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Olga Romanets

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Olga Romanets. Study of the role of measles virus receptor CD150 in viral immunopathogenesis and characterization of novel CD150 isoform. Molecular biology. Ecole normale supérieure de lyon - ENS LYON; R. E. Kavetsky institute of experimental pathology, oncology and radiology (Kiev), 2012. English. NNT: 2012ENSL0789 . tel-01124046

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# THÈSE

en vue de l'obtention du grade de

**Docteur de l'École Normale Supérieure de Lyon - Université de Lyon**

**Discipline** : Sciences de la vie

**Laboratoires** INSERM U758-ENS de Lyon et  
R.E. Kavetsky Institut de la Pathologie, Oncologie et Radiobiologie Expérimentale de l'Académie  
Nationale des Sciences (Kiev, Ukraine)

**École Doctorale** l'École Doctorale BMIC (biologie moléculaire intégrative et cellulaire)

présentée et soutenue publiquement le **14 Décembre 2012**

par **Olga ROMANETS**

## **Study of the role of measles virus receptor CD150 in viral immunopathogenesis and characterization of novel CD150 isoform**

*Directeurs de thèse :* **Mme Branka HORVAT**

**Mme Svetlana SIDORENKO**

*Après l'avis de :* **Mr Pablo ENGEL**

**Mr Pierre-Olivier VIDALAIN**

*Devant la commission d'examen formée de :*

*Mme Christine DELPRAT, LBMC-ENS-Lyon, Lyon, France (Président)*

*Mr Pablo ENGEL, Université de Barcelone, Barcelone, Espagne (Rapporteur)*

*Mme Branka HORVAT, INSERM U758-ENS-Lyon, Lyon, France (Directeur)*

*Mr Sergij OSYNSKYY, IEPOR, Kiev, Ukraine (Membre)*

*Mme Svetlana SIDORENKO, IEPOR, Kiev, Ukraine (Directeur)*

*Mr Pierre-Olivier VIDALAIN, Institut Pasteur, Paris, France (Rapporteur)*

## ACKNOWLEDGMENTS

*Finally I am writing this last piece of my PhD thesis, and, of course, I am happy to do this: happy because it's the last one ☺, and happy to remember last four years, the nice events and nice people I've met on the way. However, writing these lines also means that the big paragraph or, I'd better say chapter ☺, of my life is going to the end. Looking back – it was for sure the hardest time, but at the same time the best one, the best in terms of experience, knowledge and impressions obtained, but also great people - teachers, colleges and friends. They had a huge impact on making this work, on where I am and who I am now; and to all of them I would like to say big THANK YOU!!!*

*Foremost, I want to say thanks of course to my PhD supervisors, Dr Svetlana Sidorenko and Dr Branka Horvat. Dear Svitlana Pavlivna, you were my first teacher in scientific work, and largely because of you I have started this thesis, and for sure thanks to you I have finished it. I am very grateful for your enthusiasm and encouragement, for belief in me, for sharing your knowledge and teaching me to think independently. Thank you for giving the conception vision of studied problems, critical view in analysis and so important tips in experiments that actually made the experiments working. For me you were also in big part a “life teacher”, I will always remember your everyday support and worries that you give not only to me, and because of what we use to call you “our Mom” in the lab, as you probably know ☺.*

*Dear Branka, because of you I have got in this co-tutelle thesis project and had the opportunity to get a very nice experience of scientific work in France and particularly in your lab. I am very grateful for motivation, your knowledge, guidance of experimental work and analyzing the results. Thank you for the lessons of how to organize work, be more efficient and for pushing me with deadlines – without this I would never finish the manuscript in time ☺.*

*I am also very grateful to Dr Denis Gerlier for his scientific advices and knowledge, insightful discussions and suggestions.*

*I am very appreciative to the reviewers of my thesis, Dr Pablo Engel and Dr Pierre-Olivier Vidalain, for very useful discussions, good questions and suggestions that helped me to improve largely the manuscript, and of course for the positive reports about my work.*

*Another huge set of thanks I would like to dedicate to both of my labs, ukrainian, in Kavetsky institute of experimental pathology, oncology and radiobiology, and french, in INSERM U758-ENS de Lyon. Larysa Mykolayivna, Anna Grygorivna and Svitlana Vitaliyivna, thank you very much for having given me the scientific experimental background, encouraging, caring and concern. Masha (you were my example when I was a student), Lesia (your fun will make a dead man laughing) and Marjana (in the worst moments you always have an ability to show the good points), girls, we have been working together shoulder to shoulder since my third year at the university, and it was always a pleasure!!! ☺ I thank all of you for the support in work and just the support itself!!!*

*And of course my French lab... I start with the closest and those who is waiting forward to this chapter ☺!! Alors, Josephine-Rosepine (you've done so much for me during this time, probably more than anybody, but I thank not only for that, but for being you, my friend whom I like and*

appreciate so much), Joanna-Jonana (and another name we both know ☺, thanks for support in lab and life, being my freetime-mate in lots of activities and my good friend all this time), Marie-sushi!! (thanks for being “perpetual motion” ☺, so much helpful any time I need, and your friendship), Louis-Luis-Leon (we all know when and why we found this name for you ☺, thank you for staying in the lab always longer than anybody and for the “let the force be with you”, for helpful discussions and fun time outside the lab), Jeremy (despite my French and your English, our conversations were always very positive ☺, and of course thank you for help with confocal microscopy), Carine (thanks for bench help, positivity and great example of efficient lab work ☺), Gé (thanks for the bons de commande, always nice attitude to me and efforts to speak in English ☺), Kevin (thanks for interesting conversations and your creativity for the lab and outside-the-lab events ☺), Jade (thanks for the help in lab-related issues and contagious enthusiasm ☺), Vanessa (thank you for discussions and your smile on my French-speaking efforts ☺), Boyan (thanks for lots of experiments advises and some Russian chats in culture room ☺), Phil (thanks for “good mornings” ☺, your great sense of humor and of course help in the lab), Jessica (I think we never talked a lot but liked each other from the beginning ☺), Cyrille (thanks for always being ready to help and a lot of bench tips ☺). Also I am grateful to people who already left the lab but with whom I have started here: Celine, Anne, Fahrnan, Cristine... With all of you the lab time turned to the enjoyable experience! I would like to say thanks to all of you for your warm hearts, lots of fun and sometimes silliness, for Charlie-unicorn, gloves on the ceiling, birthday gifts full of meanings ☺, questioner-scale on the whiteboard...for the lunches, dinners and parties ☺

I am also very grateful to people in both institutes who helped me during experimental work for thesis: girls from the cell bank in IEPOR institute in Kiev, Dr Tetjana Malysheva from Neurosurgery institute in Kiev, who helped me with tumor samples and analysis of IHC results, Sébastien Dussurgey and Thibault Andrieu from Plateau technique/Cytométrie en Flux, Isabelle Grosjean from CelluloNet/IFR128, people from PLATIM and PBES of ENS-Lyon.

And the last, but the most important, I want to say huge thanks to my family and close friends. Because of you, I managed to finish this work; you believed in me on each step, you supported me and gave rise to new plans, ideas, you enriched me with the enthusiasm not to stop but work to get these 205 pages of 4 years long work presented here ☺. I am grateful to you and I love you!



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## ABBREVIATIONS

Akt - v-Akt murine thymoma viral oncogen  
CDV – canine distemper virus  
CNS – central nervous system  
CPE – cytopathic effect  
CHO – chinese hamster ovarian  
CHT – contact hypersensitivity test  
DCs – dendritic cells  
DNA – desoxiribonucleic acid  
ERK - extracellular signal-regulated kinase  
GFAP – glial fibrillary acidic protein  
GM-CSF - granulocyte macrophage colony-stimulating factor  
GSK3 – glycogen synthase kinase-3  
HLA – human leucocyte antigen  
JNK - c-Jun N-terminal kinase  
IFN - interferon  
Ig - immunoglobulin  
IRF – interferon regulating factor  
LCs – Langerhans cells  
LPS - lipopolysacharide  
mAb – monoclonal antibodies  
MAPK – mitogen-activated kinase  
MAPKK – mitogen-activated kinases kinase  
MAPKKK - mitogen-activated kinases kinases kinase  
MHC – major histocompatibility complex  
MLR – mixed lymphocyte reaction  
MOI – multiplicity of infection  
MV – measles virus  
MV-H – measles virus hemagglutinin  
NF- $\kappa$ B – nuclear factor  $\kappa$ B  
PBMC – peripheral blood mononuclear cells

PFU – plaque forming unit

PI3K – phosphatidylinositol 3-kinase

PKB – protein kinase B

PPR – pattern recognition receptor

PVRL4 – poliovirus receptor related 4

RIG-I – retinoic acid inducible gene-I

RNA – ribonucleic acid

SLAM – signaling lymphocyte activation molecule

TLR – Toll-like receptor

VSV – vesicular stomatitis virus

## THESIS SUMMARY

Measles virus (MV) causes an acute childhood disease, associated in certain cases with the infection of the central nervous system (CNS). MV induces a profound immunosuppression, resulting in high infant mortality. The major cellular receptor for MV is CD150, which binds MV hemagglutinin (MV-H). As dendritic cell (DC) dysfunction is considered to be essential for the MV immunopathogenesis, we analyzed consequences of MV-H interaction with DCs. We developed an experimental model allowing us to analyze the direct CD150-MV-H interaction in the absence of infectious context. This interaction caused the downregulation of surface expression of CD80, CD83, CD86 and HLA-DR molecules and inhibition of IL-12 production in DCs. DCs also failed to activate T cell proliferation. The CD150-MV-H interaction in DCs and B cells decreased the phosphorylation of JNK1/2, but not ERK1/2 kinases, after subsequent CD150 ligation with anti-CD150 antibodies. Moreover, MV-H by itself induced Akt phosphorylation via CD150 in DCs and B cells. Engagement of CD150 by MV-H in mice transgenic for human CD150 decreased the inflammatory reaction, contact hypersensitivity response, confirming the immunosuppressive effect of CD150-MV-H interaction *in vivo*. Furthermore, our studies revealed the CD150 expression in CNS tumors and identified the novel CD150 isoform, containing an additional 83bp exon expressed in lymphoid cells, DCs and CNS tumors. Although its isoforms remain intracellular in tumor cells, CD150 may represent a new marker for human brain tumors. Novel mechanism of CD150-induced immunosuppression and new CD150 isoform identified in these studies shed new light on its immunoregulatory role and CD150 isoform diversity and open perspectives for their clinical applications.

## RÉSUMÉ DE THÈSE

Le virus de la rougeole (MV) provoque une maladie sévère chez les enfants qui induit une forte immunosuppression et peut dans certains cas infecter le système nerveux central (SNC). La protéine CD150, principal récepteur cellulaire du virus, permet l'entrée du MV en se liant à l'hémagglutinine (MV-H). L'altération fonctionnelle des cellules dendritiques (DC) étant considérée comme essentielle dans l'immunopathogénèse du MV, nous avons analysé les conséquences de l'interaction de MV-H avec les DC. Nous avons développé un modèle expérimental qui nous permet d'étudier l'interaction directe entre CD150 et MV-H hors contexte infectieux. Nos résultats montrent que cette interaction provoque une diminution de l'expression des molécules de surface CD80, CD83, CD86, et HLA-DR, de la production d'IL-12 par les DC, et de la capacité des DC à stimuler la prolifération des lymphocytes T. L'interaction CD150-MV-H a inhibé la phosphorylation des protéines kinases JNK1/2 dans les DC et les lymphocytes B (LB) induite par la stimulation de CD150 par un anticorps spécifique, mais pas celle des kinases ERK1/2. Par ailleurs MV-H seule induit la phosphorylation d'Akt via CD150 dans les DC et les LB. La liaison de CD150 par MV-H a réduit la réponse inflammatoire chez les souris transgéniques exprimant CD150 humain, ce qui confirme l'effet de l'interaction de CD150 et MV-H *in vivo*. Nos études ont révélé l'expression de CD150 dans les tumeurs du SNC et l'existence d'une nouvelle isoforme de CD150. Cette isoforme contient un exon supplémentaire de 83 pb et est exprimée dans les cellules lymphoïdes et les DC en plus des tumeurs du SNC. Bien que l'expression de CD150 soit uniquement intracellulaire dans les cellules tumorales, elle peut représenter un nouveau marqueur pour les tumeurs cérébrales humaines. Cette étude apporte un éclairage nouveau sur le rôle immunorégulateur de CD150 et sur la diversité de ses isoformes, ouvrent ainsi de nouvelles perspectives pour leurs applications thérapeutiques.

# **I. INTRODUCTION**

# **1. MEASLES VIRUS**

## **1.1. MEASLES EPIDEMIOLOGY**

Measles is a highly infectious and potentially fatal disease. In the last 12 months of 2012 , 24116 measles cases were reported to WHO regional office for Europe, similar number to 2011 (30 567 cases)and 2010 (30 264 cases), but considerably more cases than in 2009 (7 175 cases) and 2008 (7 817 cases). Ukraine reported the most cases as well as the highest notification rate per 1 million population, and accounted for more than half of the cases (WHO, 2012).

The causative agent for measles is measles virus (MV), a member of the family *Paramyxoviridae*, genus *Morbillivirus*. *Morbilliviruses* also include rinderpest virus (RPV), canine distemper virus (CDV), peste des petits ruminants virus (PPRV), phocine distemper virus, cetacean morbillivirus virus and seal distemper virus (Lamb & Parks, 2007). RPV affects several species of wild and domestic cloven-hoofed animals. The mortality rate can reach nearly 100% in highly susceptible cattle or buffalo herds, therefore rinderpest had caused significant economic damage since records began. CDV is a cause of fatal disease in many species of carnivores. Fatal CDV infection has been reported in other species such as large felids (Appel et al., 1994), javelinas (Appel et al., 1991), and freshwater and marine seals (Visser et al., 1990).

Historically, the first scientific description of measles-like syndrome was provided by Abu Becr, known as Rhazes, in the 9th century. Epidemics identified as measles were recorded in the 11th and 12th centuries (Furuse et al., 2010) Genetically and antigenetically, MV is most closely related to rinderpest virus (RPV), which is a pathogen of cattle. MV is assumed to have evolved in an environment where cattle and humans lived in close proximity (Barrett, 1999). MV probably evolved after commencement of livestock farming in the early centers of civilization in the Middle East and its divergence from RPV occurred around the 11th to 12th centuries. The population size at that time was sufficient for maintaining MV (Furuse et al., 2010). The virus was introduced into the Americas in the sixteenth century as a result of European exploration of the New World, resulting in thousands of deaths in native Amerindian populations (Moss & Griffin, 2006).

MV is one of the most highly contagious infectious agents and outbreaks can occur in populations in which less than 10% of individuals are susceptible. The contagiousness of MV is best expressed by the basic reproductive number ( $R_0$ ), which is the mean number of secondary

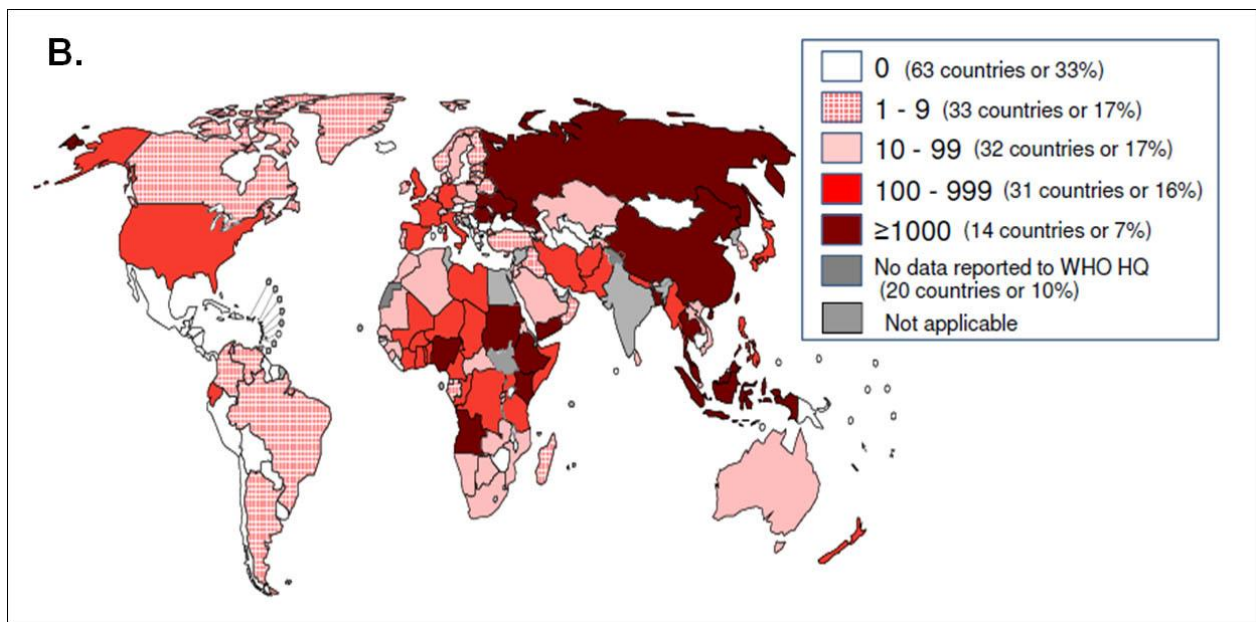
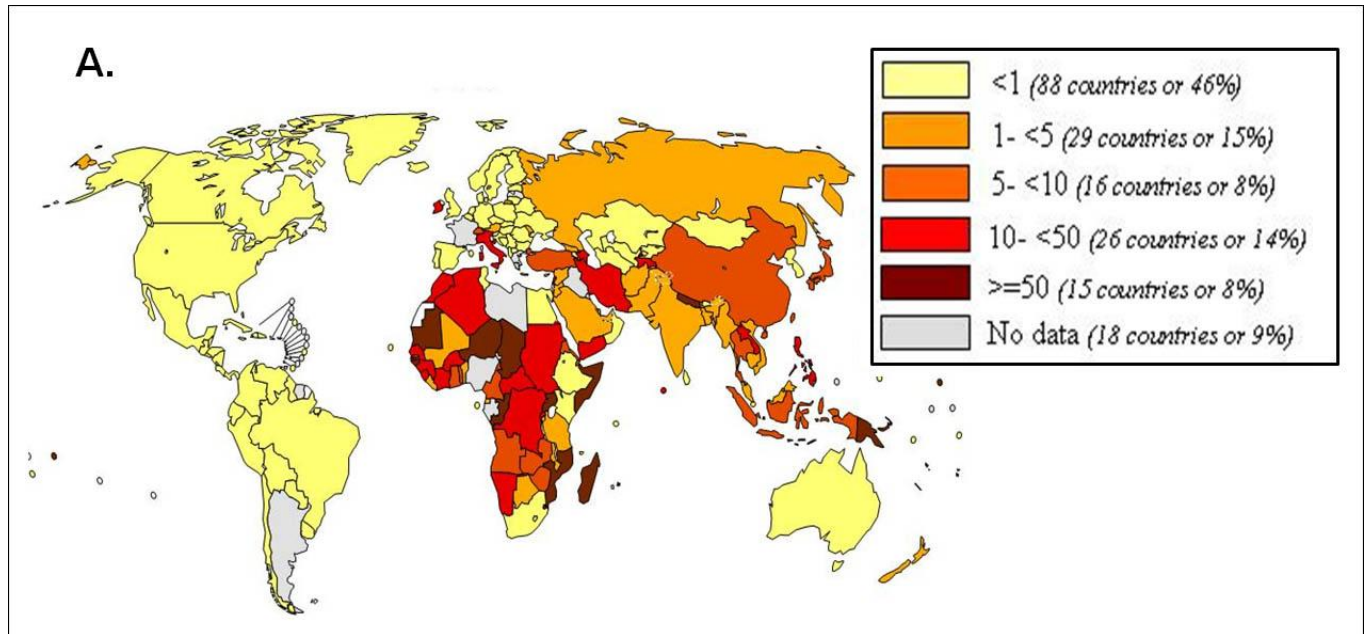


cases that would arise if an infectious agent were introduced into a completely susceptible population. The estimated  $R_0$  for MV is generally assumed to be 12–18, in contrast to only 5–7 for smallpox virus and 2–3 for SARS corona-virus (Moss & Griffin, 2006).

More than 40 years ago live attenuated and effective vaccines were developed to limit these severe and fatal diseases of *Morbilliviruses*. Great advances in the development and distribution of vaccines mean that some diseases can be eradicated ("The case of measles," 2011) In particular, international campaigns have been conducted to eradicate both MV and RPV globally. As a result of vaccination efforts and culling of infected animals, eradication of rinderpest in all 198 countries and territories was declared by OIE (2011) and FAO (2011) (OIE, 25 May 2011). Rinderpest became the second viral disease, after smallpox, to be eradicated through human efforts (WHO, 2011a).

In the case of measles, in 1980, before vaccination was widespread, there were around 4 million cases of measles and an estimated 2.6 million deaths from the disease worldwide ("The case of measles," 2011). After a global campaign for vaccination, estimated global measles mortality decreased by 74% from 535,300 deaths in 2000 to 139,300 in 2010. Measles mortality was reduced by more than three-quarters in all WHO regions except the WHO Southeast Asia region. India accounted for 47% of estimated measles mortality in 2010, and the WHO African region accounted for 36% (Simons et al., 2012). (Fig. 1A, 2). Ideally, 95% of children need to receive two doses of a measles-containing vaccine to interrupt disease transmission. By 2009, almost 60% of countries had achieved 90% coverage with at least one dose — but some are still far below this, and some are slipping backwards ("The case of measles," 2011). In the United States measles was officially eliminated in 2000, but in 2011 more cases, imported from elsewhere, have been registered than in any year since 1996 ("The case of measles," 2011; Fig. 1B, 2). For these outbreaks, genotyping continues to be critical in defining sources of importation and understanding transmission pathways, and monitoring indigenous and imported measles virus, which is required to document progress towards elimination (WHO, 2011a).

The Dahlem Conference on Disease Eradication (1997) defined eradication as the permanent reduction to zero of the global incidence of infection, caused by a specific pathogen as a result of deliberate (Moss & Griffin, 2006). Three criteria were deemed necessary for a disease to be considered eradicable: first, humans must be crucial for transmission; second,



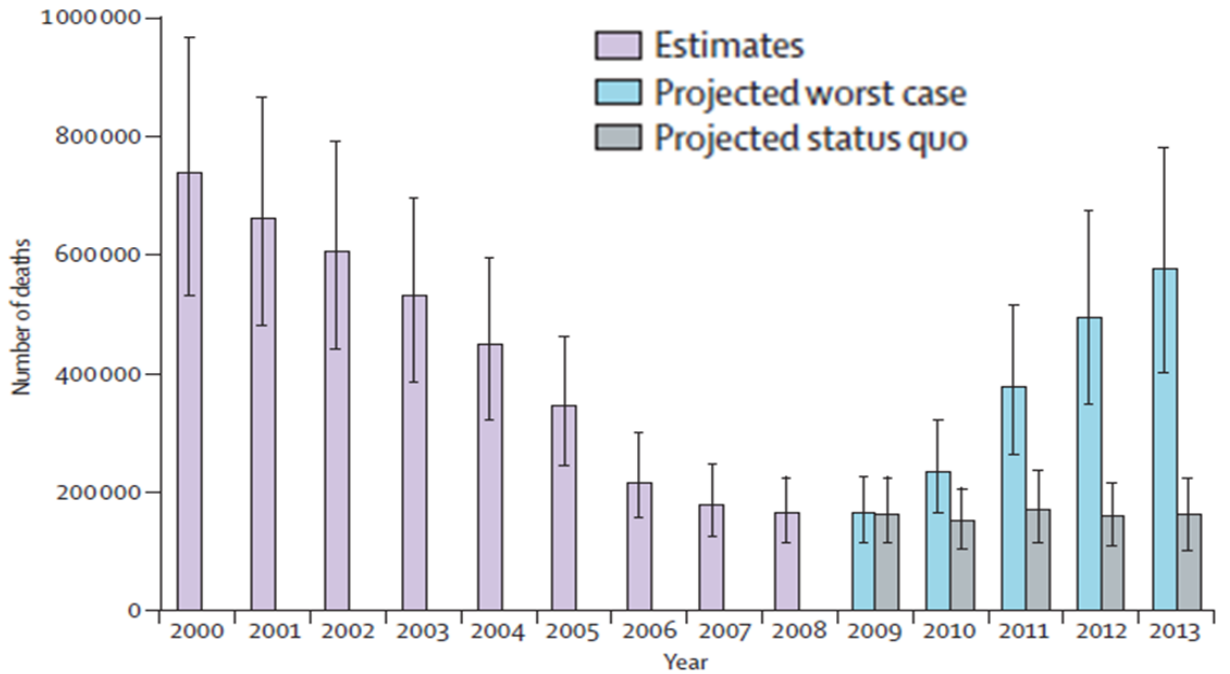
**Figure.1.** Changes in measles incidence rate between 2003 and 2012 worldwide. **A.** Measles incidence rate per 100,000 population in 2003. **B.** Number of measles cases from December 2011 to June 2012 (WHO report).

sensitive and specific diagnostic tools must exist; and finally, an effective intervention must be available (Moss & Griffin, 2006).

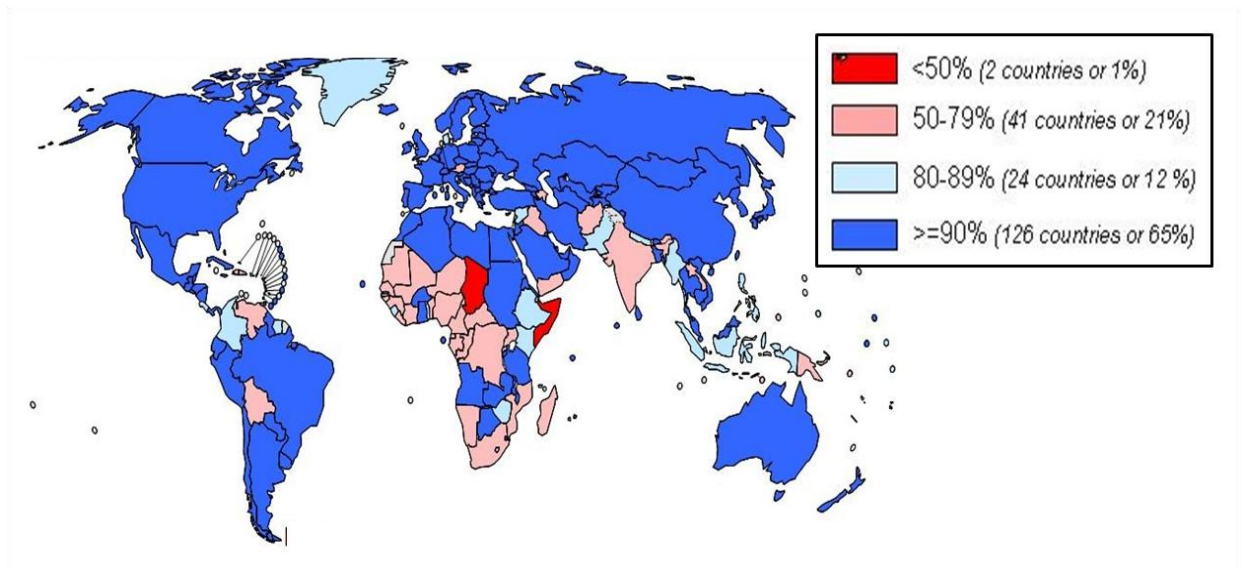
Humans are the only reservoir for MV. Non-human primates can be infected with MV and develop an illness similar to measles in humans. Populations of wild primates are not of a sufficient size to maintain MV transmission. However measles outbreaks have also been described in colonies of captive nonhuman primates (van Binnendijk et al., 1995). To provide a sufficient number of new susceptible individuals through births to maintain MV transmission, a population size of several hundred thousand individuals with 5,000 to 10,000 births per year is required (Black, 1966).

Measles is specifically diagnosed and several attenuated measles vaccines are available worldwide, either as single-virus vaccines or in combination with other vaccine viruses (commonly rubella and mumps) (Fig. 3). Most of the currently used measles vaccines were derived from the Edmonston strain of MV isolated by Enders and Peebles in 1954 (Enders & Peebles, 1954). These vaccines have undergone different passage histories in cell culture, but nucleotide sequence analyses show genetic differences of less than 0.6% between most vaccine strains (Moss & Griffin, 2006). Despite it has a lot of errors in genome, as other RNA viruses, they do not appear in proteins, which makes MV not as plastic as for example influenza virus and allows having a stable live attenuated vaccine. The reasons for such phenomena are not clear. One of the most variable regions of the MV genome is the 450-nucleotide sequence at the carboxyl terminus of the N protein, with up to 12% variability between wild-type viruses. The WHO recognizes 8 clades of MV (designated A to H) and 23 genotypes.

All vaccine strains currently in use derive from five different type A wild-type MV, including Edmonston, and have been passed several times in embryonated eggs and/or quail embryonic cells and/or most often in CEF (Rota et al., 1994). These MV strains shared four common properties that distinguish them from the currently wild-type MV strains: they belong to the type A strain, they can use human CD46 as a cellular receptor, they grow well in Vero cells and CEF, and they are strongly attenuated both in humans and monkeys. Some vaccine strains, such as AIK-C, Zagreb and Moraten, differ in their thymic pathogenicity, as evaluated in a human thymus xenograft model in SCID mice (Gerlier & Valentin, 2009; Valsamakis et al., 2001).



**Figure 2.** Number of measles death cases during last decade (WHO report).



**Figure 3.** Immunization coverage with measles containing vaccines in infants in 2010 (WHO report).

When propagated *in vitro* in their established cell host, the vaccine strains are remarkably stable, with little or no change in sequence with time passages and maintenance in different laboratories all around the world (Parks et al., 2001a; Rota et al., 1994). There are few mutations in noncoding regions, some of them being common to all Edmonston-derived vaccine strains (Parks et al., 2001a), but their functional impact is unknown. The vaccine strains also display changes in coding sequence in all viral proteins, few of them being common to all vaccine strains (Parks et al., 2001b; Rima, et al., 1995). One notable common mutation is the N481Y mutation in H, which is absent from the so-called wild-type Edmonston strain and is associated with the usage of CD46 as alternate cellular receptor (Gerlier & Valentin, 2009).

When looking at mutations in other virus genes occurring after adaptation in Vero cells, no mutation was systematically recovered, nor was a single gene systematically found to be mutated, indicating that MV adaptation for optimal growth in Vero cells is multifactorial. Only one recent attempt to adapt a wild-type strain into CEF has been described and it is associated with mutations in internal P/V/C and M proteins (Bankamp et al., 2008; Gerlier & Valentin, 2009). How a virus with initially poor entry abilities can overcome the entry step to grow to about normal levels in most cells after adaptation remains enigmatic (Gerlier & Valentin, 2009).

Despite all predispositions of measles to be eradicated, it is still not. There are several limitations of the licensed vaccines that might be important for global measles elimination. First, attenuated measles vaccines are inactivated by light and heat, and lose about half of their potency after reconstitution if stored at 20°C for one hour and almost all potency if stored at 37°C for one hour. A cold chain must be maintained to support measles immunization activities. Second, measles vaccines must be injected subcutaneously or intramuscularly, necessitating trained healthcare workers, needles, syringes and the proper disposal of hazardous waste. Third, both maternally acquired antibodies and immunological immaturity reduce the protective efficacy of measles vaccination in early infancy, hindering effective immunization of young infants (Gans et al., 1998). Fourth, the attenuated measles vaccine has the potential to cause serious outcomes, such as lung or brain infection, in severely immunocompromised persons (Angel et al., 1998). Moreover, children with defective cell-mediated immunity might not develop the characteristic measles rash, and infection might therefore go unrecognized, with the potential for widespread transmission of MV. Fifth, a second opportunity for measles vaccination, in addition to the first dose through routine immunization services, must be provided to achieve high enough levels of population immunity to interrupt MV transmission. And finally, measles outbreaks have occurred in communities that are

opposed to vaccination on religious or philosophical grounds. Much public attention has focused on a purported association between the measles-mumps-rubella (MMR) vaccine and autism (Wakefield et al., 1998) that led to diminished vaccine coverage and provide important lessons in the misinterpretation of epidemiological evidence to the public. As a consequence, measles outbreaks became more frequent and larger in size. However, the relationship between MMR vaccination and autism was not scientifically approved (DeStefano & Thompson, 2004; Madsen et al., 2002).

