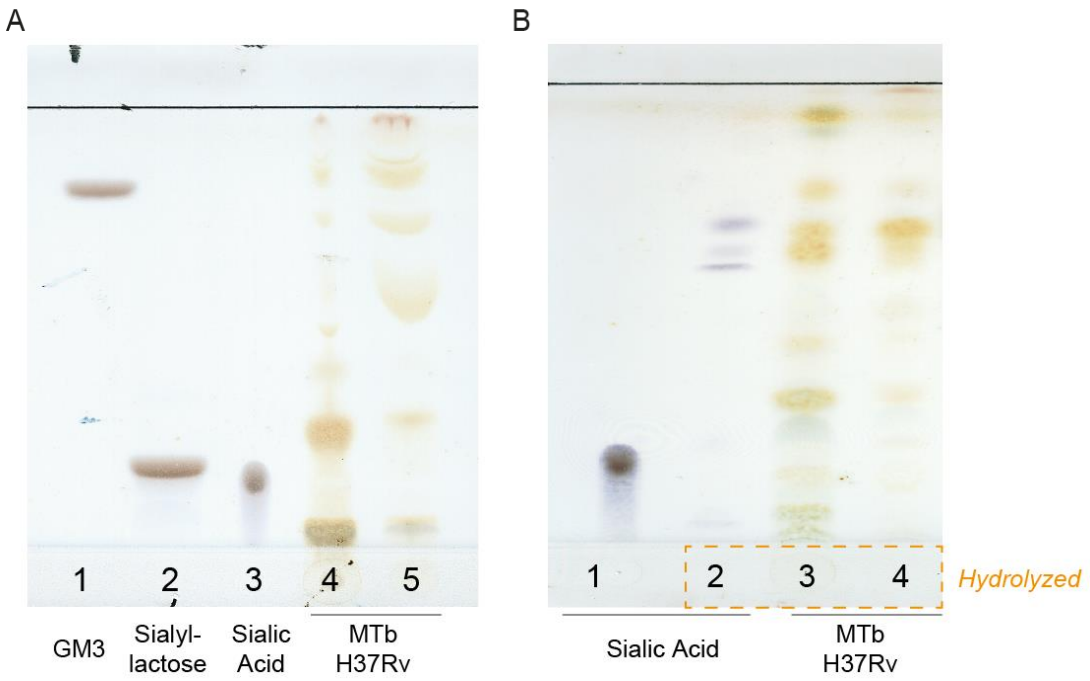

Figure 5.3 Sialylated ligands were not detected in the cell wall of *Mtb* by thin-layer chromatography. A. Thin-layer chromatography of GM3 standard (lane 1); Sialyllactose standard (lane 2); Sialic acid standard (lane 3); aqueous extracts of *Mtb* H37Rv (lane 4) and chloroform extracts of *Mtb* H37Rv (lane 5). Plate was revealed with resorcinol spray that is specific for sialic acid and detected by brown-violet or blue-violet colors. **B.** Thin-layer

chromatography of sialic acid standard (lane 1); hydrolyzed sialic acid standard (lane 2); aqueous extracts of hydrolyzed *Mtb* H37Rv (lane 4) and chloroform extracts of hydrolyzed *Mtb* H37Rv (lane 5). Plate was revealed with resorcinol spray that detects sialic acid by brown-violet or blue-violet colors.

Taken together, our experiments suggest no direct interaction between Siglec-1 and *Mtb.* These findings indicate that beyond the direct clearance of sialylated pathogens, alternative immune mechanisms against *Mtb* might be triggered by Siglec-1 receptor and could be altered on individuals bearing the *SIGLEC1* null variant.

3.2 Siglec-1 on antigen-presenting cells is required to induce antigen presentation via EV uptake

Siglec-1 is able to bind and capture sialylated EVs *in vitro*[261,293] and *in vivo*[294]. Vesicles secreted by infected cells or antigen-presenting cells allow for the transfer of processed antigens to dendritic cells, which amplify the initiation of immune responses[51,49,50]. We hypothesized that Siglec-1 on antigen-presenting cells could be critical for trapping EVs that are involved in the initiation or amplification of the immune response against *Mtb*[90,89,91,84,92,88].

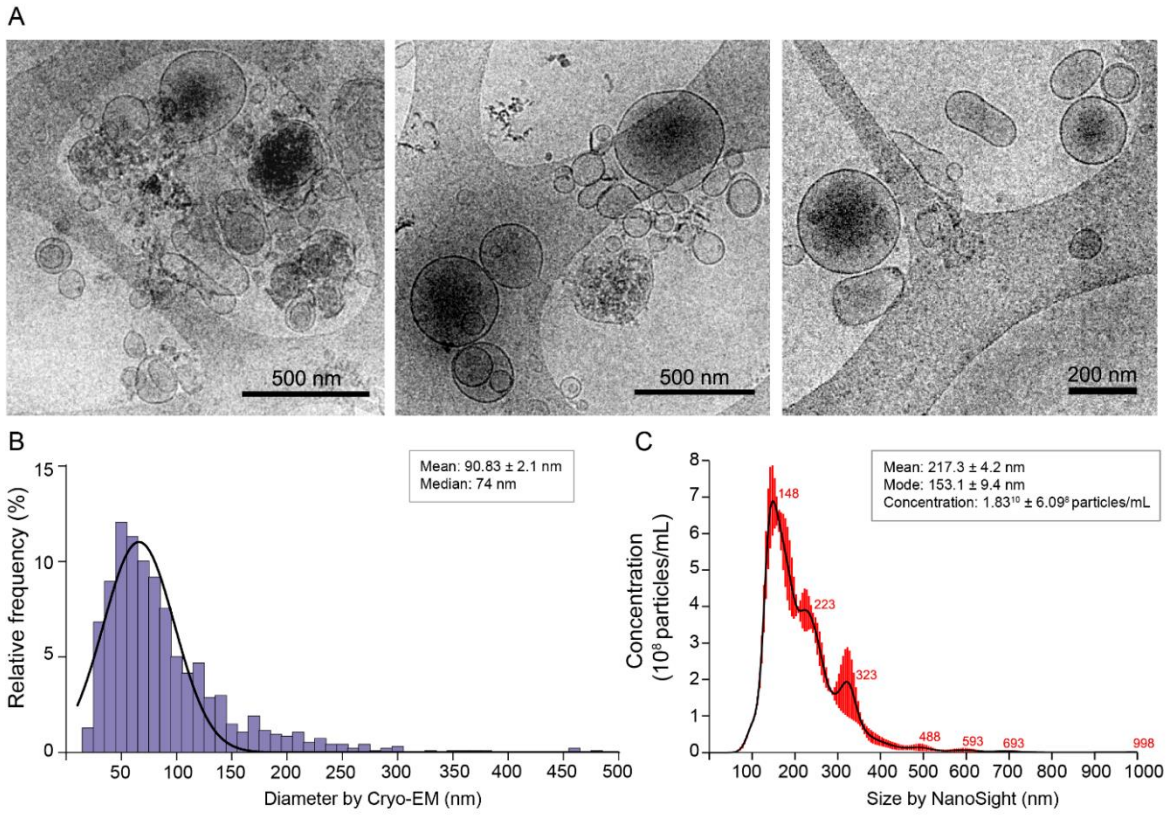

Figure 5.4 (Caption overleaf)

Figure 5.4 Size and morphological analysis confirms the presence of EVs. A. Cryogenic electron microscopy (cryo-EM) analysis of EVs purified from THP-1-derived macrophages infected with *Mtb* at a MOI of 0.1 shows round vesicles with a distinctive membrane and a diameter and size concurring to EVs, as quantified in the following panels. **B.** Histogram depicting the distribution of the diameter of the EVs according to cryo-EM. **C.** Histogram showing the size and concentration of EVs according to nanoparticle tracking analysis.

We therefore tested if EVs released by *Mtb*-infected cells could be captured by one of the most potent antigen-presenting cells located in the lymphoid tissues, such is the case of human mature dendritic cells (mDCs). Our first approach was to characterize the vesicles derived from *Mtb*-infected cells using cryo-electron microscopy and nanoparticle tracking analysis to define their diameter and size (**Figure 5.4A-C**).

Next, we generated the same EVs but this time loaded with fluorescence to assess their capture by mDCs using confocal microscopy (**Figure 5.5A-B**). These EVs were captured by mDCs and accumulated in sac-like compartments after 4h (**Figure 5.5A-B** and **Movies 1** and **2**). This is in agreement with previously described work on EVs isolated from different sources[293,103] and for very distinct sialylated viruses[324,325,269,326] that subvert this pathway[103].

The percentage of fluorescent-positive mDCs was also assessed by flow cytometry and was similar regardless of whether the vesicles were produced by cells infected with *Mtb* at a high or low multiplicity of infection or by uninfected cells (**Figure 5.6A**). Then, mDCs were incubated with fluorescent EVs in the presence or absence of a blocking monoclonal antibody (mAb) against Siglec-1 (**Figure 5.6B**). While pre-treatment with an isotype mAb control had no effect on EV uptake, treatment with an anti-Siglec-1 mAb inhibited retention of these vesicle couriers (**Figure 5.6B**; *P*-values on the graph; one sample t-test). When monocytes isolated from homozygous *SIGLEC1* null and common allele individuals were cultured in the presence of IFNα to induce Siglec-1 expression, and then exposed to fluorescent EVs, cells from the null individual which naturally lack Siglec-1 expression (**Figure 5.6C**, histograms) did not capture EVs (**Figure 5.6C**, bars). Therefore, Siglec-1 is required for EV capture by mDCs and activated monocytes, which are key cells implicated in the initiation of immunity in secondary lymphoid tissues[327,29,328].

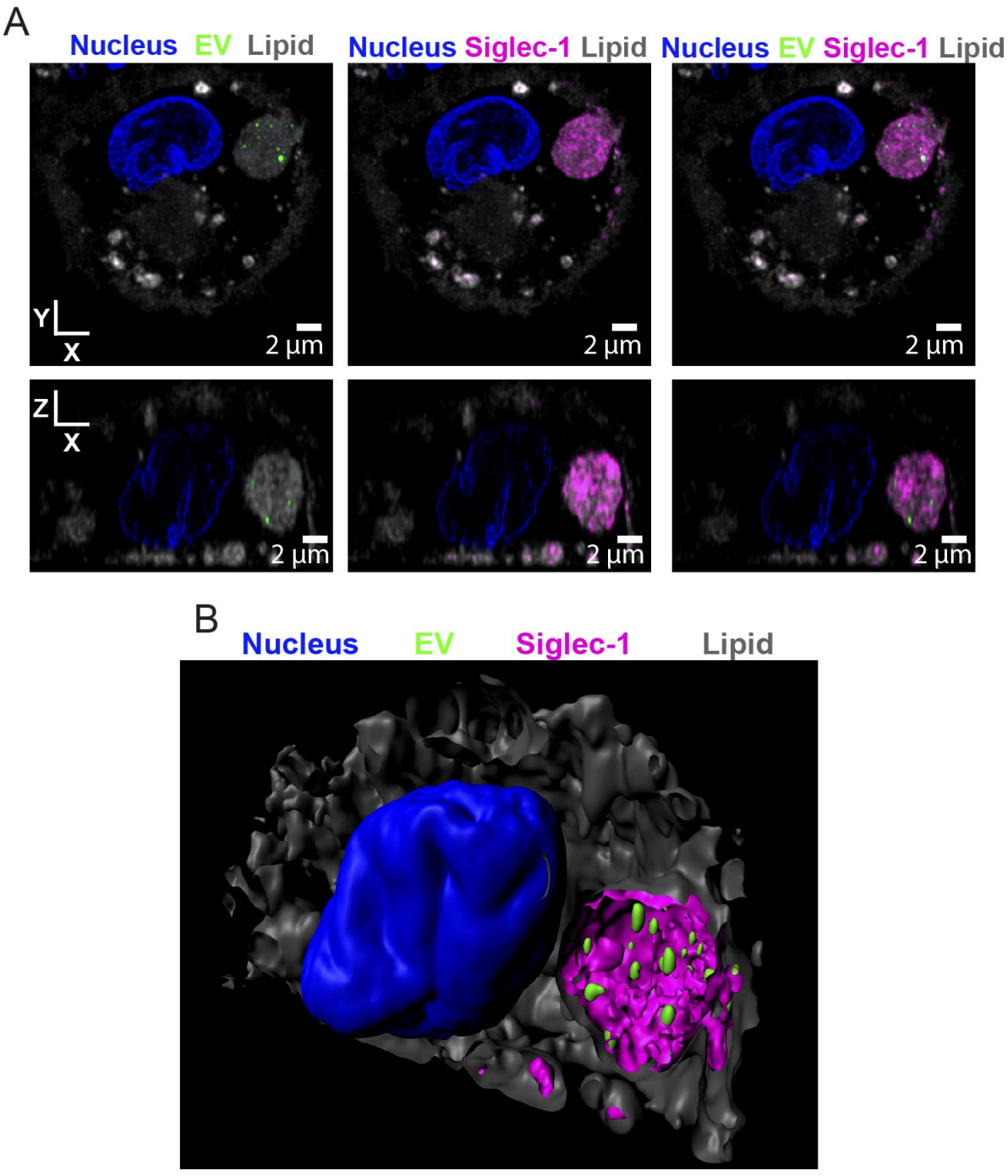

Figure 5.5 mDCs accumulate captured EVs derived from *Mtb*-infected cells in the Siglec-1-positive sac-like compartment. A. Confocal microscopy analysis of mDCs pulsed with CFSE-labelled EVs (EV; green) purified from *Mtb*-infected THP-1-derived macrophages. mDCs were stained with an anti-Siglec-1 mAb (magenta), with DAPI to detect nuclei (blue) and with lipid dyes to reveal membranes (grey). XY and XZ volume slices of a representative mDC loaded with EVs are shown. Scale bar = 2 µm. **B.** Isosurface rendering of the mDC loaded with EVs.

Finally, we assessed if antigen-presenting cells that had captured EVs through Siglec-1 could effectively trigger immune responses. We performed a functional assay in which $CD14^+$ monocytes from *Mtb*-infected individuals, cultured in the presence of IFNα to induce Siglec-1 expression and then pre-incubated or not with a mAb against Siglec-1, were exposed to EVs from *Mtb*-infected cells (**Figure 5.7A**). After extensive washing, monocytes were co-cultured with autologous peripheral blood mononuclear cells (PBMCs) depleted of monocytes, and then we measured IFNγ production by ELISpot assay (**Figure 5.7B**).