Clinical trials are conducted to combine standard measles, mumps, rubella vaccine (MMR) and subsequent varicella vaccine (MMR + V) (Czajka et al., 2009; Vesikari et al., 2012). Several new anti-measles vaccine candidates are undergoing development and testing, like naked cDNA vaccines, DNA vaccines encoding either or both of the measles H and F proteins, a different DNA vaccine, containing H, F and N genes and an IL-2 molecular adjuvant (Polack et al., 2000; Premenko-Lanier et al., 2003; Premenko-Lanier et al., 2004); alternative vectors for administering MV genes, such as alphavirus, parainfluenza virus or enteric bacteria are also under investigation. (Pan et al., 2005; Pasetti et al., 2003; Skiadopoulos et al., 2001). Novel oral immunization strategies have been developed using plant-based expression of the H protein from MV in tobacco (Moss & Griffin, 2006; Webster et al., 2005).

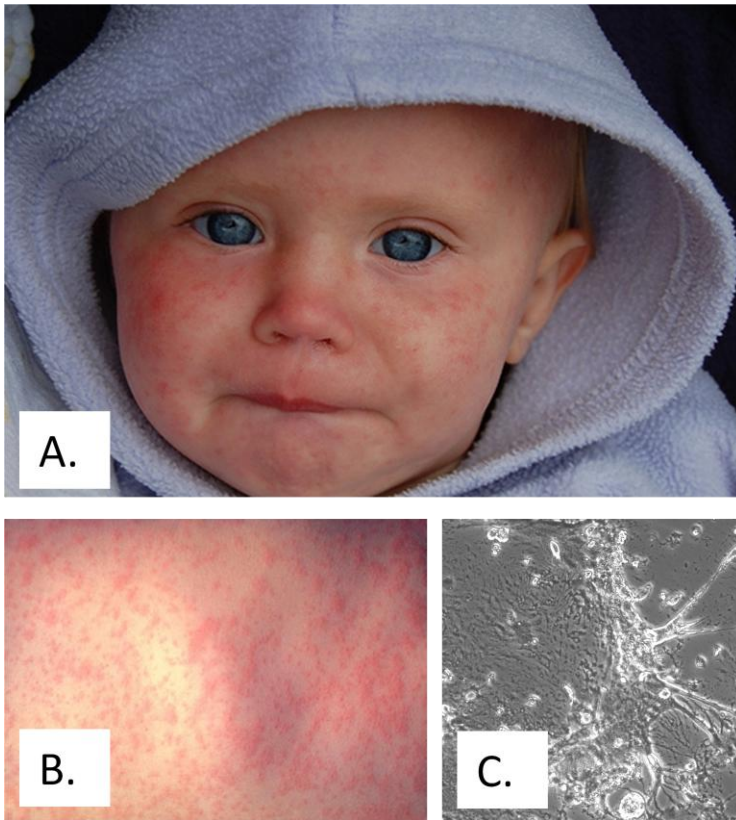
## 1.2. MEASLES CLINICAL PRESENTATION

The name "measles" comes from the Middle English "maselen" meaning "many little spots" - characteristic of the rash.

During the 10–14 day incubation period between infection and the onset of clinical signs and symptoms, MV replicates and spreads in the infected host. Clinically apparent measles begins with a prodrome characterized by fever, cough, coryza (runny nose) and conjunctivitis. Koplik's spots, which are small white lesions on the buccal mucosa inside the mouth, might be visible during the prodrome. The characteristic erythematous and maculopapular rash appears first on the face and behind the ears, and then spreads to the trunk and the extremities (Fig. 4). Skin rash is thought to be caused by the T-cell response to MV-infected cells in capillary vessels because it does not appear in children with T-cell immunodeficiency. Moreover, the studies on rhesus monkeys whose CD8<sup>+</sup> T cells were depleted showed that CD4<sup>+</sup>, but not CD8<sup>+</sup> T cells are responsible for the development of rash (Yanagi et al., 2006). The rash lasts for 3–4 days after which it fades, disappearing from the face first. The World Health Organization (WHO) clinical case definition for measles states: "any person with a generalized maculopapular rash, fever of 38°C or higher and one of the following: cough, coryza or conjunctivitis; or any person in whom a health professional suspects measles" (Robertson et al., 1997).

The laboratory diagnosis of measles is based on serology or virus detection. The most common method is the demonstration of MV-specific IgM in a single serum sample, but a more than 4-fold titer rise in paired serum samples is also formal proof of a recent MV infection. Virus detection may be performed by virus isolation or RT-PCR. Alternatives to the classic clinical specimens collected from measles patients (serum and throat swab) include oral fluid, dried blood spots collected on filter paper, and urine. Collection of these samples is less invasive, and transportation less dependent on cold chain maintenance (Featherstone et al., 2003).

In uncomplicated measles, clinical recovery begins soon after the appearance of the rash. The risk of complication is increased by extremes of age, malnutrition and vitamin A deficiency (Moss & Griffin, 2006).



**Figure. 4.** Pathogenesis of measles infection. **A, B.** The characteristic measles rash. **C.** Measles cytopathic effect.



Complications of measles are not as specific as for example for HIV, and have been described in almost every organ system. Pneumonia, the most common fatal complication of measles, occurs in 56–86% of measles-related deaths, and is caused by secondary viral or bacterial infections or by MV itself. Other respiratory complications include laryngotracheobronchitis (croup) and otitis media (ear infection). Mouth ulcers, or stomatitis, might hinder children from eating or drinking. Many children with measles develop diarrhea, further contributing to malnutrition. Eye disease (keratoconjunctivitis) is common after measles, particularly in children with vitamin A deficiency, and can cause blindness (Moss & Griffin, 2006).

Rare but serious complications of measles involve the central nervous system (CNS). Post-measles encephalomyelitis, an autoimmune disorder triggered by MV infection, occurs in approximately 1 in 1000 cases, and is mainly confined to older children and adults. Encephalomyelitis occurs within two weeks of the onset of rash and is characterized by fever, seizures and various neurological abnormalities. Other CNS complications that occur months to years after acute infection are measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). MIBE and SSPE are caused by persistent MV infection. MIBE is a rare but fatal complication that affects individuals with defective cellular immunity and typically occurs months after infection. SSPE is a slow progressive disease characterized by seizures, progressive deterioration of cognitive and motor functions and by death, which occurs 5–15 years after MV infection (Moss & Griffin, 2006).

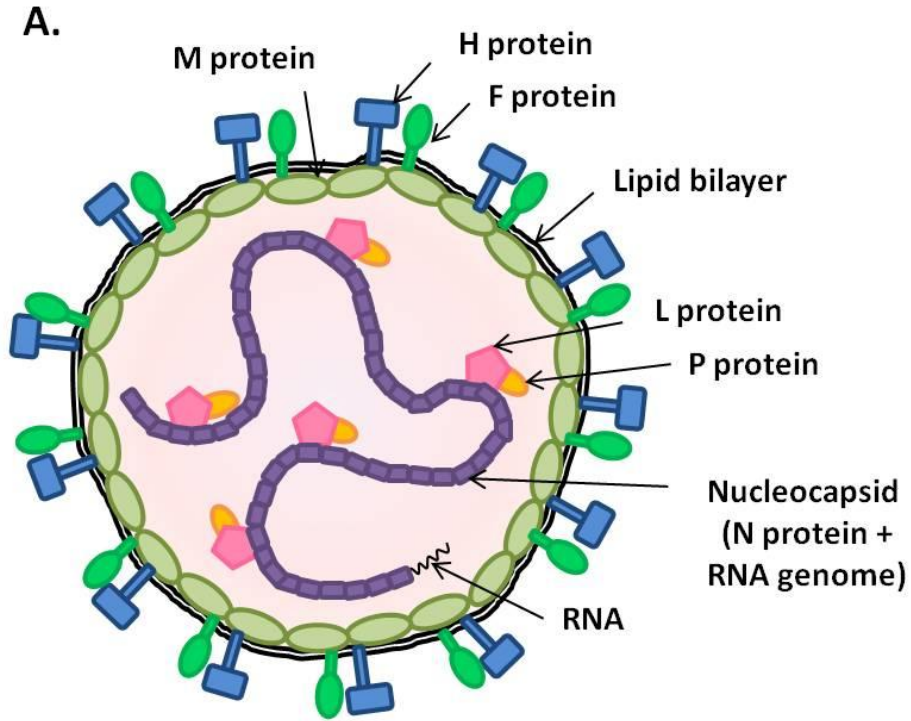
### 1.3. MEASLES VIRUS STRUCTURE AND GENOME

MV is a spherical enveloped virus with a diameter of between 100 and 300 nm. The lipid bilayer envelope is derived from the host cell. The virion is surrounded by a fringe of spikes, which in contrast to influenza viruses, appear to be uniform in size, 5 nm in diameter and up to 15 nm in length. These spikes probably consist of complexes of the fusion (F) and hemagglutinin (H) glycoproteins, two key viral proteins which unite to form the biologically active fusion complex (Moss & Griffin, 2012; Rima & Duprex, 2011; Fig. 5A).

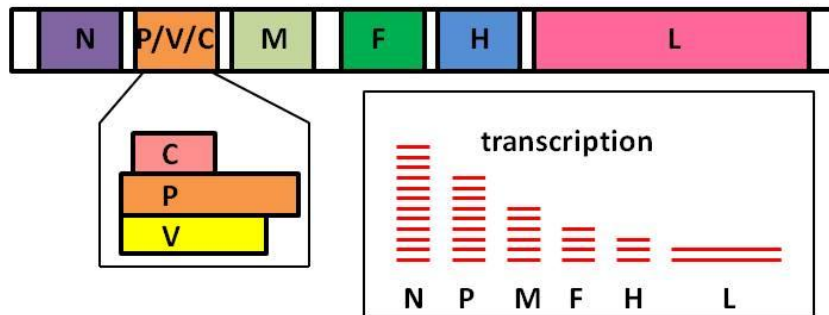
Inside the virion is a negative sense (–) ribonucleoprotein complex (RNP), the (–)RNP. It consists of the viral genome, which is 15,894 nucleotides in length, and at least three viral proteins. When observed by electron microscopy (EM) it appears as a helical structure which is 1 µm in length and 18–21 nm in diameter, with a central core, which is 5 nm in diameter (Rima & Duprex, 2011).

A lipid-containing envelope of MV derives from the host cell. The same applies to translation apparatus, as MV is an obligatory translation intracellular parasite as well as all other viruses. The MV genome containing 6 genes encodes eight proteins. V and C proteins are encoded by the P gene in addition to phosphoprotein (P) via RNA editing process and use of alternative translational frame respectively (Fig. 5B). Of the six structural proteins, P, large protein (L) and nucleoprotein (N) form the nucleocapsid that encloses the viral RNA. The haemagglutinin protein (H), fusion protein (F) and matrix protein (M), together with lipids from the host cell membrane, form the viral envelope (Griffin, 2007; Moss & Griffin, 2012).

There are thirteen nucleocapsid (N) protein molecules per helical turn (Bhella, Ralph, Murphy, & Yeo, 2002; Desfosses, Goret, Farias Estrozi, Ruigrok, & Gutsche, 2011). The genome must consist of a multiple of six nucleotides ( $6 \times 2649$ ). Each N protein may associate with exactly six nucleotides of the genomic RNA. There are approximately 204 helical turns in each RNP, a number which has been validated by direct observation (Calain & Roux, 1993; Lund, Tyrrell, Bradley, & Scraba, 1984). It also means that residues 78 ( $6 \times 13$ ) nucleotides apart would be almost adjacent to each other. This fits well with the alignment of the so called A and B boxes, which are present at the genome termini and in the 5' untranslated region (UTR) of the N and the 3' UTR of the L mRNAs which are key conserved promoter sequences essential for



**B.**



**Figure 5.** Measles virus particle and genome. **A.** The structure of measles virus particle. **B.** Schematic presentation of measles virus genome.

RNA synthesis. By analogy to other non-segmented negative strand (NNS) RNA viruses it is likely that the RNA is located on the outside of the RNP and thus available to the vRdRp, its cofactors and substrates. It is likely that the vRdRp and the nascent RNA molecule and replicative intermediates are stationary in the cell whilst the template rotates as it is being copied. The alternative would be energetically too costly (Rima & Duprex, 2011).

Mentioned above six transcription units (genes) of MV genome are separated from each other by trinucleotide intergenic sequences. These six transcription units are preceded by the leader (Le) region at the 3'-terminus encoding a short Le RNA of 56 nucleotides and are followed at the 5'-terminus by a trailer (Tr) region comprised of 40 nucleotides (Rima & Duprex, 2011).

The virus glycoprotein spikes consist of the trimeric F (Zhu et al., 2003) and the H glycoproteins (Hashiguchi et al., 2007), which are integral type 1 and type 2 membrane proteins, respectively. The H protein is a dimer or a dimer of dimers (Hashiguchi et al., 2007). The F0 glycoprotein precursor is biologically activated by proteolytic cleavage in the Golgi or post-Golgi involving furin-like proteases (Navaratnarajah et al., 2011; M. Watanabe et al., 1995). These cleave the glycoprotein into an F1–F2 complex, which is held together by a disulfide bridge between two cysteine residues which are conserved throughout the *Paramyxoviridae*. The furin proteases, which perform the cleavage, are ubiquitously expressed in a large number of cell types meaning that biological activation of the F0 glycoprotein does not constitute a limiting factor in MV egress, cell-to-cell spread and transmission. This is in contrast to other enveloped RNA viruses, such as Sendai and influenza viruses, which productively infect and spread in many fewer cell types. Simple co-expression of the H and F glycoproteins in transiently transfected cells leads to cell-to-cell fusion and the production of multinucleated syncytia, which are indistinguishable from those produced upon infection (Rima & Duprex, 2011).

In infected cells, all structural proteins including the mature F1-F2 and H proteins are enriched into the raft fraction, while the F0 precursor remains excluded from the raft fraction. Interestingly, F0 is cleaved into F1 -F2 in the trans-Golgi network, where the membrane rafts are formed (Gerlier & Valentin, 2009; Manie et al., 2000; Vincent et al., 2000).

It is proposed that membrane rafts allow co-localisation and assembly of H and F glycoproteins on one hand, and of the M–RNP complex on the other hand (Manie et al., 2000). Incidentally, the infection by MV strongly modifies receptor signaling such as CD40 and T cell receptor, which requires membrane raft integrity, thus contributing to MV-induced immunosuppression (Avota et al., 2004; Servet-Delprat et al., 2003; Vidalain et al., 2000).

Unfortunately, the viral component(s) responsible for these effects have not been identified yet (Gerlier & Valentin, 2009).

The inside of the virion membrane is coated with the matrix (M) protein, which interacts with the cytoplasmic tails of the F and H glycoproteins forming the “bridge” between these and the (-)RNP.

At least two additional proteins are generated from the gene encoding the P protein, which are non-structural in the sense that they are not detected in the virion. The C protein is translated from the P mRNA from an overlapping open reading frame (ORF) (Bellini et al., 1985). The V protein is generated by co-transcriptional editing of 30–50% of the P/V/C gene transcripts by the non-templated addition of a single guanosine residue at a slippery sequence in the gene, the so-called editing site (Bankamp et al., 2008; Cattaneo et al., 1989). The role of the viral proteins and especially the P, C and V proteins is in modifying the host environment.

## 1.4. MEASLES VIRUS RECEPTORS

The first step in any virus cell cycle is to get inside the cell. Only the viruses of fungi do not require a specific cell entry mechanism as they are spread by cell-cell fusion occurring during the mating process, but for bacterial, plant and animal viruses the entry process is much more complicated. Unlike other members of the Paramyxoviridae, which utilize sialic acid molecules present on glycoproteins and glycolipids to enter cells, *Morbilliviruses* use specific cellular receptors. Some molecules (called entry receptors) are, by themselves, capable of inducing the conformational changes of the H- and F-proteins required for membrane fusion and thus allowing MV entry, whereas others (called attachment receptors) only allow MV attachment to the cell without the ensuing membrane fusion and entry (Yanagi et al., 2009).

### 1.4.1. CD46

Measles virus was first isolated by Enders and Peebles (Enders & Peebles, 1954) from primary human kidney cells inoculated with the blood and throat washings of a child with measles. The virus strain (Edmonston) was passaged multiple times in primary human kidney and amnion cells and then adapted to eggs and multiply passaged in chick embryo cells to produce the original Edmonston B vaccine, which was licensed in 1963. Administration of the live attenuated vaccine induces both expression of the neutralizing antibody and cellular immune responses sufficient for protection (Sato et al., 2012).

Vero cells derived from the African green monkey kidney had been utilized to isolate MV as a standard cell line because it is beneficial and safe. About 40 years after MV was isolated, two groups reported in 1993 that CD46 acts as a cellular receptor for laboratory-adapted strains of MV. Nanche et al. obtained a monoclonal antibody that inhibited cell fusion induced by recombinant vaccinia virus encoding the H and F proteins of the Halle strain of MV. The antibody precipitated a cell-surface glycoprotein from human and simian cells but not from murine cells. N-terminal amino acid sequencing identified that the glycoprotein was human membrane cofactor protein CD46, a member of the regulators of the complement activation gene cluster (Nanche et al., 1993). Transfection of non-permissive murine cells with a CD46 expression vector confirmed that the human CD46 molecule serves as a MV receptor, allowing virus-cell binding, fusion, and viral replication. Dorig et al. showed independently that hamster cell lines expressing CD46 produced

syncytia and virus proteins after infection with the Edmonston strain of MV and that polyclonal antisera against CD46 inhibited virus binding and infection (Dorig et al., 1993).

CD46 is a cell-surface, type I transmembrane 57–67 kD glycoprotein that belongs to the family of complement activation regulators and is ubiquitously expressed in all nucleated human cells (Fig. 6). In monkeys, CD46 is also present on red blood cells, consistent with the observation that these strains hemagglutinate monkey red blood cells (Yanagi et al., 2009). It protects host cells from complement deposition by functioning as a cofactor for the factor-I-mediated proteolytic inactivation of C3b and C4b (Liszewski et al., 1991). In addition, CD46 has been implicated in the modulation of T-cell functions (Marie et al., 2002), generation of regulatory T-cells (Kemper et al., 2003), and control of interferon (IFN) production (Katayama et al., 2000). CD46 is also important during fertilization – it presumably promotes sperm–egg interaction (Harris et al., 2006; Riley-Vargas et al., 2005).

CD46 exists in multiple isoforms, which are generated by alternative splicing of a single gene. It has four short consensus repeats (SCR 1–4) comprising 60–64 aa each, an alternatively spliced serine/threonine/proline-rich region, a transmembrane region, and an alternatively spliced cytoplasmic tail (Sato et al., 2012; Fig. 6).

Previous studies have located the MV binding site on CD46 to the SCR1 and SCR2 domains of the receptor (Buchholz et al., 1997; Casasnovas et al., 1999; Christiansen et al., 2000; Hsu et al., 1997). Functional studies *in vitro* have suggested that signaling via CD46 is an important component of MV pathogenesis. For example, the high degree of interaction between MV-H and CD46 results in downregulation of CD46 from the surface of infected cells, rendering them more sensitive to C3b-mediated complement lysis (Schneider-Schaulies et al., 1995; Schnorr et al., 1995). Moreover, it was shown recently that CD46-Cyt-1 isoform engagement with either MV or group A *Streptococcus*, also its binding partner, is sufficient to induce autophagy through CD46-Cyt-1/GOPC pathway that presents an alternative way to control infection upon microorganism recognition (Joubert et al., 2009). Cross-linking CD46 on the surface of monocytes by MV inhibits activation-induced expression of interleukin (IL)-12, which is essential for the generation of successful effector T-cell responses (Galbraith et al., 1998; Karp, 1999; Karp et al., 1996; Kurita-Taniguchi et al., 2000). Interaction of MV-H and CD46 also induces IL-10, leading to inhibition of the contact hypersensitivity reaction (Marie et al., 2002). In contrast, MV binding to CD46 induces IFN production, which further triggers the early antiviral immune response (Naniche et al., 2000).

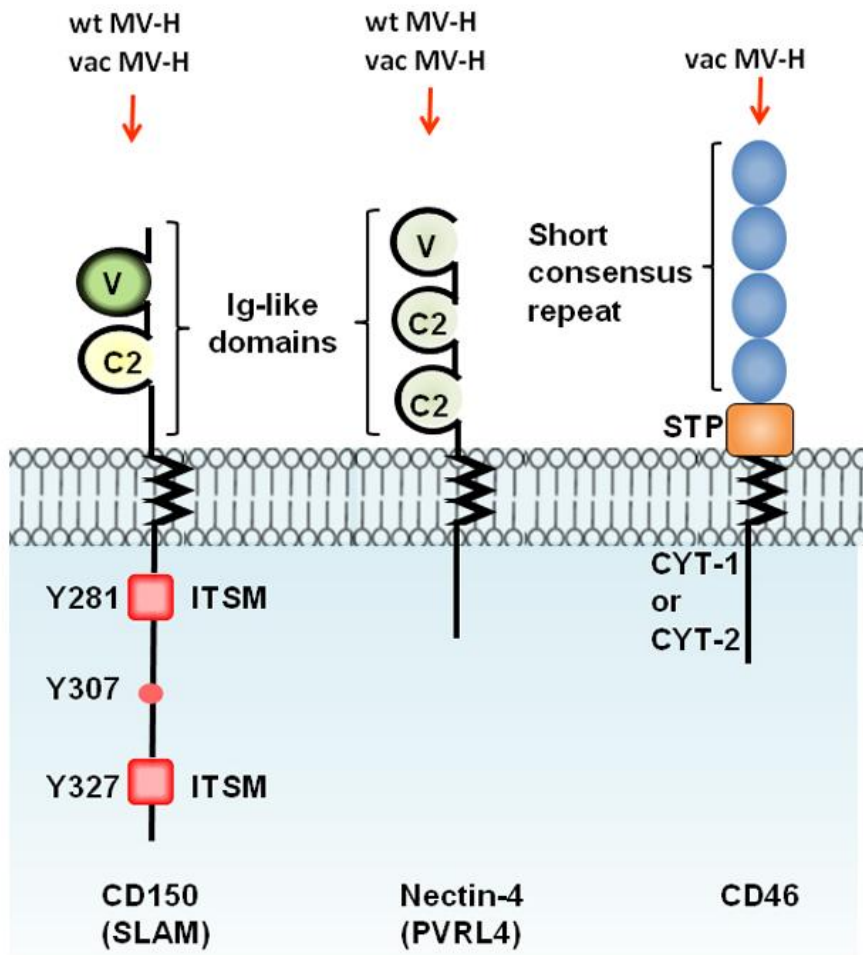
### ***1.4.2. CD150***

Although Vero cells had been used for MV isolation for a long time, the isolation was not highly efficient and usually required several blind passages. In contrast, Kobune et al. found that an Epstein–Barr-virus-transformed marmoset B-cell line, B95a, is 10,000-fold more sensitive to the MV present in clinical specimens than Vero cells. Furthermore, MVs isolated and propagated in B95a cells cause clinical signs in experimentally infected monkeys, which resemble those of human measles such as rashes and Koplik’s spots, leukopenia, and marked histological lesions in the lymphoid tissues (Kobune et al., 1990). Subsequently, Kobune et al. reported that two strains of wild MV from the same patient, one isolated in B95a cells and the other in Vero cells, had different virulence in monkeys. The former induced acute signs of MV infection, whereas the latter did not induce any clinical signs of disease and caused milder histological lesions. These findings strongly indicated that MV isolated in B95a cells maintains virulence similar to that in humans and that isolation in Vero cells leads to loss of virulence (Kobune et al., 1990; Kobune et al., 1996).

Furthermore, the H protein of MV isolated from B-cell lines neither induced downregulation of CD46 nor caused cell–cell fusion (upon coexpression of the F protein) in CD46-positive cell lines (Bartz et al., 1998; Lecouturier et al., 1996; Tanaka et al., 1998). From these observations, it had been postulated that B-cell line-isolated strains do not use the ubiquitously expressed CD46 but utilize another molecule as a receptor (Bartz et al., 1998; Buckland & Wild, 1997; Hsu et al., 1998; Lecouturier et al., 1996; Tanaka et al., 1998; Tatsuo, Okuma et al., 2000).

Tatsuo et al. performed a screening of a cDNA library of B95a cells, in which a non-susceptible human kidney cell line, 293T, was transfected with the cDNA library and then screened with a vesicular stomatitis virus pseudotype bearing the H protein of MV isolated from B-cells and F protein from the Edmonston strain. As a result, a single cDNA clone capable of making transfected 293T cells susceptible to MV-H protein bearing pseudotype was identified.





**Figure 6.** Schematic presentation of structure of measles virus receptors (part 1). Three entry MV receptors, able to interact with MV hemagglutinin have been identified: CD46 for laboratory MV strains, CD150 for both, wild type and laboratory MV strains in lymphoid cells, and nectin-4, for both MV strains again, but in epithelial cells. **CD46** consists of four short consensus repeats (SCR) on N-terminus, followed by alternatively spliced serine, threonine and proline-rich region (STP), transmembrane domain and one of two cytoplasmic tails (CYT-1 or CYT-2). **CD150** comprises two immunoglobuline(Ig)-like domains (V and C2) on N-terminus, transmembrane region and cytoplasmic tail containing three tyrosine residues which could be phosphorylated, two of which form the core of immunoreceptor tyrosine-based switch motif (ITSM). The structure of **PVRL4** (nectin-4) is defined by three extracellular Ig-like domains (V and two C2-type domains), a single transmembrane helix, and an intracellular domain.

The sequence of the clone was a homolog of signaling lymphocyte activation molecule (SLAM/CD150), and consequently, human SLAM was identified as a lymphoid cell receptor for wild-type MV (Fig. 6). Importantly, the Edmonston strain was found to utilize SLAM, in addition to CD46, as a receptor, indicating that SLAM acts as a receptor not only for B-cell line-isolated MV strains but also for vaccine and laboratory-adapted strains (Tatsuo et al., 2000; (de Vries, Lemon et al., 2010)).

Furthermore, it has been demonstrated that all CDV and RPV strains use dog and cow SLAM as a receptor, respectively, and that SLAM is a common and principal receptor for morbillivirus (Tatsuo et al., 2001).

### ***1.4.3. Nectin-4***

Distribution and functions of SLAM provide a good explanation for the lymphotropism and immunosuppressive nature of *Morbilliviruses*. However, *Morbilliviruses*, in autopsied patients and some experimentally infected animals, have also been shown to infect the epithelial cells of the trachea, bronchial tubes, lungs, oral cavity, pharynx, esophagus, intestines, liver, and bladder (Moss & Griffin, 2012). These epithelial cells do not express SLAM, but the infected cells do shed virus, suggesting that entry into these SLAM-negative cells is mediated by other cellular receptors.

In 2011, two independent groups reported identification of the EpR. Both groups utilized microarray data from susceptible versus non-susceptible cell lines and compared the membrane protein gene transcripts.

Noyce et al. described the susceptibility of many different tumor cell lines to MV infection and selected susceptible and non-susceptible cell lines. They filtered the microarray data for membrane protein genes, and produced a short list of 11 candidate receptors. Of these, only human PVRL4 (nectin-4), a tumor cell marker found on breast, lung, and ovarian carcinomas, rendered cells susceptible to MV infection. Transient knockdown of nectin-4 using siRNA abolished MV infection in these cell lines. Furthermore, antibodies specific for human nectin-4 inhibited MV infection (Noyce et al., 2011).

Mühlebach et al. performed microarray analysis of seven epithelial cell lines from human airways or bladder previously characterized as permissive (three lines) or non-permissive (four lines), and identified that nectin-4 renders CHO cells susceptible to MV. It was demonstrated that the V domain of nectin-4 binds strongly to MV-H (Muhlebach et al., 2011).

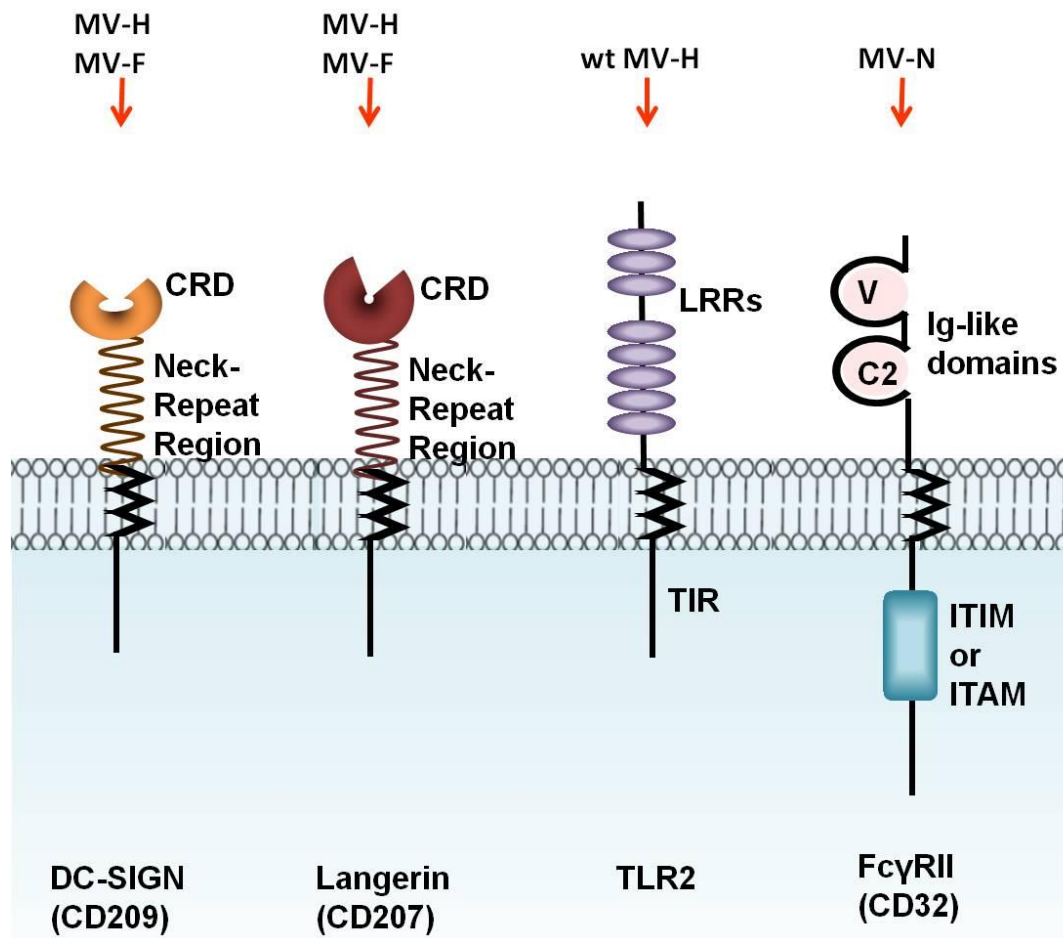
The nectin family is a cell adhesion molecule family comprising four members (nectin-1–4), and only nectin-4 functions as the EpR (Muhlebach et al., 2011; Noyce et al., 2011). Nectins contain immunoglobulin-like domains, similar to SLAM (Fig. 6). The nectin family proteins have recently been shown to be essential contributors to the formation of cell–cell adhesions and are novel regulators of cellular activities, including cell polarization, differentiation, movement, proliferation, and survival (Takai et al., 2008). Nectins are also involved in the establishment of apical–basal polarity at cell–cell adhesion sites and the formation of tight junctions in epithelial cells (Takai et al., 2008).

#### ***1.4.4. C-type lectins***

Although they do not serve as entry receptors, the C-type lectins, DC-SIGN (CD209) and Langerin (CD207) facilitate MV attachment to the myeloid dendritic cells (de Witte et al., 2008) and Langerhans cells (LCs) (van der Vlist et al., 2011) respectively (Fig. 7). DC-SIGN is considered to facilitate attachment to the cell rather than to permit delivery of the RNP into the cytoplasm. Moreover, no mutations in specific amino acids which ablate MV-H glycoprotein binding to DC-SIGN have been reported. DC-SIGN mediated transinfection of lymphocytes by DCs and cell-to-cell spread is an important means by which MV, as a highly cell-associated virus, initiates and perpetuates the infection *in vivo* (de Witte et al., 2008; Rima & Duprex, 2011). Similarly, Langerin mediates both MV strains attachment to Langerhans cells, as virus binding was blocked by anti-Langerin and mannan. However, it does not serve as entry receptor for MV as anti-Langerin did not affect infection of LCs with either MV strain. The interaction with MV is specific for the carbohydrate recognition domain of Langerin (van der Vlist et al., 2011).

#### ***1.4.5. Alternative receptors***

CD150 and nectin-4 account for the lymphotropic and epitheliotropic properties of natural MV. However, the virus is also endotheliotropic and neurotropic. Whether nectin-4 is used to infect endothelial cells and neurons remains to be determined. Previous studies using recombinant *Morbilliviruses* expressing GFP have demonstrated that cell entry independent of SLAM and CD46 (and probably nectin-4) occurs in a variety of cell lines with low infectivity



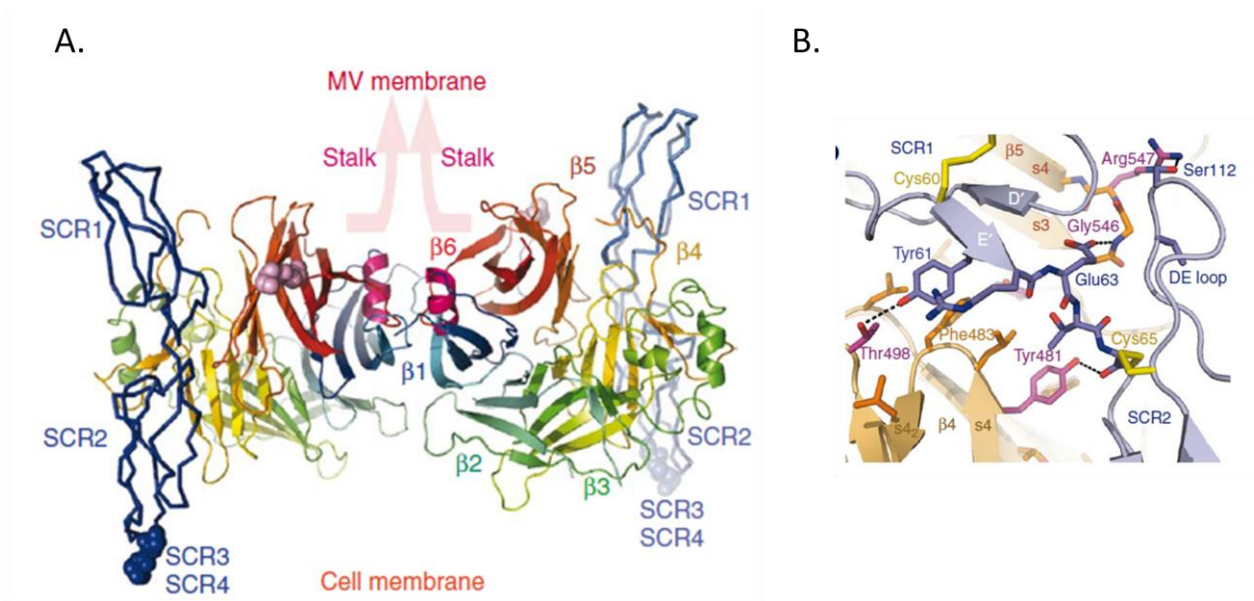
**Figure 7.** Schematic presentation of structure of measles virus receptors (part 2). Cell membrane molecules interacting with MV, but not allowing virus fusion. Four other surface molecules have been found to interact with MV proteins: DC-SIGN, Langerin, TLR2 and FcγRII. DC-SIGN (CD209) consists of carbohydrate recognition domain (CRD) and neck-repeat region in the extracellular region, after that transmembrane and cytoplasmic domains. It interacts with both MV membrane glycoproteins: H and F. Langerin (CD207) and DC-SIGN are highly homologous, share some ligands, but are expressed by different cell types. While DC-SIGN represents a trimer, Langerin is present as tetramer on cell surface. Langerin also has an additional carbohydrate-binding Ca<sup>2+</sup>-independent site in its CRD domain. TLR2 is composed of extracellular multiple leucine-rich repeats (LRRs), transmembrane domain and cytoplasmic region with Toll/IL-1R (TIR) domain and interacts only with wt MV-H. FcγRII (CD32) has two Ig-like domains on N-terminus, followed by transmembrane and cytoplasmic regions. FcγRII receptors represent two subfamilies: activatory FcγRII with immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic tail, and inhibitory FcγRII containing immunoreceptor tyrosine-based inhibitory motif (ITIM) instead of ITAM. In contrast to other described receptors, FcγRII binds measles virus N protein.

(Hashimoto et al., 2002; Terao-Muto et al., 2008). This suggests the existence of inefficient but ubiquitously expressed receptors. It has been suggested that neurokinin-1 (NK-1) may act as MV receptor in neurons although the F glycoprotein rather than the H glycoprotein is bound by NK-1 (Makhortova et al., 2007). Whether this receptor or another facilitates trans-synaptic spread, which has been suggested as a means by which the virus spreads, also remains to be determined (Rima & Duprex, 2011). It was also shown by some studies, that infection with several SLAM (and presumably nectin-4) negative cell lines with *Morbilliviruses* was inhibited by soluble heparin, and that virus bound to immobilized heparin. It suggests that ubiquitously expressed heparin-like glycosaminoglycans are involved in *Morbillivirus* infection (Terao-Muto et al., 2008). More recently it was demonstrated that MV viral particles may incorporate cellular cyclophilin (Cyp)B on their surface and bind to cellular CD147, a receptor for CypA and B, independently of MV-H. This mechanism is similar to the one utilized by HIV (A. Watanabe et al., 2010).

#### ***1.4.6. Interaction of MV-H protein with MV receptors***

Significant efforts have been expended to determine and model the three dimensional structure of the glycoprotein. The H glycoprotein is comprised of 617 aa, has an ectodomain of 559 aa, a transmembrane domain of 24 aa and a cytoplasmic tail of 34 aa (Rima & Duprex, 2006). It is a type II integral membrane glycoprotein with a globular head extending from the viral core on a long stalk. Sequence similarities of hemagglutinins in the *Paramixoviridae* family are as low as 7%. However, a sequence alignment of *Morbilliviruses* indicates that all cysteine residues forming disulphide bonds are well conserved, as are most CD150-binding residues. Residues essential for CD46 binding are conserved only among measles vaccine strains (Colf et al., 2007).

Analysis of the H proteins from different MV strains allows identifying amino acids critical for interaction with particular receptors. The extracellular region of CD46 contains four short consensus repeats, two of which, SCR1 and SCR2, participate in the interaction with the MV-H. The critical MV binding epitope includes two consecutive prolines (Pro38 and Pro39) at the base of SCR1 (Buchholz et al., 1997; Casasnovas et al., 1999; Fig. 8A) The ability to use CD46 as the receptor is linked to the presence of two distinct amino acid substitutions in the MV-H protein: N481Y and S546G (Fig. 8B). However, additional mutations such as N390I, N416D, T446S,



**Figure 8.** Crystal structure of the MV-H protein bound to the CD46 receptor. **A.** Dimeric MV-H-CD46 complex. **B.** Interactions of MV-H with the CD46 SCR1-SCR2 interdomain region (Santiago et al., 2010).

T484N and E492G also increase the viral binding affinity for CD46 (Tahara et al., 2007). MV-H binds to the CD46 receptor via a groove at the side of the  $\beta$ -propeller domain. Earlier studies have shown that a proline in the D'D loop of SCR1 is a key player in this interaction, and the crystal structure of the MV-H in complex with CD46 demonstrates that this residue is part of a hydrophobic plug that inserts into a socket at one end of the MV-H groove. The tyrosine side chain introduced by the N481Y mutation makes precise contacts with the SCR1-SCR2 interdomain interface. The S546G mutation likely results in added flexibility to the  $\beta$ 5s3- $\beta$ 5s4 region, required for CD46 binding (Santiago et al., 2010).

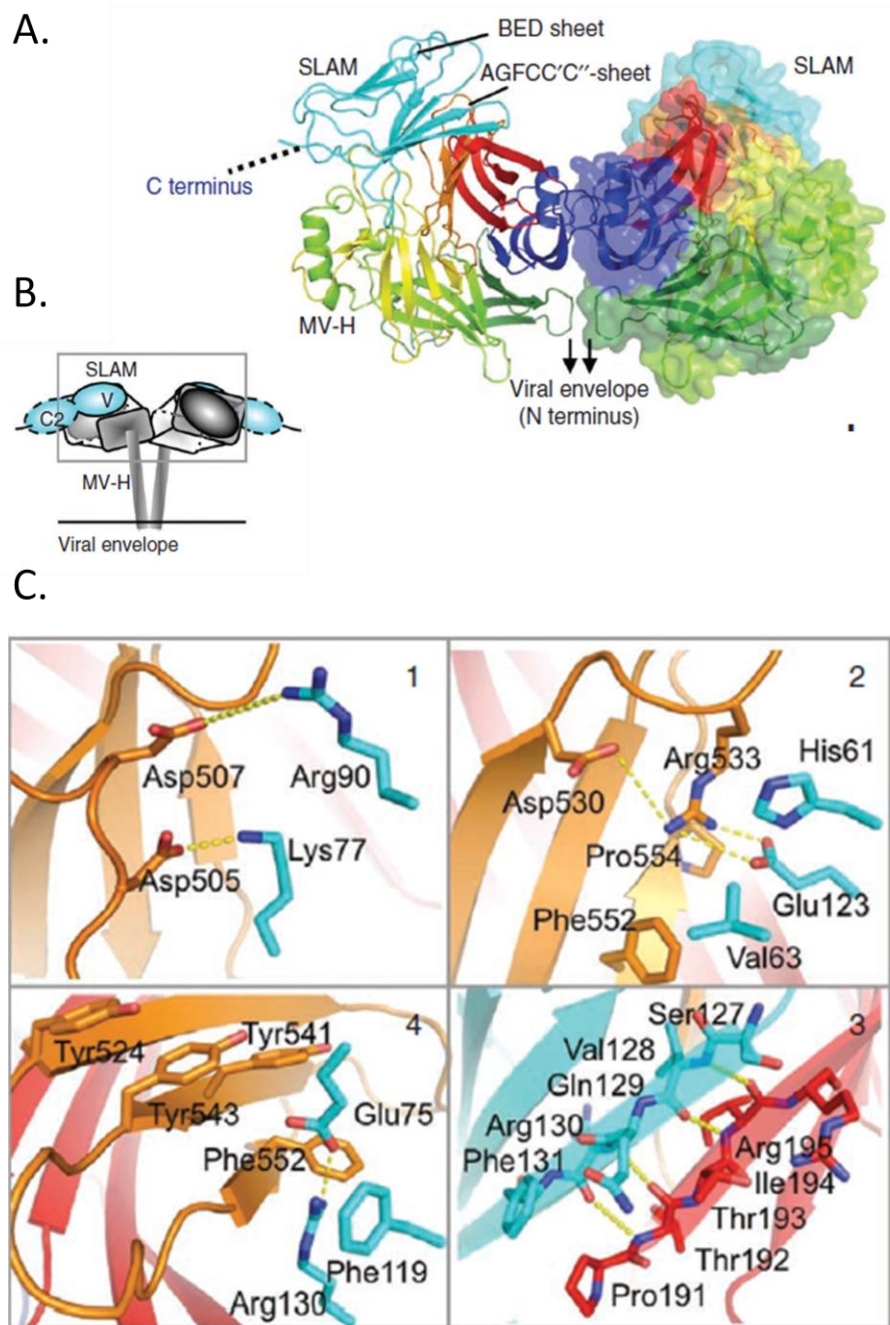
The inability to fully engage with the CD150 receptor led to the single-step attenuation of the wild-type virus. This reduction in virulence could be due to fewer CD150-positive infected, a restriction in cell-to-cell spread or due the virus having to bind to another receptor initiating the infection in a non-canonical manner (Rima & Duprex, 2011).

All of the vaccine strains belonging to the Edmonston lineage have either or both Y481 and G546 in the H protein, presumably accounting for their ability to use CD46 as a receptor. Considering the high mutation rates of RNA viruses and the ubiquitous expression of CD46, CD46-using viruses containing a single N481Y or S546G substitution in the H protein may well be generated and selected readily *in vivo*. Interestingly, that despite the high mutation rates of RNA viruses and the ubiquitous expression of CD46 receptor, CD46-using viruses containing a single N481Y or S546G substitution in the H protein, are not detected (Yanagi, Takeda, & Ohno, 2006). One proposed explanation for the lack of CD46-using viruses *in vivo* is that such viruses would downregulate CD46 from infected cells, which would then be subject to complement-mediated lysis and elimination (Schnorr et al., 1995). Another possibility is that CD46-using viruses may induce much higher levels of IFN- $\alpha/\beta$  in infected cells than viruses using SLAM only (Naniche et al., 2000).

Meanwhile, mutagenesis of the H protein based on its ability to induce SLAM-dependent cell-cell fusion has revealed that residues important for interaction with SLAM are Ile194, Asp505, Asp507, Asp530, Arg533, Phe552 and Pro554 (Masse et al., 2004; Navaratnarajah et al., 2008; Vongpunsawad et al., 2004). Three other residues of MV-H (Phe483, Tyr541 and Tyr543) are essential for infection of polarized epithelial cells, but not SLAM-positive immune cells. These residues occur in site 4 in the crystal structure and do interact with SLAM, but on the basis of the mutagenesis data their contribution is probably not critical (Leonard et al., 2008; Fig. 9).

All receptors and most neutralizing antibodies bind to overlapping or closely located regions on the limited exposed surface of MV-H. This may explain why a single antibody can inhibit measles virus infection mediated by different receptors (Hashiguchi et al., 2011).





**Figure 9.** Overall structure of the MV-H-SLAM complex. **A.** Side view of the MV-H and marmoset SLAM-V complex. **B.** Schematic of the homodimer of the MV-H-SLAM complex on the viral envelope. **C.** Detailed views of the interaction between MV-H and SLAM (Hashiguchi et al., 2010).

## 1.5. MV TRANSMISSION MODE IN INFECTED BODY

To follow the root of measles virus in host infected body, macaques, which display many of the symptoms of the human infection and become systemically infected following infection via the respiratory route, were infected with rMV KS EGFP(1) (a rMV which expresses EGFP from the promoter proximal position of the genome, position 1 (1)). This infection resulted in the observation that alveolar macrophages and DCs in the lower respiratory tract were the primary cells targeted early in the infection. They were the “vehicles” by which the infection was seeded in the deep lung. Shortly after infection of these cells MV replicated in localised areas of bronchial associated lymphoid tissue (BALT) where the close spatial proximity of CD150 positive T-, B- and myeloid cells amplified the infection.

Thereafter the virus spread first to the tracheobronchial lymph node prior to dissemination throughout the body. According to some *in vitro* study which shows that it is not possible to infect differentiated ciliated human bronchial epithelial cells with wild-type MV strains from the apical surface and extremely difficult to infect *ex vivo*-cultured human corneal rims, other than at side of the tissue where the integrity was compromised during surgical removal (Ludlow et al., 2010).

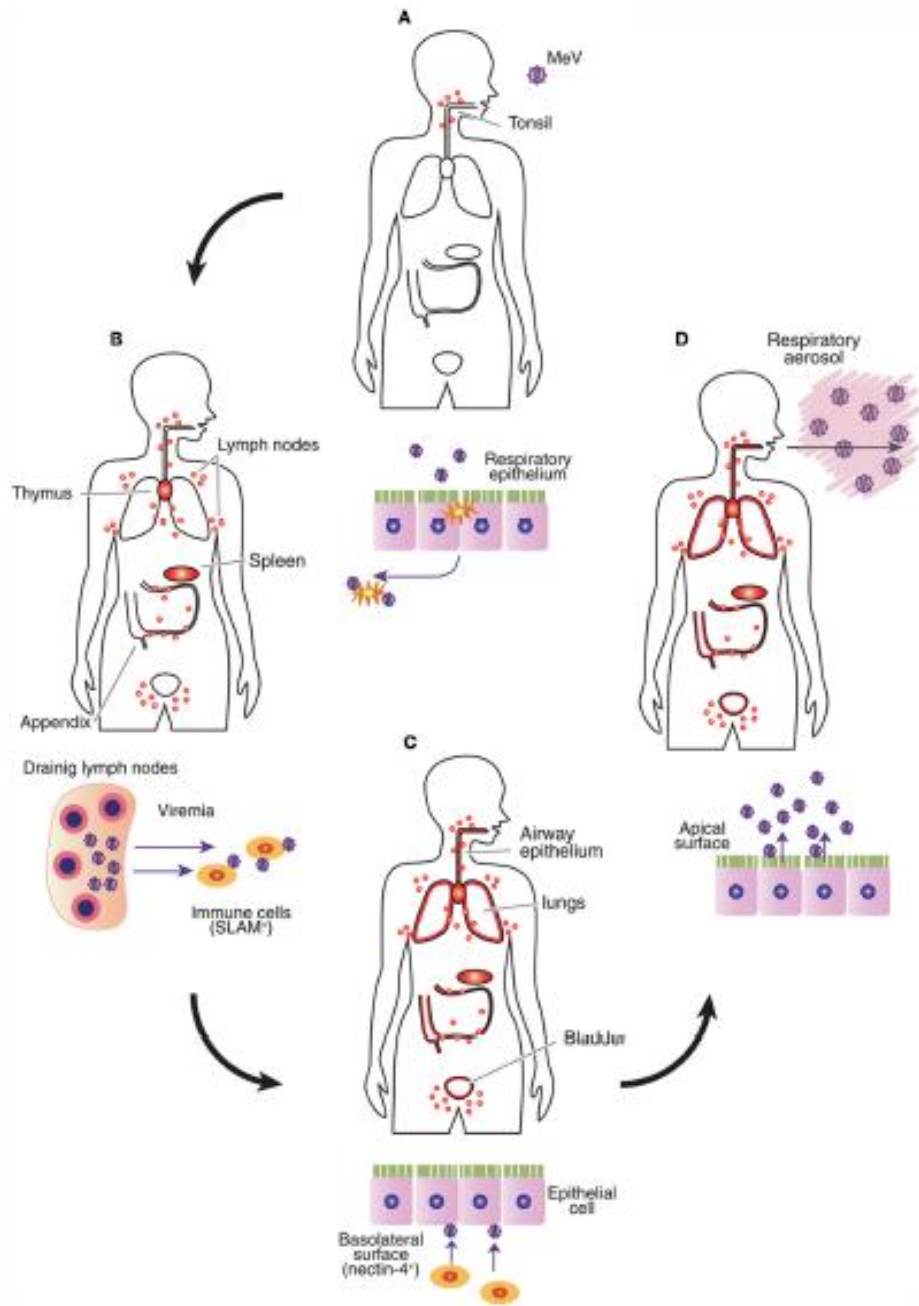
However, the virus efficiently entered and replicated in the differentiated ciliated human bronchial epithelial cells when applied basolaterally or if the cell sheets were mechanically disrupted, which might be consistent with entry using a specific epithelial receptor. Identification of mutations in the H glycoprotein which abolished binding to the receptor, generation of rMVs expressing the modified glycoprotein and infection of macaques demonstrated that the wild-type, non-epithelial receptor binding virus could still cause a systemic infection but was suggested no longer to be shed from the respiratory epithelium (Leonard et al., 2008). This suggests that there is a receptor for MV, different to SLAM and CD46 that is critical for exit of the virus and therefore vital for MV transmission. In the absence of its identification it has been termed “EpR” (Rima & Duprex, 2011).

Recent identification of nectin-4, which is present in the adherent junctions of epithelial cells below the tight junctions, as the epithelial receptor finally completes the MV pathogenesis jigsaw puzzle (Muhlebach et al., 2011; Noyce et al., 2011; Rima & Duprex, 2011).

Measles virus is spread from person to person by the respiratory route. Virus-laden droplets produced by sneezing or coughing enter the respiratory tract (Fig. 10A). There the virus multiplies, migrates to local lymph nodes, and multiplies again, leading to a viremia, the presence of virus in

the blood (Fig. 10B). The virus then replicates at many other sites, including lymphoid organs (spleen, lymph nodes), skin, kidney, lung, and liver. The characteristic rash of measles is produced by viral replication in cells of the skin. Transmission of measles virus by respiratory secretions requires that the virus replicate in cells of the respiratory tract. Early models of measles pathogenesis hypothesized that virus enters the apical domain of respiratory epithelial cells. However, in laboratory animals, epithelial cells of the respiratory tract do not become infected until late in disease. Instead, measles virus initially replicates in cells of the immune system, including alveolar macrophages, dendritic cells, and T lymphocytes. These infected cells carry the virus from the respiratory tract to local lymphatic tissues (Racaniello, 2011). Infected lymphocytes travel to the respiratory tract and transfer virus to the epithelium, where it binds nectin-4 and commences a replication cycle (Fig. 10C). Newly synthesized virus particles are released into the airway, where they are spread by aerosol droplets produced by sneezing and coughing (Fig. 10D). This mechanism of host exit is consistent with the high infectivity of measles virus-containing aerosols (Racaniello, 2011). But such model for measles virus pathogenesis leads to a question: if measles virus is carried into the host by immune cells, how does the virus get back out—into the airways of the lung—so it can be transmitted to others? One idea is that later in infection, infected lymphocytes carry virus to the basal side of the respiratory epithelium. After replication in epithelial cells, the virus is released into the airways at the apical domain, facilitating transmission (Racaniello, 2011).

Viral spread in immune cells followed by their exit through epithelial cells may not be unique to measles infection. A similar pathway is observed during infections by other viruses, including varicella-zoster virus and vaccinia virus. An aspect of measles virus pathogenesis not addressed by these findings is how infected lymphocytes deliver virus to the basal domain of the respiratory epithelium. Is free virus passed from cell to cell, or its transfer is mediated by cell-cell contact, and if so, what membrane proteins are involved? (Racaniello, 2011)



**Figure 10.** Major transmission mode of MV in infected body (A-D explanations see in the text) (Sato et al., 2012).

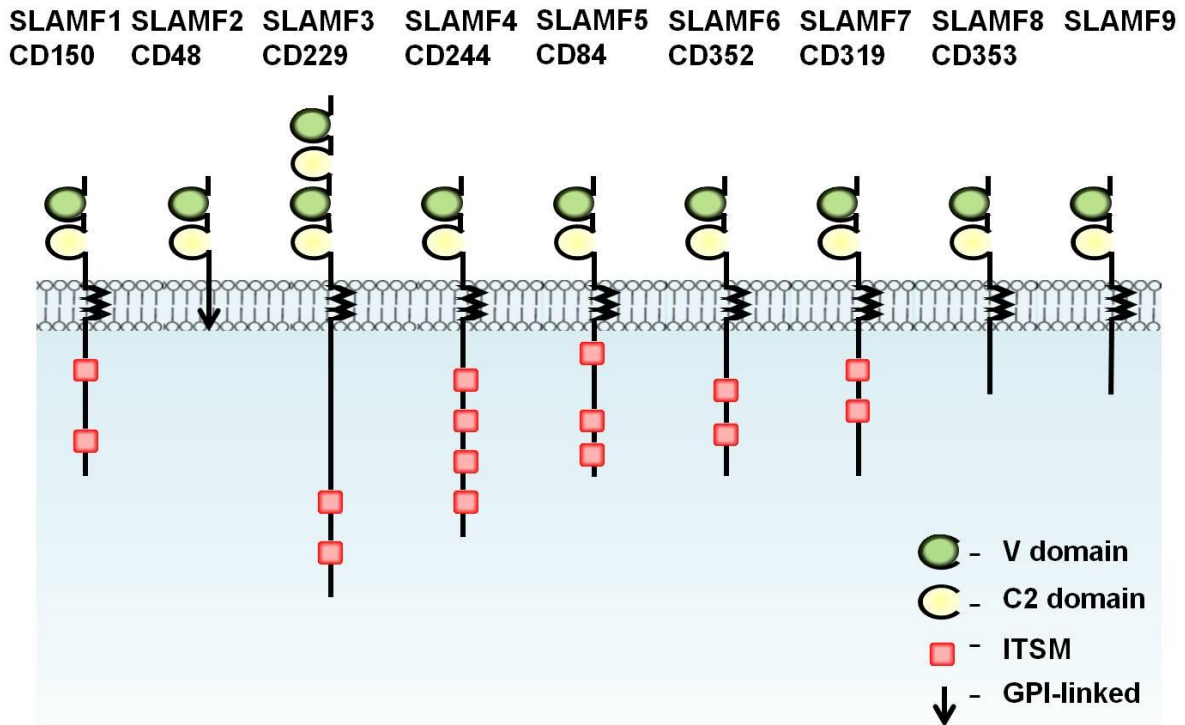
## **2. CD150 RECEPTOR**

### **2.1. CD150 STRUCTURE AND EXPRESSION**

The CD150 cell surface receptor was first described as ‘IPO-3 antigen’ (Pinchouk et al., 1988; Pinchouk et al., 1986; Sidorenko & Clark, 1993) on activated human lymphocytes. Its preferential expression on cells of hematopoietic tissues, biochemical and functional features have been characterized (Sidorenko & Clark, 1993). In 1995 the cDNA for this receptor was cloned under the name signaling lymphocyte activation molecule (SLAM) (Cocks et al., 1995). The monoclonal antibody IPO-3 was submitted for the international collaborative studies in the frame of 5-7 workshops on Human Leukocyte Differentiation Antigens (HLDA). In 1996 on the 6th workshop on HLDA (Kobe, Japan) the IPO-3 antigen received international nomenclature CDw150 (Sidorenko, 1997) that was transformed into CD150 at the 7th HLDA workshop (Harrogate, Great Britain, 2000) (Sidorenko, 2002).

CD150 (IPO-3/SLAM F1) is a member of SLAM family within the immunoglobulin superfamily of surface receptors. According to the nomenclature accepted by 9th international Workshop on Human Leukocyte Differentiation Antigens (Barcelona, 2010) SLAM family includes nine members: SLAMF1 (CD150 or IPO-3, SLAM), SLAMF2 (CD48 or BCM1, Blast-1, OX-45), SLAMF3 (CD229 or Ly9), SLAMF4 (CD244 or 2B4, NAIL), SLAMF5 (CD84 or Ly9B), SLAMF6 (CD352 or NTB-A, SF2000, Ly108), SLAMF7 (CD319 or CRACC, CS1, 19A24), SLAMF8 (CD353 or BLAME) and SLAMF9 (SF2001 or CD84-H1) (Fig. 11). The characteristic signature of CD150 family is within the extracellular region: an N-terminal variable (V) Immunoglobulin (Ig) domain lacking disulfide bonds is followed by a truncated Ig constant 2 (C2) domain with two intradomain disulfide bonds (Fig. 2A). The genes of the SLAM family are clustered close together on the long arm of chromosome 1 at bands 1q21-24 (Sidorenko & Clark, 2003). The gene structure for all CD150/SLAM family receptors is similar with the signal peptides and each of the Ig domains encoded by separate exons. All these receptors are type I transmembrane proteins with the exception of CD48 (Cannons et al., 2011).

CD150 is a single chain transmembrane phosphoglycoprotein of 75-95 kDa in size with a 42 kDa protein core. It is heavily N-glycosylated with terminal sialic acid residues on oligosaccharide chains. Moreover, it was shown that this receptor is associated with tyrosine (Y)



**Figure 11.** SLAM family receptors. SLAM family consists of 9 members. The common feature of these receptors is the structure of extracellular region that consists of variable Ig-like domain lacking disulfide bonds (shown in green), which is followed by truncated Ig constant C2 domain (in yellow). Moreover, 6 of 9 SLAM family receptors contain 2 or 4 ITSM motifs in their cytoplasmic regions (red rectangles).

and serine/threonine (S/T) kinases and is phosphorylated at tyrosine and serine residues (Sidorenko & Clark, 1993).

In addition to transmembrane form of CD150 (mCD150), activated T and B lymphocytes, CD40 ligand-activated dendritic cells and also lymphoblastoid and Hodgkin's lymphoma cell lines express mRNA encoding secreted form of CD150 (sCD150) that lacks 30 amino acids – the entire transmembrane region (TM), mRNAs for cytoplasmic form (cCD150) lacking the leader sequence, and a variant membrane CD150 (vmCD150) with truncated cytoplasmic tail (CY). However, expression of vmCD150 (tCD150) isoform was not confirmed at the protein level (Bleharski et al., 2001; Cocks et al., 1995; Punnonen et al., 1997; Sidorenko & Clark, 2003; Yurchenko et al., 2005).

CD150 receptor is mainly expressed within hematopoietic cell lineage. Its expression was detected on immature thymocytes, activated and memory T cells, CD4<sup>+</sup> T cells with much higher expression level on Th1 cells, CD4<sup>+</sup>CD25<sup>+</sup> regulatory and follicular helper T cells, peripheral blood and tonsillar B cells, mature dendritic cells and macrophages. The level of CD150 expression on subpopulations of tonsillar B cells differs with its maximum levels on plasma cells. The activation of T and B cells leads to the rapid upregulation of CD150 expression. Low level of CD150 expression was also found on NKT cells, platelets and eosinophiles. The expression of CD150 on peripheral blood monocytes could be induced by mitogen and cytokines stimulation, and also by measles virus particles (Browning et al., 2004; Cocks et al., 1995; Kruse et al., 2001; Minagawa et al., 2001; Sidorenko & Clark, 1993, 2003; Veillette et al., 2007; Yurchenko et al., 2010). CD150 expression is a distinguishing feature of hematopoietic stem cells (HSC) in mice (Hock, 2010). CD150 was also found on malignant cells of leukemias and lymphomas mainly with an activated cell phenotype (Mikhalap et al., 2004).

## 2.2 CD150 LIGANDS

With the exception of CD48 and CD244/2B4 (receptor-ligand pair), all SLAM family receptors are self-ligands. Intriguingly, there is a marked variation in the apparent affinity of these self-associations ( $K_d=0.5\text{--}200\ \mu\text{M}$ ), suggesting that the various members of the CD150 family may modulate immune cell functions to a different extent. Crystallographic determination of the structures of SLAM family members homodimers indicated that self-association is mediated by the N-terminal variable (V)-type Ig-like (IgV) domain, which enables head-to-head contact between two monomers (Mavaddat et al., 2000; Sintès & Engel, 2011; Veillette et al., 2007). CD150 binds to itself with low affinity ( $K_d\gg 200\ \mu\text{M}$ ). Although the affinity of this receptor is low, the binding avidity increases due to the redistribution or clustering of molecules after cell activation. It is probable that the binding is affected by other receptor–ligand interactions that help to bring the membranes into proximity and induce changes in the lateral mobility of receptors (Engel et al., 2003; Mavaddat et al., 2000).

CD150 was found to be the major receptor for several Morbilliviruses (Tatsuo & Yanagi, 2002). In case of MV the role of attachment protein is played by hemagglutinin (MV-H), which directly binds the cellular receptors of the virus (CD150, CD46 and nectin-4), mediating virus entry into the cell (Fig. 6). Human CD150 (hCD150) was reported as a cellular receptor for wild type (wt) measles virus (Tatsuo et al., 2000); attenuated strains were shown to use either CD150 or CD46 as a receptor (Dorig et al., 1993; Naniche et al., 1993), and nectin-4 is used by wt MV to entry epithelial cells of respiratory tract at the end of the acute phase of infection (Muhlebach et al., 2011; Noyce et al., 2011; Racaniello, 2011). The hCD150 V domain is necessary and sufficient for its function as a MV receptor; however, the other regions of hCD150, including the C2 domain, TM, and CY are not required for this function (Ono et al., 2001). Histidine (H) at position 61 appears to be critical for CD150 to act as a receptor for MV. Isoleucine (I) at position 60, Valine (V) 63, lysine (K) at position 58 and S59 also appear to contribute to receptor function of hCD150 (Ohno et al., 2003; Xu et al., 2006). The mutagenesis of the H protein based on its ability to induce SLAM-dependent cell–cell fusion has revealed that residues important for interaction with SLAM are I194, D505, D507, D530, R533, F552 and P554 (Masse et al., 2004; Navaratnarajah et al., 2008; Vongpunsawad et al., 2004).

CD150 receptors from human, mouse, dog, cow and marmoset have about 60–70% identity at the amino acid level, except human and marmoset CD150, which have 86% identity. The sequences at positions 58–67 are well conserved among these species, especially I60 and H61 which are conserved in human, marmoset, dog and cow CD150 (Ohno et al., 2003). Mouse CD150



has functional and structural similarity to human CD150 (60% identity at the amino acid level), but it cannot act as a receptor for MV, partly explaining why mice are not susceptible to MV. It has been proposed that amino acid substitutions at the positions 60, 61, 63 to human-type residues may make mouse CD150 act as an MV receptor, as introduction of changes at these positions compromises the receptor function of human CD150., while introduction of changes at these positions compromises the receptor function of human CD150 (Yanagi et al., 2009). CDV and RPV use dog and bovine CD150 respectively as cellular receptors. Furthermore, it was found that MV, CDV and RPV can use CD150 of non-host species as receptors but with lower efficiency (Yanagi et al., 2009).