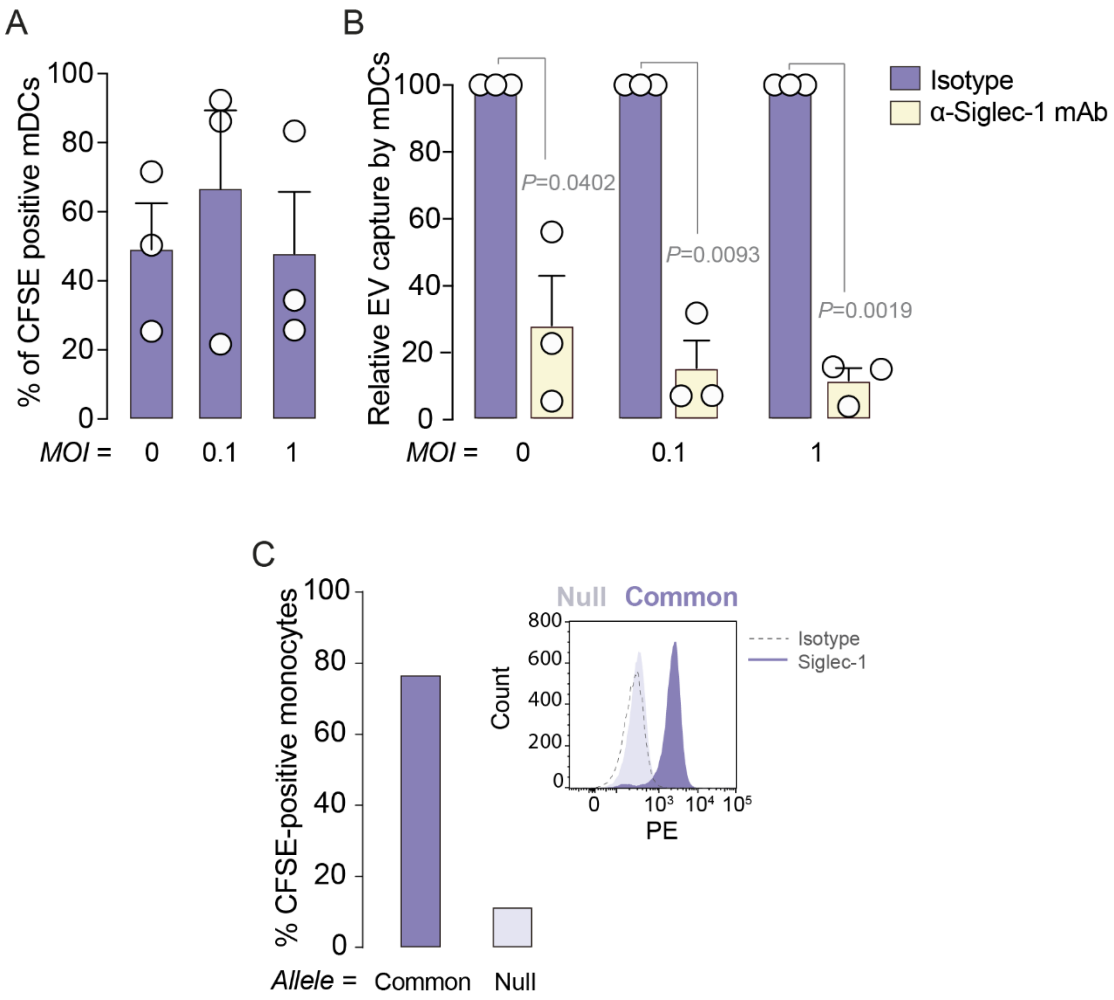

Figure 5.6 EVs capture by APCs is dependent of Siglec-1. A. Flow cytometry analysis of mDC uptake of CFSE-labelled EV purified from THP-1-derived macrophages left uninfected (MOI = 0) or infected with *Mtb* at a MOI of 0.1 or 1. Mean values and SEM from 3 independent experiments. **B**. Relative uptake of CFSE-labelled EV as in C, where mDCs were preincubated with an anti-Siglec-1 mAb or an isotype control. Values are normalized to the level of EV uptake by isotype control-treated cells (set at 100%). Statistical differences were assessed with a one sample t-test. **C.** Capture of fluorescent EV by IFNα-activated monocytes isolated

from a Siglec-1 null individual and common allele individual. Histograms show Siglec-1 expression on these activated monocytes.

We found higher IFNγ responses in monocytes that had captured EVs through Siglec-1 as compared to monocytes that had been pre-incubated with an anti-Siglec-1 mAb (**Figure 5.7B**; *P*-value 0.031; Wilcoxon test). Collectively, these results indicate that adaptive responses can be triggered via Siglec-1 uptake of EVs.

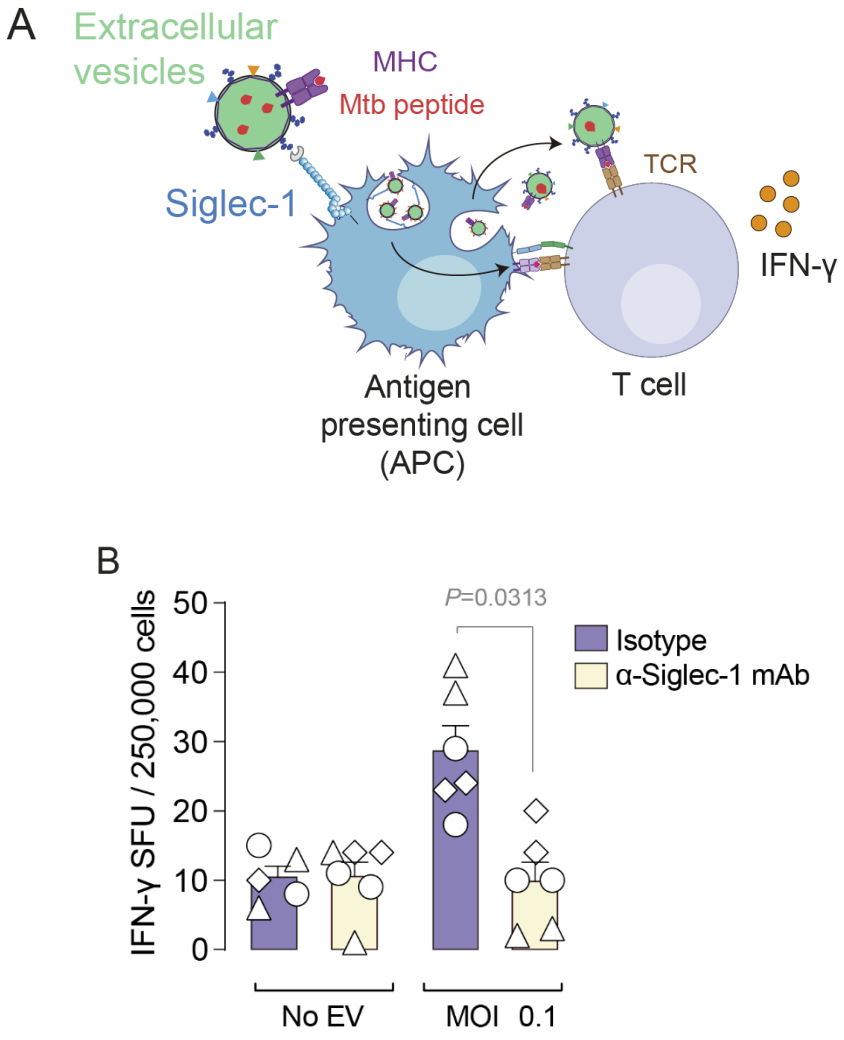

Figure 5.7 Siglec-1 on antigen-presenting cells is required to induce antigen presentation via EV uptake. A. Schematic representation of the co-culture performed in B. Antigen-presenting cells such as activated monocytes capture EV from *Mtb*-infected THP-1-derived macrophages via Siglec-1. These vesicles can either bear antigens already loaded into MHC molecules, or be re-processed and presented via MHC-I or II molecules expressed on the antigen presenting cell capturing these vesicles. Either way, these EVs can lead to the activation of T-cells. **B.** IFNγ-producing cells detected in a co-culture of PBMCs from TB-infected individuals induced by EVs

derived from *Mtb*-infected THP-1-derived macrophages. Activated monocytes previously pulsed or not with EVs derived from *Mtb*-infected cells, in the presence of an anti-Siglec-1 mAb or an isotype control, were cultured with autologous $CD14^-$ cells. The mean values and standard error of the mean (SEM) of IFNγ spot-forming cells (SFC) per 2.5 x 10^5 cells are represented. Statistical differences were assessed with a Wilcoxon test. Data from three patients assessed in duplicate are shown.

Taken together, this chapter shows that upon *Mtb* infection, Siglec-1 does not seem to promote the phagocytic clearance of the mycobacteria, as we did not detect a direct interaction between the receptor and *Mtb*. Alternatively, we showed that Siglec-1 expressed on APCs was required to trap EVs derived from *Mtb*-infected cells. Particularly, we observed that T-cell immunity was enhanced via EV capture by Siglec-1, as IFNγ responses were reduced in the presence of an anti-Siglec-1 mAb. Thus, in *SIGLEC1* homozygotes for the common allele, the initiation of immunity against *Mtb* may be mounted earlier due to the amplification of responses via antigen exchange through EVs. However, in individuals bearing the *SIGLEC1* null allele, the initiation of immunity against *Mtb* may be delayed, facilitating the extrapulmonary dissemination of *Mtb* in humans or favoring the local pulmonary spread of *Mtb* in mice, just before immunity is mounted likely through Siglec-1 independent pathways.

Collectively, through this thesis, we have performed a functional study of naturally occurring human “knockouts” in infectious contexts, which led us to identify Siglec-1 as a novel host factor that illuminates previously unknown pathways that modulate complex infections, such as TB and HIV-1. Moreover, in this thesis we have explored the concept of antagonistic pleiotropy[311] in co-infected individuals, where the potential beneficial effect of lacking Siglec-1 receptor for avoiding HIV-1 dissemination might be eclipsed by the deleterious effect of this particular null variant through suboptimal immune control of a bacterial co-infection with *Mtb*.

Chapter 6

DISCUSSION

Through the work of this thesis, we have explored how the analysis of loss-of-function genetic variants serves to illuminate particular *in vivo* mechanisms of disease. The identification and study of *SIGLEC1* human null individuals lacking functional copies of the *SIGLEC1* gene in an infectious context has allowed a better understanding of how this loss may affect HIV-1 disease progression. In addition, we have also described that Siglec-1 is a novel host factor that modulates TB immunity. In this chapter, we will discuss the role of Siglec-1 in the pathogenesis of two major infectious diseases that affect millions of people worldwide: HIV-1 and tuberculosis. Based on the results obtained in this thesis, we postulate that the potential benefits of lacking Siglec-1 receptor and avoiding HIV-1 dissemination might be eclipsed by the deleterious effect of this variant, through a suboptimal immune control of *Mtb* co-infection. We also propose a novel molecular basis to explain how the capacity of Siglec-1 to bind sialylated ligands initiates immunity against *Mtb* through the recognition of EVs that transport antigens between immune cells.

1. An insight into HIV-1 pathogenesis through the study of *SIGLEC1* null individuals

The identification of individuals with null alleles enables studying how the loss of gene function affects infection and disease progression. Particularly, the discovery of a deletion in the gene encoding the chemokine receptor CCR5, which is the main co-receptor used by HIV-1 to enter target cells, provided the first genetic evidence of protection against HIV-1 infection[156,171,172]. This particular deletion allele of the CCR5 structural gene (CCR5Δ32), which restricts CCR5-tropic HIV-1 infection in homozygous individuals and delays progression to AIDS in heterozygotes, is present at an allele frequency of ~0.10 in the Caucasian population of the United States[156]. However, since HIV-1 uses both CCR5 and CXCR4 co-receptors to enter into target cells, this particular deletion has no capacity to avoid HIV-1 infection of CXCR4-tropic viral strains.

Other major determinants for host control of HIV-1 have been confirmed by large-scale genomic analysis[163]. These genetic determinants may not protect from HIV-1 infection, but can influence disease progression. Such is the case of HLA-B*57:01, which has been shown to

be strongly associated with slow disease progression in HIV-1 infected individuals[165,166,161,163,164,167,168,169]. However, these large-scale analyses do not have the power to reveal polymorphisms that may have an impact on HIV-1 acquisition or disease progression, but are not included in the analyses or occur at a much lower frequency than the CCR5Δ32 or HLA-B*57:01 variants, and consequently, they would require studying very large sample sizes that are difficult to find.³²⁹³²⁹

Regarding susceptibility to HIV1 infection, we found two homozygous HIV-1-infected individuals with a confirmed loss-of-function variant in *SIGLEC1* gene, both of whom were infected via mucosal exposure. This indicates that Siglec-1-mediated viral *trans*-infection is not indispensable to establish HIV-1 infection through sexual contact. Thus, Siglec-1 independent mechanisms of infection are sufficient to support mucosal HIV-1 spread. However, despite the lack of impact on HIV-1 acquisition, *SIGLEC1* null variant could modulate the clinical evolution by delaying the disease progression compared to wild type individuals. Nonetheless, in our analysis, mainly focused on heterozygous individuals, we did not observe an effect of Siglec-1 truncation on progression to AIDS. Therefore, in the absence of *trans*-infection, the classical HIV-1 infectious routes, including cell-free virus infection or cell-to-cell HIV-1 transmission, compensate for the lack of Siglec-1 and are able to fuel HIV-1 dissemination within infected individuals. This could be an explanation for the lack of marked differences in clinical evolution of individuals with one or two copies of the *SIGLEC1* null variant.

These results in humans are in contrast to those observed in the bone marrow, liver and thymus (BLT) humanized mouse model, where Siglec-1 blockade was associated to a lower HIV-1 infection of splenocytes[286]. Discrepancies could be attributed to the fact that our study mainly focused on heterozygous Siglec-1 Glu88Ter individuals, which maintain partial receptor expression and function (**Chapter 3, Figure 3.2**). Yet, the clinical course of the two homozygous individuals did not show any obvious benefit of the absence of Siglec-1 (**Chapter 3, Figure 3.4**). Alternatively, differences with the murine model could be due to the distinct timeframes analyzed in each case. The murine study was limited to the early dynamics of HIV-1 infection[286], a phase that is missing from the clinical records of most patients, including the two null-homozygous individuals identified here. Hence, it will be important to determine if the lack of Siglec-1 in humans impacts early viral kinetics. If that is the case, current

antiretroviral treatments implemented early after infection could include Siglec-1 blocking agents to tackle multiple infectious routes simultaneously and limit the settlement of viral reservoirs. Importantly, the identification of *SIGLEC1* null individuals *in natura* indicates that Siglec-1 could represent a safe therapeutic target.

Several challenging limitations explain the lack of conclusive results in the study of the *SIGLEC1* genetic variant[329] (**Figure 6.1**). Since the *SIGLEC1* null variant is rare, we would require to study much larger cohorts to measure its potential effect. Power estimation analyses identified that to detect a similar effect of B*57:01 on HIV-1 control[163,165,330,167], more than 10,000 individuals should be genotyped. This sample size is even larger than the one analyzed in the largest genome-wide analysis, which includes ~6,300 individuals[169]. Thus, given the available sample size of our study, comprising 3,733 individuals, we can only discard a major effect of this genetic variant on HIV-1 disease progression.

Another important limitation we had was the fact that the seroconversion date of most individuals from the SHCS HIV-1 cohort included in the analyses was unknown, as occurs in the majority of the clinical cohorts of HIV-1 infected individuals[329]. Accordingly, the effect of *SIGLEC1* null variant on progression to AIDS was analyzed only from the date of diagnosis, which could have happened years after the infection date, underestimating the expected beneficial effect of this genetic variant on the clinical evolution of HIV-1 infected individuals. Furthermore, in some cases, we missed important clinical data that hampered clear interpretation. This was the case of one of the homozygous individuals, who lacked clinical information regarding the nine years of follow-up before starting antiretroviral treatment. Once therapy is started, drugs interfere with the natural course of the disease, ending the period suitable for the analysis of the impact of the *SIGLEC1* null variant on HIV-1 disease progression.

Current clinical guidelines recommend treatment introduction immediately after HIV-1 diagnosis, based on results from START[185]. This large-scale randomized clinical trial that showed that, in HIV-infected individuals with a $CD4^+$ T cell count higher than 500 cells/mm^3, initiation of antiretroviral therapy at the time of diagnosis provided net benefits over deferring therapy after the $CD4^+$ T cell count had declined below 350 cells/mm^3. Consequently, future genetic studies of Siglec-1 will be only possible in retrospective cohorts. This is due to the fact

that to investigate the natural course of HIV-1 infection in the absence of Siglec-1, it will be required to focus only in periods when infected individuals are off therapy. Thus, only clinical cohorts including individuals recruited before the year 2015, when the clinical guidelines were adapted to the study START, would be available for the study of the *SIGLEC1* null variant and other genetic variants[329].

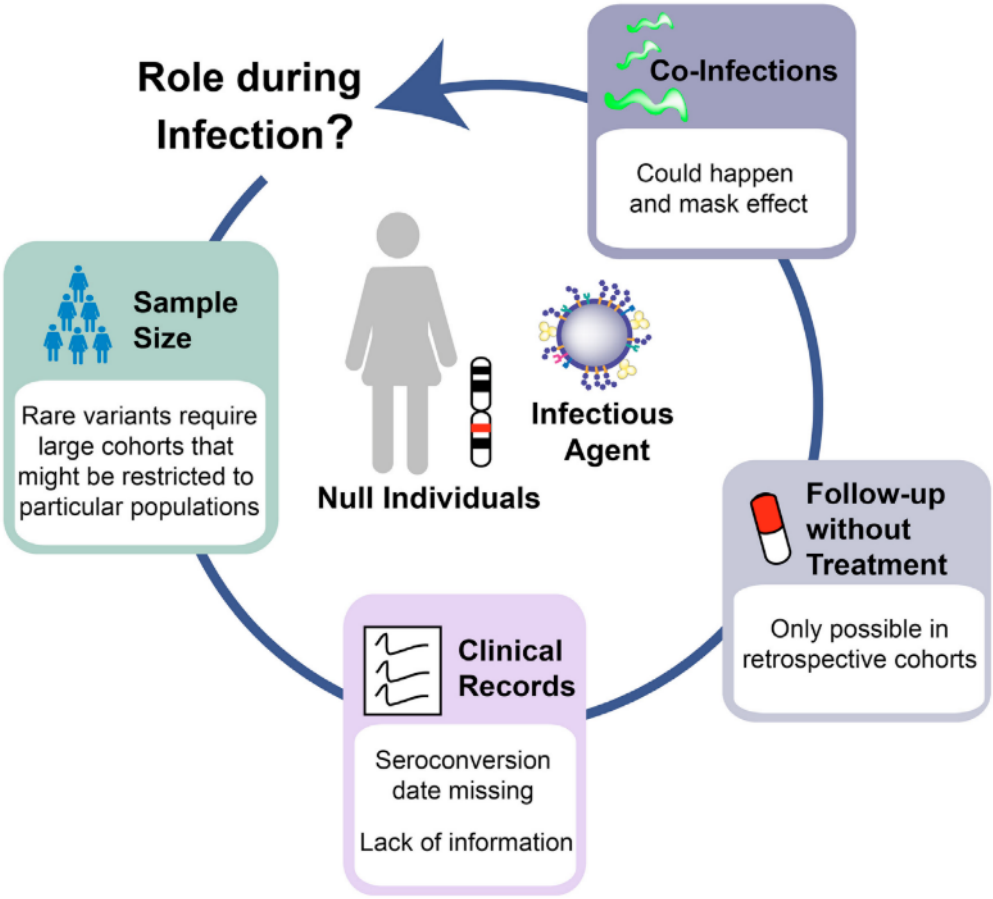

Figure 6.1 Factors involved in the analysis of the biological function of knockout genes in infectious diseases. To unravel the phenotypic consequences derived from the *SIGLEC1* null variant in HIV-1 infection, several factors must be considered, such as: the sample size, which must be consistent with the frequency of the variant; the clinical information of all individuals analyzed, which should be accurately collected throughout the years; the period of study should be in the absence of antiretroviral therapy to avoid the effect of antiviral drugs on the interpretation of the impact of the study variant; and the co-infection with other pathogens that can interfere and modify the clinical course of HIV-1 mono-infected individuals. Adapted from reference[329].

Finally, to accurately interpret the effect of a genetic variant in infectious diseases, co-infections with additional pathogens that could change the clinical course of infection, should

be considered in the analysis[329]. Indeed, one of the homozygous individuals identified in our study for the rare *SIGLEC1* allele had a good immune control until TB was diagnosed, when $CD4^+$ T cell counts dropped despite the initiation of antiretroviral treatment (**Chapter 4, Figure 4.2**). This observation led us to think that upon *Mtb* infection, the lack of Siglec-1 could have hampered the immune control of the *Mtb*, worsening the clinical prognosis of this co-infected individual. Thus, if Siglec-1 was promoting the immunity against *Mtb*, the lack of beneficial impact of *SIGLEC1* null variant on HIV-1 progression in this particular case could have been be due to a worse prognosis in the presence of and *Mtb* infection.

As we have discussed, several factors influence the analysis of the biological function of knockout genes *in vivo*, complicating the understanding of the phenotypic consequences of particular gene variants. Complexity on the analysis of the resulting phenotype can also be affected by the effect of other genetic variants, but also by the exposure to particular environmental conditions[331], including the occurrence of co-infections. That was the reason why we addressed the association of the *SIGLEC1* null variant with co-infections with additional pathogens, which is a common scenario in the natural course of HIV-1 infection. Moreover, we also explored how this putative association could modulate the clinical evolution of HIV-1 disease progression.

2. Siglec-1 is implicated on the pulmonary containment of *Mtb*

Given that individuals harboring the *SIGLEC1* null variant could be exposed to distinct infections at the same time, we investigated the possible effect of this variant in HIV-1 related co-infections. Among the HIV-1 infected individuals from the SHCS cohort with reported bacterial, fungal, viral or protozoal co-infections, the *SIGLEC1* null variant was only significantly associated to *Mtb* infection. The observation of a homozygous individual with reported HIV-1 and TB co-infection (**Chapter 4, Figure 4.2**) indicated that Siglec-1 is dispensable for becoming infected with *Mtb*. The significant association found between the *SIGLEC1* null allele and TB was linked to an extrapulmonary dissemination of *Mtb* (**Chapter 4, table 4.2**). These results were further confirmed when we analyzed an independent cohort of individuals with TB from Russia (**Chapter 4, Figure 4.3**). Of note, TB diagnosis in individuals from the Russian cohort was

initially based on clinical manifestations, presence of acid-fast bacilli in sputum and chest X-ray compatible images, thus limiting the capacity to enroll patients presenting only extrapulmonary/disseminated TB. The enrollment of subjects in the Russian cohort favored the inclusion of individuals with pulmonary TB, ignoring cases of restricted extrapulmonary disease. Still, we found a significant association in a cohort that underrepresented extrapulmonary TB. Overall, the reproducibility of a significant association between dissemination of TB and the *SIGLEC1* truncation in two independent cohorts strengthen our findings.

Two different polymorphisms in the *SIGLEC* gene family have been previously reported for TB: particularly the *SIGLEC14* null allele has been associated with protection from TB due to higher BCG-specific IL-2 responses[332], and the *SIGLEC1* rs3859664 SNP has been associated with active pulmonary TB in a small sized cohort, in which it was proposed that a decreased inflammasome activation and the consequent IL-1β production could be associated to this phenotype[333]. However, the functional consequences of the *SIGLEC1* rs3859664 SNP on Siglec-1 activity remain to be elucidated.

The association between the *SIGLEC1* null variant and *Mtb* dissemination we found in human, was further characterized using a murine C57BL/6 model infected with *Mtb,* in which we compared *SIGLEC1* knockout versus wild type mice. Results in the mouse model indicated that the absence of Siglec-1 did not avoid *Mtb* acquisition, but could hinder bacterial containment within the granulomatous infiltration, thus allowing for *Mtb* spread towards distant pulmonary areas. Although no bacillary burden differences were detected between WT and *SIGLEC1* knockout mice (**Chapter 4, Figure 4.4**), it was still important to analyze if there were significant differences in the *Mtb* antigen-specific T-cell activation in mice. For this reason, we performed an extensive analysis of cytokines in both WT and *SIGLEC1* knockout mice, not only in their serum, but also in pulmonary homogenates (**Chapter 4, Figures 4.5 and 4.6**). However, no significant differences were detected in any of the 14 cytokines that we tested at week 3 or 4 post-infection. We believe that this lack of differences was due to the mouse strain used in our study, which is the only strain where a *SIGLEC1* knockout has been generated and carefully characterized[316]. Unfortunately, this C57BL/6 strain is highly resistant to TB progression once specific immune responses are mounted 4 weeks post-infection in the

lungs[317]. Therefore, the strong immune responses mounted upon *Mtb* infection in this murine strain will not accurately reflect what happens in humans, and the expected differences we aimed to measure were not effectively detected in the C57BL/6 strain. For this reason, we moved to the *in vitro* system described in **Chapter 5**, in order to work with human cells, and performed all the functional studies with distinct *SIGLEC1* "knockout cells" generated by alternative strategies, such as RNA interference described in **Chapter 5, Figure 5.2**, and the use of blocking mAbs and cells derived from a null subject, both described in **Chapter 5, Figure 5.6**.

Despite the fact that we had to analyze the effects of a *SIGLEC1* null variant in a TB resistant murine model, *SIGLEC1* knockout mice displayed higher pulmonary damage compared to wt mice in the absence of significant differences on bacillary load in lungs or spleen, nor cytokine profile at the time points we measured them. Although this did not represent a difference in the bacterial dissemination towards the secondary lymphoid tissues at the early stages analyzed in the murine model, we cannot exclude that this local spread may favor later dissemination in chronic phases of infection in a disease context similar to humans, where *Mtb* containment is not always achieved.

Our results show how the pathogenesis of *Mtb* infection can be modulated by host genetics. In particular, through the study of *SIGLEC1* null individuals and knockout mice, we revealed a novel role for Siglec-1 receptor favoring *Mtb* containment within the lung. Thereby, in the context of HIV-1 infected individuals co-infected with *Mtb,* the lack of Siglec-1 could favor the spread of the *Mtb*, worsening their clinical prognosis. If that was the case, the expected beneficial effects on HIV-1 progression of the *SIGLEC1* truncated variant would be masked by the detrimental consequences of *Mtb* dissemination. However, the mechanism by which Siglec-1 could promote host protection against *Mtb* spread was not defined when we obtained these results.

3. Lack of EVs capture via Siglec-1 may facilitate *Mtb* migration in *SIGLEC1* knockout mice and *SIGLEC1* null individuals

The immune system has evolved highly sophisticated mechanisms to recognize and eliminate a diverse array of pathogens. Siglec-1 contributes to this process by regulating host immune responses to infection through the interaction with sialylated ligands[9] that can be present on the membrane of some pathogens and EVs. Since Siglec-1 is a sialic acid binding scavenger receptor implicated in the uptake and clearance of distinct sialylated bacteria like *Campylobacter jejuni*, group B Streptococcus or certain meningococcus[289,290,291,287], we first tested whether *Mtb* could be phagocytosed through Siglec-1. However, we did not detect a direct interaction between Siglec-1 and *Mtb* (**Chapter 5, Figure 5.3**). These results are further supported by the lack of differences in bacillary load that would be expected if Siglec-1 was degrading bacteria. Indeed, the similar bacillary load found in the presence or absence of Siglec-1 in human monocyte-derived macrophages (**Chapter 5, Figure 5.2**) is in accordance with the *Mtb* burden found in the lungs of wt and knockout mice described previously (**Chapter 4, results 3.3**). Similar results were reported by Santos AA *et al.* using a P2X7 knockout mice infected by *Mtb*[334]. A null polymorphism in the P2X7 purinergic receptor had been previously associated to extrapulmonary dissemination of *Mtb*[335]. In the absence of P2X7 receptor, the inability of *Mtb*-infected macrophages to appropriately kill mycobacteria might predispose a disseminated disease. However, as observed for *SIGLEC1* knockout mice infected by *Mtb,* they found that the bacillary load in lungs of P2X7 knockout mice was only partially reduced when compared to WT mice[334], suggesting that impairment of bacterial killing might not be the key mechanism involved in extrapulmonary dissemination of *Mtb*. Taken together, our data point that neither the local pulmonary *Mtb* spread in the mice model nor the extrapulmonary dissemination of *Mtb* in humans could be attributed to the absence of phagocytic *Mtb* clearance via Siglec-1 receptor. Thus, we next explored the potential for Siglec-1 to mediate immune responses against *Mtb* via antigen capture through EVs.

Previous studies demonstrated that EVs were captured by Siglec-1 expressing APCs through the recognition of sialic acid, present on the surface of these vesicles[261,293,294]. Specifically, Jurkat T cell lines and human reticulocyte-derived EVs were efficiently captured by mDCs[261,293]. This capture was specifically inhibited in the presence of a mAb that blocks Siglec-1, confirming

that EVs capture was mediated by Siglec-1[261,293]. Moreover, Saunderson *et al*. showed *in vivo* that EVs were bound by Siglec-1 positive macrophages in the spleen and lymph node from wt C57BL/6 mice but not Siglec-1 knockout mice[294]. Interestingly, they also found that Siglec-1 expression on these cells was restricted to subpopulations specialized on antigen capture and direct presentation or transfer of antigen to other APCs[336]. In addition, Siglec-1 positive macrophages were also strategically situated at antigen entry points into the spleen and lymph node, suggesting an important role of Siglec-1-mediated capture of EVs in the immune response[294].

EVs captured by Siglec-1 positive APCs is critical to boost immune responses. Once APCs have encountered a pathogen, these cells can release EVs containing peptide-MHC complexes that can sensitize other APCs, which have not been in direct contact with the pathogen. As a result, EVs production would increase the number of APCs bearing the antigen-MHC complexes, thus leading to the amplification of the initiation of immune responses[49,50,83]. Importantly, to induce antigen-specific naïve T-cell activation *in vitro*, these EVs require the presence of activated DCs to deliver the co-stimulatory signals to T cells[83]. Thereby, EVs capture by APCs via Siglec-1 can spread antigen-specific signals between different DCs amplifying immune responses.

In the case of *Mtb* infection, we found that Siglec-1 expressed on APCs was required to trap EVs derived from *Mtb*-infected cells, which accumulate in the same cellular compartment as HIV-1 and Ebola viruses[269,103]. Importantly, Siglec-1 expression is potently up-regulated on APCs in the presence of type I IFNs, such as IFNα[262,267], a predominant cytokine present in pulmonary active TB[270]. Consequently, IFNα produced during *Mtb* infection could increase the levels of Siglec-1, what could boost to a greater extent the Siglec-1-mediated capture of EVs and the subsequent T-cell activation. Our results indicate that T-cell immunity was enhanced via EV capture by Siglec-1, and that IFNγ responses were reduced in the presence of an anti-Siglec-1 mAb (**Chapter 5, Figure 5.7**). Thus, in *SIGLEC1* homozygotes for the common allele or heterozygotes for the *SIGLEC1* null allele, the initiation of immunity against *Mtb* may be mounted earlier due to the amplification of responses via antigen exchange through EVs. However, in individuals homozygotes for the *SIGLEC1* null allele, the initiation of immunity against *Mtb* may be delayed, facilitating the extrapulmonary dissemination of *Mtb* in humans

or favoring the local pulmonary spread of *Mtb* in mice, just before immunity is mounted likely through Siglec-1 independent pathways. This EV-exchange mechanism may be especially relevant for infections in which antigen presenting cells are productively infected and bacteria actively inhibit antigen presentation, as is the case of *Mtb*[337]. Indeed, cell cooperation between infected migratory DCs and resident lymph node DCs is necessary for optimal activation of naïve T cells during TB infection, and the active transfer of mycobacterial antigens between these cells is required for T-cell priming[338].