CD150 is not only a self ligand and a receptor for *Morbilliviruses*, but also a bacterial sensor that regulates intracellular enzyme activities involved in the removal of Gram-negative bacteria (Berger et al., 2010; Sintes & Engel, 2011).

### 2.3 CD150-MEDIATED SIGNALING

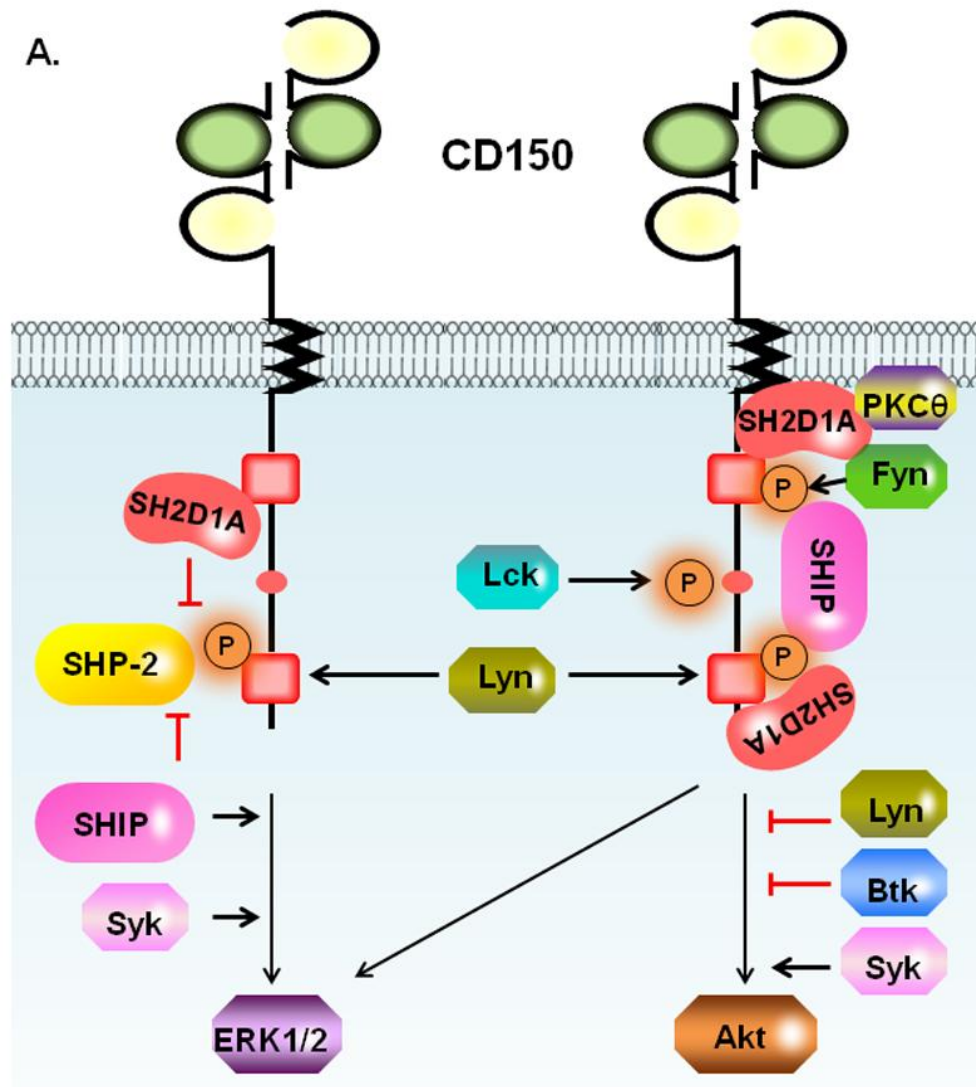
CD150 receptor functions in cells are mediated by its signaling properties. All available information regarding signaling via CD150 is concerning mCD150 isoform while nothing is known about signaling properties of other CD150 isoforms.

Engagement of CD150 on cell membrane results in numerous intracellular signaling events. CD150-mediated signals can be divided into receptor-proximal, including immediate binding partners (Fig. 12), and distal – signal transduction pathways (Fig. 13). CD150 receptor interacts with intracellular partners through its cytoplasmic tail (CD150ct), specifically via three key tyrosine residues which could be phosphorylated: Y281, Y307 and Y327 (Y288, Y315 and Y335 in mice respectively). The first and the last tyrosine residues form the core for the unique immunoreceptor tyrosine-based switch motifs (ITSMs) (Shlapatska et al., 2001; Fig. 6). Six members of SLAM family receptors contain 1 to 4 ITSMs. The cytoplasmic tail of CD150 has 2 ITSMs. Upon the tyrosine phosphorylation by Src family kinases ITSMs bind key SH2-containing components of signal transduction pathways, including the adaptor proteins SH2D1A, SH2D1B (EAT-2) and SH2D1C (ERT) (Shlapatska et al., 2001; Sidorenko & Clark, 2003).

Upon antigen receptor cross-linking the cytoplasmic tail of CD150 becomes phosphorylated, while after CD150 ligation with the IPO-3 antibodies – the opposite effect is observed (Mikhalap et al., 1999). It was suggested that A12 antibody is antagonistic, while the outcome of IPO-3 cross-linking is similar to the effect of hemophilic stimulation with CD150 recombinant protein (Punnonen et al., 1997; Veillette, 2004). Several Src-family kinases, namely Lck phosphorylates Y307, Lyn – Y327 and Fyn – Y281, were found to be able to phosphorylate key tyrosine residues of CD150 (Howie et al., 2002; Mikhalap et al., 2004; Fig. 12).

The signaling events downstream of CD150 could be divided on those that are dependent on CD150-SH2D1A interaction and those which are not. SH2D1A binds Y281 of CD150 cytoplasmic tail independently of phosphorylation, does not bind Y307, and binds Y327 only after phosphorylation (Howie et al., 2002). The SH2D1A interaction with Y281 of SLAM is mediated via R32 of SH2D1A (Sayos et al., 1998).

The coupling of CD150 to signal transduction pathways is provided by two SH2D1A functions: recruitment of enzymes or, alternatively, blocking binding of SH2-containing proteins. SH2D1A SH2 domain binds to the SH3 domain of FynT and indirectly couples FynT to CD150.



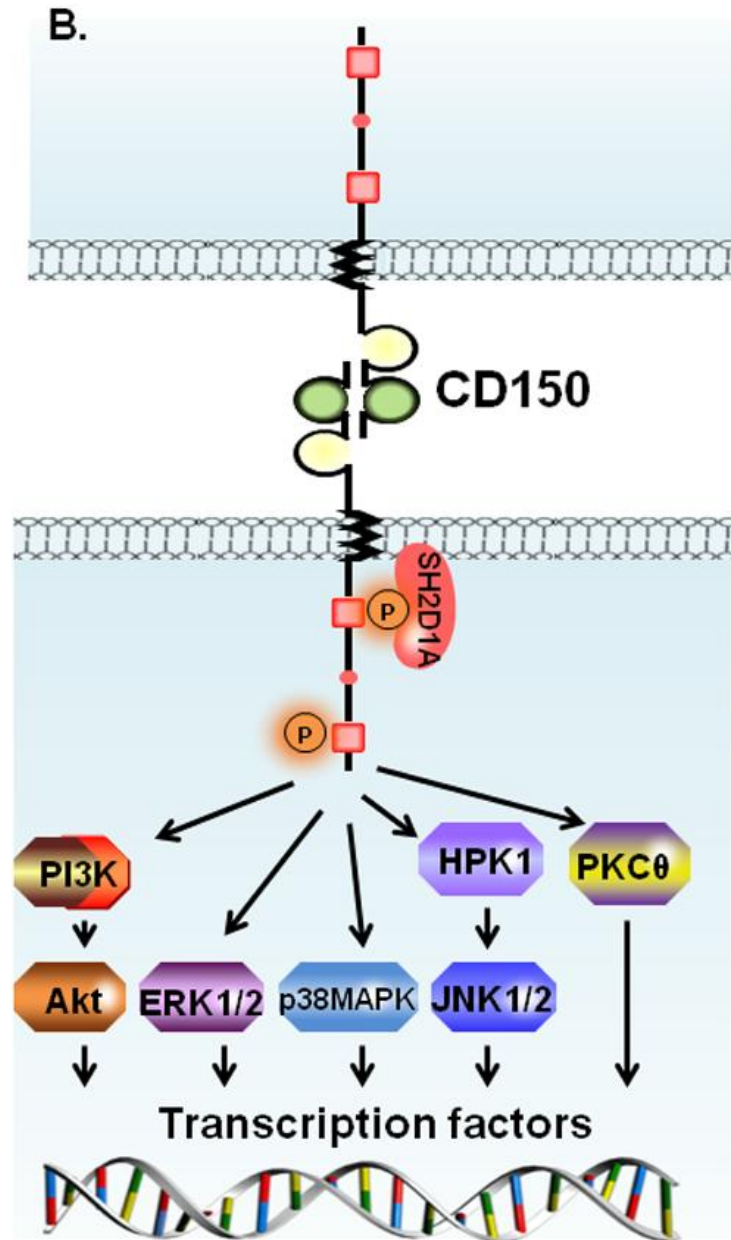
**Figure 12.** CD150-mediated signaling (part 1). Receptor-proximal signaling events triggered by CD150 ligation could be divided on those which are dependent on CD150-SH2D1A interaction and those which are not. Upon ligation, three tyrosine residues in cytoplasmic tail could be phosphorylated. Lck, Lyn and Fyn kinases were shown to be able to do it. Afterward several SH2- and SH3-containing molecules were shown to be able to bind to these tyrosines either via SH2D1A (Fyn, SHIP, PKCθ) or not (SHP-2). CD150 ligation mediates the activation of Akt signaling pathway (requires Syk and SH2D1A and is negatively regulated by Lyn and Btk) and ERK signaling pathway (requires SHIP and Syk, but not SH2D1A).

The crystal structure of a ternary CD150-SH2D1A-Fyn-SH3 complex reveals that SH2D1A SH2 domain binds the FynT SH3 domain through a R78-based motif which does not involve canonical SH3 or SH2 binding interactions (Chan et al., 2003; Latour et al., 2001; Latour et al., 2003). The observed mode of binding to the Fyn-SH3 domain is expected to preclude the auto-inhibited conformation of Fyn, thereby promoting activation of the kinase after recruitment (Chan et al., 2003). SHIP also is recruited in CD150-SH2D1A signaling complex via binding of its SH2 domain to phosphorylated Y315 and/or Y335 of mouse CD150 and appears to be the intermediate molecule that links CD150-SH2D1A to Dok-related adaptors and RasGAP (Latour et al., 2001). This signaling cascade is dependent on the generation of a ternary CD150-SH2D1A-Fyn complex for CD150 phosphorylation. The presence of SH2D1A facilitates binding of SHIP to CD150 (Latour et al., 2001; Shlapatska et al., 2001). Although SHIP possesses only one SH2 domain, both Y281 and Y327 in CD150 are important for its association with SHIP (Shlapatska et al., 2001).

R78 residue of SH2D1A is also critical for its constitutive association with PKC $\theta$  in T cells. This SH2D1A-PKC $\theta$  interaction is Fyn independent. Moreover, CD150 engagement increases TCR-induced PKC $\theta$  recruitment to the site of T cell stimulation (Cannons et al., 2010). As PKC $\theta$  could be detected as part of the CD150-SH2D1A complex following TCR ligation, CD150 engagement can facilitate PKC $\theta$  recruitment, nuclear p50 NF- $\kappa$ B levels, and IL-4 production via a ternary CD150-SH2D1A-PKC $\theta$  complex (Cannons et al., 2004; Cannons et al., 2010).

SH2D1A is capable of not only bridging the interaction of FynT, PKC $\theta$  or SHIP with CD150 but also inhibiting the binding of SHP-2 to phosphorylated CD150 (Sayos et al., 1998; Shlapatska et al., 2001). SH2 domains of SHIP and SHP-2 bind to the same sites in the receptors of SLAM family as does SH2D1A strongly suggesting that these three proteins are competing binding partners. Apparently, displacement of SHP-2 by SH2D1A makes Y281 and Y327 available for binding by SHIP, which also requires Y281 and Y327 (Liet al., 2003; Shlapatska et al., 2001).

CD150 also associates with CD45 in human B lymphoblastoid cell lines MP-1 and CESS that express SH2D1A, however it is not clear whether CD150-CD45 association is direct, or is mediated via SH2D1A (Mikhalap et al., 1999). It should be noted that in SH2D1A negative B cell lines CD150 is strongly associated with SHP-2 that binds only tyrosine phosphorylated



**Figure 13.** CD150-mediated signaling (part 2). Receptor-distal signaling events after CD150 ligation: activation of Akt, ERK1/2, JNK1/2, p38MAPK signaling pathways that lead to the activation of transcription factors, e.g. FoxO1, c-Jun and NF- $\kappa$ B.

ITSMs (Shlapatska et al., 2001). Since BCR engagement induces CD150 tyrosine phosphorylation (Mikhalap et al., 1999) it could be that activation of B cells is required for SHP-2 binding to CD150. In B cells CD150 is associated with Src-family kinases Fgr and Lyn (Mikhalap et al., 1999), however only Lyn is able to phosphorylate CD150 in vitro (Mikhalap et al., 2004).

CD150-binding partners link it to several signal transduction pathways, among them are MAPK and Akt/PKB signaling networks (Fig. 13). In T and B cells CD150 crosslinking alone results in an increase in serine phosphorylation of Akt/PKB on S473, which is the hallmark of kinase activation state (Mikhalap et al., 1999; Howie et al., 2002). CD150-mediated Akt phosphorylation requires Syk and SH2D1A, is negatively regulated by Lyn and Btk, but is SHIP independent as was shown in the model of DT40 chicken knockout cell sublines transfected with SH2D1A and/or CD150 (Mikhalap et al. 2004). In B cells CD150-induced ERK phosphorylation requires SHIP and Syk but not SH2D1A. Lyn, Btk, and SHP-2 appear to be involved in the regulation of constitutive levels of ERK1/2-phosphorylation. These data allow proposing of the hypothesis that CD150 and SH2D1A are co-expressed during a narrow window of B-cell maturation and SH2D1A may be involved in regulation of B-cell differentiation via switching the CD150-mediated signaling pathways (Mikhalap et al., 2004; Fig. 12).

It was also shown that Akt kinase could be phosphorylated via CD150 both in naïve and activated primary tonsillar B cells. CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction followed by phosphorylation of CD150<sup>ct</sup> and attraction of p85 $\alpha$  regulatory subunit of phosphatidylinositol-3-kinase (PI3K). As was demonstrated by surface plasmon resonance, p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150<sup>ct</sup>, and this interaction takes place in normal tonsillar B cells and lymphoblastoid cell line MP-1. One of the downstream targets of Akt kinase in B cells is transcription factor FoxO1. Despite the high basal level of pFoxO1, CD150 mediates FoxO1 phosphorylation in normal tonsillar B cells and MP-1 cell line (Yurchenko et al., 2011).

In primary tonsillar B cells and Hodgkin's lymphoma cell lines CD150 signaling not only induces the activation of ERK1/2 kinases but also regulates the activation of two other families of MAPK kinases – p38 MAPK and JNK1/2. CD150 mediates the activation of JNK1/2 p54 and JNK2- $\gamma$  isoforms in all tested human B cells. Furthermore, the CD150-mediated JNK activation is significantly enhanced after HPK1 overexpression and HPK1 is co-precipitated with CD150, suggesting that HPK1 is downstream of CD150 in this signaling pathway (Yurchenko et al., 2010).

All these facts demonstrate that signaling properties of CD150 depend on the cell type and availability of other components of signal transduction networks. However, it is clear that downstream of CD150-mediated signaling pathways are transcription factors that regulate cell biology and could modulate immune response.

## 2.4 CD150 FUNCTIONS

Several models were used to study CD150 functions: ligation of CD150 with monoclonal antibodies or CD150 recombinant protein in human and murine in vitro system and CD150 knockout mice, where the expression of murine CD150 was genetically deleted. These experimental approaches revealed numerous roles of CD150 in the functions of different immune cell subsets.

### 2.4.1 CD150 functions in lymphocytes

CD150 is involved in the regulation of interferon-gamma (IFN- $\gamma$ ) response. Initial studies showed that the engagement of human CD150 with mAb A12 enhances the antigen-specific proliferation, cytokine production by CD4<sup>+</sup> T cells, particularly IFN- $\gamma$ , and direct the proliferating cells to a T0/Th1 phenotype (Aversa et al., 1997; Cocks et al., 1995). Similarly, antibodies against mouse CD150 also enhanced IFN- $\gamma$  production (Howie et al., 2002; Castro et al., 1999). However, it seems that some anti-CD150 antibodies, including A12 mAb, are antagonistic, since CD150 ligation on T cells inhibits IFN- $\gamma$  production rather than stimulate it. It was shown that expression of full-length CD150 and SH2D1A in BI-141 T cell transfectants, activated with anti-CD3, abolished the production of IFN- $\gamma$  (Latour et al., 2001). In addition, homophilic CD150-CD150 interactions between T cells and artificial CD150 expressing APC (antigen-presenting cell) line reduced IFN- $\gamma$  expression (Cannons et al., 2004, Fig. 14). It was also shown that SH2D1A deficient T cells produce more IFN- $\gamma$  in response to CD3 triggering (Cannons et al., 2004; Howie et al., 2002).

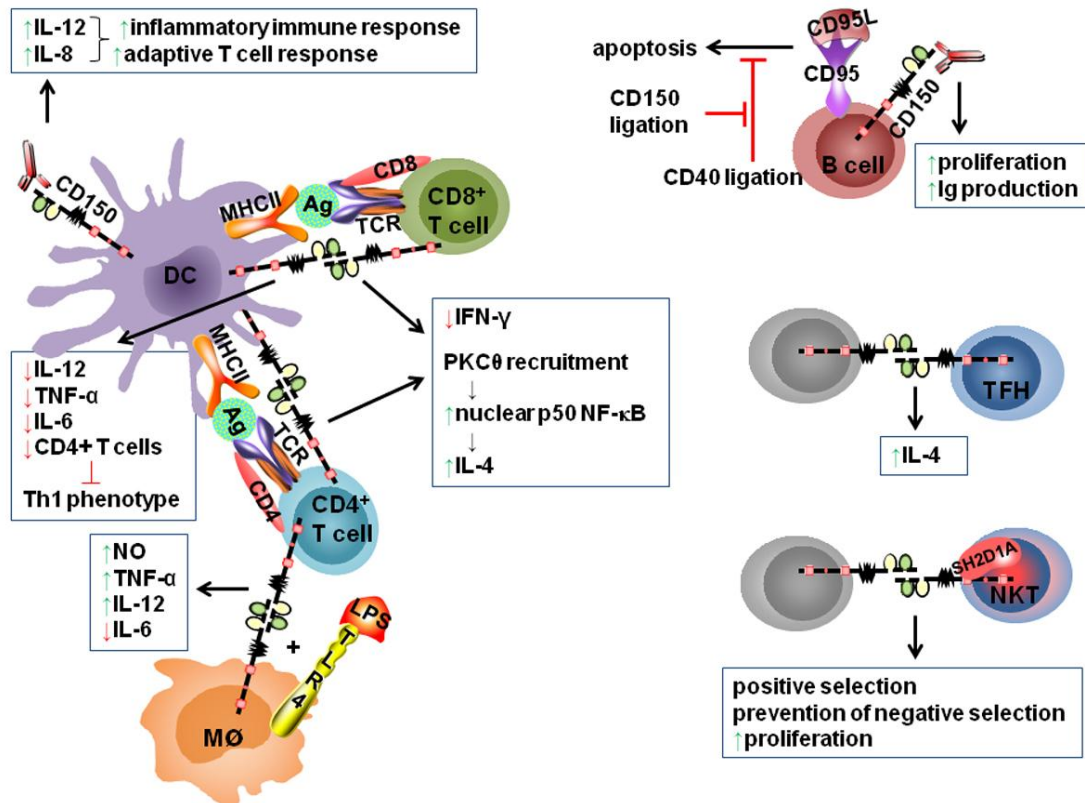
Studies of CD150-knockout mice revealed an important role for CD150 in regulation of Th2 development. In CD150-deficient CD4<sup>+</sup> T cells a significant decrease in TCR-mediated IL-4 production and slight upregulation in IFN- $\gamma$  secretion was observed (Wang et al., 2004). Moreover, CD150 receptor positively regulates TCR-induced IL-4 production by naive and activated CD4<sup>+</sup> T cells, but negatively regulates IFN- $\gamma$  secretion by CD8<sup>+</sup> and to a lesser extent by CD4<sup>+</sup> cells (Wang et al., 2004). SH2D1A expression may be a limiting factor coordinating the TCR and CD150-mediated signaling pathways contributing to IL-4 regulation, as overexpression of SH2D1A potentiates the effects of CD150 on IL-4 production (Cannons et al., 2010).

CD150 is also specifically required for IL-4 production by germinal center (GC) T follicular helper (TFH) cells, CD4<sup>+</sup> T cells, specialized in B cell help. On the model of CD150 knockout mice it was shown, that for optimal help to B cells TFH cells require CD150 signaling to produce IL-4 (Yusuf et al., 2010; Fig. 14).



Studies with knockout mice revealed that CD150-SH2D1A mediated signaling may contribute to some functions of innate-like lymphocytes (Griewank et al., 2007; Jordan et al., 2007; Nichols et al., 2005; Veillette et al., 2007). Innate-like lymphocytes are subsets of lymphocytes which, when activated, exhibit faster and more robust effector functions. The prototypical innate-like lymphocytes are the NKT cells (Godfrey & Berzins, 2007; Veillette et al., 2007). It was demonstrated that SH2D1A-driven signals may be necessary for the positive selection, proliferation, and/or prevention of negative selection of immature NKT cells. One possibility is that SH2D1A-mediated signals are triggered by homotypic interactions between SLAM family receptors expressed on double-positive thymocytes. CD150 and Ly108 seem to be the predominant SLAM family receptors triggering the function of SH2D1A during NKT cell development (Griewank et al., 2007). Another indication of CD150 role in NKT differentiation comes from the characterization of the *Nkt1* locus on mouse chromosome 1. The differential expression of *Slamf1* and *Slamf6* genes mediates the control of NKT cell numbers attributed to the *Nkt1* gene (Jordan et al., 2007; Fig. 14).

CD150 is also involved in the regulation of cell proliferation and survival. Ligation of CD150 with monoclonal antibody IPO-3, signaling properties of which are opposite to A12 mAb, as well as homotypic CD150-CD150 interactions augment proliferation of resting human B lymphocytes, induced by BCR or CD40 ligation, mitogens or cytokines, especially IL-4. Ligation of CD150 also enhances immunoglobulin production by CD40-activated B cells (Punnonen et al., 1997; Sidorenko & Clark, 1993). CD150-induced signals can both synergize with and augment CD95-mediated apoptosis in human B cells and T cells (Mikhalap et al., 1999). In human B cell lines MP-1, RPMI-1788, and Raji, signaling via CD40 rescues cells from CD95-mediated apoptosis, but ligation of CD150 can block CD40-mediated protection. Conversely, CD40 ligation cancels out the synergistic effect of CD95 and CD150 (Mikhalap et al., 1999; Fig. 4). Besides, CD150 could inhibit cell proliferation of Hodgkin's lymphoma cell lines and induce apoptosis in one of them (Yurchenko et al., 2010).



**Figure 14.** CD150 functions. CD150 is expressed on several cell populations in immune system: T and B lymphocytes, dendritic cells, macrophages, follicular helper T cells and NKT lymphocytes. It has been shown that CD150 ligation mediates the functional changes in these cell types. In CD4<sup>+</sup> and CD8<sup>+</sup> T cells CD150 engagement mediates the inhibition of IFN- $\gamma$ , but activation of IL-4 production via increase of nuclear p50 NF- $\kappa$ B levels in PKC $\theta$ -dependent manner. In DCs, CD150 ligation with different ligands had (exerted) the opposite effects. Stimulation with anti-CD150 antibodies resulted in activation of inflammatory and adaptive T cell responses due to the increase of IL-12 and IL-18 production, while CD150 self-ligation mediated inhibition of IL-12, TNF- $\alpha$  and IL-6 production by DCs, leading to the inhibition of naïve CD4<sup>+</sup> T cell differentiation towards Th1 phenotype. In macrophages CD150 acts as a co-receptor that regulates signals transduced by the major LPS receptor TLR4, controlling the production of TNF- $\alpha$ , NO, IL-12 and IL-6. CD150 ligation blocks the rescue of B cells by CD40 ligation from CD95-mediated apoptosis. Besides, it augments B cell proliferation and enhances Ig production by CD40-activated B cells. In follicular helper T cells (TFH) CD150 engagement leads to upregulation of IL-4 production, and finally in NKT cells it promotes positive selection and enhances their proliferation. Ag - antigen; M $\phi$  - macrophage; MHCII – major histocompatibility complex class II; TCR – T cell receptor.

## 2.4.2 CD150 functions in antigen-presenting cells

CD150 also plays an important role in immune responses of macrophages. CD150 acts as a co-receptor that regulates signals transduced by Toll-like receptor (TLR) 4, the major lipopolysaccharide (LPS) receptor on the surface of mouse macrophages (Wang et al., 2004). In CD150<sup>-/-</sup> macrophages LPS-induced IL-12 and TNF secretion is impaired, while IL-6 is overproduced even in the absence of any extraneous stimuli. Moreover, CD150<sup>-/-</sup>-macrophages produce reduced amounts of nitric oxide (NO) upon stimulation with LPS (Fig. 14). However, CD150 does not regulate phagocytosis or responses to peptidoglycan or CpG (Wang et al., 2004). In SLAM-deficient (Slamf1<sup>-/-</sup>) mice macrophages also exhibit impaired LPS-induced production of TNF- $\alpha$  and nitric oxide. These experiments allow to conclude that CD150 acts as a vital regulator in the innate immune defense against Gram-negative bacteria in macrophages controlling two main independent bactericidal processes: phagosome maturation and the production of free radical species by the NOX2 complex (Berger et al., 2010).

Macrophages of CD150<sup>-/-</sup> C57Bl/6 mice are defective at clearing the parasite *Leishmania major* (Wang et al., 2004). The activation of CD150 may also promote the cell-mediated immune response mediated by IFN- $\gamma$  to intracellular bacterial pathogens, namely to *Mycobacterium leprae* (Garcia et al., 2001). Finally, CD150 is required for the replication of *Trypanosoma cruzi* (T.cruzi) in macrophages and DCs (Calderon et al., 2012). T. cruzi, the protozoan parasite responsible for Chagas' disease, causes severe myocarditis. In the absence of CD150, macrophages and DCs are less susceptible to T. cruzi infection and produce less myeloid cell specific factors that are keys in influencing the host response to parasite and the outcome of the infection. Consequently, CD150 deficient mice are resistant to T. cruzi infection.

These findings clearly define CD150 as a novel bacterial sensor (Sintes & Engel, 2011) and suggest its role in the regulation of some parasite infections.

CD150 is highly expressed on mature dendritic cells (DCs) but only at a low level on immature DCs (Polacino et al., 1996). CD150 surface expression is strongly up-regulated by IL-1 $\beta$  that leads to an increased DC-induced T cell response (Kruse et al., 2001). CD150 expression is rapidly increased on DCs during the maturation process (Bleharski et al., 2001; Polacino et al., 1996). CD150 could be upregulated on DCs with CD40L, poly(I:C) (polyinosinic polycytidylic acid) or LPS. mRNA encoding both membrane-bound and soluble secreted isoforms of CD150 was detected in CD40 ligand-activated DCs. The functional outcome of CD150 ligation on DCs surface depends on the nature of its ligands. Particularly, engagement of CD150 with anti-CD150 mAbs

IPO-3 enhances production of IL-12 and IL-8 by DC, but has no effect on the production of IL-10 (Bleharski et al., 2001). At the same time, another group of scientists has obtained the opposite results with the stimulation of CD150 on the surface of DCs with its self-ligand, CD150, expressed on transfected cells (Rethi et al., 2006). This may suggest that the differential functional outcome of CD150 signaling in DCs depends on the receptor region, engaged by its ligand. CD150/CD150 interactions did not interfere with sCD40L induced DC maturation. CD150/CD150 associations inhibited IL-12, TNF- $\alpha$ , and IL-6 secretion by CD40L-activated DCs. CD150 signaling also modulated the function of CD40L-activated DCs by reducing their ability to promote naïve T-cell differentiation toward IFN- $\gamma$ -producing effector cells (Fig. 4). CD150 inhibited the differentiation of CD4<sup>+</sup> naive T cells into Th1-type effector cells without promoting their differentiation into Th2 cells which was shown by the lack of IL-4 production and the low levels of IL-13 in the presence of high concentration of IFN- $\gamma$  (Rethi et al., 2006).

### **2.4.3 CD150 functions in other cell types**

CD150 has a lateral mobility in the plasma membrane and forms adhesion contacts between cells, presumably due to homophilic interaction (Howie et al., 2002). Moreover, CD150 is expressed by platelets and signaling of SLAM family adhesion molecules, namely CD84 and CD150, activated by proximity during aggregation, further stabilize platelet-platelet interactions in thrombosis (Nanda et al., 2005). Analysis of CD150-deficient mice revealed an overall defect in platelet aggregation *in vitro* and a delayed arterial thrombotic process *in vivo* (Nanda et al., 2005).

Thus, CD150 isoforms are expressed on a wide range of lymphoid cell types. With its extracellular part, CD150 binds to several types of ligands, including CD150 by itself, glycoproteins of Morbilliviruses and bacterial antigens, while its cytoplasmic tail may interact with key signaling molecules that connect CD150 to several intracellular signal transduction pathways. This allows CD150 to be involved in the regulation of cell transcription programs and gene expression and therefore control cell proliferation, differentiation and survival. In the context of all these findings CD150 has attracted a lot of interest in terms of its involvement in the measles virus induced immunosuppression. As the major measles virus receptor and a dual-function signaling lymphocyte receptor, CD150 may play an important role in mediating the inhibition of immune response by measles virus and its proteins.

### **3. DENDRITIC CELL DIFFERENTIATION, SIGNALING AND FUNCTION**

Dendritic cells (DCs) are critical regulators of immunity to pathogens, specialized for the uptake, transport, processing and presentation of antigens to T cells. DCs in addition to residing in lymphoid tissues are widely distributed throughout the body consistent with a role as sentinels for pathogens (Shortman & Liu, 2002; Tisch, 2010).

DCs can be subdivided into two types: plasmacytoid DC (pDC) and conventional DC (cDC) (Heath, 2009). pDCs are noted for rapid production of large amounts of type I interferons, especially IFN- $\alpha$ , in response to viral infections. Upon stimulation the precursors of pDC, which are found in blood, traffic to lymphoid organs and non-lymphoid sites of inflammation. Conventional DCs in turn could be further subdivided to resident cDCs, found in T cell areas of lymphoid tissues, and migratory cDCs that inhabit peripheral tissues and traffic to the local lymph nodes after stimulation. cDCs subsets differ in uptake, processing, and presentation of antigens (Tisch, 2010).