Prior reports showed that EVs derived from *Mtb*-infected cells contain antigenic bacterial proteins[86,87], which modulate antigen presentation during *Mtb* infection[84,88,89,90,91,92]. Indeed, *Mtb* EVs inhibit macrophage functions, but promote MHC-II antigen presentation by DCs[339]. The role of EVs in antigen presentation was first described in a study using exosomes secreted by a human B cell line[57]. Since then, the immune modulatory properties of EVs derived from other cells have been demonstrated[50]. In addition to *Mtb,* EVs release from cells infected with other intracellular pathogens such as *Salmonella typhimurium* and *Toxoplasma gondii* have been also implicated in the induction of immune responses aimed at the containment of these pathogens[84,85]. Importantly, the role of EVs as regulators of immune function further supports their potential role boosting immune responses against *Mtb* through Siglec-1 interaction. Overall, the particular immune activity of EVs, which are efficiently trapped by Siglec-1[261,293,294], provides a molecular basis to explain the genetic association found between the *SIGLEC1* null variant and the *Mtb* dissemination.

Here we provide a plausible molecular model to explain how Siglec-1 capacity to bind EVs can initiate immune responses against TB (**Figure 6.2**), and how its absence impacts *Mtb* dissemination. Delayed onset of those responses in individuals lacking this particular receptor allows for extrapulmonary dissemination of *Mtb* in humans or local pulmonary spread of *Mtb* in resistant mice. While this hypothesis is compatible with all the observations of this study, additional mechanisms may also be at play. Siglec-1 is an adhesion receptor that favors attachment between immune cells[9], and therefore, within pulmonary lesions, it could be involved in anchoring infected Siglec-1-expressing macrophages with other cells or the extracellular matrix of the granuloma. In this case, its total or partial absence could facilitate infected-cell migration, leading to a more widespread pulmonary damage. Yet, if Siglec-1 was

just acting as a docking molecule, we should have found higher bacillary loads in the spleen of knockout mice at week 3, when immune responses are still being mounted.

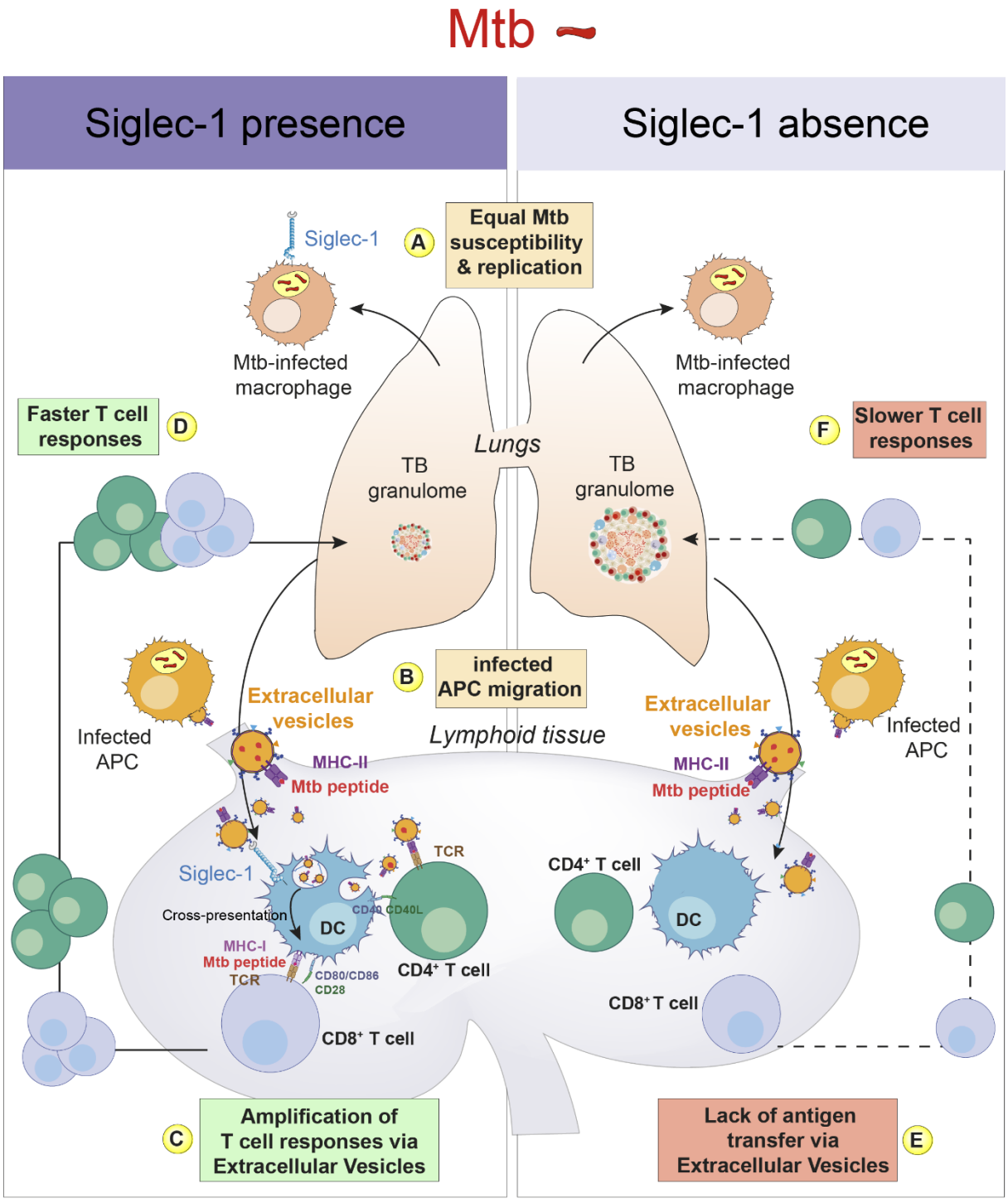

Figure 6.2 Hypothetical model explaining the early induction of immunity against *Mtb* in the presence or in the absence of Siglec-1. A. Susceptibility to *Mtb* infection is similar in both cases, as human and mice are equally infected in the absence of Siglec-1. Moreover, *Mtb* replication rates are also equivalent in murine models and human cells with varying Siglec-1 expression levels. **B**. Once antigen-presenting cells are infected, these cells migrate to

secondary lymphoid tissues, where they are not competent for direct antigen presentation as they are productively infected with *Mtb,* but can transfer antigens to competent uninfected antigen presenting cells through EV release, which are captured via Siglec-1. **C**. Antigen uptake by Siglec-1 amplifies the initiation of T cell responses. **D**. These T-cell responses are mounted faster and contain the pulmonary damage. **E**. In sharp contrast, the lack of Siglec-1 compromises antigen exchange via EVs. **F**. Thus, in the absence of Siglec-1, T-cell responses are mounted later and are not able to control the early pulmonary damage leading to bacterial dissemination.

Future work should dissect the relative contribution of these Siglec-1 related pathways to the local pulmonary and extrapulmonary dissemination of *Mtb*. In turn, the functional study of naturally occurring "human knockouts" in infectious contexts may identify novel host factors, such as Siglec-1, which illuminate previously unknown pathways that modulate complex infections such as TB.

Overall, through this thesis we have explored the concept of antagonistic pleiotropy[311] in co-infected individuals, where the potential beneficial effect of lacking Siglec-1 receptor for avoiding HIV-1 dissemination might be eclipsed by the deleterious effect of this particular null variant through suboptimal immune control of a bacterial co-infection with *Mtb.*

Chapter 7

CONCLUSIONS

AIM 1. To identify and study individuals with a loss-of-function variant in the *SIGLEC1* gene to determine the contribution of *trans*-infection to HIV-1 pathogenesis *in vivo*

- The identification of two homozygous HIV-1-infected individuals with a confirmed loss-of-function variant in *SIGLEC1*, both of whom were infected via mucosal exposure, indicates that Siglec-1 *trans*-infection is not indispensable to establish a productive HIV-1 infection via sexual transmission.
- Although *SIGLEC1* null individuals can be infected with HIV-1, their cells have lost the capacity for HIV-1 capture and *trans*-infection via Siglec-1 *in vitro*.
- *SIGLEC1* null variant limits HIV-1 capture and transmission *ex vivo*, but the effect of this variant on progression to AIDS was not conclusive, due to the limited sample size of our study, the lack of critical clinical information such as the seroconversion date, and the restriction to study only off-therapy periods.
- Since the identification of *SIGLEC1* null individuals *in natura* indicates that Siglec-1 could represent a safe therapeutic target, future interventional strategies blocking Siglec-1 could aid to unambiguously dissect its contribution.

AIM 2. To investigate the effect of the *SIGLEC1* null variant in HIV-1 related co-infections.

- Among the HIV-1 infected individuals studied with reported bacterial, fungal, viral or protozoal co-infections, the *SIGLEC1* null variant was only significantly associated to TB infection.
- The significant association found between the *SIGLEC1* null allele and TB was linked to an extrapulmonary dissemination of *Mtb*.
- In a resistant murine model infected with *Mtb,* the *SIGLEC1* knockout mice had lower capacity to contain the granulomatous infiltration in the lung compared to wild type mice, despite having similar bacterial load or cytokine environment.

AIM 3. To assess which is the mechanism that facilitates the extrapulmonary dissemination of *Mtb* in *SIGLEC1* null humans and the local pulmonary Mtb spread in the absence of Siglec-1 in mice.

- Local or distant *Mtb* migration could not be attributed to an absence of bacteria promoted by a phagocytic clearance of Siglec-1 receptor.
- Siglec-1 expressed on APCs was necessary to induce EV uptake and T cell responses.
- Finally, we hypothesize that in *SIGLEC1* homozygotes for the common allele, the initiation of immunity against *Mtb* may be mounted earlier due to the amplification of responses via antigen exchange through EVs. However, in individuals bearing the *SIGLEC1* null allele, the initiation of immunity against *Mtb* may be delayed, facilitating the extrapulmonary dissemination of *Mtb* in humans or favoring the local pulmonary spread of *Mtb* in mice.

GENERAL CONCLUSION

Overall, our findings suggest that Siglec-1 might help protect the host against disseminated tuberculosis while simultaneously playing a deleterious role in enhancing *trans*-infection of HIV-1, exemplifying the biological phenomenon known as antagonistic pleiotropy. The functional study of naturally occurring “human knockouts” in infectious contexts may identify novel host factors, such as Siglec-1, which help explain previously unknown pathways that modulate complex infections such as TB and HIV-1.

Chapter 8

REFERENCES

1. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* 197–216 (2002). doi:10.1146/annurev.immunol.20.083001.084359

2. Mario Riera Romo, Dayana Pérez-Martínez, C. C. F. Innate immunity in vertebrates : an overview. *Immunology* **148**, 125–139 (2016).

3. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).

4. Medzhitov, R. & Janeway, C. A. Innate immunity: The virtues of a nonclonal system of recognition. *Cell* **91**, 295–298 (1997).

5. Medzhitov Ruslan, J. C. Innate immunity. *N. Engl. J. Med.* **343**, 338–344 (2000).

6. Maximiliano Javier Jiménez-Dalmaroni, M. Eric Gerswhin, and I. E. A. Mechanisms and pathways of innate immune activation and regulation in health and cancer. *Hum. Vaccin. Immunother.* **10**, 3270–3285 (2014).

7. Janeway, C. A. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today* **13**, 11–16 (1992).

8. Crocker, P. R. Siglecs in innate immunity. *Curr. Opin. Pharmacol.* **5**, 431–437 (2005).

9. MacAuley, M. S., Crocker, P. R. & Paulson, J. C. Siglec-mediated regulation of immune cell function in disease. *Nat. Rev. Immunol.* **14**, 653–666 (2014).

10. Kelm, S. *et al.* Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Curr. Biol.* **4**, 965–972 (1994).

11. Crocker, P. R. & Varki, A. Siglecs, sialic acids and innate immunity. *Trends Immunol.* **22**, 337–342 (2001).

12. Crocker, P. R. Siglecs: Sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling. *Curr. Opin. Struct. Biol.* **12**, 609–615 (2002).

13. Crocker, P. R., Paulson, J. C. & Varki, A. Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* **7**, 255–266 (2007).

14. Varki, A. & Angata, T. Siglecs - The major subfamily of I-type lectins. *Glycobiology* **16**, 1–27 (2006).

15. Neill, L. A. J. O., Golenbock, D. & Bowie, A. G. The history of Toll-like receptors — redefining innate immunity. *Nat. Rev. Immunol.* **13**, 453–460 (2013).

16. Gerswhin, M. E., Iannis, E. & Plata, L. The critical role of toll-like receptors - From microbial recognition to autoimmunity: A comprehensive review. *Autoimmun. Rev.* **15**, 1–8 (2016).

17. Underhill, D. M., Ozinsky, A., Smith, K. D. & Aderem, A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14459–14463 (1999).

18. Harding, C. V. & Boom, W. H. Regulation of antigen presentation by Mycobacterium tuberculosis: A role for Toll-like receptors. *Nat. Rev. Microbiol.* **8**, 296–307 (2010).

19. Medzhitov, R., Preston-hurlburt, P. & Jr, C. A. J. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394–397 (1997).

20. Steinman, R. M., Gutchinov, B., Witmer, M. D. & Nussenzweig, M. C. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* **157**, 613–627 (1983).

21. Banchereau, J. *et al.* IMMUNOBIOLOGY OF DENDRITIC CELLS. *Annu. Rev. Immunol.* **18**, 767–811 (2000).

22. Inaba, K. *et al.* The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J. Exp. Med.* **191**, 927–936 (2000).

23. Turley, S. J. *et al.* Transport of peptide-MHC class II complexes in developing dendritic cells. *Science (80-.).* **288**, 522–527 (2000).

24. Mellman, I. & Steinman, R. M. Dendritic cells: Specialized and regulated antigen processing machines. *Cell* **106**, 255–258 (2001).

25. Worbs, T., Hammerschmidt, S. I. & Förster, R. Dendritic cell migration in health and disease. *Nat. Rev. Immunol.* **17**, 30–48 (2017).

26. Trombetta, E. S. & Mellman, I. Cell biology of antigen processing in vitro and in vivo. *Annu. Rev. Immunol.* **23**, 975–1028 (2005).

27. Fujii, S., Liu, K., Smith, C., Bonito, A. J. & Steinman, R. M. The Linkage of Innate to Adaptive Immunity via Maturing Dendritic Cells In Vivo Requires CD40 Ligation in Addition to Antigen Presentation and CD80 / 86 Costimulation. *J. Exp. Med.* **199**, (2004).

28. Spörri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat. Immunol.* **6**, 163–170 (2005).

29. Steinman, R. M. & Banchereau, J. Taking dendritic cells into medicine. *Nature* **449**, 419–426 (2007).

30. Michael L. Dustin, Arup K. Chakraborty, and A. S. S. Understanding the Structure and Function of the Immunological Synapse. *Cold Spring Harb Perspect Biol* **2**, 1–14 (2010).

31. Huse, R. B. and M. Mechanical communication at the immunological synapse. *Trands Cell Biol.* **27**, 241–254 (2017).

32. Santana, M. A. & Esquivel-Guadarrama, F. Cell Biology of T Cell Activation and Differentiation. *Int. Rev. Cytol.* **250**, 217–274 (2006).

33. Norbury, C. C., Malide, D., Gibbs, J. S., Bennink, J. R. & Yewdell, J. W. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat. Immunol.* **3**, 265–271 (2002).

34. Wilson, N. S. & Villadangos, J. A. Regulation of antigen presentation and cross-presentation in the dendritic cell network: Facts, hypothesis, and immunological implications. *Adv. Immunol.* **86**, 241–305 (2005).

35. Mantegazza, A. R., Magalhaes, J. G., Amigorena, S. & Marks, M. S. Presentation of Phagocytosed Antigens by MHC Class I and II. *Traffic* **14**, 135–152 (2013).

36. Gagliani, N. & Huber, S. Basic aspects of T helper cell differentiation. *Methods Mol. Biol.* **1514**, 19–30 (2017).

37. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12**, 557–569 (2012).

38. Dennis, M. K. *et al.* In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* **17**, 221–220 (2002).

39. Majlessi, L. *et al.* Inhibition of Phagosome Maturation by Mycobacteria Does Not Interfere with Presentation of Mycobacterial Antigens by MHC Molecules. *J. Immunol.* **179**, 1825–1833 (2007).

40. F Buseyne, S Le Gall, C Boccaccio, J P Abastado, J D Lifson, L O Arthur, Y Rivière, J M Heard, O. S. MHC-I – restricted presentation of HIV-1 virion antigens without viral replication. *Nat. Med.* **7**, 344–349 (2001).

41. Larsson, M. *et al.* Activation of HIV-1 specific CD4 and CD8 T cells by human dendritic cells: Roles for cross-presentation and non-infectious HIV-1 virus. *Aids* **16**, 1319–1329 (2002).

42. Sabado, R. L. *et al.* Pathways utilized by dendritic cells for binding, uptake, processing and presentation of antigens derived from HIV-1. *Eur. J. Immunol.* **37**, 1752–1763 (2007).

43. Miller, M. A., Ganesan, A. P. V., Luckashenak, N., Mendonca, M. & Eisenlohr, L. C. Endogenous antigen processing drives the primary CD4+ T cell response to influenza. *Nat. Med.* **21**, 1216–1222 (2015).

44. Eisenlohr, L. C., Luckashenak, N., Apcher, S., Miller, M. A. & Sinnathamby, G. Beyond the classical: Influenza virus and the elucidation of alternative MHC class II-restricted antigen processing pathways. *Immunol. Res.* **51**, 237–248 (2011).

45. Coulon, P.-G. *et al.* HIV-Infected Dendritic Cells Present Endogenous MHC Class II–Restricted Antigens to HIV-Specific CD4 + T Cells. *J. Immunol.* **197**, 517–532 (2016).

46. Villadangos, J. A. & Schnorrer, P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat. Rev. Immunol.* **7**, 543–555 (2007).

47. Inaba, K. *et al.* Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* **188**, 2163–2173 (1998).

48. Théry, C. *et al.* Molecular characterization of dendritic cell-derived exosomes: Selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* **147**, 599–610 (1999).

49. Théry, C., Zitvogel, L. & Amigorena, S. Exosomes: Composition, biogenesis and function. *Nat. Rev. Immunol.* **2**, 569–579 (2002).

50. Théry, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9**, 581–593 (2009).

51. Yáñez-Mó, M. *et al.* Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **4**, 27066 (2015).

52. Harding, C., Heuser, J. & Stahl, P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* **97**, 329–339 (1983).

53. Pan, B. T., Teng, K., Wu, C., Adam, M. & Johnstone, R. M. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* **101**, 942–948 (1985).

54. Pan, B. T. & Johnstone, R. M. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. *Cell* **33**, 967–978 (1983).

55. Johnstone, R. M. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem. Cell Biol.* **70**, 179–190 (1992).

56. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L. & Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **262**, 9412–9420 (1987).

57. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161–1172 (1996).

58. Zitvogel, L. *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* **4**, 594–600 (1998).

59. Van Niel, G., D'Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* **19**, 213–228 (2018).

60. Monguió-Tortajada, M., Gálvez-Montón, C., Bayes-Genis, A., Roura, S. & Borràs, F. E. Extracellular vesicle isolation methods: rising impact of size-exclusion chromatography. *Cell. Mol. Life Sci.* **76**, 2369–2382 (2019).

61. Hurley, J. H. ESCRT complexes and the biogenesis of multivesicular bodies. *Curr. Opin. Cell Biol.* **20**, 4–11 (2008).

62. Katarina Trajkovic, Chieh Hsu, Salvatore Chiantia, Lawrence Rajendran, Dirk Wenzel, Felix Wieland, Petra Schwille, Britta Brügger, M. S. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science (80-.).* **319**, 1244–1247 (2008).

63. Guillaume van Niel, Stéphanie Charrin, Sabrina Simoes, Maryse Romao, Leila Rochin, Paul Saftig, Michael S. Marks, Eric Rubinstein, and G. R. The tetraspanin CD63 regulates ESCRT-independent and dependent endosomal sorting during melanogenesis. *Dev. Cell* **21**, 708–721 (2011).

64. Perez-Hernandez, D. *et al.* The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes. *J. Biol. Chem.* **288**, 11649–11661 (2013).

65. Kowal, J., Tkach, M. & Théry, C. Biogenesis and secretion of exosomes. *Curr. Opin. Cell Biol.* **29**, 116–125 (2014).

66. Colombo, M., Raposo, G. & Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* **30**, 255–289 (2014).

67. Raposo, G. & Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J. Cell Biol.* **200**, 373–383 (2013).

68. Tricarico, C., Clancy, J. & D'Souza-Schorey, C. Biology and biogenesis of shed microvesicles. *Small GTPases* **8**, 220–232 (2017).

69. Minciacchi, V. R., Freeman, M. R. & Di Vizio, D. Extracellular Vesicles in Cancer: Exosomes, Microvesicles and the Emerging Role of Large Oncosomes. *Semin. Cell Dev. Biol.* **40**, 41–51 (2015).

70. Hugel, B., Martínez, M. C., Kunzelmann, C. & Freyssinet, J. M. Membrane microparticles: Two sides of the coin. *Physiology* **20**, 22–27 (2005).

71. Booth, A. M. *et al.* Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. *J. Cell Biol.* **172**, 923–935 (2006).

72. Wang, T. *et al.* Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc. Natl. Acad. Sci. U. S. A.* **111**, (2014).

73. Hristov, M., Erl, W., Linder, S. & Weber, P. C. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood* **104**, 2761–2766 (2004).

74. Taylor, D. D. & Gercel-Taylor, C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* **110**, 13–21 (2008).

75. Simpson, R. J., Lim, J. W. E., Moritz, R. L. & Mathivanan, S. Exosomes: Proteomic insights and diagnostic potential. *Expert Rev. Proteomics* **6**, 267–283 (2009).

76. Gallo, A., Tandon, M., Alevizos, I. & Illei, G. G. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One* **7**, 1–5 (2012).

77. Giri, P. K. & Schorey, J. S. Exosomes derived from M. bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo. *PLoS One* **3**, (2008).

78. Mignot, G., Roux, S., Thery, C., Ségura, E. & Zitvogel, L. Prospects for exosomes in immunotherapy of cancer. *J. Cell. Mol. Med.* **10**, 376–388 (2006).

79. Shimoda, A., Tahara, Y., Sawada, S. ichi, Sasaki, Y. & Akiyoshi, K. Glycan profiling analysis using evanescent-field fluorescence-assisted lectin array: Importance of sugar recognition for cellular uptake of exosomes from mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* **491**, 701–707 (2017).

80. Williams, C. *et al.* Glycosylation of extracellular vesicles: current knowledge, tools and clinical perspectives. *J. Extracell. Vesicles* **7**, (2018).

81. Williams, C. *et al.* Assessing the role of surface glycans of extracellular vesicles on cellular uptake. *Sci. Rep.* **9**, 1–14 (2019).

82. Lee, Y., El Andaloussi, S. & Wood, M. J. A. Exosomes and microvesicles: Extracellular vesicles for genetic information transfer and gene therapy. *Hum. Mol. Genet.* **21**, 125–134 (2012).

83. Théry, C. *et al.* Indirect activation of naïve CD4+T cells by dendritic cell-derived exosomes. *Nat. Immunol.* **3**, 1156–1162 (2002).

84. S., B., K., S., F.J., C. & J.S., S. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood* **110**, 3234–3244 (2007).

85. Aline, F., Bout, D., Amigorena, S., Roingeard, P. & Dimier-Poisson, I. Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. *Infect. Immun.* **72**, 4127–4137 (2004).

86. Pramod K. Giri, Nicole A. Kruh, Karen M. Dobos, and J. S. S. Proteomic analysis identifies highly antigenic proteins on exosomes from M. tuberculosis-infected and culture filtrate protein-treated macrophages. **71**, 233–236 (2013).

87. Mehaffy, C. *et al.* Identification of Mycobacterium tuberculosis peptides in serum extracellular vesicles from persons with latent tuberculosis infection . *J. Clin. Microbiol.* 1–13 (2020). doi:10.1128/jcm.00393-20

88. Prachi P. Singh, Victoria L. Smith, Petros C. Karakousis, and J. S. S. Exosomes isolated from mycobacteria-infected mice or cultured macrophages can recruit and activate immune cells in vitro and in vivo. *J Immunol.* **189**, 777–785 (2012).

89. Cheng, Y. & Schorey, J. S. Exosomes carrying mycobacterial antigens can protect mice against Mycobacterium tuberculosis infection. *Eur. J. Immunol.* **43**, 3279–3290 (2013).

90. Smith, V. L., Cheng, Y., Bryant, B. R. & Schorey, J. S. Exosomes function in antigen presentation during an in vivo Mycobacterium tuberculosis infection. *Sci. Rep.* **7**, 1–12 (2017).

91. Cheng, Y. & Schorey, J. S. Extracellular vesicles deliver Mycobacterium RNA to promote host immunity and bacterial killing. *EMBO Rep.* **20**, e46613 (2019).

92. Bhatnagar, S. & Schorey, J. S. Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. *J. Biol. Chem.* **282**, 25779–25789 (2007).

93. Chaput, N. & Théry, C. Exosomes: Immune properties and potential clinical implementations. *Semin. Immunopathol.* **33**, 419–440 (2011).

94. Montaner-Tarbes, S. *et al.* Targeted-pig trial on safety and immunogenicity of serum-derived extracellular vesicles enriched fractions obtained from Porcine Respiratory and Reproductive virus infections. *Sci. Rep.* **8**, 1–10 (2018).

95. Schnitzer, J. K., Berzel, S., Fajardo-Moser, M., Remer, K. A. & Moll, H. Fragments of antigen-loaded dendritic cells (DC) and DC-derived exosomes induce protective immunity against Leishmania major. *Vaccine* **28**, 5785–5793 (2010).

96. Martin-Jaular, L., Nakayasu, E. S., Ferrer, M., Almeida, I. C. & del Portillo, H. A. Exosomes

from Plasmodium yoelii-infected reticulocytes protect mice from lethal infections. *PLoS One* **6**, 1–10 (2011).

97. Marcilla, A. *et al.* Extracellular vesicles in parasitic diseases. *J. Extracell. Vesicles* **3**, (2014).

98. Urbanelli, L. *et al.* The Role of Extracellular Vesicles in Viral Infection and Transmission. *Vaccines* **7**, 1–20 (2019).

99. Cameron, P. U. *et al.* Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. *Science (80-.).* **257**, 383–387 (1992).

100. Spira, A. I. *et al.* Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. *J. Exp. Med.* **183**, 215–225 (1996).

101. Izquierdo-Useros, N. *et al.* HIV and mature dendritic cells: Trojan exosomes riding the Trojan horse? *PLoS Pathog.* **6**, 1–9 (2010).

102. Knight, S. C. & Patterson, S. Bone marrow-derived dendritic cells, infection with human immunodeficiency virus, and immunopathology. *Annu. Rev. Immunol.* **15**, 593–615 (1997).

103. Izquierdo-Useros, N. *et al.* Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway. *Blood* **113**, 2732–2741 (2009).

104. Michael S. Gottlieb, Robert Schroff, Howard M. Schanker, Joel D. Weisman, Peng Thim Fan, R. A. W. and A. S. Pneumocystis carinii Pneumonia and Mucosal Candidiasis in Previously Healthy Homosexual Men — Evidence of a New Acquired Cellular Immunodeficiency. *N. Engl. J. Med.* **305**, 1425–1431 (1981).

105. Barré-Sinoussi, F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Rev. Investig. Clin.* **220**, 868–871 (1983).

106. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science (80-.).* **224**, 497–500 (1984).

107. Hahn, B. H. *et al.* Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature* **312**, 166–169 (1984).

108. Luciw, P. A., Potter, S. J., Steimer, K., Dina, D. & Levy, J. A. Molecular cloning of AIDS-associated retrovirus. *Nature* **312**, 760–763 (1984).

109. Alizon, M. *et al.* Molecular cloning of lymphadenopathy-associated virus. *Nature* **312**, 757–760 (1984).

110. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. Nucleotide sequence of the AIDS virus, LAV. *Cell* **40**, 9–17 (1985).

111. Sanchez-Pescador, R. *et al.* Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science (80-.).* **227**, 484–492 (1985).

112. Rabson, A. B. & Martin, M. A. Molecular organization of the AIDS retrovirus. *Cell* **40**, 477–480 (1985).

113. M D Daniel, N L Letvin, N W King, M Kannagi, P K Sehgal, R D Hunt, P J Kanki, M Essex, R. C. D. Isolation of T-Cell Tropic HTLV-III-Like Retrovirus from Macaques. *Science (80-.).* **228**, 1201–1204 (1985).

114. Hebb, 1, Singer, D. O. & Gray, W. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Annu. Rev. Neurosci* **18**, 555–586 (1999).

115. Vernooy, R., Shrestha, P. & Sthapit, B. Origins and evolution of AIDS viruses: estimating the time-scale. *Biochem. Soc. Trans.* **28**, 275–282 (2000).

116. Preston A. Marx, Cristian Apetrei, E. D. AIDS as a zoonosis ? Confusion over the origin of the virus and the origin of the epidemics. *J. Med. Primatol.* **33**, 220–226 (2004).

117. UNAIDS Data Report. 2020. (2020).

118. *WHO Global Statistics. 2019*.

119. International Committee on Taxonomy of Viruses, A. M. K. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press: San Diego, 2012*. (2012).

120. H, Whittle, J, Morris, J, Todd, T, Corrah, S, Sabally, J, Bangali, PT, Ngom, M, Rolfe, Wilkins, A. W. HIV-2-infected patients survive longer than HIV-1-infected patients. *AIDS* **8**, 1617–1620 (1994).

121. Eberle, J. & Gürtler, L. HIV types, groups, subtypes and recombinant forms: Errors in replication, selection pressure and quasispecies. *Intervirology* **55**, 79–83 (2012).

122. Korber, B. *et al.* Timing the ancestor of the HIV-1 pandemic strains. *Science (80-.).* **288**, 1789–1796 (2000).

123. Abbas AK, Lichtman AH, P. S. *Cellular and Molecular Immunology. Ninth Edit. Elservier; 2018*.

124. Malim, M. E. and M. H. HIV-1 Regulatory/Accessory Genes: Keys to Unraveling Viral and Host Cell Biology. *Science (80-.).* **280**, 1880–1884 (1998).

125. Emanuele Fanales-Belasio, Mariangela Raimondo, B. S. and S. B. HIV virology and pathogenetic mechanisms of infection: a brief overview. *Ann Ist Super Sanità* **46**, 5–14 (2010).