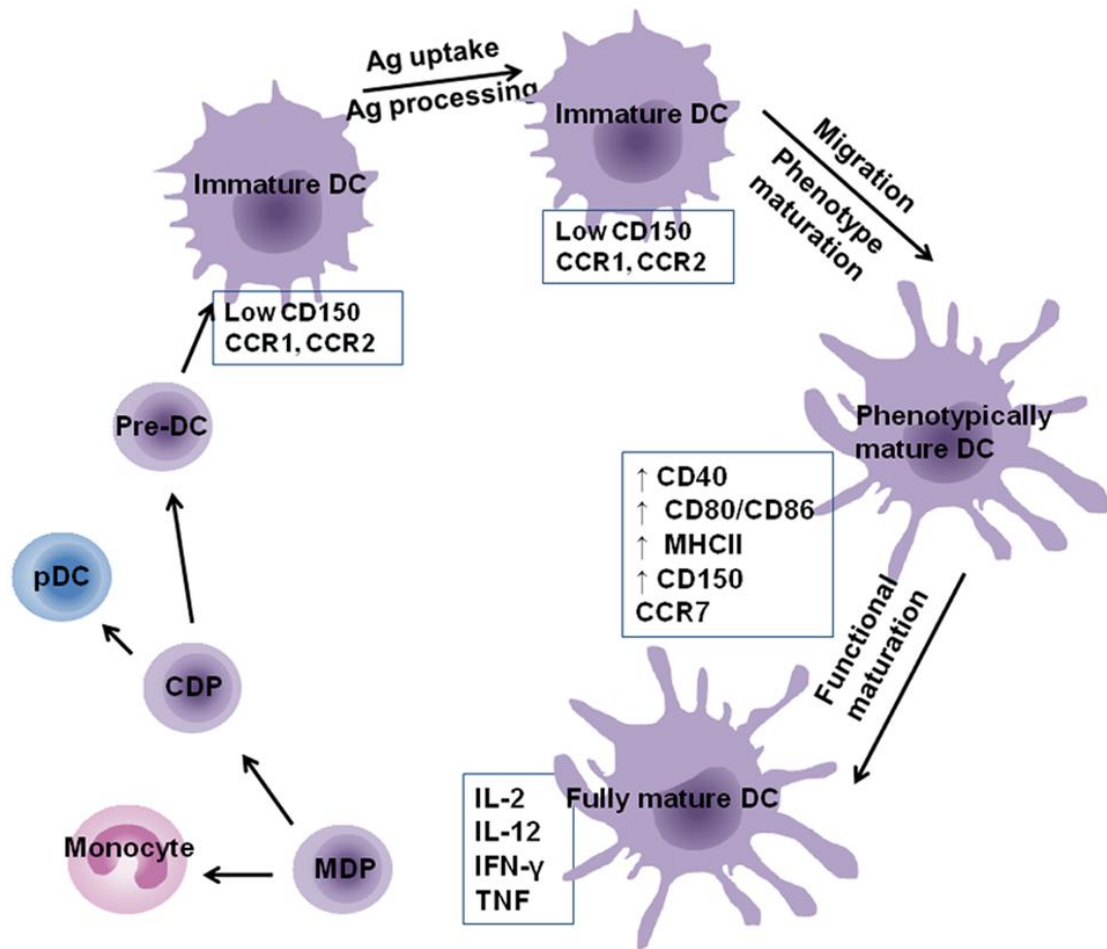
The human Langerhans-cell DCs are recognized as a separate DC subtype with distinct markers, including the presence of Birbeck granules and the expression of CD1a and langerin. Most human thymic DCs are CD11c<sup>+</sup>CD11b<sup>-</sup>CD45RO<sup>lo</sup> and lack myeloid markers, so resemble mouse thymic CD8<sup>+</sup> DCs. A minority of human thymic DCs are CD11c<sup>hi</sup>CD11b<sup>+</sup>CD45RO<sup>hi</sup> and express many myeloid markers. The second pathway of development from CD34<sup>+</sup> precursors leads to DCs resembling intestinal DCs, lacking Birbeck granules and the Langerhans cell antigens but expressing CD9 (a tetraspan molecule), CD68 (macrosialin) and coagulation factor XIIIa. Blood monocytes are the most commonly used precursor cells for generating human DCs in culture. In the presence of GM-CSF and IL-4, DCs are produced after six days (Romani et al., 1996). Final maturation to CD14<sup>-</sup>CD38<sup>+</sup>CD86<sup>+</sup>-surface MHC-II<sup>hi</sup> DCs is achieved by stimulating with proinflammatory cytokines such as TNF- $\alpha$  or microbial products such as LPS. Helper CD4<sup>+</sup> T cells, signalling by means of CD154, can also mature and activate these DCs (Shortman & Liu, 2002).

The pDCs and cDCs express different sets of pattern-recognition receptors (Kadowaki et al., 2001) and show corresponding differences in reactivity to different microbial products. Monocytes, which are the most commonly used precursor cells for generating human DCs in culture, therefore express TLR1, TLR2, TLR4, TLR5 and TLR8, and respond to the appropriate microbial ligands, including peptidoglycan, lipoteichoic acid and LPS, whereas pDC2 cells express TLR7 and TLR9,

and respond to CpG oligonucleotides. The CD11c<sup>+</sup> iDCs found in blood express yet another set of TLRs, and their corresponding reactivity differs. In particular, they selectively express TLR3 and, accordingly, selectively respond to double-stranded RNA (Kadowaki et al., 2001; Shortman & Liu, 2002).

Under steady state conditions, peripheral DCs exhibit an immature phenotype characterized by a high capacity for antigen uptake, and a relatively low T cell stimulatory function. Immature DCs reside in almost all tissues, where they can capture and process antigens (Fig. 14). Thereafter, they migrate toward the T cell areas of secondary lymphoid organs via the afferent lymphatics (Fig. 14). During this migration, they lose their ability to internalize antigens and they acquire the capacity to present antigens to naive T cells, a process referred to as DC maturation or activation (Nakahara et al., 2006). Upon stimulation, DC are activated and undergo a number of phenotypic and functional changes referred to as maturation. A series of microbial products, like CPG motifs in bacterial DNA, double-stranded viral RNA, lipopolysaccharides (LPS) and necrotic cell products (such as heat-shock proteins), activate DCs. In addition, DCs distinguish between tissue cells that die by the normal process of apoptosis and those that die by externally generated necrosis — antigens from both types of dead cells are taken up and presented to T cells, but only the latter activate the DC (Gallucci et al., 1999; Sparwasser et al., 1998; Verdijk et al., 1999).

In a mature state, DC gain competency to induce efficient T cell activation, proliferation, and effector differentiation. Activation of immature DC is often initiated by pattern recognition receptors (PPRs) that bind to conserved molecular signatures of microbial pathogens. Immature DCs express several PPRs, which include the well studied Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptor, retinoic acid inducible gene-I-like receptors, and C-type lectin receptors (Medzhitov, 2007). DC maturation enhances antigen processing and presentation, in addition to increasing expression of pathogen-derived, peptide-bound major histocompatibility complex (MHC) molecules (Fig. 15). APCs usually present exogenous antigens on MHC class II (MHC II) molecules, whereas they usually present endogenous antigens (from self-components or a viral infection) on MHC class I (MHC I) molecules.



**Figure 15.** Dendritic cell maturation. Upon antigen recognition dendritic cells undergo maturation process which is accompanied by the upregulation of some cell surface markers (CD40, CD80, CD86, MHCII, CD150), induction of cytokine production (IL-2, IL-12, IFN- $\gamma$ , TNF) and morphological changes. MDC – monocyte and dendritic cell precursor; CDP – common DC precursor; pDC - plasmacytoid DC; Ag - antigen; MHCII - major histocompatibility complex class II.

Binding of these peptide-MHC complexes by the T cell receptor leads to transduction of “signal 1” required for T cell activation. Elevated expression of various co-stimulatory molecules, such as CD80 and CD86, leads to transduction of “signal 2” that drives T cell proliferation (Fig. 15). Secretion of cytokines by mature DC provide “signal 3” that is essential for differentiation of T effector subsets. DC maturation also entails upregulation of lymphoid chemokine receptors, such as CCR7, required for directed migration of DC to the lymph nodes (Tisch, 2010; Fig. 15). Thus, fully mature DCs show high surface expression of both costimulatory and adhesion molecules, such as CD40, CD54, CD80, and CD86, as well as MHC class II antigens, but have a decreased capacity for antigen uptake and processing. Up-regulation of CD83, a specific marker for DC maturation, also occurs in mature DCs (Nakahara et al., 2006).

DC–T-cell interaction results in T-cell activation and proliferation that might lead to immunity or to tolerance, to the generation/activation of effector T cells or regulatory T cells, and to T cells that secrete different patterns of cytokines, including the extreme cytokine-polarized T helper 1 (TH1) and TH2 responses. Although the signals from DCs that govern T-cell cytokine polarization have not all been determined, the production of the TH1-inducing cytokine interleukin-12 (IL-12) is an important factor (Fig. 15). Several factors are needed to induce IL-12 production by DCs, including microbial products, the CD40-ligand stimulation from activated T cells and the appropriate cytokine milieu (Moser & Murphy, 2000).

In addition to these interactions with T cells in the lymphoid organs, DCs interact with other cell types, including B cells and natural killer (NK) cells. They might also remain in peripheral tissues as inflammatory mediators rather than migrating to LNs. The several and often opposing roles now ascribed to DCs cannot all be carried out at once by the same cell, so there should be different sets of DCs that perform different functions (Shortman & Liu, 2002).

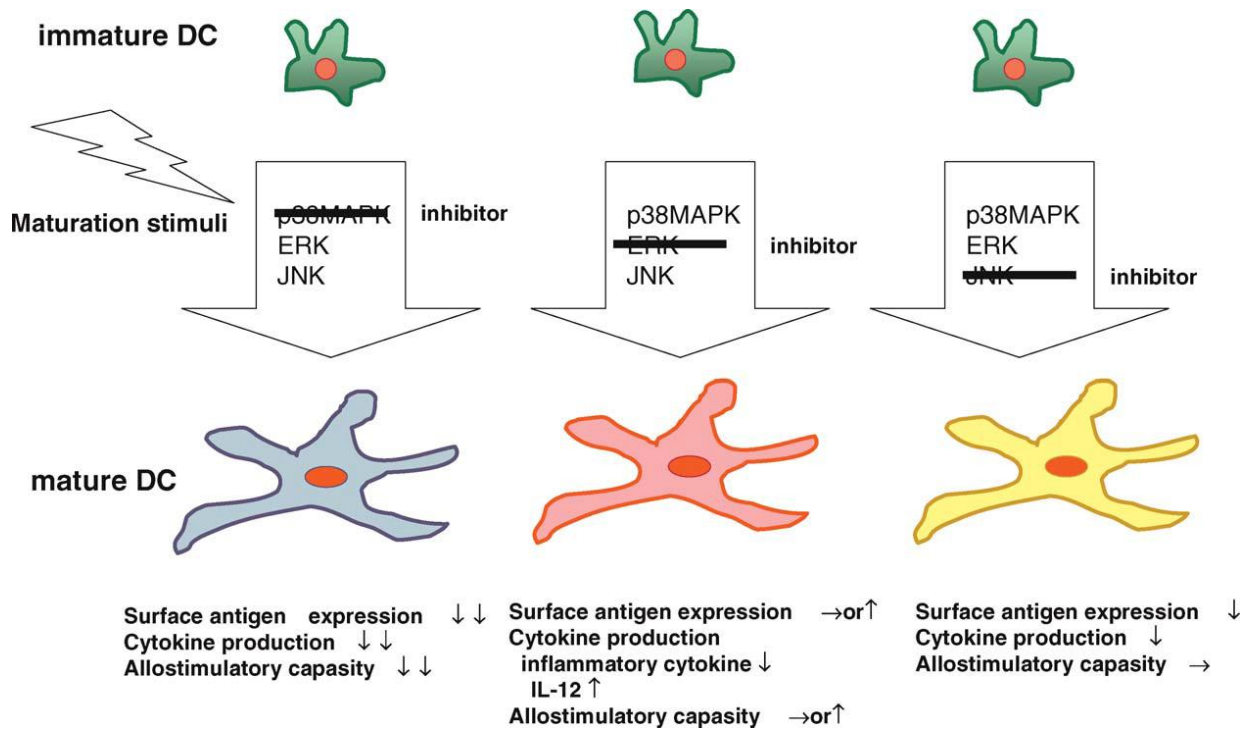
However, DCs are also key effectors of self-tolerance. Central tolerance of T cells in the thymus, which is achieved by inducing the apoptotic death of potentially self-reactive T cells, is mediated by thymic DCs (Brocker et al., 1997). The evidence so far indicates that it is the stage of T-cell differentiation, rather than the type of DC, that determines the negative outcome of this interaction (Matzinger & Guerder, 1989). Nevertheless, thymic DCs do have a life history that differs from the standard model, because most of them derive from an intrathymic precursor and develop and die within the thymus (Ardavin et al., 1993). A nonmigratory behaviour seems to be more appropriate to such DCs, the function of which is to present self antigens rather than to collect foreign antigens (Shortman & Liu, 2002).



Upon stimulation with extracellular stimuli the maturation signals are regulated via several signaling transduction pathways. Many of such maturation signals are mediated with the phosphorylation of mitogen-activated protein kinases (MAPKs).

The MAPK signaling pathway is a highly conserved pathway that is involved in diverse cellular functions, including cell proliferation, cell differentiation and apoptosis. This MAPK family includes several subgroups such as JNK, ERK, and p38 MAPK. All share the common property of being activated via three-module phosphorylation cascade, which in turn allows them to phosphorylate a wide range of substrates including other protein kinases, phospholipases, and transcription factors. The MAPK-activating phosphorylation cascade is initiated by the so-called MAPK kinase kinases (MAPKKKs), which activate the downstream MAPK kinases (MAPKKs) by phosphorylating them on specific serine and threonine amino acid residues. Subsequently, the MAPKKs phosphorylate and activate some threonine and tyrosine residues of MAPKs, which then become able to interact with their cytoplasmic substrates and also to translocate into the cell nucleus, where many MAPK targets, such as transcription factors, are found (Nakahara et al., 2006).

The role of MAPKs in the maturation of human DCs is now revealed with the help of kinase-specific inhibitors. As follows, preincubating of immature DCs with p38 MAPK inhibitor (SB203580) prevents the up-regulation of CD40, CD80, CD86, HLA-DR and CD83 as well as inhibits the production of IL-12 p40 and IL-12 p70, induced by TNF- $\alpha$  or LPS, and decrease of antigen uptake (Arrighi et al., 2001; Nakahara et al., 2004). Similarly, the LPS-induced up-regulation of CD80, CD86, CD83 and CD54 is slightly inhibited by JNK inhibitor (SP600125), while the expression of LPS-induced HLA-DR and CD40 remains unaffected. SP600125 inhibits also the production of TNF- $\alpha$  and IL-12 p70 from mature DCs in response to LPS, but not the production of IL-6 and IL-12 p40 (Nakahara et al., 2004). Quite the opposite, a MEK1 inhibitor, usually used as the inhibitor of the ERK signaling pathway, does not down regulate the expression of CD80, CD86, CD83, HLA-DR or CD40 during TNF- $\alpha$ - or LPS-induced DC maturation, but even slightly increase the level of CD86, CD83 and HLA-DR induced by UVB, DNCB or NiCl<sub>2</sub> (Aiba et al., 2003; Nakagawa et al., 2004; Fig. 16).



**Figure 16.** Differential role of MAPK signaling in human dendritic cell. p38 MAPK, ERK (MEK1/2) and JNK inhibitors have different effects on the maturation of human monocyte-derived dendritic cells, which is seen in the phenotype changes, modulation of cytokine production and allostimulatory capacities (modified from Nakahara et al., 2006).

Thus, three classes of MAPK kinases differently regulate the maturation pathway of dendritic cells: while p38 MAPK and JNK promote this process, ERK is observed to rather inhibit it. Various maturation stimuli may activate these three MAPKs at different time points promoting diverse cytokine responses in DCs to stimulate different Th responses.

## **4. MEASLES VIRUS IMMUNOPATHOGENESIS**

### **4.1 MEASLES VIRUS MAJOR CELL TARGETS AND SPREAD**

Measles represents a typical childhood disease, which is contracted only once in the lifetime of an individual because the anti-viral immune responses are efficiently induced and persist (Avota et al., 2010). But paradoxically measles virus infection induces both an efficient MV-specific immune response and a transient but profound immunosuppression. This depression of host immune response leads to an increased susceptibility to secondary infections following measles, which are responsible for its high mortality rate.

It is currently postulated that the primary cell targets for MV are CD150-positive alveolar macrophages, dendritic cells and lymphocytes in the respiratory tract. This concept is also supported with the fact that almost all CD14+ monocytes in human tonsils express CD150. DC infection has been observed in experimentally infected cotton rats (Niewiesk et al., 1997), transgenic mice (Shingai et al., 2005; (Ferreira et al., 2010)), and macaques (de Swart et al., 2007; (Lemon et al., 2011)). Both cultured CD1a+ DCs and epidermal Langerhans cells can be infected in vitro by both vaccine and wild type strains of MV. Both vaccine and wild-type strains of MV undergo a complete replication cycle in DCs (Grosjean et al., 1997).

In peripheral tissues DCs are found in immature state but in response to danger signals they undergo maturation and upregulate co-stimulatory molecules as well as MHC class I and II. MV replication induces maturation of immature DCs, causing down-regulation of CD1a, CD11c, and CD32 expression; up-regulation of MHCI, MHCII, CD80, and CD40 expression; and induction of CD25, CD69, CD71, CD86, and CD83 expression, alteration in the expression of chemokines, chemokine receptors, and chemotaxis (Abt et al., 2009; Schnorr et al., 1997; Servet-Delprat et al., 2000; Zilliox et al., 2006). Such dramatic changes allow DCs to increase their migratory potential and effectively realize their function – antigen presentation and activation of effector cells. All these events are also supported with the production of respective cytokines by DCs. Infection of DCs results in rapid induction of IFN- $\beta$  mRNA and protein and multiple IFN- $\alpha$  mRNAs, as well as upregulation of IL-10 and IL-12 secretion and acquisition of MIP-3 $\beta$  responsiveness, which provides the migratory capacity to DCs (Dubois et al., 2001; Zilliox et al., 2006). DC infection with vaccine strains of MV also induces their functional maturation (Schnorr et al., 1997).

Infection spreads in DC cultures, but release of infectious virus is minimal unless cells are activated (Fugier-Vivier et al., 1997; Grosjean et al., 1997). Immature DCs do not express CD150 receptor at a high level. However, DC maturation is followed by the upregulation of CD150 expression level on their surface which could be used for viral spreading. Immature DCs form a network within all epithelia and mucosa, including the respiratory tract (Servet-Delprat et al., 2003). MV may exploit the DC network to travel to secondary lymphoid organs by infecting immature DCs, inducing their maturation and migration to these organs (Hahm et al., 2004). Moreover, the virus could be transported attached to DC-SIGN molecule on the surface of uninfected dendritic cells. DCs expressing either CD150 or DC-SIGN can transmit MV to co-cultured T cells (de Witte et al., 2008). The interaction of lymphocytes with MV-infected DCs results in lymphocyte infection, DC activation, and enhanced MV replication, leading to DC death (Fugier-Vivier et al., 1997; Murabayashi et al., 2002; Servet-Delprat et al., 2000). Thus, some of dendritic cells get infected but some carry the virus to the lymph nodes where MV meets its main lymphoid targets – B and T cells (Condack et al., 2007; de Swart et al., 2007). However, MV transmission to T cells occurs most efficiently from cis-infected DCs, and this involves the formation of polyconjugates and an organized virological synapse (Koethe et al., 2012).

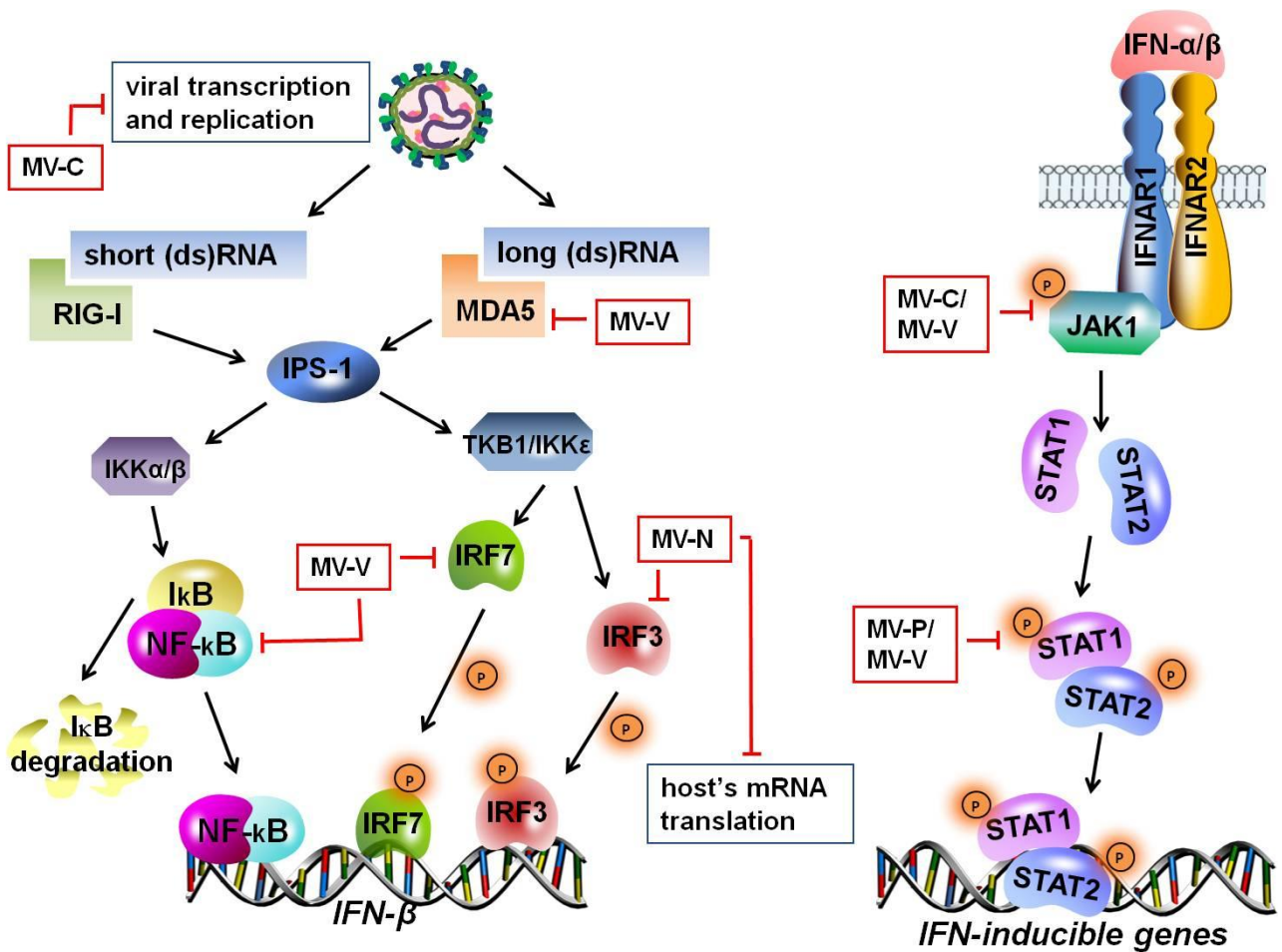
## 4.2 INDUCTION OF INNATE AND ADAPTIVE IMMUNE RESPONSE BY MEASLES VIRUS

MV infection activates the innate immunity with the induction of a type I interferon (IFN) response *in vitro* and *in vivo* (Devaux et al., 2008; Gerlier & Valentin, 2009; Herschke et al., 2007; Plumet et al., 2007). Type I interferons (IFN- $\alpha/\beta$ ) are an important part of innate immunity to viral infections because they induce an antiviral response and limit viral replication until the adaptive response clears the infection. Type I IFNs are induced upon pathogen recognition by the pattern recognition receptors which include TLRs, RIG-I like receptors (RLRs) and Nod-like receptors (NLRs). RLRs are RNA helicases, expressed by all nucleated cells, that recognize RNA molecules, which are absent in cell cytoplasm in normal conditions. Three types of RLRs, namely RIG-I, MDA5 and LGP2, could be distinguished depending on their ligands. MDA5 recognizes long double-stranded RNAs, RIG-I detects short double-stranded RNAs with a 5'-triphosphate residue, while LGP2 has greater affinity for dsRNA and regulates function of RIG-I and MDA5. This RNA detection induces IFN- $\beta$  gene transcription and activation of the transcription factors: IFN regulatory factor 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Griffin, 2010; Fig. 17).

During MV replication MV RNA interacts with RIG-I and less with MDA5 and thus stimulates IFN- $\beta$  transcription in responsive cells. Moreover, measles N protein can interact with and activate IRF3 by itself (Herschke et al., 2007; Plumet et al., 2007; Fig. 17). However, MV infection usually suppresses type I IFN production in PBMCs (Naniche et al., 2000), CD4+ T cells (Sato et al., 2008; Fig. 17) and has a variable effect in plasmacytoid DCs (Druelle et al., 2008; Schlender et al., 2005). In addition wild type MV is less able to induce IFN, than MV vaccine strains most of the time (Naniche et al., 2000).

Besides of RNA helicases, measles viral components or replication intermediates could also be recognized by pattern-recognizing host machinery of TLRs. Pattern recognition by antigen-presenting cells via TLRs is an important element of innate immunity (Beutler, 2004). TLR2 was found to interact with the hemagglutinin of wild-type measles virus (Fig. 7). This effect of MV-H could be abolished by mutation of a single amino acid, asparagine at position 481 to tyrosine, which is found in attenuated strains and is important for interaction with CD46. TLR2 activation by MV wild-type H protein induces surface CD150 expression in human monocytes and stimulates IL-6 production (Bieback et al., 2002).

The induction of adaptive immune response during measles infection coincides with the appearance of its clinical manifestations, including skin rash. At the time of the rash, both MV-specific antibody and activated T cells are detectable in circulation, however in few days after its appearance the virus is cleared from peripheral blood cells. The central role in viral clearance belongs to CD8+ T cells that is supported by studies in infected macaques (Permar et al., 2003; (de Vries, Yuksel, Osterhaus, & de Swart, 2010)). In early immune response, CD4+ T cells produce type 1 cytokines, including IFN- $\gamma$  and IL-2, while after clearance of infectious virus they switch to the production of type 2 cytokines, like IL-4, IL-10, and IL-13 (Moss et al., 2002; Fig. 18). Th2 cytokine skewing after acute measles infection facilitates the development of humoral responses important for lifelong memory protection against any new measles infection, but depresses the induction of type 1 responses that is necessary for fighting new pathogens. However, recent studies on macaque model showed that humoral responses could also be abrogated upon MV infection. MV efficiently replicates in B lymphocytes, resulting in follicular exhaustion and disorganization of the germinal centers, which are essential in actively ongoing humoral immune responses (de Vries et al., 2012; Fig. 18).



**Figure 17.** Innate anti-measles immune response and viral escape from its detection. MV leader RNA interacts with and activates RIG-I and to a lesser extent MDA5. Both pathways lead to the induction of signal transduction pathways with the activation of several transcription factors that regulate the expression of IFN genes. MV has developed several strategies to escape the induction of IFN response, some of which do not depend on viral replication but only the presence of viral proteins and their interaction with host cell proteins.

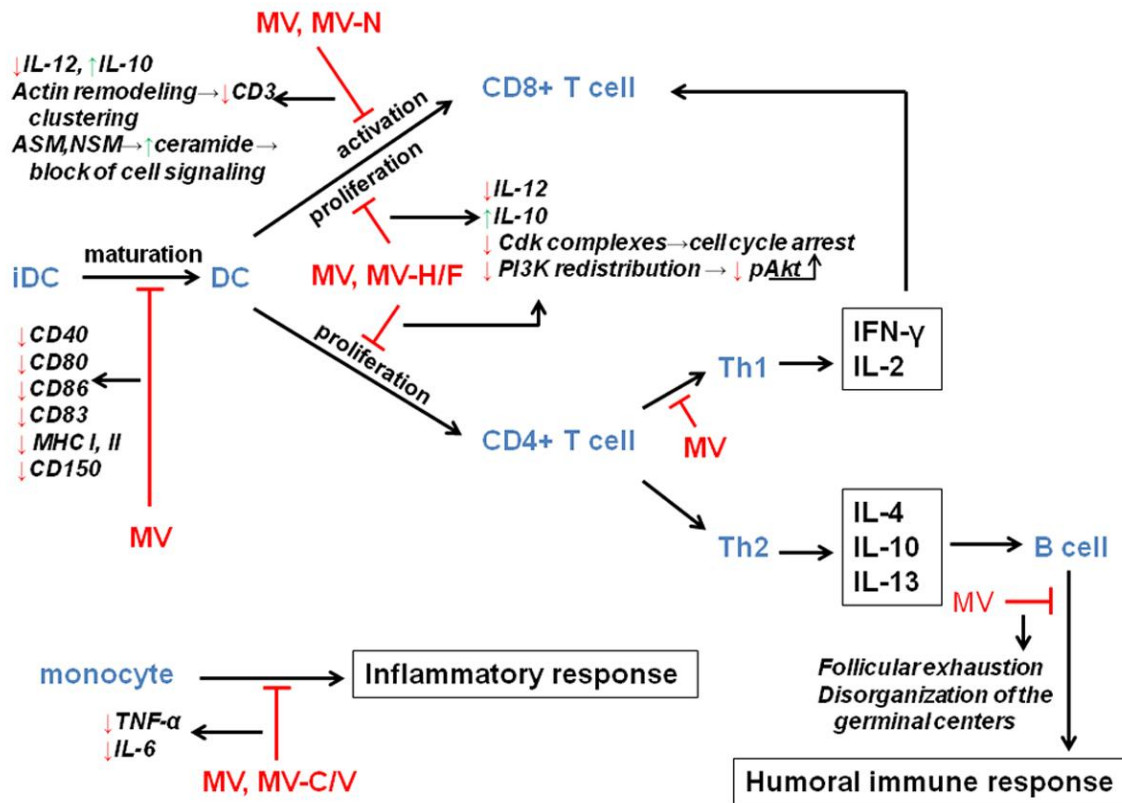


### **4.3 MEASLES VIRUS STRATEGIES FOR SUPPRESSION OF IMMUNE RESPONSE**

The immunosuppressive effects of measles were first recognized and quantified by von Pirquet in the 19th century, in his study of delayed type hypersensitivity (DTH) skin test responses during a measles outbreak in a tuberculosis sanitarium (Von Pirquet, 1908). In addition to depressed immune responses to recall antigens (such as tuberculin), the cellular immune responses and antibody production to new antigens are impaired during and after the acute measles (Moss et al., 2004). The fact that just a small proportion of peripheral blood cells are infected during measles allows us to speculate about alternative mechanisms of immunosuppression that do not involve direct viral infection of lymphoid cells. During several weeks after resolution of the rash and visible recovery, infectious virus is no longer detectable, but viral RNA continues to be present in PBMCs, as well as in respiratory secretions and urine (Permar et al., 2001; Riddell et al., 2007). A number of different potential mechanisms for MV-induced immunosuppression have been proposed, including lymphopenia, prolonged cytokine imbalance, leading to inhibition of cellular immunity, suppression of lymphocyte proliferation and compromised DC functions (Avota et al., 2010; Griffin, 2010). The interaction of measles viral proteins with cellular receptors and other intracellular elements involved in the induction of immune response may play an important role in MV-induced immunosuppression.

#### **4.3.1 The role of viral glycoproteins in measles immunosuppression**

Measles virus glycoproteins are highly implicated in the suppression of lymphocyte proliferation during and after measles acute infection even in the absence of MV replication. In cotton rat model transiently transfected cells expressing the viral glycoproteins (hemagglutinin and fusion protein) inhibit lymphocyte proliferation after intraperitoneal inoculation (Niewiesk et al., 1997). This is not connected to cell fusion or unresponsiveness to IL-2, but rather to the retardation of the cell cycle that correlates with inhibition of proliferation. This inhibitory signal prevents entry of T cells into S phase and results in the reduction of cyclin-dependent kinase complexes and delayed degradation of p27Kip leading to cell cycle retardation and accumulation of cells in the G0/G1 phase (Engelking et al., 1999; Niewiesk et al., 1999; Schnorr et al., 1997). Similarly, the complex of both MV glycoproteins, F and H, is critical in triggering MV-induced



**Figure 18.** MV evasion strategies to avoid host adaptive immune response. Dendritic cells lay in the center of regulation of antiviral immune responses. By abrogation of DC maturation via down-regulation of surface markers like CD40, CD80, CD86, CD83, MHC I/II, CD150, and deregulation of cytokine production (IL-12 and IL-10), MV influences different stages of DC life cycle, and inhibits not only DC function, but also other immune cell responses. The mechanisms of such action include the disruption of cell signaling (PI3K/Akt pathway), cytoskeletal remodeling that crashes the stable immunological synapses, ceramide accumulation, etc.

suppression of mitogen-dependent proliferation of human PBLs in vitro (Dubois et al., 2001; Schlender et al., 1996). It is also supported by the data that human DCs expressing MV glycoproteins in vitro fail to deliver a stimulation signal for proliferation to T cells, despite the presence of co-stimulatory molecules on their surface (Klagge et al., 2000). However, the nature of the receptor responsible for mediating MV glycoprotein-induced immunosuppression has not been identified so far.