126. Wilen, C. B., Tilton, J. C. & Doms, R. W. HIV: Cell Binding and Entry. *Cold Spring Harb Perspect Med* **2**, (2012).

127. E. A. Berger, R. W. Doms, E.-M. Fenyö, B. T. M. Korber, D. R. Littman, J. P. Moore, Q. J. Sattentau, H. Schuitemaker, J. S. & R. A. W. A new classification for HIV-1. *Nature* **391**, (1998).

128. Sarafianos, S. G. *et al.* Structure and Function of HIV-1 Reverse Transcriptase: Molecular Mechanisms of Polymerization and Inhibition. *J. Mol. Biol.* **385**, 693–713 (2009).

129. Cherepanov, A. E. and P. The structural biology of HIV-1: mechanistic and therapeutic

insights. *Nat. Rev. Microbiol.* **10**, 279–290 (2012).

130. Karn, J. & Stoltzfus, C. M. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harb. Perspect. Med.* **2**, 1–17 (2012).

131. Chun, T. *et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183–188 (1997).

132. Kraüsslich, W. I. S. and H.-G. HIV-1 Assembly, Budding, and Maturation. *Cold Spring Harb Perspect Med* **2**, (2012).

133. Nguyen, D. H. & Hildreth, J. E. K. Evidence for Budding of Human Immunodeficiency Virus Type 1 Selectively from Glycolipid-Enriched Membrane Lipid Rafts. *J. Virol.* **74**, 3264–3272 (2000).

134. Hunter, E. Macromolecular interactions in the assembly of HIV and other retroviruses. *Seminars in Virology* **5**, 71–83 (1994).

135. Barré-Sinoussi, F., Ross, A. L. & Delfraissy, J. F. Past, present and future: 30 years of HIV research. *Nat. Rev. Microbiol.* **11**, 877–883 (2013).

136. Shaw, G. M. & Hunter, E. HIV transmission. *Cold Spring Harb. Perspect. Med.* **2**, 1–23 (2012).

137. T C Quinn, M J Wawer, N Sewankambo, D Serwadda, C Li, F Wabwire-Mangen, M O Meehan, T Lutalo and R H Gray, for the R. P. S. G. Viral load and heterosexual transmission of human immunodeficiency virus type 1. *N. Engl. J. Med.* **342**, 921–929 (2000).

138. Keele, B. F. *et al.* Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 7552–7557 (2008).

139. Haase, A. T. Early events in sexual transmission of hiv and siv and opportunities for interventions. *Annu. Rev. Med.* **62**, 127–139 (2011).

140. Ames, J., Ahn, O. K., Ruce, B. & Alker, D. W. Acute human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **339**, 33–39 (1998).

141. Miller, C. J. *et al.* Propagation and Dissemination of Infection after Vaginal Transmission of Simian Immunodeficiency Virus. *J. Virol.* **79**, 9217–9227 (2005).

142. Masurier, C. *et al.* Dendritic Cells Route Human Immunodeficiency Virus to Lymph Nodes after Vaginal or Intravenous Administration to Mice. *J. Virol.* **72**, 7822–7829 (1998).

143. Hu, J., Gardner, M. B. & Miller, C. J. Simian Immunodeficiency Virus Rapidly Penetrates the Cervicovaginal Mucosa after Intravaginal Inoculation and Infects Intraepithelial Dendritic Cells. *J. Virol.* **74**, 6087–6095 (2000).

144. Coffin, J. & Swanstrom, R. HIV pathogenesis: Dynamics and genetics of viral populations and infected cells. *Cold Spring Harb. Perspect. Med.* **3**, (2013).

145. Simon, V., Ho, D. D. & Abdool Karim, Q. HIV/AIDS epidemiology, pathogenesis,

prevention, and treatment. *Lancet* **368**, 489–504 (2006).

146. Maartens, G., Celum, C. & Lewin, S. R. HIV infection: Epidemiology, pathogenesis, treatment, and prevention. *Lancet* **384**, 258–271 (2014).

147. Brenchley, J. M. *et al.* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12**, 1365–1371 (2006).

148. Estes, J. D. *et al.* Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. *PLoS Pathog.* **6**, (2010).

149. Schacker, T., Collier, A. C., Hughes, J., Shea, T. & Corey, L. Annals of Internal Medicine: Clinical and Epidemiologic Features of Primary HIV Infection. *Ann. Intern. Med.* **125**, 257–264 (1996).

150. Pantaleo, G. *et al.* HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362**, 355–358 (1993).

151. Iribarren, J. A. *et al.* Prevention and treatment of opportunistic infections and other coinfections in HIV-infected patients: May 2015. *Enferm. Infecc. Microbiol. Clin.* **25**, 23–31 (2007).

152. Klein, M. R. *et al.* Kinetics of gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181**, 1365–1372 (1995).

153. Yunzhen Cao, M.D., Limo Qin, M.D., Linqi Zhang, Ph.D., Jeffrey Safrit, Ph.D., and David D. Ho, M. . Virologic and Immunologic Characterization of Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection. *N. Engl. J. Med.* **332**, 201–208 (1995).

154. Rinaldo, C. *et al.* High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J. Virol.* **69**, 5838–5842 (1995).

155. Rosenberg, E. S. *et al.* Vigorous HIV-1-Specific CD4+ T Cell Responses Associated with Control of Viremia. *Science (80-.).* **278**, 1447–1450 (1997).

156. Dean, M. *et al.* Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. *Science (80-.).* **273**, 1856–1862 (1996).

157. Dalmau, J. *et al.* Highly pathogenic adapted HIV-1 strains limit host immunity and dictate rapid disease progression. *Aids* **28**, 1261–1272 (2014).

158. Kaslow, R. A. *et al.* Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* **2**, 405–411 (1996).

159. Migueles, S. A. & Connors, M. The role of CD4+ and CD8+ T cells in controlling HIV infection. *Curr. Infect. Dis. Rep.* **4**, 461–467 (2002).

160. Migueles, S. A. *et al.* HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2709–2714 (2000).

161. Vogel, M. *et al.* Influence of HLA-B57 on clinical presentation and viral control during

acute HIV-1 infection. *AIDS* **17**, 2581–2591 (2003).

162. Mothe, B., Ibarrondo, J., Llano, A. & Brander, C. Virological, immune and host genetics markers in the control of HIV infection. *Dis. Markers* **27**, 105–120 (2009).

163. Fellay, J. *et al.* A whole-genome association study of major determinants for host control of HIV-1. *Science (80-.).* **317**, 944–947 (2007).

164. O'Brien, S. J., Gao, X. & Carrington, M. HLA and AIDS: A cautionary tale. *Trends Mol. Med.* **7**, 379–381 (2001).

165. Kaslow RA, Carrington M, Apple R, Park L, Muñoz A, Saah AJ, Goedert JJ, Winkler C, O'Brien SJ, Rinaldo C, Detels R, Blattner W, Phair J, Erlich H, M. D. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* **2**, 405–411 (1996).

166. Goulder, P. J. R. *et al.* Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. *AIDS Res. Hum. Retroviruses* **12**, 1691–1698 (1996).

167. International HIV Controllers Study, Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI, Walker BD, Ripke S, Brumme CJ, Pulit SL, Carrington M, Kadie CM, Carlson JM, Heckerman D, Graham RR, Plenge RM, Deeks SG, Gianniny L, Crawford G, Sullivan J, ... Zhao M. The Major Genetic Determinants of HIV-1 Control Affect HLA Class I Peptide Presentation. *Science (80-.).* **330**, 1551–1557 (2010).

168. Fellay, J., Shianna, K. V, Telenti, A. & Goldstein, D. B. Host Genetics and HIV-1 : The Final Phase ? *PLoS Pathog.* **6**, 1–9 (2010).

169. Mclaren, P. J. *et al.* Polymorphisms of large effect explain the majority of the host genetic contribution to variation of HIV-1 virus load. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 14658–14663 (2015).

170. M Carrington, G W Nelson, M P Martin, T Kissner, D Vlahov, J J Goedert, R Kaslow, S Buchbinder, K Hoots, S. J. O. HLA and HIV-1: Heterozygote Advantage and B*35-Cw*04 Disadvantage. *Science (80-.).* **283**, 1748–1752 (1999).

171. Liu, R. *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367–377 (1996).

172. Rappaport, J. *et al.* 32 bp CCR-5 gene deletion and resistance to fast progression in HIV-1 infected heterozygotes [3]. *Lancet* **349**, 922–923 (1997).

173. S M Hammer, K E Squires, M D Hughes, J M Grimes, L M Demeter, J S Currier, J J Eron Jr, J E Feinberg, H H Balfour Jr, L R Deyton, J A Chodakewitz, M. A. F. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N. Engl. J. Med.* **337**, 725–733 (1997).

174. F J Palella Jr 1 , K M Delaney, A C Moorman, M O Loveless, J Fuhrer, G A Satten, D J Aschman, S. D. H. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *N. Engl. J. Med.* **338**, 853–860 (1998).

175. Margaret A. Fischl, M.D., Douglas D. Richman, M.D., Michael H. Grieco, M.D., J.D., Michael S. Gottlieb, M.D., Paul A. Volberding, M.D., Oscar L. Laskin, M.D., John M. Leedom, M.D., Jerome E. Groopman, M.D., Donna Mildvan, M.D., Robert T. Schooley, M.D., G, and T. A. C. W. G. The efficacy of azidothumidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* **317**, (1987).

176. Gallo, R. C., Bolognesit, D., Barryt, D. W. & Broder, S. 3 ' -Azido-3 ' -deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III / lymphadenopathy-associated virus in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7096–7100 (1985).

177. Arts, E. J. & Hazuda, D. J. HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harb Perspect Med* **2**, (2012).

178. B. Autran, G. Carcelain, T. S. Li, C. Blanc, D. Mathez, R. Tubiana, C. Katlama, P. Debré, J. L. Positive Effects of Com bined An tiretroviral Therapy on CD4 + T Cell Homeostasis and Function in Advanced HIV Disease. *Science (80-.).* **277**, 112–116 (1997).

179. Brinkman, K., Smeitink, J. A., Romijn, J. A. & Reiss, P. Mitochondrial toxicity induced by nucleoside-analogue rev e rs e - transcriptase inhibitors is a key factor in the pathogenesis of a n t i r e t r ov i r a l - t h e r a py-related lipodystrophy. *Lancet* **354**, 1112–1115 (1999).

180. Chesney, M. Adherence to HAART regimens. *AIDS Patient Care STDS* **17**, 169–177 (2003).

181. David R Bangsberg, Kathleen Ragland, A. M. and S. G. D. A single tablet regimen is associated with higher adherence andviral suppression than multiple tablet regimens in HIV+homeless and marginally housed people. *AIDS* **24**, 2835–2840 (2010).

182. Nachega, J. B. *et al.* Lower pill burden and once-daily antiretroviral treatment regimens for HIV infection: A meta-analysis of randomized controlled trials. *Clin. Infect. Dis.* **58**, 1297–1307 (2014).

183. Dejesus, E. *et al.* Simplification of antiretroviral therapy to a single-tablet regimen consisting of efavirenz, emtricitabine, and tenofovir disoproxil fumarate versus unmodified antiretroviral therapy in virologically suppressed HIV-1-infected patients. *J. Acquir. Immune Defic. Syndr.* **51**, 163–174 (2009).

184. Rodger, A. J. *et al.* Risk of HIV transmission through condomless sex in serodifferent gay couples with the HIV-positive partner taking suppressive antiretroviral therapy (PARTNER): final results of a multicentre, prospective, observational study. *Lancet* **393**, 2428–2438 (2019).

185. INSIGHT START Study Group *et al.* Initiation of antiretroviral therapy in early asymptomatic HIV infection. *N. Engl. J. Med.* **373**, 795–807 (2015).

186. https://hivinfo.nih.gov/understanding-hiv/infographics/fda-approval-hiv-medicines. *FDA Approval of HIV Medicines*

187. Cahn, P. *et al.* Dolutegravir plus lamivudine versus dolutegravir plus tenofovir disoproxil fumarate and emtricitabine in antiretroviral-naive adults with HIV-1 infection (GEMINI-

1 and GEMINI-2): week 48 results from two multicentre, double-blind, randomised, non-inferior. *Lancet* **393**, 143–155 (2019).

188. Aboud, M. *et al.* Efficacy and safety of dolutegravir–rilpivirine for maintenance of virological suppression in adults with HIV-1: 100-week data from the randomised, open-label, phase 3 SWORD-1 and SWORD-2 studies. *Lancet HIV* **6**, 576–587 (2019).

189. Overton, E. T. *et al.* Long-acting cabotegravir and rilpivirine dosed every 2 months in adults with HIV-1 infection (ATLAS-2M), 48-week results : a randomised , multicentre , open-label , phase 3b , non-inferiority study. *Lancet* **396**, 1994–2005 (2020).

190. Martin, A. R. & Siliciano, R. F. Progress toward HIV eradication: Case reports, current efforts, and the challenges associated with cure. *Annu. Rev. Med.* **67**, 215–228 (2016).

191. Harrigan, P. R., Whaley, M. & Montaner, J. S. G. Rate of HIV-1 RNA rebound upon stopping antiretroviral therapy. *Aids* **13**, (1999).

192. Masur, H. *et al.* An Outbreak of Community-Acquired Pneumocystis carinii Pneumonia. Initial manifestation of cellular immune dysfunction. *N. Engl. J. Med.* **305**, 1431–1438 (1981).

193. Siegal, F. P. *et al.* Severe Acquired Immunodeficiency in Male Homosexuals, Manifested by Chronic Perianal Ulcerative Herpes Simplex Lesions. *N. Engl. J. Med.* **305**, 1439–1444 (1981).

194. H C Lane, H Masur, L C Edgar, G Whalen, A H Rook, A. S. F. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **309**, 453–458 (1983).

195. Moderator: Lane, H. C. D. *et al.* Recent Advances in the Management of AIDS-related Opportunistic Infections. *Ann. Intern. Med.* **120**, 945–955 (1994).

196. Ana María Caro-Murillo, Jesús Castilla, Santiago Pérez-Hoyos, José M. Miró, Daniel Podzamczer, Rafael Rubio, Melchor Riera, Pompeyo Viciana, José López Aldeguer, José Antonio Iribarren, Ignacio de los Santos-Gil, Juan Luis Gómez-Sirvent, Juan Berenguer, F, S. M. y G. de trabajo de la C. de la R. de I. en S. (CoRIS). Cohorte RIS de pacientes con infección por VIH sin tratamiento antirretroviral previo (CoRIS): metodología y primeros resultados. *Enferm. Infecc. Microbiol. Clin.* **25**, 23–31 (2007).

197. *WHO 'Global HIV/AIDS report 2020'*.

198. *WHO" Global Tuberculosis Report 2020"*.

199. Koch, R. The etiology of tuberculosis. *Am J Med Sci* **84**, 109–115 (1882).

200. Barry, C. E. *et al.* The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol.* **7**, pages845–855 (2009).

201. Esmail, H., Barry, C. E., Young, D. B. & Wilkinson, R. J. The ongoing challenge of latent tuberculosis. *Philos Trans R Soc L. B Biol Sci* **369**, (2014).

202. Pai, M. *et al.* Tuberculosis. *Nat. Rev. Dis. Prim.* **2**, (2016).

203. Russell, D. G. Mycobacterium tuberculosis and the intimate discourse of a chronic

infection. *Immunol. Rev.* **240**, 252–268 (2011).