Some immunosuppressive effects of measles virus were shown to be caused by direct interaction of its glycoproteins with CD46 receptor. Thus, upon the intraperitoneal injection of UV-inactivated MV in hapten-sensitized CD46-transgenic mice a significant decrease in dendritic cell IL-12 production from draining lymph nodes was observed, providing in vivo evidence of the systemic modulation of IL-12 production by MV proteins (Marie et al., 2001). Down-regulation of IL-12 production in primary human monocytes, could be induced in the absence of viral replication by cross-linking of CD46 with an antibody or with the complement activation product C3b (Karp et al., 1996). Moreover, the engagement of distinct CD46 isoforms (CD46-1 and CD46-2) induces different effects in immune response. CD46-2 induces increased generation of specific CD8<sup>+</sup> T cells and decreases proliferation of CD4<sup>+</sup> T cells, which is associated with strongly suppressed IL-10 secretion. In contrast, CD46-1 engagement decreases the inflammatory response as well as the generation of specific CD8<sup>+</sup> T cells. Moreover, it markedly increases the proliferation of CD4<sup>+</sup> T cells which are capable of producing the anti-inflammatory cytokine IL-10 (Marie et al., 2002).

CD46 downregulation can be rapidly induced in uninfected cells after surface contact with MV particles or MV-infected cells. Contact-mediated CD46 modulation can lead to complement-mediated lysis of uninfected cells that also may cause lymphopenia (Schneider-Schaulies et al., 1996; Schnorr et al., 1995). Recent discovery of Notch ligand Jagged1, as a natural ligand for CD46, which induces Th1 differentiation (Le Fricc et al., 2012), opens new possibilities to explain the importance of CD46-pathogen interaction in the modulation of the immune response. However, the effects of CD46 are most probably mediated by vaccine strains of measles virus as most of wild type MV strains do not use this receptor.

#### **4.3.2 The impact of measles non-structural proteins on antiviral immune response**

MV non-structural proteins can block the induction of IFN response using multiple strategies. The MV V protein interacts with RNA helicase MDA5 and inhibits the IFN response (Andrejeva et al., 2004; Childs et al., 2007; Fig. 17). Moreover, in plasmacytoid dendritic cells MV

V can be a substrate for IKK $\alpha$ , which results in competition between MV-V and IRF7 for the phosphorylation by IKK $\alpha$ . In addition MV V can bind to IRF7 and inhibit its transcriptional activity. Binding to both IKK $\alpha$  and IRF7 requires the 68-amino-acid unique C-terminal domain of V (Pfaller & Conzelmann, 2008; Fig. 17). Both P and V proteins can inhibit the phosphorylation of STAT1 and the signal cascade downstream from the IFNAR (Caignard et al., 2007; Devaux et al., 2007). In MV-infected cells, C and V proteins of measles virus form a complex containing IFNAR1, RACK1, and STAT1 and inhibit the Jak1 phosphorylation (Yokota et al., 2003). Moreover, MV V protein could act as an inhibitor of cell death by preventing the activation of the apoptosis regulator PUMA (Cruz et al., 2006). The nonstructural C protein inhibits type I IFN induction and signaling, in part by decreasing viral RNA synthesis, and has been implicated in prevention of cell death (Escoffier et al., 1999; Reutter et al., 2001; Shaffer et al., 2003; Fig. 17). Recombinant MV lacking the C protein grows poorly in cells possessing the intact IFN pathway and this growth defect is associated with reduced viral translation and genome replication. The translational inhibition correlates with phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (Nakatsu et al., 2006). In addition, the protein kinase PKR plays an important role as an enhancer of IFN- $\beta$  induction during measles virus infection via activation of mitogen-activated protein kinase and NF- $\kappa$ B (McAllister et al., 2010). Besides the anti-IFN- $\alpha/\beta$  properties of V and C proteins in vivo, both C and V proteins are required for MV to strongly inhibit the inflammatory response in the peripheral blood monocyctic cells of infected monkeys. In the absence of V or C protein, virus-induced TNF- $\alpha$  downregulation is alleviated and IL-6 is upregulated (Devaux et al., 2008).

In addition, MV P efficiently stabilizes the expression and prevents the ubiquitination of the human p53-induced-RING-H2 (PIRH2), an ubiquitin E3 ligase belonging to the RING-like family. PIRH2 is involved in the ubiquitination of p53 and the  $\epsilon$ -subunit of the coatmer complex,  $\epsilon$ -COP, which is part of the COP-I secretion complex. As such, PIRH2 can regulate cell proliferation and the secretion machinery (Chen et al., 2005).

#### **4.3.3 The effect of MV nucleoprotein in the regulation of immune response**

Measles virus nucleoprotein (N) interacts with Fc $\gamma$ R receptor on dendritic cells via its C-terminal part (Laine et al., 2003; Ravel et al., 1997); Fig. 7). This interaction is critical for MV-induced immunosuppression of inflammatory responses in vivo that was shown by the inhibition of hypersensitivity responses in CD46-transgenic mice. MV N protein impairs the APC function

affecting the priming of naïve CD8<sup>+</sup> lymphocytes during sensitization as well as the development of the effector phase following secondary antigen contact (Marie et al., 2001). It has been difficult to understand how this cytosolic viral protein could leave an infected cell and then perturb the immune response. Finally it was shown that intracellularly synthesized nucleoprotein enters the late endocytic compartment, where it recruits its cellular ligand, the Fc $\gamma$ R. Nucleoprotein is then expressed at the surfaces of infected leukocytes associated with the Fc $\gamma$ R and is secreted into the extracellular compartment, allowing its interaction with uninfected cells. Cell-derived nucleoprotein inhibits the secretion of IL-12 and the generation of the inflammatory reaction, both shown to be impaired during measles (Marie et al., 2004). MV N was also shown to induce an anti-inflammatory regulatory immune response and its repetitive administration into apolipoprotein E-deficient mice resulted in the reduction of atherosclerotic lesions (Ait-Oufella et al., 2007). Finally, the immunosuppressive properties of MV N seem to be shared with the other members of Morbilliviruses, since both PPRV and CDV nucleoproteins bind Fc $\gamma$ R and suppress delayed hypersensitivity responses (Kerdiles et al., 2006).

Interestingly, N protein also binds to the eukaryotic initiation factor 3 (eIF3-p40). The interaction between MV-N and eIF3-p40 in vitro inhibits the translation of a reporter mRNAs (Sato et al., 2007). This is the first observation indicating that MV can repress the translation in host cells. Therefore, viral mRNAs may selectively escape requirement for eIF3-p40 initiation factor for translation of viral proteins (Gerlier & Valentin, 2009).

#### **4.3.4 The effect of measles virus infection on host pathophysiology**

MV infection is associated with lymphopenia with decreased numbers of T cells and B cells in circulation during the rash. Increased surface expression of CD95 (Fas) and Annexin V staining on both CD4<sup>+</sup> and CD8<sup>+</sup>T cells during acute measles suggests that apoptosis of uninfected lymphocytes may account for some of the lymphopenia. While B cell numbers may remain low for weeks, T cell counts return to normal within few days (Ryonet al., 2002). MV-induced lymphopenia could be due to loss of precursors, albeit MV interaction with CD34<sup>+</sup> human hematopoietic stem cells (HSCs) or thymocytes did not affect viability and/or differentiation in vitro (Avota et al., 2010).

Recent studies on macaque model proposed the alternative explanation for MV-induced lymphopenia and its relation to immunosuppression. It showed that MV preferentially infects CD45RA<sup>-</sup> memory T lymphocytes and follicular B lymphocytes, resulting in high infection levels

in these populations, which causes temporary immunological amnesia thus explaining the short duration of measles lymphopenia yet long duration of immune suppression. Therefore, MV infection wipes immunological memory, resulting in increased susceptibility to opportunistic infections (de Vries et al., 2012). The influence of measles virus infection on gene expression by human peripheral blood mononuclear cells (PBMCs) was examined with cDNA microarrays. MV infection up-regulated the NF- $\kappa$ B p52 subunit and B cell lymphoma protein-3 (Bcl-3). This finding suggests the modulation of the activity of NF- $\kappa$ B, transcription factor that regulates the expression of a wide range of genes involved in inducing and controlling immune responses (Bolt et al., 2002). MV also upregulated the activating transcription factor 4 (ATF-4) and the pro-apoptotic and growth arrest-inducing CHOP/GADD153 genes indicated that MV infection induces the endoplasmic reticulum (ER) stress response in PBMCs. It is possible that the ER stress response is involved in the MV-induced reduction of lymphocyte proliferation through the induction of CHOP/GADD153 (Bolt et al., 2002).

It was shown that MV infection induces downregulation of human receptor CD150 (hCD150) in transgenic mice, inhibits cell division and proliferation of hCD150<sup>+</sup> but not hCD150<sup>-</sup> T cells, and makes them unresponsive to mitogenic stimulation. The role of CD150 in this process was studied on the transgenic mice expressing hCD150 under the control of the *lck* proximal promoter. The use of the *lck* proximal promoter in producing transgenic mice results in specific expression of CD150 protein on immature and mature T lymphocytes in blood, spleen, and thymus that partially mimics the CD150 expression in human T cells (Hahm et al., 2003).

Activation of the PI3K/Akt kinase pathway is an important target for MV interference in T cells. Shortly after MV exposure *in vivo* and *in vitro* this pathway was shown to be efficiently inhibited after IL-2R or CD3/CD28 ligation in T cells (Avota et al., 2001; Avota et al., 2004). In the absence of MV replication, MV H/F can inhibit T cell proliferation *in vitro* and the intracellular pathway involves the impairment of the Akt kinase activation (Avota et al., 2001). Downstream effectors of Akt kinase include molecules essentially involved in S-phase entry such as subunits of cyclin-dependent kinases, and consequently, these were found deregulated in T-cell cultures exposed to MV (Engelking et al., 1999; Fig. 18). Active Akt kinase largely abolished MV-induced proliferation arrest (Avota et al., 2001). The regulatory subunit of the PI3K, p85, which acts upstream Akt kinase activation, was tyrosine phosphorylated shortly after TCR ligation in MV-exposed T cells, yet failed to redistribute to cholesterol-rich detergent-resistant membranes (DRMs) and this correlated with a lack of TCR-stimulated degradation of Cbl-b protein (Avota et al., 2004).

This signal also impairs actin cytoskeletal remodeling in T cells, which lose their ability to adhere to and promote microvilli formation (Muller et al., 2006). The role of Akt pathway in the MV infection may be cell dependent, as its specific inhibitor strongly reduces the replication of several Paramyxoviruses, including MV (Sun et al., 2008). In this study Akt signaling was shown to support the viral replication either directly or by promoting host cell survival and cell cycle entry in nonlymphoid cell types.

Another measles strategy to influence the intracellular signaling in immunocompetent cells is to cause ceramide accumulation in human T cells in a neutral (NSM) and acid (ASM) sphingomyelinase–dependent manner (Avota et al., 2011; Fig. 18). Ceramides promote formation of membrane microdomains and are essential for clustering of receptors and signaling platforms in the plasma membrane. They also act as signaling modulators, namely by regulating relay of PI3K signaling. In T cells ceramides, induced by MV, downmodulate chemokine-induced T cell motility on fibronectin that mimics extracellular matrix interaction (Avota et al., 2011; Gassert et al., 2009). In human DCs SMase activation and subsequent ceramide accumulation are directly linked to c-Raf-1 and ERK activation in response to DC-SIGN ligation. DC-SIGN signaling in turn may weaken TLR4 signaling, thereby downregulating inflammatory responses. SMase activation also promotes surface translocation and compartmentalization of CD150 receptor, promoting MV fusion (Avota et al., 2011).

MV exposure also results in an almost complete collapse of membrane protrusions associated with reduced phosphorylation levels of cofilin and ezrin/radixin/moesin (ERM) proteins. The signal elicited by MV prevents efficient clustering and redistribution of CD3 to the central region of the immunological synapse in T cells. Thus, by inducing microvillar collapse and interfering with cytoskeletal actin remodeling, MV signaling disturbs the ability of T cells to adhere, spread, and cluster receptors essential for sustained T-cell activation (Muller et al., 2006). Together, these findings highlight an as yet unrecognized concept of pathogenesis where a virus causes membrane ceramide accumulation or cytoskeletal remodeling to reorganize the immunological synapses and to target essential processes in T cell activation.

Different murine models have been generated to analyze the effect of MV infection in vivo. Suckling mice ubiquitously expressing CD150 were highly susceptible to the intranasal MV infection (Sellin et al., 2006) and generated FoxP3<sup>+</sup> T regulatory cells. After being crossed to IFN-I deficient background, adult mice developed generalized immunosuppression with strong reduction of hypersensitivity responses (Sellin et al., 2009). In double transgenic mice, expressing both

CD150 and CD46 in the IFN-I deficient background, wild-type MV induced infection of DCs, but functional immunological defect was not further analyzed (Shingai et al., 2005). Finally, the transgenic murine model, capable of reproducing several aspects of measles-induced immunosuppression was made by introducing the V domain of human CD150 to mouse CD150 that allows MV infection in mice. The infected mice developed lymphopenia, impaired T cell proliferation and cytokine imbalance (Koga, Ohno, Ikegame, & Yanagi, 2010) illustrating potential in vivo role of CD150 in measles immunopathogenesis, although the effect on MV replication and its interaction with CD150 on the modulation of immune responses was not clearly dissociated in any of these transgenic models.

#### **4.3.5 MV interference with the function of dendritic cells**

The initiation of adaptive immune responses to viruses and bacterial infections relies on the activation of T and B cells by professional antigen-presenting cells, dendritic cells (Trifilo et al., 2006). Maturation of DCs includes the up-regulation of co-stimulatory molecules and expression of proinflammatory cytokines, shown to be impaired during measles infection (Trifilo et al., 2006). At initial stages of infection, MV replication induces maturation of immature DCs. However, CD40-dependent maturation of DCs is inhibited by MV replication, which is demonstrated by repressed induction of co-stimulatory membrane molecules (CD40, CD80, CD86) and a lack of activation (CD25, CD69, CD71) and maturation (CD83) marker expressions (Fig. 18). The inhibition of CD80 and CD86 expression could be related to the impairment of CD40 signaling, which is demonstrated by inhibition of tyrosine-phosphorylation in MV-infected CD40-activated DCs (Servet-Delprat et al., 2000). The same effect of MV infection was observed in transgenic mice expressing hCD150 on dendritic cells under the CD11c promoter. After infection with wt MV, murine splenic hCD150+ DCs decreased the expression level of B7-1 (CD80), B7-2 (CD86), CD40, MHC class I, and MHC class II molecules and increased the rate of apoptosis (Fig. 18). Furthermore, MV-infected DCs failed to stimulate allogeneic T cells. Analysis of CD8+ T cells showed that they were poorly or even not activated, as judged by their low expression levels of CD44, CD25, and CD69 molecules and by their inability to proliferate (Hahm et al., 2004).

After the first contact with pathogen antigens DCs undergo morphological changes and increase migratory capacities. Upon measles virus infection antigen-presenting function of DCs is partly damaged. Antigen uptake, as measured by mannose receptor-mediated endocytosis, is not affected (Grosjean et al., 1997). However, the ability of MV-infected DCs to stimulate proliferation



of heterologous CD4<sup>+</sup> T cells is affected that was shown in mixed leukocyte reaction (MLR) (Dubois et al., 2001; Fugier-Vivier et al., 1997; Grosjean et al., 1997; Schnorr et al., 1997). MV infection of DCs also prevents CD40L-dependent CD8<sup>+</sup> T cell proliferation (Servet-Delprat et al., 2000). The full T-cell activation does not occur probably because T-cell conjugation with DCs is unstable and short (Shishkova et al., 2007). MV-exposed T cells are recruited into conjugates with DCs in vitro but have impaired clustering of receptors needed for sustained T-cell activation, in part due to accumulation of ceramide and preventing stimulated actin cytoskeletal dynamics and recruitment of surface receptors and membrane-proximal signaling complexes (Gassert et al., 2009).

Measles virus attached to DC-SIGN (de Witte et al., 2008) or Langerin (Van der Vlist et al., 2011) probably uses DCs as the vehicle to the secondary lymphoid organs. However DCs are not found in efferent lymphatic vessels as they probably undergo apoptosis in the lymph nodes. MV-infected DCs develop increased sensitivity to CD95L and undergo CD95-mediated apoptosis when co-cultured with activated T cells. Moreover, CD95-dependent DC apoptosis participates in MV release (Servet-Delprat et al., 2000). IFN- $\alpha/\beta$  production induced by MV in human DCs, triggers the synthesis of functional TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) on their surface. TRAIL belongs to TNF family receptors and was shown to be involved in apoptosis of several tumor cell lines. Moreover, MV was found to induce functional TRAIL expression also in monocytes after activation of IFN- $\alpha/\beta$  expression (Vidalain et al., 2000). Thus, IFN- $\alpha/\beta$ -activated DCs and monocytes may exert an innate TRAIL-mediated cytotoxic activity towards surrounding MV-infected cells (Servet-Delprat et al., 2003).

Prolonged suppression of IL-12 production has been observed in children with measles that is critical for the orchestration of cellular immunity (Atabani et al., 2001). CD40-induced cytokine pattern in DCs is also modified in vitro by MV replication: IL-12 and IL-1 $\alpha/\beta$  mRNA are decreased, whereas IL-10 mRNA is induced (Fugier-Vivier et al., 1997; Servet-Delprat et al., 2000; Fig. 18).

When infected with MV, transgenic mice, which express human CD150 receptor on DCs are defective in the selective synthesis of IL-12 in response to stimulation of TLR4 signaling, but not to engagements of TLR2, 3, 7 or 9. MV suppressed the TLR4-mediated IL-12 induction in DCs even in the presence of co-stimulation with other ligands for TLR2, 3, 7 or 9. MV V and C proteins are not responsible for IL-12 suppression mediated TLR4 signaling, while the interaction of MV-H with human CD150 facilitates this suppression. IL-12 is a key cytokine inducing cellular immune responses to protect host from the pathogenic infections. It is possible that expressed on MV-infected cells MV-H, interacts with CD150 receptor on uninfected cells to cause the inhibition of

TLR4-mediated IL-12 synthesis. Similarly, MV abrogates the function of DCs in detecting and delivering of danger signals by pathogenic components via TLRs to the host immune system (Hahm et al., 2007). Modulation of TLR signaling by MV also occurs upon co-ligation of DC-SIGN, as reflected by induction of DC-SIGN-dependent Raf-1 activation, which in turn promotes IL-10 transcription in immature DCs by inducing acetylation of the NF- $\kappa$ B subunit p65. Measles virus induces IL-10 in the absence of LPS, because it binds to both TLR2 and DC-SIGN (Gringhuis et al., 2007).

IL-10, an immunoregulatory and immunosuppressive cytokine, is also elevated for weeks in the plasma of children with measles (Moss et al., 2002). It downregulates the synthesis of cytokines, suppresses macrophage activation and T cell proliferation, and inhibits delayed-type hypersensitivity responses, which could contribute to the increased susceptibility to the secondary infections), often observed during measles.

CD150 surface receptor is expressed on the main measles virus cell targets: DCs, T and B cells, activated monocytes and macrophages. Its interaction with MV hemagglutinin mediates the viral entry and therefore plays an important role in the primary steps of the infection. CD150 is described as a dual-function receptor, involved in the regulation of immune cell functions. While in B cells CD150 stimulation promotes cell activation, proliferation and Ig production, in T cells its ligation has mostly inhibitory effect (particularly, the inhibition of IFN- $\gamma$  production). Moreover, by mediating the inhibition of IL-12, IL-6 and TNF- $\alpha$  secretion by DCs and induction of IL-4 production by NKT cells, CD150 shifts the differentiation of CD4+ T cells towards Th2 phenotype, thus inhibiting the generation of IFN- $\gamma$  producing Th1 cells. Engagement of CD150 was shown to regulate the activation of several signaling pathways, including MAPK and Akt/PKB. Measles virus infection is associated with dramatic suppression of cellular immune response, which is accountable for the risk of secondary infections and high mortality rate after measles infection. MV-induced immunopathogenesis is linked to the aberrant DC maturation, modulation of IL-12 production and inhibition of T cell proliferation. At the same time, MV induces virus-specific lifelong immunity as humoral immune responses remain almost intact, presenting an immunological paradox associated with this infectious disease. It is possible, that measles virus uses CD150-mediated signaling machinery to inhibit cellular immune response allowing successful viral propagation.

## **II. OBJECTIVES**

Measles virus (MV) is among the most contagious pathogens for humans. This virus is responsible for an acute childhood disease, which could be associated with severe complications. MV induces a profound suppression of the immune system responsible for the high mortality rate of this infection. Measles infection in certain cases can be also followed by invasion of central nervous system (CNS). Our work aims to better understand the molecular and cellular mechanisms of these most important measles complications.

Measles virus envelope glycoprotein, hemagglutinin, directly interacts with viral major entry receptor CD150, which is a signaling receptor, widely expressed in the hematopoietic tissues. CD150 mediates the activation of several signal transduction pathways that regulate immune cell differentiation and function. Therefore, the interaction between CD150 and MV hemagglutinin (H) *in vivo* may be implicated in MV-induced immunosuppression. Thus, **the first objective** of my thesis was to study the role of CD150-MV-H interaction in measles immunopathogenesis. Dendritic cells (DCs) orchestrate the immune response to pathogens. We therefore focused our studies on the analysis of the effect of CD150-MV-H interaction on the modulation of DCs' function. For modeling of MV-H expression on the surface of infected cells, we generated a cell line, stably transfected with wild type MV-H. Using this cell line, we have studied the effect of MV-H on DCs maturation and function. Furthermore, to find out the molecular mechanisms, that underlie the MV-induced immunosuppression, we have monitored the signaling events upon CD150 ligation with anti-CD150 mAb and/or MV-H. Finally, we looked for the *in vivo* outcome of this interaction in human CD150-transgenic mice.

To analyze the mechanism of MV entry into the cells of CNS, **the second objective** of my thesis was to study the expression of CD150 in human brain and CNS tumors. We studied CD150 expression in cells of glial origin, including primary human CNS tumors and glioma cell lines. While CD150 is not expressed in brain tissues, we found intracellular, but not cell surface CD150 protein expression in glioma cells. Using RT-PCR analysis, followed by sequencing of obtained fragments, in parallel with classical membrane CD150 we identified the novel CD150 isoform, expressed in glioma cells, as well as in several subsets of normal and malignant hematopoietic cells. Altogether, our results revealed that CD150 could be a potential diagnostic marker for CNS tumors and may be considered as a new target for glioma treatment.

In summary, the thesis was focused on two complementary axis of research with the main goals to 1) analyze the role of CD150-MV-H interaction in MV-induced immunosuppression and 2) study CD150 expression in human glioma cells that potentially could be used as a novel diagnostic marker as well as a target for MV-based oncolytic treatment.

## **III. RESULTS**

# **Article 1.**

## **Interaction of Measles virus hemagglutinin with CD150-expressing dendritic cells is sufficient to induce immunosuppression**

Olga Romanets<sup>1,2</sup>, Larysa M. Kovalevska<sup>2</sup>, Tsukasa Seya<sup>3</sup>, Svetlana P. Sidorenko<sup>2</sup> and Branka Horvat<sup>1\*</sup>

<sup>1</sup>INSERM, U758; Ecole Normale Supérieure de Lyon, Lyon, F-69007 France; University of Lyon 1; 69365 Lyon, France

<sup>2</sup>R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU, Kiev 03022, Ukraine.

<sup>3</sup>Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638 Japan.

\*Address correspondence to Branka Horvat, INSERM U758, 21 Avenue Tony Garnier, 69365 Lyon cedex 07, France. Phone: 33.437.282.392; Fax: 33.437.282.391; Email: branka.horvat@inserm.fr

**Running title:** Measles virus-CD150 interaction and immunosuppression

## Abstract

Measles virus (MV) is a highly contagious pathogen, which could cause a profound immunosuppression, resulting in high infant mortality. MV infects dendritic cells (DCs) after binding of MV hemagglutinin (MV-H) to the CD150 receptor. As DC dysfunction is considered essential for the MV immunopathogenesis, we analyzed here the phenotypic and functional consequences of MV-H interaction with DCs. We demonstrate that MV-H in the absence of an infectious context is sufficient to inhibit IL-12 production, downregulate expression CD80, CD83, CD86 and HLA-DR on DCs and suppress DC-mediated T-cell alloproliferation. Interaction with MV-H inhibited CD150-induced JNK1/2 but not ERK phosphorylation in DCs and B cells. Moreover, MV-H stimulated Akt phosphorylation on S473 in CD150<sup>+</sup> DCs and B cells. Engagement of CD150 by MV-H in mice transgenic for human CD150 decreased the inflammatory response, confirming the immunosuppressive effect of CD150-MV-H interaction *in vivo*. Altogether, our results reveal a novel mechanism of MV-induced immunosuppression and shed new light on CD150-mediated immunoregulation.

## Introduction

Measles remains one of the most deadly vaccine-preventable diseases. Although vaccination has controlled measles in many countries, the disease remains an important cause of child mortality (52). This virus induces a respiratory infection, and may cause a profound suppression of the immune system that permits opportunistic infections, leading to high infant morbidity and mortality. Immune responses induced by measles virus infection are paradoxically associated with depressed responses to non-measles-virus antigens—an effect that continues for several weeks to months after resolution of the acute illness. This measles-virus-induced immune suppression renders individuals more susceptible to secondary bacterial and viral infections, which can cause pneumonia and diarrhea and is responsible for much measles-related morbidity and mortality (33).

Myeloid dendritic cells (DCs) and lymphocytes were shown to be principal targets of MV infection in peripheral tissues (11). Different types of DCs, including skin Langerhans cells (17), peripheral blood DCs (45), CD34<sup>+</sup>-derived DCs (17) and monocyte-derived DCs (15) are permissive to MV infection. Viral infection induces formation of DC syncytia, followed by the loss of the DC capacity to stimulate naïve CD4<sup>+</sup>T cells (15, 17) and acquisition of an active inhibitory function on CD4<sup>+</sup> T-cell proliferation in response to allogeneic non-infected DCs (17) or mitogens (45). MV-induced inhibition of T-cell functions could be mediated through either transmission of infectious virus to T cells, leading to a block in the cell cycle (29, 38) and/or delivery of inhibitory signals via infected DCs (14, 17). MV infection was shown to enhance apoptosis of DCs and to inhibit their CD40 ligand-induced terminal differentiation (46, 47, 55). In addition, it MV can induce cytotoxic activity by activation of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) synthesis in DCs and monocytes (46). Due to the crucial role of DCs in the immunoregulation, those studies suggest that MV interference with DC function may represent an important mechanism in the induction of immunosuppression. In most of these studies DCs were infected with MV to alter DC function. Although the infection of DCs is an attractive hypothesis to explain MV-induced immunosuppression, implication of viral proteins interacting with DC membrane proteins may play a role and has not been clearly elucidated so far.

Three different cell surface receptors have been identified to interact with MV hemagglutinin (H) permitting viral entry into cells: CD46 for laboratory MV strains, CD150, for both, wild-type and laboratory MV strains, and finally nectin-4, recently discovered to allow viral egress from the respiratory tract (13, 34, 39, 41, 54). CD150 (or SLAM, for Signaling Lymphocytic Activation Molecule) is a membrane protein that in humans is encoded by the *SLAMF1* gene and is expressed on activated T and B lymphocytes, dendritic cells and monocytes (10, 32, 50, 51). CD150 functions as a co-receptor molecule that mediates signalling via



antigen receptors and could modulate function of macrophages (56). The cytoplasmic tail of CD150 can bind key SH2-containing components of signal transduction pathways, such as SHP-1, SHP-2 and SHIP, also adaptor molecules SH2D1A (SAP) and EAT-2, as well as to Src-family kinases, including Fyn, FynT, Lyn and Fgr and the p85 regulatory subunit of phosphatidylinositol-3 kinase (23, 26, 30, 31, 49). CD150 was shown to regulate Akt and MAPK (mitogen-activated protein kinase) signaling pathways in human primary B lymphocytes, lymphoblastoid and Hodgkin's lymphoma cell lines (58, 59). CD150 cross-linking on CD4<sup>+</sup> T cells also induces phosphorylation of Akt (23). By binding to the CD150 cytoplasmic tail, the adaptor protein SH2D1A/SAP works as a molecular switch that regulates CD150-mediated signaling pathways. CD150 engagement increases T-cell antigen receptor induced protein kinase C  $\theta$  (PKC $\theta$ ) recruitment, nuclear p50 NF- $\kappa$ B levels, NF- $\kappa$ B1 activation and interleukin IL-4 production in an SH2D1A-dependent but Fyn-independent fashion (8, 9). However, CD150-induced cell signaling in DCs is currently unknown.

Interaction between MV and CD150, in addition to mediating virus entry, may thus induce cell signaling leading to the modulation of immune cell functions. Although this is not completely unexpected from the known functions of CD150, a formal proof is missing and intracellular pathways which CD150 may engage in DCs have not been determined. To better understand the cellular and molecular basis of MV-induced regulation of DC function we used a model that allowed us to stimulate CD150 with MV-H in the absence of infectious context. We analyzed the changes of DC phenotype, functions and signaling pathways *in vitro*, including JNK1/2, ERK1/2 and Akt phosphorylation, using the DCs generated from human monocytes, and *in vivo*, by analyzing the effect of MV-H induced CD150 engagement on the generation of the immune inflammatory response in mice transgenic for human CD150.

## **Materials and Methods**

### ***Cell culture***

The B lymphoblastoid cell line MP-1 was maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 10 mM Hepes and antibiotics. CHO (Chinese hamster ovary) cells and CHO transfectants were cultivated in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2mM MEM NEAA, 10 mM Hepes and antibiotics. Human peripheral blood was obtained from healthy donors from the Blood Transfusion Centre (Lyon, France). PBMC were isolated by density Ficoll/Hypaque gradient centrifugation, then centrifuged through a 50 % Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) for 20 min at 400 g. Peripheral blood lymphocytes (PBLs) were recovered from the high density fraction and monocytes from the light density fraction at the interface. CD3<sup>+</sup> lymphocytes were isolated from high density fraction, using microbeads (Miltenyi Biotech) and magnetic cell separation with MACS Separator. DCs were generated *in vitro* from the adherent fraction of purified monocytes, treated for 6 days at 5 x 10<sup>5</sup> monocytes/ml with IL-4 (250U/ml, Peprotech) and GM-CSF (500U/ml, Peprotech). Obtained DCs were CD1a<sup>+</sup> and both DCs and T cells were further cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 10mM Hepes and antibiotics.

### ***Production of stably transfected CHO-H cell line, cell stimulation and IL-12 detection***

The plasmid pCXN2-KA-H containing H protein from wild type MV strain KA was kindly provided by Dr. Y. Yanagi (Kyushu University, Japon). CHO cells were transfected with pCXN2-KA-H plasmid using Lipofectamine Transfection Reagent (Life Technologies) following the manufacturer's protocol. The selective antibiotic G418 was added 48h later and cells were cultured until separate clones appeared. The polyclonal cell line was cloned and single-cell-colonies were amplified and analyzed on flow cytometry using anti-HA antibodies. The obtained cell line, designated as CHO-H, along with CHO, was used further for MP-1 and DC stimulations. All stimulations lasted 24 hours and were performed in 1:5 (DC/MP-1 : CHO/CHO-H) cell ratio. The production of IL-12 cytokine by DCs was measured in supernatants using human IL-12 (p70) ELISA kit (BD Biosciences), according to the manufacturer's instructions.

### ***Flow cytometry analysis and cytokine detection with ELISA***

DCs were stimulated with CHO/CHO-H cells as described, and stained with anti-CD80 and HLA-DR (FITC labeled), CD1a, CD83 (PeCy5 labeled) and CD86 (PECy7-labeled) (all from BD-Science), anti-CD46 (MCI 20.6) (40) and anti-MV-H C155 (16), followed with goat anti-mouse IgG conjugated to PE (BD Biosciences). Viable cells were acquired on FACSCalibur 3C cytometer (Becton Dickinson) and FACS analysis was performed using CellQuestPro software followed by FlowJo software analysis.

### ***Allogeneic MLR and T cell proliferation***

Human DCs, differentiated from peripheral blood monocytes, were stimulated with CHO/CHO-H cells as described above and UV-irradiated at 0.25 J/cm<sup>2</sup> for 30 min. Freshly generated or CHO/CHO-H co-cultured DCs at 10<sup>4</sup> cells per well were added in round-bottomed 96-well plates to 2×10<sup>4</sup> CD3<sup>+</sup> T cells, isolated from allogeneic PBLs. After 6 days, the cells were pulsed with 1μCi of [<sup>3</sup>H]thymidine and harvested after 16 h. Thymidine incorporation was determined using a β counter. Results were expressed as mean cpm ± SD of quadruplicate wells.

### ***Cell stimulation and immunoblot analysis***

DCs and MP-1 cells were stimulated with anti-CD150 mAb, following or not pre-culture with CHO or CHO-H cells for 24 hours as described above. For the stimulation with anti-CD150 antibodies cells were pelleted, resuspended at 10<sup>7</sup> per 1 ml in fully supplemented RPMI 1640 medium and equilibrated at 37°C for 10 to 15 minutes. Then the cells were stimulated using 10 μg/mL anti-CD150 mAb (IgG1, clone IPO-3, IEPOR NASU, Kiev, Ukraine) to ligate CD150. Stimulations were stopped at various time points by diluting of cell suspensions into more than 10 volumes of ice-cold phosphate-buffered saline (PBS) with 0.1% NaN<sub>3</sub>. Cells were pelleted and washed with ice-cold PBS before lysis in 1% Triton lysis buffer supplemented with inhibitors of proteases and phosphatases. Then the samples were boiled for 5 min in Laemly buffer with 2-ME and analyzed by standard SDS-PAGE on 10% gels, followed by western blotting, using rabbit antisera against phospho-Akt (Ser473) and phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA), mouse monoclonal Ab against phospho-JNK1/2 (Thr183/Tyr185) (Santa Cruz Biotechnology) and CD45 (produced in IEPOR NASU, Kiev, Ukraine). Protein expression was detected by horseradish peroxidase (HRP) conjugated secondary antibodies either anti-mouse (Amersham) or anti-rabbit (Promega), visualized using Chemiluminescent Reagent Kit Covalight (Covalab).

### ***Virus***

Recombinant vesicular stomatitis virus (VSV) expressing the MV hemagglutinin (Edmonston strain) (44), and the recombinant VSV control strain were kindly provided by Dr J.K. Rose. All viruses were propagated on Vero cells and harvested when a strong cytopathic effect was observed. Virus titers were determined by PFU assaying on Vero cell monolayers. A mock preparation contained virus-free supernatant from uninfected Vero cells, prepared under the same conditions as a virus. All viruses were inactivated by 30 min exposure at 4°C to 254 nm UV irradiation and inactivation was confirmed by the plaque assay on Vero cells. 5×10<sup>6</sup> PFU of UV-inactivated virus was injected in mice i.p. in all experiments.

### ***Contact hypersensitivity assay***

Mice transgenic for the human CD150 with the expression pattern in tissues closely correlating to humans, were generated as described previously (48) and crossed into C57BL/6 background more than 10 generations. Eight to twelve weeks old CD150tg mice or C57Bl/6 mice (Charles River, France) were injected i.p. with mock preparation, VSV or VSV-H UV-irradiated viruses at  $5 \times 10^6$  PFU and contact hypersensitivity to dinitrofluorobenzene (DNFB) was analyzed as described (27). Briefly, 6h after injection mice were sensitized with 25 $\mu$ l of 0.5% DNFB, diluted in acetone : olive oil (4:1), on shaved ventral skin or left unsensitized (sensitization phase). After 5 days mice received 10 $\mu$ l of nonirritant concentration of DNFB on both sides of the left ear and the solvent alone on the right ear (effector phase). Ear thickness was monitored before challenge and every day after challenge for 4 to 6 days. Ear swelling was calculated as [(T-To) left ear] – [(T-To) right ear], where T and To are ear thickness after and before challenge respectively. The results are expressed as mean ear swelling ( $\pm$ SD). The protocol was approved by the Regional Animal Care Committee (CREEA).

### ***Statistical analysis***

All statistical analyses were performed with GraphPad Prism 5 software. Two groups were compared using unpaired two-tailed Student's t test and three groups were compared using 1 way ANOVA. Differences were considered to be statistically significant when  $p < 0.05$ . Graph results are shown as the mean  $\pm$  SD.

## Results and Discussion

### *MV-H induces phenotypic and functional changes in human DCs*

Measles virus infection implicates complex process of production of several viral proteins and RNA, which could all interfere with cell functions (25, 33). However, the importance of the direct interaction between MV-H with CD150 expressing DCs and its potential consequences in the immunoregulation has not been clarified. To be able to analyze this interaction in the absence of infectious context, we have generated CHO cell line stably expressing MV-H from the wild type (wt) MV strain KA (CHO-H cells). In accord to previous data (12, 35), DCs generated from human monocytes express CD150 (Fig. 1A). This expression was slightly increased after 24 h co-culture with CHO cells, probably because of further DC maturation. However, co-culture with CHO-H cells induced an important downregulation of CD150, but not CD46, which corresponds to the utilization of CD150 receptor by the wt MV (Fig. 1A). Furthermore, DCs co-cultured with CHO-H cells had decreased expression of all analyzed DC activation markers: HLA-DR, CD80, CD83 and CD86, suggesting that the CD150-MV-H interaction may affect the phenotype of DCs and decrease the level of the expression of different co-stimulatory molecules, important for the immunostimulatory role of DCs. Similarly to our results, MV downregulated costimulatory molecules and MHC proteins on splenic DCs from mice expressing human SLAM receptor (19). Infection of activated DCs was shown to induce aberrant maturation of DCs and increase the expression of DC costimulatory molecules (1, 45, 47, 60). We did not observe the effect of MV-H on CD40 ligand-activated DCs (three independent experiments, data not shown), suggesting that MV-infection of DCs does not have the same final outcome as interaction with MV-H.

Since production of immunoregulatory cytokine IL-12 is an important function of DCs, orienting the immune response towards Th1, we further analyzed whether DC-CHO-H co-culture may influence the production of this cytokine (Fig. 1B). The level of IL-12 production by nonstimulated DCs was rather low and was upregulated during co-cultivation with CHO cells. However, DCs co-cultivation with CHO-H significantly reduced the level of IL-12 production. MV infection was shown to skew host immune response towards Th2 phenotype (57) and suppression of IL-12 was reported in MV-infected children (4), in rhesus macaques during measles (42), in MV-infected transgenic mice expressing CD46 receptor (28) and in DCs generated from human PBMC when infected by MV (15, 47). CD46 and MV nucleoprotein were suggested to play a role in the inhibition of IL-12 production from monocytes/DCs (24, 28). Furthermore, MV infection of CD150 tg murine DCs decreased TLR4-induced IL-12 production and the effect was seen with the UV-inactivated MV, although to the lower extent (20). Our results demonstrate for the first time that interaction between MV-H and human DCs may also lead to the inhibition of IL-12 production during measles and thus participate in the generation of the immunosuppressive state. Although the engagement of CD150 with mAb

IPO3 was shown to enhance DC production of IL-12 (7), the homotypic CD150/CD150 interaction strongly inhibited IL-12 production (43), suggesting that different CD150 ligands may have the opposite effect on DC function.

Finally, to evaluate the effect of MV-H in the DC-induced T-cell stimulation, we measured proliferation of CD3<sup>+</sup> lymphocytes after stimulation with allogeneic DCs. Although co-culture of DCs with CHO did not affect their immunostimulatory properties, co-culture with CHO-H decreased significantly the allostimulation (Fig. 1C). Several studies have shown decreased lymphocyte proliferative responses in children with measles (22) as well as with *in vitro* infected human DCs (15, 17) and murine CD150 transgenic DCs (18). In addition, mouse genetics studies showed that individual expression of human SLAMF7, type I IFN receptor, TNF- $\alpha$ , lymphotoxin- $\alpha$  or  $\beta$  from T cells was not required for MV-infected DCs to inhibit the proliferation of T cells (19). Finally, the expression of full-length CD150 and SH2D1A in BI-141 T cell, activated with anti-CD3, abolished the production of IFN- $\gamma$  (26). Altogether, these data suggests that interaction between MV-H and CD150 could induce signaling in DCs, important for the negative regulation of their immunological function, which could be involved in MV-induced immunosuppression.

#### ***MV-H modulates signaling pathways in DCs***

Maturation of human DCs is regulated by the modulation of the balance of three mitogen-activated protein kinases (MAPK): JNK (c-Jun N-terminal kinases), ERK (extracellular signal-regulated kinases) and p38 MAPK (36). Since it was shown that CD150 mediates activation of these kinases in B cells (31, 58, 59), we have explored the effect of MV-H on the activation of JNK and ERK. The phosphorylation of JNK1/2 on T183/Y185 residues reflects the activation state of these kinases. For comparative evaluation of pJNK1/2 levels in human B cells and DCs after co-cultivation with hamster CHO cells we used normalization on CD45 that was not expressed in CHO cells. We did not detect any JNK phosphorylation in CHO cells (data not shown), excluding thus any contamination from the CHO par of the culture. Human DCs and B cell line MP1 were co-cultured for 24h with either CHO or CHO-H and then CD150 was engaged with anti-CD150 mAb (Fig. 2A-C). Similarly to B cells (Fig. 2A, B), stimulation of human DCs with anti-CD150 antibodies triggers the activation of JNK1/2 p54 kinase (Fig. 2C, D). However, kinetics is slightly different; the pick of JNK1/2 p54 activation in B cells was detected at time point of 30 minutes, while in DCs the highest level of phosphorylation was at 90 minutes after CD150 crosslinking. Moreover, in contrast to B cells, in DCs CD150 ligation with antibody induced JNK1/2 p46 phosphorylation in addition to JNK1/2p54. After co-culture of B cells with CHO, kinetics and amplitude of CD150-mediated JNK1/2 p54 activation remained similar, with strong phosphorylation both p54 and p46 JNK1/2 and only slightly enhanced the level of p54 JNK1/2 phosphorylation after 5 minutes of ligation with Abs. In contrast, pre-culture of B cells with CHO-H

significantly abolished the level of JNK1/2 p54 phosphorylation (Fig 2C-3). Similarly, pre-incubation of dendritic cells with CHO-H drastically reduced the level of p54 JNK1/2 phosphorylation but, interestingly, did not affect p46 JNK1/2 phosphorylation. In contrast to JNK1/2, we did not observe any changes in the in ERK1/2 activation, in any tested condition (Fig. 2E). Although the effect of MV-H on CD150-mediated JNK phosphorylation could be linked to reduction of CD150 surface levels and consequent less efficient ligation of CD150, the inhibition of only pJNKp54, without affecting pJNKp46 and pERK1/2 phosphorylation in cells, makes it unlikely. Members of JNK family play a crucial role in regulation of responses to various stresses and inflammation. JNK activation is more complex than that of ERK1/2, owing to the input of bigger number of MAPKKs (Mitogen-Activated Protein Kinase Kinase Kinases) (21, 36) and may be more affected by CD150 stimulation, with differential regulation of p46 and p54 subunit phosphorylation.

The studies using specific inhibitors for different MAPK families suggest that three MAPK signaling pathways differently regulate all aspects of phenotypic and functional maturation of monocyte-derived dendritic cells (36). JNK and p38 MAPK positively regulate DC phenotypic maturation and cytokine production which is seen in the downregulation of CD80, CD86, CD83 and HLA-DR surface expression and inhibition of IL-12 production after DC treatment with specific inhibitors of any of these kinases (3, 37). On the contrary, the inhibition of ERK kinase does not reduce but rather slightly increase the expression of DC markers and IL-12 production (2). Thus, various stimuli mediate DC responses via differential regulation of the three MAPKs. Therefore, the immunosuppressive effect of MV-H in lymphoid tissue, which is manifested as the downregulation of DC markers, cytokine imbalance, skewing of immune response towards Th2, may be attributed, at least in part, to the inhibition of JNK pathway.

Akt (v-Akt Murine Thymoma Viral Oncogen)/PKB (Protein Kinase B) is a serine /threonine kinase that is involved in various biological processes including cell proliferation and apoptosis and was shown to be implicated in MV-induced suppression of lymphocyte function (5). In addition, CD150 crosslinking on T and B lymphocytes induces Akt phosphorylation (23, 30).

CD150 ligation with antibody IPO-3 rapidly induced Akt phosphorylation on S473 in MP-1 cells with maximum of kinase activation at 30 min (Fig. 3A). Pre-incubation with CHO cells alone slightly elevated the level of Akt phosphorylation while practically did not affect CD150-mediated kinetics of Akt phosphorylation. However, pre-incubation with CHO-H cells significantly enhanced both initial level of Akt phosphorylation in B cells and CD150-mediated kinetics of Akt phosphorylation. In contrast to B cells, CD150 ligation with IPO-3 mAb did not induce Akt phosphorylation on S473 in DCs (Fig. 3B). Pre-incubation of DCs with CHO slightly enhanced basal level of pAkt and crosslinking with anti CD150 mAb further induced Akt phosphorylation, suggesting that for CD150-mediated Akt phosphorylation in DCs requires pre-activation with some additional stimuli, which B cells do not require. This is in accord with our

flow cytometry data on DC surface CD150 increase, after pre-incubation with CHO. Similarly to B cells, pre-cultivation with CHO-H strongly elevated both basal level and CD150-mediated Akt phosphorylation (Fig. 3C). So, MV-H by itself may activate signaling pathways linked to Akt, presumably via CD150.

CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction followed by phosphorylation of CD150 and attraction of p85 $\alpha$  regulatory subunit of PI3K. It was demonstrated by SPR that p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150ct, and that this interaction takes place in normal tonsillar B cells and lymphoblastoid cell line MP-1 (58). One of the downstream targets of Akt kinase in B cells is transcription factor FoxO1. Despite the high basal level of pFoxO1, CD150 mediates FoxO1 phosphorylation in normal tonsillar B cells and lymphoblastoid cell line (LCL) MP-1 (58). Whether it could have the same effect in DCs remains to be determined.

#### ***MV-H-CD150 interaction inhibits inflammatory immune response***

MV infection results with a suppression of delayed-type hypersensitivity responses to recall antigens, such as tuberculin (53). We have thus analyzed whether interaction between MV-H and human CD150 may be involved in the inhibition of inflammatory response, hypersensitivity reaction to the hapten DNFB. We used transgenic mice expressing CD150 for this test in the similar pattern as humans (48) and engaged CD150 by injecting UV-inactivated vesicular stomatitis virus (VSV) expressing or not MV-H (44). This approach has already been shown to successfully engage transgenic membrane receptors on immune cells (27). While mice injected with VSV developed good inflammatory response, the reaction was significantly reduced when they were injected with VSV-H (Fig. 4). These results strongly suggest that MV-H-CD150 interaction may play an important role in the inhibition of cellular immune response, characteristic for measles. MV-H from wild type strains was reported to activate TLR2 signaling (6). However, the utilisation of VSV expressing MV-H from the Edmonston strain, shown not to be capable of interacting with TLR2, excludes this ligand from the observed effect in mice. It has been demonstrated that MV-H as well as MV fusion protein F interacts with DC-SIGN, which could mediate transinfection of MV by DCs (12). Nevertheless, the absence of the effect of VSV-H in non-transgenic mice (27), suggests that anti-inflammatory effect of MV-H passes via the interaction with CD150, rather than DC-SIGN or TLR2.

Altogether, these results present an evidence for a novel mechanism of MV-induced immunosuppression, where direct contact between MV-H and CD150<sup>+</sup> DCs is sufficient to transmit intracellular signals via MAPK and Akt pathway to alter DC functions in the absence of any infection. As MV-H is expressed on all infected cells in addition to free viral particles and therefore can easily come in contact with circulating DCs, this interaction could thus play an important role in measles immunosuppression and amplify the effect of viral replication in the inhibition of the



immune response. Furthermore, obtained results reveal the immunosuppressive capacity of CD150, known largely as an activating molecule, when it is engaged with a viral ligand, expanding thus the immunomodulatory properties of this molecule.

### **Acknowledgements**

The work was supported by INSERM, Ligue contre le Cancer, Ministère des affaires Etrangères, Partenariat Curien franco-ukrainien «Dnipro», The State Fund for Fundamental Researches of Ukraine, National Academy of Sciences of Ukraine, FEBS Summer and Collaborative Fellowships, Bourse de Gouvernement Français and “AccueilDoc from Region Rhone Alpes. Authors thank to Dr. D. Gerlier and members of the INSERM-U758 group “Immunobiology of viral infections” for their help in the achievement of this study.

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### Figure legends:

**Figure 1.** Effect of MV-H-CD150 interaction on DCs phenotype and functions. (A) DCs were co-cultured with either CHO (solid line) or CHO-H cells (dashed line) for 24 hours, and analyzed by cytofluorometry for the cell membrane expression of CD150, CD46, CD80, HLA-DR, CD83 and CD86 on gated CD1c DCs. Light gray filled histogram represents non-stained control, dark grey filled histogram – the expression level of DC markers before CHO/CHO-H co-cultures. Data represent one of four independent experiments with different donors. (B) IL-12 production by DCs was measured in duplicates by ELISA in supernatants of freshly generated DCs (■), or DCs co-cultured for 24H with CHO (■) or CHO-H (■) cells. Data represent one of four independent experiments with different donors (\*P<0.05, unpaired two-tailed Student's *t* test). (C) Human peripheral blood CD3<sup>+</sup> T cells (2×10<sup>4</sup>) were cultured in quadruplicates for 6 days either in culture medium (□), co-cultured with 10<sup>4</sup> DCs (■), 10<sup>4</sup> DCs, either CHO- (■) or CHO-H- (■) pre co-cultured for 24 hours. T cell proliferation was measured after 6 days by [<sup>3</sup>H]thymidine uptake. The results, expressed as mean cpm (±SD), are from one of six representative experiments. (\*\*\*P<0.001, unpaired two-tailed Student's *t* test).

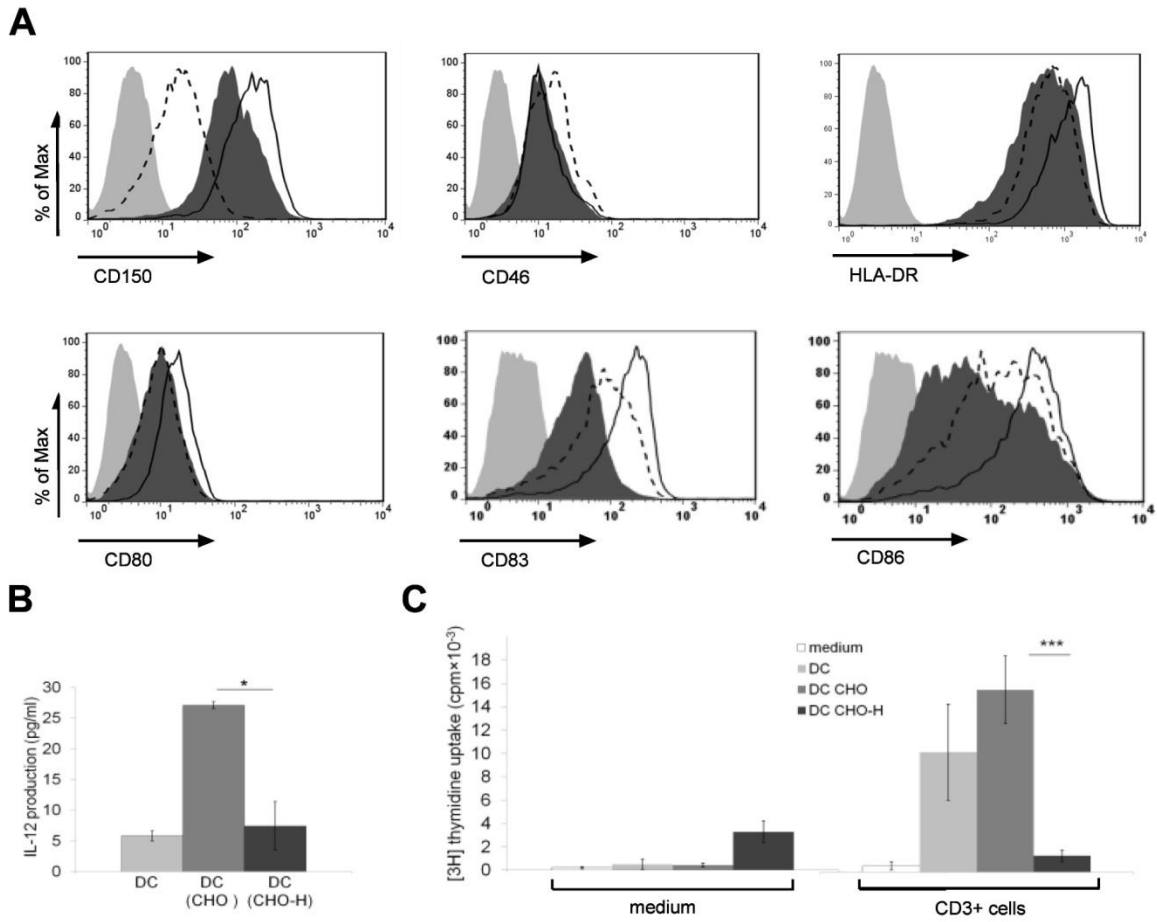
**Figure 2.** MV-H modulates kinetics of CD150-mediated phosphorylation of JNK1/2 but not ERK kinases in B cell line and DCs. (A, B) MP-1 cells or (C, D) DCs were either directly stimulated with 10 µg/ml of anti-CD150 IPO-3 antibody at different time points (1), or co-cultured with CHO (2) or CHO-H (3) cells for 24 hours and then stimulated with IPO-3 at different time points. Cell lysates were analyzed for pJNK1/2 (T183/Y185) expression by western blot, using CD45 as loading control. (B, D) The level of pJNK1/2 in cells directly stimulated with anti-CD150 antibodies (◆), or cells co-cultured before with CHO (■) and CHO-H (▲) cells, was normalized against the level of CD45 using TotalLab program. (E, F) ERK1/2 (Thr202/Tyr204) phosphorylation was analyzed in MP-1 and DC cells using the same experimental set up as above. Results are representative of three independent experiments.

**Figure 3.** MV-H induces Akt phosphorylation in B cell line and DCs. (A) MP-1 cells or (B, C) dendritic cells were either directly stimulated with 10 µg/ml of anti-CD150 IPO-3 antibody at different time points (1) or co-cultured with CHO (2) or CHO-H (3) cells for 24 hours and subsequently stimulated with anti-CD150 mAb IPO-3 at different time points. Cell lysates were analyzed for the level of Akt phosphorylation (S473) using western blot with CD45 as loading control. One of three experiments is presented. (C) The level of pAkt in DCs, co-cultured pre-cultured with CHO (□) or CHO-H (■) cells for 24 hours, was normalized against the level of CD45 in three different experiments (1, 2, 3) using TotalLab program.

**Figure 4.** CD150 engagement inhibits inflammatory immune response *in vivo*. Contact hypersensitivity response to the DNFB was analyzed in groups of five CD150tg or C57B/6 (control) mice, injected i.p. with

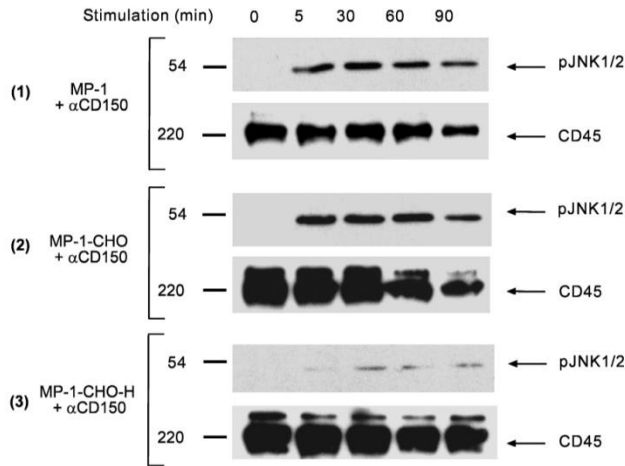
$5 \times 10^6$  PFU of VSV (□) or VSV-H (△). Mice were sensitized 6h later with DNFB or left unsensitized (○) and challenged after 5 days. Results are expressed as mean ear swelling ( $\pm$ SD) of three experiments at indicated time points after challenge. A statistical difference between VSV and VSV-H injected groups is indicated (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 1 way ANOVA).

**Figure 1.**

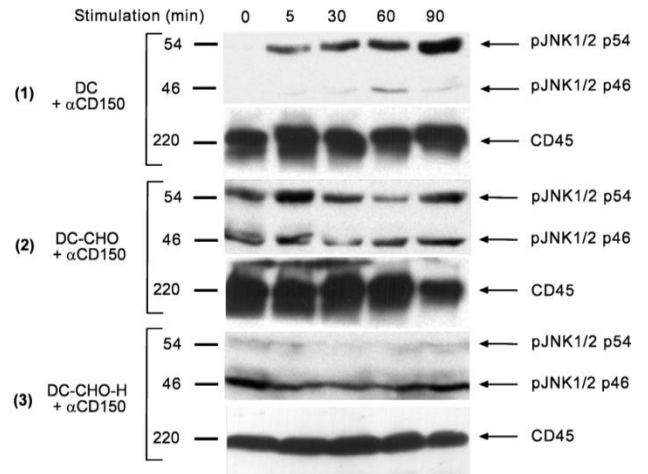


**Figure 2.**

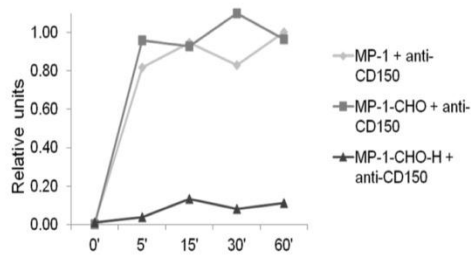
**A**



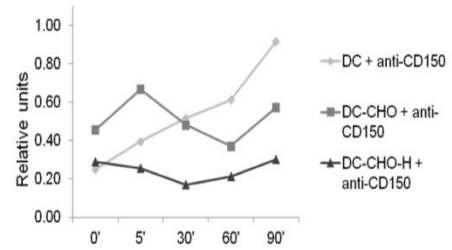
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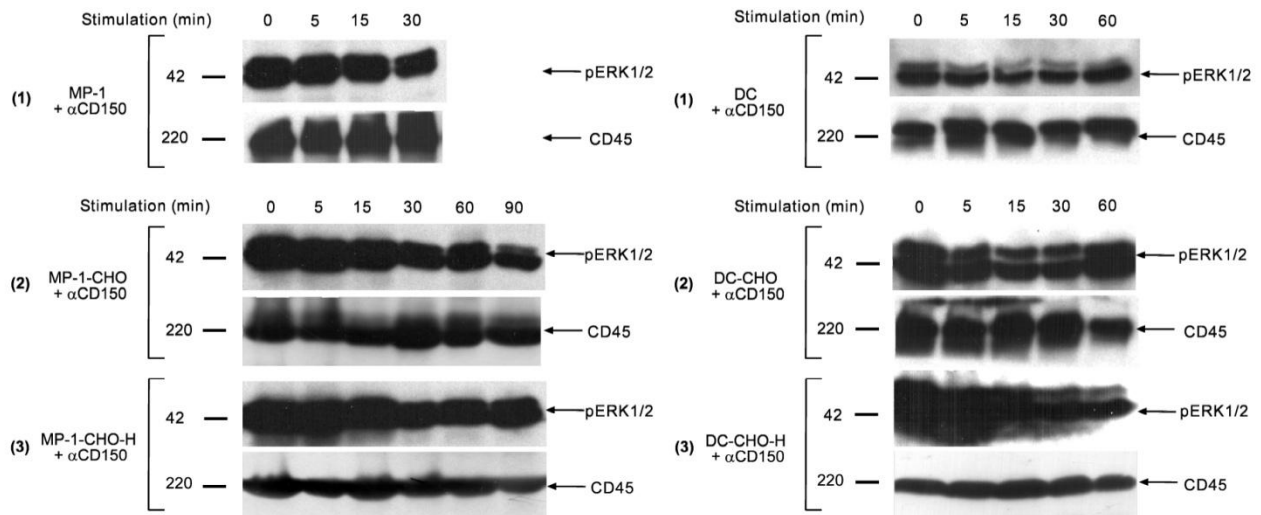
**B**



**D**

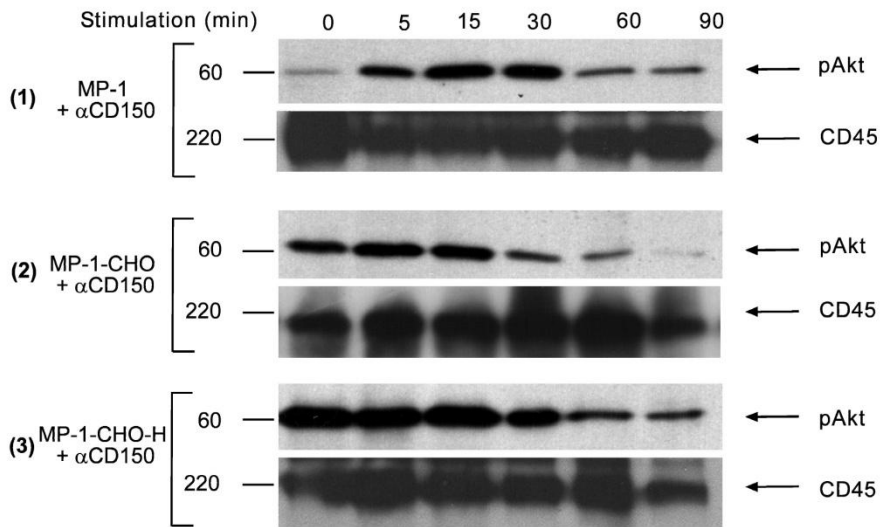


**E**

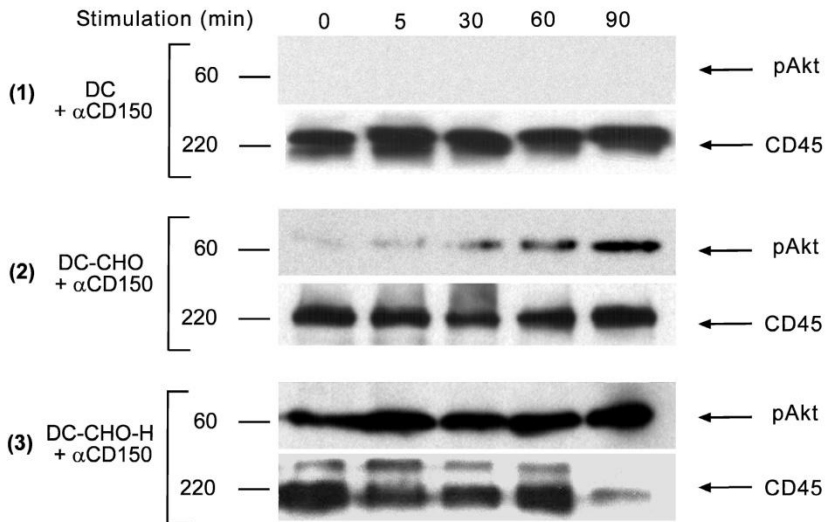


**Figure 3.**

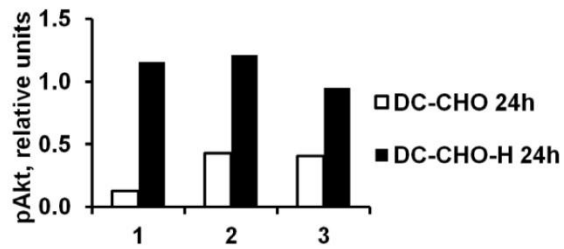
**A**



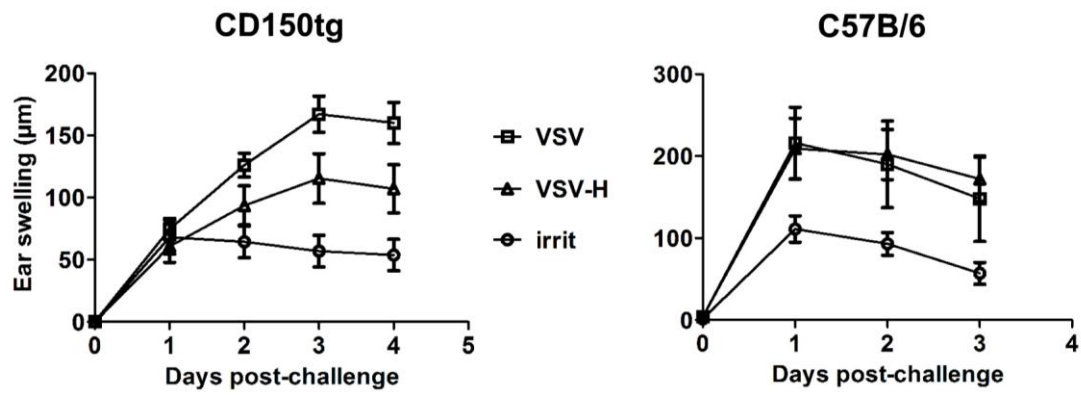
**B**



**C**



**Figure 4.**



## **Article 2.**

### **Expression of CD150 isoforms in tumors of the central nervous system**

Romanets O.<sup>1,2</sup>, Najakshin A. M.<sup>3</sup>, Yurchenko M.<sup>1</sup>, Malysheva T.<sup>4</sup>, Kovalevska L.<sup>1</sup>, Shlapatska L. M.<sup>1</sup>, Zozulya Yu. A.<sup>4</sup>, Taranin A. V.<sup>3</sup>, Horvat B.<sup>2</sup>, Sidorenko S. P.<sup>1</sup>

<sup>1</sup> R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine, Kyiv, Ukraine

<sup>2</sup> INSERM U758, Human Virology, F-69365 France; Ecole Normale Supérieure de Lyon, University of Lyon 1, France

<sup>3</sup> Institute of Molecular and Cellular Biology, Novosibirsk, Russia

<sup>4</sup> Romodanov Institute of Neurosurgery of AMS of Ukraine, Kyiv, Ukraine

#### **Abstract**

CD150 (IPO3/SLAM) belongs to SLAM family of surface receptors and serves as major entry receptor for measles virus (MV). CD150 is expressed on activated T and B lymphocytes, dendritic cells, NKT cells, macrophages, and also on malignant cells with activated cell phenotype at leukemias and lymphomas. However, little is known about its expression outside the hematopoietic system, especially tumors. Our immunohistochemical studies of human CNS tumors for the first time showed CD150 expression in glioblastoma, anaplastic astrocytoma, diffuse astrocytoma, anaplastic ependymoma, ependymoma, anaplastic oligodendroglioma, and others. Since CD150 expression was not observed in different regions of brain tissues from patients with non-tumor pathology, CD150 could be considered as a potential diagnostic marker for CNS tumors. CD150 was not expressed on the surface of glioma cell lines, however, it was detected in cytoplasm and colocalized with endoplasmic reticulum and Golgi complex. Besides full length mCD150 splice isoform, in glioma cells we found a novel CD150 transcript (nCD150) containing 83 bp insert. This insert derived from a previously unrecognized Cyt-new exon located 510 bp downstream of the exon for the transmembrane region. Both mCD150 and nCD150 cDNAs did not contain any mutations, and leader sequence was unmodified. We showed that nCD150 isoform is expressed on mRNA level in glioma cells, normal and malignant B lymphocytes, primary T cells, dendritic cells and macrophages. Promotion of CD150 vertical segregation from intracellular storage compartments in glioma cells could open new perspectives for anti-tumor treatment, including MV based oncolytic therapy of CNS tumors.