204. Cardona, P. J. Pathogenesis of tuberculosis and other mycobacteriosis. *Enferm. Infecc. Microbiol. Clin.* **36**, 38–46 (2018).

205. Russell, D. G., Cardona, P. J., Kim, M. J., Allain, S. & Altare, F. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat. Immunol.* **10**, 943–948 (2009).

206. Thomas R Frieden, Timothy R Sterling, Sonal S Munsiff, Catherine J Watt, C. D. Tuberculosis. *Lancet* **362**, 887–889 (2003).

207. A G Bean, D R Roach, H Briscoe, M P France, H Korner, J D Sedgwick, W. J. B. Structural Deficiencies in Granuloma Formation in TNFGene-Targeted Mice Underlie the Heightened Susceptibility toAerosolMycobacterium tuberculosisInfection, Which Is NotCompensated for by Lymphotoxin. *J. Immunol.* **162**, 3504–3511 (1999).

208. Schaible, U. E., Sturgill-Koszycki, S., Schlesinger, P. H. & Russell, D. G. Cytokine activation leads to acidification and increases maturation of Mycobacterium avium-containing phagosomes in murine macrophages. *J. Immunol.* **160**, 1290–6 (1998).

209. Ulrichs, T. *et al.* Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *J. Pathol.* **204**, 217–228 (2004).

210. Ehlers, S. & Schaible, U. E. The granuloma in tuberculosis: Dynamics of a host-pathogen collusion. *Front. Immunol.* **3**, 1–9 (2013).

211. Guirado, E. & Schlesinger, L. S. Modeling the Mycobacterium tuberculosis granuloma - the critical battlefield in host immunity and disease. *Front. Immunol.* **4**, (2013).

212. Mattila, J. T. *et al.* Microenvironments in Tuberculous Granulomas Are Delineated by Distinct Populations of Macrophage Subsets and Expression of Nitric Oxide Synthase and Arginase Isoforms. *J. Immunol.* **191**, 773–784 (2013).

213. Ramakrishnan, L. Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* **12**, 352–366 (2012).

214. Marakalala, M. J. *et al.* Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat. Med.* **22**, 531–538 (2016).

215. Lin, P. L. *et al.* Sterilization of granulomas is common in both active and latent tuberculosis despite extensive within-host variability in bacterial killing. *Nat. Med.* **20**, 75–79 (2014).

216. Cardona, P. J. A spotlight on liquefaction: Evidence from clinical settings and experimental models in tuberculosis. *Clin. Dev. Immunol.* (2011). doi:10.1155/2011/868246

217. Kim, M. J. *et al.* Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol. Med.* **2**, 258–274 (2010).

218. Kaplan, G. *et al.* Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. *Infect. Immun.* **71**, 7099–7108 (2003).

219. Subbian, S. *et al.* Lesion-specific immune response in granulomas of patients with pulmonary tuberculosis: A pilot study. *PLoS One* **10**, (2015).

220. Golden, M. P. & Vikram, H. R. Extrapulmonary tuberculosis: An overview. *Am. Fam. Physician* **72**, 1761–1768 (2005).

221. Zumla, A., Raviglione, M., Hafner, R. & Fordham von Reyn, C. Tuberculosis. *N. Engl. J. Med.* **368**, 745–755 (2013).

222. Souriant, S. *et al.* Tuberculosis Exacerbates HIV-1 Infection through IL-10/STAT3-Dependent Tunneling Nanotube Formation in Macrophages. *Cell Rep.* **26**, 3586–3599 (2019).

223. Toossi, Z. Virological and Immunological Impact of Tuberculosis on Human Immunodeficiency Virus Type 1 Disease. *J. Infect. Dis.* **188**, 1146–1155 (2003).

224. D Goletti, D Weissman, R W Jackson, N M Graham, D Vlahov, R S Klein, S S Munsiff, L Ortona, R. C. and A. S. F. Effect of Mycobacterium tuberculosis on HIV replication. Role of immune activation. *J. Immunol.* **157**, 1271–1278 (1996).

225. Vanham, G. *et al.* Generalized immune activation in pulmonary tuberculosis: Co-activation with HIV infection. *Clin. Exp. Immunol.* **103**, 30–34 (1996).

226. Hinterberg, K. *et al.* Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science (80-.).* **286**, 1353–1357 (1999).

227. Z Toossi, K Nicolacakis, L Xia, N A Ferrari, E. A. R. Activation of latent HIV-1 by Mycobacterium tuberculosis and its purified protein derivative in alveolar macrophages from HIV-infected individuals in vitro. *J Acquir Immune Defic Syndr Hum Retrovirol* **15**, 325–331 (1997).

228. V Garrait, J Cadranel, H Esvant, I Herry, P Morinet, C Mayaud, D. I.-B. Tuberculosis generates a microenvironment enhancing the productive infection of local lymphocytes by HIV. *J. Immunol.* **159**, 2824–2830 (1997).

229. Loddick, S. A. & Rothwell, N. J. Mechanisms of tumor necrosis factor α action on neurodegeneration: Interaction with insulin-like growth factor-1. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9449–9451 (1999).

230. Gray, C. M. *et al.* Identification of cell subsets expressing intracytoplasmic cytokines within HIV-1-infected lymph nodes. *Aids* **10**, 1467–1475 (1996).

231. Doms, R. W. & Moore, J. P. HIV-1 Membrane Fusion: Targets of Opportunity. *J. Cell Biol.* **151**, (2000).

232. Dimitrov, D. S. *et al.* Quantitation of human immunodeficiency virus type 1 infection kinetics. *J. Virol.* **67**, 2182–2190 (1993).

233. Dixit, N. M. & Perelson, A. S. Multiplicity of Human Immunodeficiency Virus Infections in Lymphoid Tissue. *J. Virol.* **78**, 8942–8945 (2004).

234. Patterson, S. & Knight, S. C. Susceptibility of human peripheral blood dendritic cells to infection by human immunodeficiency virus. *J. Gen. Virol.* **68**, 1177–1181 (1987).

235. Macatonia, S. E., Lau, R., Patterson, S., Pinching, A. J. & Knight, S. C. Dendritic cell infection, depletion and dysfunction in HIV-infected individuals. *Immunology* **71**, 38–45 (1990).

236. Langhoff, E. *et al.* Replication of human immunodeficieacy virus type 1 in primary dendritic cell cultures. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7998–8002 (1991).

237. Cameron PU, Forsum U, Teppler H, Granelli-Piperno A, S. R. During HIV-1 infection most blood dendritic cells are not productively infected and can induce allogeneic CD4+ T cells clonal expansion. *Clin. Exp. Immunol.* **88**, 226–236 (1992).

238. McIlroy, D. *et al.* Infection frequency of dendritic cells and CD4+ T lymphocytes in spleens of human immunodeficiency virus-positive patients. *J. Virol.* **69**, 4737–4745 (1995).

239. Granelli-Piperno, A., Delgado, E., Finkel, V., Paxton, W. & Steinman, R. M. Immature Dendritic Cells Selectively Replicate Macrophagetropic (M-Tropic) Human Immunodeficiency Virus Type 1, while Mature Cells Efficiently Transmit both M- and T-Tropic Virus to T Cells. *J. Virol.* **72**, 2733–2737 (1998).

240. Granelli-Piperno, A., Finkel, V., Delgado, E. & Steinman, R. M. Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells. *Curr. Biol.* **9**, 21–29 (1999).

241. Moris, A. *et al.* DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation. *Blood* **103**, 2648–2654 (2004).

242. Laguette, N., Sobhian, B., Casartelli, N. & Ringeard, M. SAMHD1 is the dendritic – and myeloid – cell – specific HIV – 1 restriction factor counteracted by Vpx. *Nature* **474**, 654–657 (2011).

243. Kasia Hrecka, Caili Hao, Magda Gierszewska, Selene K. Swanson, Malgorzata Kesik-Brodacka, Smita Srivastava, Laurence Florens, M. P. W. and J. S. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* **474**, 658–661 (2011).

244. Lahouassa, H. *et al.* SAMHD1 restricts HIV-1 by reducing the intracellular pool of deoxynucleotide triphosphates. *Nat. Immunol.* **13**, 223–228 (2013).

245. Ann M. Sheehy, Nathan C. Gaddis, J. D. C. & M. H. M. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**, 646–650 (2002).

246. Mariani, R. *et al.* Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* **114**, 21–31 (2003).

247. Mangeat, B. *et al.* Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**, 99–103 (2003).

248. Geijtenbeek, T. B. H. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587–597 (2000).

249. McDonald, D. *et al.* Recruitment of HIV and its receptors to dendritic cell-T cell

junctions. *Science (80-.).* **300**, 1295–1297 (2003).

250. Hyun, J. Y., Reuter, M. A. & McDonald, D. HIV traffics through a specialized, surface-accessible intracellular compartment during trans-infection of T cells by mature dendritic cells. *PLoS Pathog.* **4**, (2008).

251. Izquierdo-Useros, N. *et al.* Dynamic Imaging of Cell-Free and Cell-Associated Viral Capture in Mature Dendritic Cells. *Traffic* **12**, 1702–1713 (2011).

252. Akiyama, H., Ramirez, N. G. P., Gudheti, M. V. & Gummuluru, S. CD169-Mediated Trafficking of HIV to Plasma Membrane Invaginations in Dendritic Cells Attenuates Efficacy of Anti-gp120 Broadly Neutralizing Antibodies. *PLoS Pathog.* **11**, 1–23 (2015).

253. Garcia, E. *et al.* HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse. *Traffic* **6**, 488–501 (2005).

254. Moris, A. *et al.* Dendritic cells and HIV-specific CD4+ T cells: HIV antigen presentation, T-cell activation, and viral transfer. *Blood* **108**, 1643–1651 (2006).

255. Trumpfheller, C., Park, C. G., Finke, J., Steinman, R. M. & Granelli-Piperno, A. Cell type-dependent retention and transmission of HIV-1 by DC-SIGN. *Int. Immunol.* **15**, 289–298 (2003).

256. Wu, L. *et al.* Rhesus macaque dendritic cells efficiently transmit primate lentiviruses independently of DC-SIGN. *Proc. Natl. Acad. Sci.* **99**, 1568–1573 (2002).

257. Gummuluru, S., Rogel, M., Stamatatos, L. & Emerman, M. Binding of Human Immunodeficiency Virus Type 1 to Immature Dendritic Cells Can Occur Independently of DC-SIGN and Mannose Binding C-Type Lectin Receptors via a Cholesterol-Dependent Pathway. *J. Virol.* **77**, 12865–12874 (2003).

258. Granelli-piperno, A. *et al.* Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. *J. Immunol.* **175**, 4265–4273 (2005).

259. Boggiano, C., Manel, N. & Littman, D. R. Dendritic Cell-Mediated trans-Enhancement of Human Immunodeficiency Virus Type 1 Infectivity Is Independent of DC-SIGN. *J. Virol.* **81**, 2519–2523 (2007).

260. Wang, J.-H., Janas, A. M., Olson, W. J., KewalRamani, V. N. & Wu, L. CD4 Coexpression Regulates DC-SIGN-Mediated Transmission of Human Immunodeficiency Virus Type 1. *J. Virol.* **81**, 2497–2507 (2007).

261. Izquierdo-Useros, N. *et al.* Siglec-1 Is a Novel Dendritic Cell Receptor That Mediates HIV-1 Trans-Infection Through Recognition of Viral Membrane Gangliosides. *PLoS Biol.* **10**, e1001448 (2012).

262. Puryear, W. B. *et al.* Interferon-Inducible Mechanism of Dendritic Cell-Mediated HIV-1 Dissemination Is Dependent on Siglec-1/CD169. *PLoS Pathog.* **9**, e1003291 (2013).

263. Dupont, M. *et al.* Tuberculosis-associated IFN-I induces Siglec-1 on tunneling nanotubes

and favors HIV-1 spread in macrophages. *Elife* **9**, (2020).

264. Crocker, P. R. *et al.* Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. *EMBO J.* **13**, 4490–4503 (1994).

265. Crocker, P. R., Vinson, M., Kelm, S. & Drickamer, K. Molecular analysis of sialoside binding to sialoadhesin by NMR and site-directed mutagenesis. *Biochem. J.* **341**, 355–361 (1999).

266. Pino, M. *et al.* HIV-1 immune activation induces Siglec-1 expression and enhances viral trans-infection in blood and tissue myeloid cells. *Retrovirology* **12**, 1–15 (2015).

267. Rempel, H., Calosing, C., Sun, B. & Pulliam, L. Sialoadhesin expressed on IFN-induced monocytes binds HIV-1 and enhances infectivity. *PLoS One* **3**, e1967 (2008).

268. Hartnell, A. *et al.* Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood* **97**, 288–296 (2001).

269. Perez-Zsolt, D. *et al.* Anti-Siglec-1 antibodies block Ebola viral uptake and decrease cytoplasmic viral entry. *Nat. Microbiol.* (2019). doi:10.1038/s41564-019-0453-2

270. Berry, M. P. R. *et al.* An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* **466**, 973–977 (2010).

271. Malhotra, S. *et al.* SIGLEC1 and SIGLEC7 expression in circulating monocytes of patients with multiple sclerosis. *Mult. Scler. J.* **19**, 524–531 (2013).

272. York, M. R. *et al.* A macrophage marker, siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and toll-like receptor agonists. *Arthritis Rheum.* **56**, 1010–1020 (2007).

273. Biesen, R. *et al.* Sialic acid-binding Ig-like lectin 1 expression in inflammatory and resident monocytes is a potential biomarker for monitoring disease activity and success of therapy in systemic lupus erythematosus. *Arthritis Rheum.* **58**, 1136–1145 (2008).

274. Xiong, Y. S. *et al.* Increased expression of Siglec-1 on peripheral blood monocytes and its role in mononuclear cell reactivity to autoantigen in rheumatoid arthritis. *Rheumatol. (United Kingdom)* **53**, 250–259 (2014).

275. Fahy, E. *et al.* A comprehensive classification system for lipids. *J. Lipid Res.* **46**, 839–861 (2005).

276. Lkonen, K. S. and E. How Cells Handle Cholestero. *Science (80-.).* **290**, 1721–1726 (2000).

277. Brown, D. A. & London, E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275**, 17221–17224 (2000).

278. Rajendran, L. & Simons, K. Lipid rafts and membrane dynamics. *J. Cell Sci.* **118**, 1099–1102 (2005).

279. Lorizate, M. & Kräusslich, H. G. Role of lipids in virus replication. *Cold Spring Harb.*

Perspect. Biol. **3**, 1–20 (2011).

280. Muniz-Trabudua, X. *et al.* Siglec-1 Expressed on Dendritic Cells is a New Receptor Implicated in Arenavirus Uptake. *Proceedings* **50**, (2020).

281. Izquierdo-Useros, N. *et al.* Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mature dendritic cell capture of HIV-1. *PLoS Biol.* **10**, e1001315 (2012).

282. Puryear, W. B., Yu, X., Ramirez, N. P., Reinhard, B. M. & Gummuluru, S. HIV-1 incorporation of host-cell-derived glycosphingolipid GM3 allows for capture by mature dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7475–7480 (2012).

283. Blixt, O., Collins, B. E., Van den Nieuwenhof, I. M., Crocker, P. R. & Paulson, J. C. Sialoside specificity of the siglec family assessed using novel multivalent probes: Identification of potent inhibitors of myelin-associated glycoprotein. *J. Biol. Chem.* **278**, 31007–31019 (2003).

284. Munday, J., Floyd, H. & Crocker, P. R. Sialic acid binding receptors (siglecs) expressed by macrophages. *J. Leukoc. Biol.* **66**, 705–711 (1999).

285. Izquierdo-Useros, N. *et al.* HIV-1 Capture and Transmission by Dendritic Cells: The Role of Viral Glycolipids and the Cellular Receptor Siglec-1. *PLoS Pathog.* **10**, (2014).

286. Sewald, X. *et al.* Retroviruses use CD169-mediated trans-infection of permissive lymphocytes to establish infection. *Science (80-.).* **350**, 563–567 (2016).

287. Chang, Y. C. *et al.* Role of macrophage sialoadhesin in host defense against the sialylated pathogen group B Streptococcus. *J. Mol. Med.* **92**, 951–959 (2014).

288. Chang, Y. C. & Nizet, V. The interplay between Siglecs and sialylated pathogens. *Glycobiology* **24**, 818–825 (2014).

289. Klaas, M. *et al.* Sialoadhesin Promotes Rapid Proinflammatory and Type I IFN Responses to a Sialylated Pathogen, Campylobacter jejuni. *J. Immunol.* **189**, 2414–2422 (2012).

290. Jones, C., Virji, M. & Crocker, P. R. Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Mol. Microbiol.* **49**, 1213–1225 (2003).

291. Heikema, A. P. *et al.* Characterization of the specific interaction between sialoadhesin and sialylated Campylobacter jejuni lipooligosaccharides. *Infect. Immun.* **78**, 3237–3246 (2010).

292. Vimr, E. & Lichtensteiger, C. To sialylate, or not to sialylate: That is the question. *Trends Microbiol.* **10**, 254–257 (2002).

293. Díaz-Varela, M. *et al.* Proteomics study of human cord blood reticulocyte-derived exosomes. *Sci. Rep.* **8**, 14046 (2018).

294. Saunderson, S. C., Dunn, A. C., Crocker, P. R. & McLellan, A. D. CD169 mediates the capture of exosomes in spleen and lymph node. *Blood* **123**, 208–216 (2014).

295. Perez-Zsolt, D., Martinez-Picado, J. & Izquierdo-Useros, N. When dendritic cells go viral:

The role of Siglec-1 in host defense and dissemination of enveloped viruses. *Viruses* **12**, (2019).

296. Turville, S. G. *et al.* Diversity of receptors binding HIV on dendritic cell subsets. *Nat. Immunol.* **3**, 975–983 (2002).

297. Izquierdo-Useros, N. *et al.* Maturation of Blood-Derived Dendritic Cells Enhances Human Immunodeficiency Virus Type 1 Capture and Transmission. *J. Virol.* **81**, 7559–7570 (2007).

298. Quintana-Murci, L., Alcaïs, A., Abel, L. & Casanova, J. L. Immunology in natura: Clinical, epidemiological and evolutionary genetics of infectious diseases. *Nat. Immunol.* **8**, 1165–1171 (2007).

299. MacArthur, D. G. *et al.* A systematic survey of loss-of-function variants in human protein-coding genes. *Science (80-.).* **335**, 823–828 (2012).

300. Sulem, P. *et al.* Identification of a large set of rare complete human knockouts. *Nat. Genet.* **47**, 448–452 (2015).

301. Lim, E. T. *et al.* Distribution and Medical Impact of Loss-of-Function Variants in the Finnish Founder Population. *PLoS Genet.* **10**, e1004494 (2014).

302. Bartha, I. *et al.* The Characteristics of Heterozygous Protein Truncating Variants in the Human Genome. *PLoS Comput. Biol.* **11**, 1–14 (2015).

303. Rausell, A. *et al.* Analysis of Stop-Gain and Frameshift Variants in Human Innate Immunity Genes. *PLoS Comput. Biol.* **10**, e1003757 (2014).

304. Rivas, M. A. *et al.* Impact of predicted protein-truncating genetic variants on the human transcriptome. *Science (80-.).* **348**, 666–669 (2015).

305. Schoeni-Affolter, F. *et al.* Cohort profile: The Swiss HIV cohort study. *Int. J. Epidemiol.* **39**, 1179–1189 (2010).

306. Phair, J. *et al.* Acquired immune deficiency syndrome occurring within 5 years of infection with human immunodeficiency virus type-1: the Multicenter AIDS Cohort Study. *J. Acquir. Immune Defic. Syndr.* **5**, 490–6 (1992).

307. Irwin Jungreis, Michael F. Lin, Rebecca Spokony, Clara S. Chan, Nicolas Negre, Alec Victorsen, K. P. W. and M. K. Evidence of abundant stop codon readthrough in Drosophila and other metazoa. *Genome Res.* **21**, 2096–2113 (2011).

308. Ramachandra, L. *et al.* Mycobacterium tuberculosis synergizes with ATP to induce release of microvesicles and exosomes containing major histocompatibility complex class II molecules capable of antigen presentation. *Infect. Immun.* **78**, 5116–5125 (2010).

309. van Dinther, D. *et al.* Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8 + T Cell Cross-Priming. *Cell Rep.* **22**, 1484–1495 (2018).

310. Kidder, D., Richards, H. E., Ziltener, H. J., Garden, O. A. & Crocker, P. R. Sialoadhesin Ligand Expression Identifies a Subset of CD4 + Foxp3 − T Cells with a Distinct Activation and Glycosylation Profile . *J. Immunol.* **190**, 2593–2602 (2013).

311. Carter, A. J. R. & Nguyen, A. Q. Antagonistic pleiotropy as a widespread mechanism for the maintenance of polymorphic disease alleles. *BMC Med. Genet.* **12**, 160 (2011).

312. Curtis, J. *et al.* Susceptibility to tuberculosis is associated with variants in the ASAP1 gene encoding a regulator of dendritic cell migration. *Nat. Genet.* **47**, 523–527 (2015).

313. Szeszko, J. S. *et al.* Resequencing and association analysis of the SP110 gene in adult pulmonary tuberculosis. *Hum. Genet.* **121**, 155–160 (2007).

314. Casali, N. *et al.* Evolution and transmission of drug resistant tuberculosis in a Russian population. *Nat. Genet.* **46**, 279–286 (2014).

315. Martinez-Picado, J. *et al.* Identification of siglec-1 null individuals infected with HIV-1. *Nat. Commun.* **7**, 1–7 (2016).

316. Oetke, C., Vinson, M. C., Jones, C. & Crocker, P. R. Sialoadhesin-Deficient Mice Exhibit Subtle Changes in B- and T-Cell Populations and Reduced Immunoglobulin M Levels. *Mol. Cell. Biol.* **26**, 1549–1557 (2006).

317. Medina, E. & North, R. J. Resistance ranking of some common inbred mouse strains to Mycobacterium tuberculosis and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology* **93**, 270–274 (1998).

318. Troegeler, A. *et al.* An efficient siRNA-mediated gene silencing in primary human monocytes, dendritic cells and macrophages. *Immunol. Cell Biol.* **92**, 699–708 (2014).

319. Pennock, P. J. W. E. L. and C. A. Measurement of sialic acid in serum and urine: Clinical applications and limitations. *Ann. Clin. Biochem.* **29**, 625–637 (1992).

320. Monguió-Tortajada, M. *et al.* Nanosized UCMSC-derived extracellular vesicles but not conditioned medium exclusively inhibit the inflammatory response of stimulated T cells: implications for nanomedicine. *Theranostics* **7**, 270–284 (2017).

321. Van Deun, J. *et al.* EV-TRACK: Transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat. Methods* **14**, 228–232 (2017).

322. Morales-Kastresana, A. *et al.* Labeling extracellular vesicles for nanoscale flow cytometry. *Sci. Rep.* **7**, 1–10 (2017).

323. Monguió-Tortajada, M. *et al.* Extracellular-Vesicle Isolation from Different Biological Fluids by Size-Exclusion Chromatography. *Curr. Protoc. Stem Cell Biol.* **49**, 1–24 (2019).

324. Erikson, E. *et al.* Mouse siglec-1 mediates trans-infection of surface-bound murine leukemia virus in a sialic acid N-acyl side chain-dependent manner. *J. Biol. Chem.* **290**, 27345–27359 (2015).

325. Suzanne D. G. Kijewski, Hisashi Akiyama, Amin Feizpour, Caitlin Miller, N.- & Guadalupe P. Ramirez, Björn M. Reinhard, and S. G. Access of HIV-2 to CD169-Dependent Dendritic Cell-Mediated Trans Infection Pathway is Attenuated. *Virology* **497**, 328–336 (2016).

326. Akiyama, H. *et al.* Virus Particle Release from Glycosphingolipid-Enriched Microdomains Is Essential for Dendritic Cell-Mediated Capture and Transfer of HIV-1 and Henipavirus. *J. Virol.* **88**, 8813–8825 (2014).

327. Steinman, R. M. Dendritic cells: Versatile controllers of the immune system. *Nat. Med.* **13**, 1155–1159 (2007).

328. León, B., López-Bravo, M. & Ardavín, C. Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. *Immunity* **26**, 519–531 (2007).

329. Martinez-Picado, J., McLaren, P. J., Telenti, A. & Izquierdo-Useros, N. Retroviruses as myeloid cell riders: What natural human Siglec-1 'knockouts' tell us about pathogenesis. *Front. Immunol.* **8**, 6–11 (2017).

330. Fellay, J. *et al.* Common genetic variation and the control of HIV-1 in humans. *PLoS Genet.* **5**, e1000791 (2009).

331. Narasimhan, V. M., Xue, Y. & Tyler-Smith, C. Human Knockout Carriers: Dead, Diseased, Healthy, or Improved? *Trends Mol. Med.* **22**, 341–351 (2016).

332. Graustein, A. D. *et al.* The SIGLEC14 null allele is associated with Mycobacterium tuberculosis- and BCG-induced clinical and immunologic outcomes. *Tuberculosis* **104**, 38–45 (2017).

333. Souza de Lima, D. *et al.* Polymorphisms in SIGLEC1 contribute to susceptibility to pulmonary active tuberculosis possibly through the modulation of IL-1ß. *Infect. Genet. Evol.* **55**, 313–317 (2017).

334. Santos, A. A. *et al.* Implication of purinergic P2X7 receptor in M. tuberculosis infection and host interaction mechanisms: A mouse model study. *Immunobiology* **218**, 1104–1112 (2013).

335. Fernando, S. L. *et al.* A polymorphism in the P2X7 gene increases susceptibility to extrapulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* **175**, 360–366 (2007).

336. Martinez-Pomares, L. & Gordon, S. CD169 + macrophages at the crossroads of antigen presentation. *Trends Immunol.* **33**, 66–70 (2012).

337. Baena, A. & Porcelli, S. A. Evasion and subversion of antigen presentation by Mycobacterium tuberculosis: REVIEW ARTICLE. *Tissue Antigens* **74**, 189–204 (2009).

338. Srivastava, S. & Ernst, J. D. Cell-to-cell transfer of M. tuberculosis antigens optimizes CD4 T cell priming. *Cell Host Microbe* **15**, 741–752 (2014).

339. Jurkoshek, K. S., Wang, Y., Athman, J. J., Barton, M. R. & Wearsch, P. A. Interspecies communication between pathogens and immune cells via bacterial membrane vesicles. *Front. Cell Dev. Biol.* **4**, 1–8 (2016).

Chapter 9

PUBLICATIONS

1. Susana Benet, Cristina Gálvez, Francis Drobniewski, Irina Kontsevaya, Lilibeth Arias, Marta Monguió-Tortajada, Itziar Erkizia, Victor Urrea, Ruo-Yan Ong, Marina Luquin, Maeva Dupont, Jakub Chojnacki, Judith Dalmau, Paula Cardona, Olivier Neyrolles, Geanncarlo Lugo-Villarino, Christel Vérollet, Esther Julián, Hansjakob Furrer, Huldrych F. Günthard, Paul R. Crocker, Gustavo Tapia, Francesc E. Borràs, Jacques Fellay, Paul J. McLaren, Amalio Telenti, Pere-Joan Cardona, Bonaventura Clotet, Cristina Vilaplana, Javier Martinez-Picado and Nuria Izquierdo-Useros. **Dissemination of *Mycobacterium tuberculosis* is associated to a *SIGLEC1* null variant that limits antigen exchange via trafficking extracellular vesicles.** *Journal of Extracellular Vesicles.* 2020 (accepted).

2. Maeva Dupont, Shanti Souriant, Luciana Balboa, Thien-Phong Vu Manh, Karine Pingris, Stella Rousset, Céline Cougoule, Yoann Rombouts, Renaud Poincloux, Myriam Ben Neji, Carolina Allers, Deepak Kaushal, Marcelo J Kuroda, Susana Benet, Javier Martinez-Picado, Nuria Izquierdo-Useros, Maria del Carmen Sasiain, Isabelle Maridonneau-Parini, Olivier Neyrolles, Christel Vérollet and Geanncarlo Lugo-Villarino. **Tuberculosis-associated IFN-I induces Siglec-1 on tunneling nanotubes and favors HIV-1 spread in macrophages.** *eLife.* 2020. 9:e52535. doi: 10.7554/eLife.52535.

3. Daniel Perez-Zsolt, Itziar Erkizia, Maria Pino, Mónica García-Gallo, Maria Teresa Martin, Susana Benet, Jakub Chojnacki, María Teresa Fernández-Figueras Dolores Guerrero, Victor Urrea, Xabier Muñiz-Trabudua, Leonor Kremer, Javier Martinez-Picado and Nuria Izquierdo-Useros. **Anti-Siglec-1 antibodies block Ebola viral uptake and decrease cytoplasmic viral entry.** *Nature Microbiology.* 2019. 4(9):1558-1570. doi: 10.1038/s41564-019-0453-2

4. Daniel Perez-Zsolt, Jon Cantero-Pérez, Itziar Erkizia, Susana Benet, Maria Pino, Carla Serra-Peinado, Alba Hernández-Gallego, Josep Castellví, Gustavo Tapia, Vicent Arnau-Saz, Julio Garrido, Antoni Tarrats, Maria J. Buzón, Javier Martinez-Picado, Nuria Izquierdo-Useros and Meritxell Genescà. **Dendritic cells from the cervical mucosa capture and transfer HIV1 via Siglec-1.** *Frontiers in Immunology.* 2019. 10:825. doi: 10.3389/fimmu.2019.00825.

5. Javier Martinez-Picado, Paul J. McLaren, Itziar Erkizia, Maureen P. Martin, Susana Benet, Margalida Rotger, Judith Dalmau, Dan Ouchi, Steven M. Wolinsky, Sudhir Penugonda, Huldrych F. Günthard, Jacques Fellay, Mary Carrington, Nuria Izquierdo-Useros and Amalio

Telenti. **Identification of Siglec-1 null individuals infected with HIV-1.** *Nature Communications.* 2016. 7:12412. doi: 10.1038/ncomms12412.

6. Maria Pino, Itziar Erkizia, Susana Benet, Elina Erikson, Maria Teresa Fernández-Figueras, Dolores Guerrero, Judith Dalmau, Dan Ouchi, Antonio Rausell, Angela Ciuffi, Oliver T Keppler, Amalio Telenti, Hans-Georg Kräusslich, Javier Martinez-Picado and Nuria Izquierdo-Useros. **HIV-1 immune activation induces Siglec-1 expression and enhances viral trans-infection in blood and tissue myeloid cells.** *Retrovirology.* 2015. 12:37. doi: 10.1186/s12977-015-0160-x

Chapter 10

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