## Introduction

CD150 (IPO3/SLAM, for Signaling Lymphocytic Activation Molecule) is a membrane protein that belongs to the SLAM family within the immunoglobulin superfamily of surface receptors. In humans it is encoded by the *SLAMF1* gene [1,2,3]. CD150 is mainly expressed within hematopoietic cell lineage: on thymocytes, activated T and B lymphocytes, dendritic cells, macrophages and monocytes [3,4,5,6,7,8]. CD150 was also found on malignant cells of lymphoid origin [9], however, little is known about CD150 expression outside of hematopoietic system, particularly in tumors. In addition to the transmembrane form of CD150 (mCD150), cells of hematopoietic lineage express mRNA encoding the secreted form of CD150 (sCD150), that lacks 30 amino acids – the entire transmembrane region [4,10,11], mRNAs of the cytoplasmic form (cCD150), lacking the leader sequence, and a variant membrane CD150 (vmCD150 or tCD150) with truncated cytoplasmic tail [12]. However, expression of vmCD150 isoform was not confirmed even on mRNA level [11].

CD150 receptor is a self-ligand and functions as a co-receptor molecule that regulates signals via antigen receptors [13]. It is also associated with several elements of the bacterial killing machinery, which defines it as a novel bacterial sensor [14]. Moreover, CD150 was found to be the major receptor for several Morbilliviruses, enveloped viruses with non-segmented negative-strand RNA genomes, which constitute a genus within the family Paramyxoviridae [15,16]. They include measles virus (MV), canine distemper virus (CDV), rinderpest virus and several others. Measles virus is among the most contagious pathogens for humans, responsible for an acute childhood disease. MV infection is dangerous because of its complications, especially in the central nervous system (CNS). MV is implicated in the pathogenesis of several types of encephalitis: acute postinfectious measles encephalomyelitis, measles inclusion body encephalitis and subacute sclerosing panencephalitis [17]. However, currently identified cellular receptors for wild type MV allow its entry only in lymphoid (through CD150) or epithelial (through nectin 4) cells [15,18,19]. Its third receptor, CD46, is expressed on all human nucleated cells, but is able to mediate the entry of only laboratory adapted and vaccine strains of the virus [20,21]. Thus, it is currently unknown, how does MV enter the cells of nervous system as well as if its receptor CD150 is expressed on nervous cells or not.

Moreover, MV attracts a lot of interest as potential oncolytic virus, as (1) it is a cytopathic virus, (2) a safe attenuated anti-measles vaccine is available and (3) it showed a potent antitumor



activity against multiple tumor lines [22,23]. One of the objects of that kind of studies are CNS tumors, including gliomas, which constitute an important health problem because of infiltrative, aggressive and recurrent nature with only 5 to 15% of 5 year survival. Current therapy includes surgery plus radiotherapy, although the high invasiveness and intracranial localization of these tumors largely limits the effectiveness and even safety of surgery. Oncolytic viruses can replicate preferentially in cancer cells, thereby leading to oncolysis, such that no therapeutic transgene is need as the virus infection itself destroys the tumor mass. Only vaccine strains of MV were used in glioma oncolytic therapy studies [24,25] as wild type MV receptor has not been found on the cells of nervous system yet.

Human central nervous system derives from ectoderma, and CD150 protein expression was found in tumors of ectodermal origin, including basalioma, squamous cell carcinoma of uterine cervix, rectum and oral cavity, but not their normal counterparts [26]. Therefore, the aim of our work was to study the expression of CD150 in human CNS tumors.

Here we show that CD150 is expressed in tumors of central nervous system and glioma cell lines. This antigen is not expressed on glioma cell surface, but was found in the cytoplasm where it was colocalised with endoplasmic reticulum and Golgi complex. We identified a novel CD150 isoform that is expressed not only in glioma cells but also in cells of hematopoietic system. nCD150 transcript contain 83 bp insert derived from a previously unrecognized exon downstream from transmembrane domain. Thus, for the first time we showed that CD150 could be a potential diagnostic marker for CNS tumors. Promotion of CD150 vertical segregation from intracellular storage compartments in glioma cells will open new perspectives for measles virus based oncolytic therapy of gliomas.

## RESULTS

**CD150 is expressed in tumors of central nervous system.** Our immunohistochemical studies of primary human CNS tumors (108 cases) revealed CD150 expression in tumor cells (Fig. 1). The level of expression and the number of positive cases varied for tumors of different histogenesis and grade. Immunostaining of all samples with the antibodies to glial fibrillary acidic protein (GFAP), nestin (neural stem/progenitor cell marker) and the marker of macrophages CD68 validated CD150 expression in malignant cells.

The highest level of CD150 expression was found in glioblastoma that also have the top incidence of CD150 positive cases (88.9% of cases) (Fig.1A, Table 1). The product of immunohistochemical reaction was detected in the cytoplasm of 70-90% of tumor cells with staining of cell processes. Two cases of glioblastoma that were CD150 negative, were characterized by high level of nestin and GFAP expression.

Tumor cells of anaplastic astrocytoma also often expressed CD150 (81.3% of cases), however, demonstrated low to moderate immunoreaction. In one case, CD150 expression was found only in giant tumor cells. Nestin expression was detected only in scattered cells, while anaplastic astrocytoma cells expressed high level of GFAP (Fig.1A).

CD150 expression was found in the majority of tumor cells in 66.7% cases of diffuse astrocytoma. Positive cytoplasmic reaction was observed both in perinuclear zone and cell processes. The fibrillary matrix, which consists of a network of neoplastic cell processes, formed a diffuse CD150 positive background (Fig. 1A). GFAP was consistently expressed, and one third of cells showed immunostaining for nestin (Fig. 1A).

The frequency of CD150 positive cases for ependymoma and anaplastic ependymoma was similar (66.7% and 75.0% respectively). Moderate to high immunostaining was observed in cytoplasm. These tumors were characterized by high level of GFAP as well as nestin expression (Fig. 1A). It should be noted that anaplastic oligodendroglioma (4 cases) demonstrated moderate to high level of immunostaining for CD150 with reaction not only in cytoplasm, but also in the nucleus (Fig. 1A)

Expression of CD150 was also found in anaplastic oligoastrocytoma, anaplastic medulloblastoma, CNS primitive neuroectodermal tumour, subependymal giant cell astrocytoma (Tuberous sclerosis), CNS neuroblastoma, central neurocytoma. At the same time

oligodendroglioma, medulloblastoma and dysembryoplastic neuroepithelial tumour were CD150 negative (data not shown).

To answer the question whether CD150 expression is a characteristic feature of CNS tumors, we studied also sections of seven different regions of brain tissues from patients with non-tumor pathology. Expression of CD150 in different regions of human brain was not observed (Fig. 1B).

Taking together, in 77.6% cases of CNS tumors we found CD150 expression, and it was specific for tumor cells. CD150 was detected mainly in the cytoplasm of tumor cells; however, immunohistochemical approach cannot give the answer whether this antigen is expressed on the cell surface.

### **CD150 is expressed in the cytoplasm but not on the surface of human glioma cell lines.**

To clarify the topography of CD150 expression in glial tumor cells we performed studies of CD150 expression in glioma cell lines. Several live astrocytoma/glioblastoma (A172, U87, U343), medulloblastoma (TE671) and primary glioblastoma (NCH 89 and NCH 92) cell lines were stained with anti-CD150 antibodies and visualized with flow cytometry and fluorescent microscopy. We did not detect CD150 positive cells with this approach (data not shown). However the usage of immunofluorescent microscopic analysis of cells, fixed in methanol-acetone, revealed CD150 expression in the cytoplasm of U87, U343, TE671, NCH89, NCH92, but not A172 cells (Fig. 2A).

To evaluate the expression of potentially very low level of CD150 on the cell surface, we next tested all available glioma cell lines for the susceptibility to the infection with measles virus, which presents very sensitive assay to determine low level of receptor expression. Glioma cell lines were infected either with G954 strain of wild type MV (wt MV), or attenuated (Edmonston) MV strain at MOI of 1. The Vero cells, stably transfected with human CD150 (VeroSLAM), were used as a positive control of infection. Supernatants were taken daily from cultures and virus production was analyzed. We did not observe any cytopathic effect in any of glioma cell lines, infected with wt MV (uses CD150 for cell entry) that was concluded from the absence of syncytia formation (Fig. 2B) and viral particle production (Fig. 2C upper panel). The only susceptible to wt MV cell line was VeroSLAM. In opposite, Edmonston strain of MV, which also can bind CD46 for entry, showed high production of viral particles (Fig. 2C down panel) and strong cytopathic effect in all analyzed cell lines (Fig. 2B). It can be easily explained by CD46 expression on the surface of all glioma cell lines (data not shown). These results support our conclusion that in glioma cell lines CD150 is expressed in the cytoplasm, but not on the cell surface. The possible explanations for the lack of

CD150 cell surface expression are: aberrant transcription of mCD150 or the presence of an alternative CD150 isoform(s).

**Expression of CD150 domains on mRNA level in glioma cell lines.** To check the domain structure of CD150 from glioma cell lines RT-PCR was performed with several sets of primers, designed to detect different parts of CD150 receptor (extracellular, transmembrane and cytoplasmic).

Extracellular part of CD150 (Extra CD150) was detected in glial cell lines U87, U343, A172 (Fig. 3B), TE671(not shown) at almost the same level as in lymphoblastoid cells (LCLs), and at low level in primary glioma cells NCH 89 and NCH 92 (Fig. 3A). The presence of cytoplasmic domain of mCD150 (Cyto CD150) was shown only in U87 and A172 cells, while U343, NCH 89, NCH 92 (Fig. 3B) and TE671 (not shown) cells did not express Cyto CD150 part. PCR with sense primer in transmembrane domain and antisense primer downstream of non-coding RNA region showed that all glial cells expressed transmembrane domain (TM CD150) of the receptor (Fig. 3A). Overall, we found that only A172 and U87 cells expressed all domains of mCD150 splice isoform (Extra, TM and Cyto) on mRNA level (Fig. 3A). All RT-PCR and protein expression data for glial cell lines are summarized in Fig. 3B. The obtained results allowed us to assume that glial cells could express aberrantly transcribed CD150, cCD150 isoform without leader sequence or other splice isoform(s) of CD150 receptor with alternative cytoplasmic tail.

**Identification of a novel splice isoform of CD150.** In several RT-PCR sets with TM primers (Table 1) by modifying PCR conditions (elevating annealing temperature) we were able to get a PCR product of approximately 500 bp length from U87 mRNA. The expected size of PCR fragment with TM primers for mCD150 splice isoform is 426 bp, and we have got such band from LCL MP-1 where conventional mCD150 is expressed on high level [11]. The sequence of RT-PCR product, obtained using U87 mRNA, revealed the CD150 fragment with insert of 83 bp downstream of transmembrane domain of the receptor (Fig. 4A).

To examine if the *CD150* gene is aberrantly transcribed in the U87 cell line, cDNA from U87 cells was amplified using specific primers to the whole coding sequence of *CD150*, cloned to pCI-neo vector and sequenced. Two variants of full-length cDNA were found among the obtained clones. The first was identical to the conventional mCD150 cDNA. The second was a novel CD150 transcript containing 83 bp insert between TM and Cyt1 exons (Fig. 4A). Both variants of cDNA did not contain any mutations, and leader sequence was present and not modified as well.

Alignment with the human genomic sequences demonstrated that the novel transcript contains a previously unrecognized exon located 510 bp downstream of the exon for the transmembrane region. The exon designated Cyt-new is flanked with canonical splice sites AG and GT (Fig. 4A). The insert of the Cyt-new exon results in the reading frame shift in the exons Cyt1-Cyt3 that leads to the formation of premature stop codon in the Cyt3 and production of a novel isoform (nCD150) with cytoplasm tail lacking any known signaling motifs.

To determine whether this exon is specific for the human CD150 genes, we searched for its homologs in the EST (expressed sequence tag) and genomic databases. The search demonstrated the presence of the exon in the CD150 genes of primate species, including marmoset and tarsier, but not in the genomes of other mammals. This fact shows that the novel exon is a specific feature of primate CD150 gene. Its maintenance in different primate species suggests that this nCD150 isoform may play some so far unknown functional role. It should be emphasized, however, that we did not find any mRNA for nCD150 isoform among the human and primate EST sequences.

**nCD150 expression.** Specific primers for nCD150 were designed to follow up nCD150 mRNA expression in human cell of different origin, including primary hematopoietic cells and cell lines (Table 2). We found nCD150 mRNA in all studied glial cell lines and in the primary glioma cells NCH 89 and NCH 92 (Fig. 4B, upper part). nCD150 mRNA was also detected in primary human tonsillar CD38<sup>+</sup> B cells, lymphoblastoid cell line T5-1, Burkitt's lymphoma cell line BJAB, and Hodgkin's lymphoma cell line L1236 (Fig. 4B, middle part). Cells of human acute monocytic leukemia cell line THP-1, normal T cells (CD3<sup>+</sup>), primary dendritic cells and macrophages from peripheral blood, but not monocytes, expressed nCD150 as well (Fig. 4B, lower part). The specificity of all bands presented on Fig. 4B was verified by sequencing.

In this study we showed that CD150 protein remained in the cytoplasm of glial cells and was not found on cell surface (Fig. 2). The reason for such cellular localization is unknown, because both splice isoforms – mCD150 and nCD150 cloned from glial cells U87 contained leader sequence essential for surface expression of the receptor. It is possible, that the retention of nCD150 as well as mCD150 in cytoplasm is a specific feature of glioma cells, but it could be expressed on the surface of cells of other origin. To address this possibility, we transiently transfected U87 and HEK293T cell lines with nCD150 or mCD150 isoforms to follow the difference of CD150 isoforms topology in glioma cells (U87) and cells of non-glioma origin (HEK293T). To our surprise, flow cytometry analysis of CD150 expression in live and permeabilized cells showed surface expression

of nCD150 and mCD150 isoforms in both cell lines (Fig. 5). In addition, these isoforms were also present in cytoplasm of both cell lines (data not shown). The percentage of CD150-positive cells after transfection differed largely because of the different susceptibility of U87 and HEK293T cells for the transfection. Thus, novel and membrane CD150 isoforms transfection, resulted in its surface expression in both glial and non-glial cell lines. These data once more confirm that the artificial transgene expression using plasmids did not completely model the intrinsic gene expression.

**In glioma cells CD150 is colocalized with the endoplasmic reticulum and Golgi complex.** The sequencing of CD150 isoforms isolated from glioma cell line, as well as CD150 overexpression in U87 cells after transfection did not answer the question why in glioma cells CD150 is exclusively localized in the cytoplasm. Therefore we analyzed the colocalisation of CD150 expression with the markers of endoplasmic reticulum (ER) and Golgi apparatus to follow its intracellular trafficking. We compared CD150 intracellular distribution in glioma cell line (U87) and B-lymphoblastoid cell line (MP-1), where it is expressed on the cell surface. For this, U87 and MP-1 cells were stained in parallel with the antibodies to CD150 and markers of endoplasmic reticulum (kinectin-1, GRP78) or Golgi apparatus (furin), followed by the analysis on confocal microscope (Fig. 6). The colocalisation analysis was provided using the Manders coefficients, which ranges from 0 to 1, where 0 is defined as no colocalisation and 1 as perfect colocalisation. It revealed that in the glioma cells CD150 was colocalized with the endoplasmic reticulum similarly to B cells for both of ER markers, kinectin-1 (Manders coefficients 0.65 and 0.69 respectively) and GRP78 (Manders coefficients 0.61 and 0.68 respectively). Concerning Golgi complex – in the glioma cell line CD150 was colocalized with the Golgi marker with almost twice lower colocalisation coefficient, comparing to MP-1 B cell line (Manders coefficients 0.43 and 0.69 respectively).

## DISCUSSION

The majority of the cell surface receptors, first described as leukocyte differentiation antigens, are also expressed on cells of different origin. The well known example is lymphocyte activation antigen CD95, which was found on cells in diverse tissues and tumors of different histogenesis [27]. CD150 was also first described as antigen of activated lymphocytes [7,28,29]. It is expressed on T and B lymphocytes, dendritic cells, thrombocytes, activated monocytes and macrophages [1,2]. It is very little known about expression and functions of this antigen outside of the hematopoietic system. Previously, using immunohistochemical approach we found CD150 expression in several tumors of ectodermal origin, e.g. squamous cell carcinoma of uterine cervix, rectum and oral cavity, basaloma, but not their normal counterparts [26]. In current paper CD150 expression was shown in malignant cells of CNS tumors (Fig. 1A). Since CD150 was not detected in normal brain tissues, expression of this antigen could be considered as a potential diagnostic marker for CNS tumors. The number of CD150-positive cases varied for different histological types of CNS tumors, nevertheless, a clear tendency persisted: the number of CD150-positive cases directly correlated with the tumor grade. Therefore, further broad studies of CD150 expression in CNS tumors in conjunction with evaluation of clinical outcome will give the answer whether CD150 expression can be used as a prognostic marker.

Our immunohistochemical studies clearly showed CD150 expression in cytoplasm of tumor cells, however, were not able to clarify whether this antigen is expressed on the cell surface of malignant cells. Flow cytometry and immunofluorescent microscopy approaches did not reveal CD150 expression on the cell surface of glioma cell lines, while confirmed CD150 cytoplasmic expression (Fig. 2 and data not shown). RT-PCR analysis detected CD150 transmembrane domain in all tested glioma cells (Fig. 3). However, mCD150 expression was limited to A172 and U87 cells, since other glioma cell lines and primary glioma cells expressed only extracellular and transmembrane parts of the receptor, and conventional cytoplasmic part was detected only in A172 and at low level in U87 cells (Figure 3B,C). This allowed us to suppose that in glioma cells CD150 could be aberrantly transcribed or a novel splice isoform with alternative cytoplasmic tail is present in these cells. Indeed, sequencing of 500 bp PCR product from U87 cells obtained with TM primers (Figure 4A) revealed the expression of a novel splice isoform nCD150 with 83 bp insert between transmembrane and cytoplasmic parts. Bioinformatic analysis showed that nCD150 isoform was formed due to alternative splicing, which intergrated an additional previously unrecognized exon

Cyt-new (Fig. 4B). This exon is an exclusive feature of CD150 in primates and was not found in other mammals. It should be emphasized, that nCD150 was expressed in all tested glioma cells, as well as in the majority of hematopoietic cells tested (Fig. 4B).

The absence/modification of leader sequence in glioma cells could potentially lead to the cytoplasmic expression of CD150 splice isoforms in these cells. We obtained the full sequences of mCD150 and nCD150 from U87 cells. However, besides the additional exon, there were no other differences in the sequences of mCD150 and nCD150. Both mCD150 and nCD150 splice isoforms did not contain mutations in the extracellular part and had original leader sequence. Thus, both these isoforms could be exposed on the cell surface. Indeed, transfection of mCD150 and nCD150 to HEK293T or U87 cells resulted in the surface expression of CD150 receptor (Fig. 5), contrary to endogenous CD150 (Fig. 2). So, it should be the intrinsic mechanism for regulation of CD150 surface recruitment in glial cells. Confocal microscopy studies of CD150 compartmentalization in glial cells demonstrated that CD150 localized both in endoplasmic reticulum and Golgi complex, however the colocalization coefficient for Golgi was lower than for ER (Fig. 6). Moreover, CD150 colocalization coefficient for Golgi in glial cells was much lower than in B cell line with high level of surface CD150 expression. It was shown recently that in dendritic cells CD150 is colocalized with Golgi complex and Lamp-1 positive lysosomal compartment [30]. Moreover, CD150 shares an intracytoplasmic lysosomal compartment with acid sphingomyelinase and they are co-transported with CD150 from lysosomal compartments on cell surface upon DC-SIGN ligation [30]. DC-SIGN ligation led to rapid activation of neutral and acid sphingomyelinases followed by accumulation of ceramides in the outer membrane leaflet. This initiates formation of ceramide-enriched membrane microdomains which promote vertical segregation of CD150 from intracellular storage compartments [30]. Thus, ceramide is involved in upregulation of CD150 expression on cell surface. Glial tumors are characterized by significantly lower levels of ceramide compared with peritumoral tissue [31]. Furthermore, ceramide is inversely associated with malignant progression of human astrocytomas and poor prognosis, and also determines glioma cell resistance to chemotherapy [31,32]. PI3K-Akt-PTEN pathway is among the dysregulated oncogenic pathways in gliomas [33]. In these tumors, the activation of the PI3K/Akt pathway is crucial to control ER to Golgi vesicular transport of ceramide, coordinate the biosynthesis of membrane complex sphingolipids with cell proliferation and growth and/or to maintain low ceramide levels. So, the PI3K/Akt pathway could contribute to ceramide downregulation, as a means of avoiding cell growth arrest and cell death in gliomas [34]. It is possible that downregulation of ceramide levels in



glial tumors could contribute to the inability of CD150 to surface relocalization. This block may be overcome in the case of CD150 overexpression.

Our data also disclose another aspect of CD150 expression in glioma cells. Due to the lack of the CD150 cell surface expression, glioma cell lines are resistant to MV entry and consequent oncolysis (Fig. 2). Presumably, an induction of CD150 surface expression on glioma cells could make this antigen an attractive target for oncolytic therapy of gliomas, thus, opening perspectives for its therapeutic application. Altogether, these data show CD150 splice isoform diversity and suggest that CD150 may represent a new diagnostical marker for human brain tumors,

## **MATERIALS AND METHODS**

**Cell lines.**The glioma cell lines A-172 (glioblastoma), U87 (glioblastoma; astrocytoma), U343 (anaplastic glioma), TE671 (medulloblastoma) and primary glioblastoma cell lines from adult patients on the advanced stage of disease NCH 89 and NCH 92 [35] were obtained from Dr. Karsten Geletneky (Department of Neurosurgery, University Hospital Heidelberg, Germany) and Prof. Joerg R. Schlehofer (Department of Applied Tumor Virology, German Cancer Research Center, Heidelberg, Germany). Glioma cell lines and primary glioblastoma cells, HEK293T (human embryonic kidney fibroblasts) were maintained in DMEM medium containing 10% FCS, 2 mM L-glutamine, and antibiotics. Burkitt's lymphoma cell line BJAB, Hodgkin's lymphoma cell line L1236, B lymphoblastoid cell lines LCL, MP-1 and T5-1, human acute monocytic leukemia cell line THP-1 were maintained in RPMI medium containing 10% FCS, 2 mM L-glutamine, and antibiotics.

**Human tissue specimens and primary human cells.** Immunohistochemical analyses were performed on sections of surgical specimens of human primary CNS tumors obtained from the Romodanov Institute of Neurosurgery of AMS of Ukraine after receiving informed consent in accordance with the Declaration of Helsinki (108 cases). The usage of archived biopsy specimens was approved by the Institutional Review Board and Research Ethics Committees of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine and Romodanov Institute of Neurosurgery of AMS of Ukraine. CNS tumors were classified according to the World Health Organization (WHO) classification. Verification of the diagnosis was performed based on a combination of morphologic, topographic and clinical characteristics. Paraffin-embedded sections were stained with mouse anti-CD150 mAb IPO-3, rabbit anti-GFAP, glial fibrillary acidic protein (DakoCytomation, DK) and anti-nestin (Sigma, USA) antibodies followed by the dextran polymeric conjugate (EnVision) technique (DakoCytomation, DK). Immunohistochemical reactions on peroxidase were developed with diaminobenzidine tetrahydrochloride (DAB) substrate (DakoCytomation, DK). Sections were examined documented using Leica DC 150 microscope complex (Germany).

Tonsils were obtained from patients undergoing tonsillectomy with informed consent in accordance with the Declaration of Helsinki. Tonsillar B cells were isolated by density fractionation on discontinuous Lymphoprep (Axis-Shield PoC AS, Norway) and Percoll (Sigma, USA) gradients

as described [36]. 96.5-99.6% of cells in CD2<sup>-</sup> populations were of B cell lineage since expressed CD19 and/or CD138 antigens. Human peripheral blood was obtained from healthy donors from the Blood Transfusion Centre (Lyon, France). PBMC were isolated by density Ficoll/Hypaque gradient centrifugation, and then centrifuged through a 50 % Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) for 20 min at 400 g. Peripheral blood lymphocytes (PBLs) were recovered from the high density fraction and monocytes from the light density fraction at the interface. CD3<sup>+</sup> lymphocytes were isolated from high density fraction, using microbeads (Miltenyi Biotech) and magnetic cell separation with MACS Separator. DCs were generated *in vitro* from the adherent fraction of purified monocytes, treated for 6 days at 5 x 10<sup>5</sup> monocytes/ml with IL-4 (250U/ml, Peprotech) and GM-CSF (500U/ml, Peprotech), macrophages – from the adherent fraction of purified monocytes, treated for 6 days at 5 x 10<sup>5</sup> monocytes/ml with M-CSF (250 U/ml, Peprotech). Obtained DCs were CD1a<sup>+</sup> and both DCs and T cells were further cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 10mM Hepes and antibiotics.

**Reverse-Transcriptase PCR.** Total RNA was isolated from cell lines using TRIzol reagent (GibcoBRL, USA) according to manufacturer's instructions. 5 x 10<sup>6</sup> cells of cell lines or primary cells were homogenized in 1 ml of TRIzol reagent (GibcoBRL, USA), and processed according to the manufacturer's instructions. Reverse transcriptase reactions were performed with M-MLV Reverse Transcriptase and Ribonuclease Inhibitor RNaseOUT<sup>TM</sup> (Invitrogen, USA). Obtained cDNAs were amplified by PCR using Taq DNA polymerase (Invitrogen, USA), and sense and antisense primers indicated below (Table 2) applying the listed programs (Table 3). PCR products were resolved in agarose gels and visualized after staining with ethidium bromide. For sequencing, PCR products were isolated from gel using Quigen gel isolation kit (Quigen, USA). Alignment of sequenced PCR products with CD150 cDNA (gi:176865712) was performed using nucleotide-nucleotide alignment option (blastn) in BLAST internet program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

**Splice isoforms cloning from U87 cells.** mRNA was isolated from U87 cells using ToTally RNA kit (Ambion, USA, TX). First strand was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) according to manufacture instructions. cDNA was amplified using Fusion polymerase (Finnzymes, USA) and 5'-catctcgagCCTTCTCCTCATTGGCTGATGG-3' (329-350, CD150 mRNA sequence GI:176865712) as forward primer and 5'-cacgcggccGCAGCATGTCTGCCAGAGGAA-3' (1436-1456) as reverse primer. PCR fragment of

nCD150 splice isoforms was eluted from the gel with MiniElute Gel Extraction Kit (Qiagen, USA), digested by XhoI and NotI, and ligated into pCI-neo vector (Promega, USA). Transformation was performed using *E. coli* XL-Blue MRF' electrocompetent cells and clones with inserts were selected and sequenced as described elsewhere.

**Transfection of HEK293T and U87 cells.** The plasmid pRRLsin-CMV-IRES-puro containing mCD150 was kindly provided by Dr. Denis Gerlier (INSERM U758, Lyon, France). HEK293T and U87 cells were transfected with pRRLsin-CMV-IRES-mCD150 and pCI-neo-nCD150 plasmids using JetPRIME reagent (Polyplus transfection) following the manufacturer's protocol. The cells were harvested 72 h after transfection and the expression of transgene was studied.

**Cell staining.** For the immunostaining adherent glioma cell lines were grown on the cover slips, while suspensial B cell line MP-1 cells were attached to the microscope slides using Cytospin. All cell lines were fixed/permeabilized with methanol:acetone (1:1) for 1 h at -20°C following overnight rehydration with PBS at +4°C. The cells were stained with anti-CD150 mouse monoclonal antibodies (IgG1, clone IPO-3, IEPOR NASU, Kiev, Ukraine), followed with rabbit anti-mouse IgG antibodies Alexa 546 (Molecular Probes, Invitrogen, USA) or rabbit anti-Kinectin (Santa Cruz Biotechnology, USA), anti-GRP78 and anti-furin antibodies (Tebu-bio, France), followed with donkey anti-rabbit IgG Alexa 647 (Molecular Probes, Invitrogen, USA). The incubation with primary antibodies lasted for 1 h, followed by three washes in PBS, secondary antibody incubation for 30 min, three washes in PBS and one brief wash in water. DAPI (4',6-diamidino-2-phenylindole) was added to the last secondary antibody for DNA staining. Finally the microscope slides were mounted and analyzed using Zeiss LSM-710 fluorescence microscope equipped with Zeiss Zen software for immunofluorescence analysis and Leica SP5 for the confocal analysis. Colocalisation coefficients were counted using ImageJ (MacBiophotonics, USA) software.

**Cell infection with measles virus.** The wild-type MV strain, G954 (genotype B3.2), was isolated in Gambia in 1993, and was propagated on VeroSLAM cells. MV vaccine strain Edmonston was obtained from ATCC (VR-24). Viruses were titrated by assaying PFU on Vero-SLAM cell monolayers. Glioma cell lines and VeroSLAM cells were infected with G954 and Edmonston measles virus strains at MOI of 1. Virus titer was determined using PFU assay. For this,

different dilutions were cultured on Vero-SLAM cell monolayers for 4 days of culture, fixed, and then stained with methylene blue. The number of lysed-cell dots corresponded to the number of plaque forming units and number of primary infectious viral particles applied.

**Flow cytometry.** To study the expression of CD150 glioma cell lines were stained with the mouse monoclonal antibodies (IgG1, clone IPO-3, IEPOR NASU, Kiev, Ukraine) followed with goat anti-mouse IgG conjugated to PE (BD Biosciences). To observe the intracellular expression of the antigen cells were permeabilized with Fixation/Permeabilisation buffers from eBioscience. Stained cells were acquired on FACSCalibur 3C cytometer (Becton Dickinson, USA) and FACS analysis was performed using CellQuestPro software followed by FlowJo (USA) software analysis.

### **ACKNOWLEDGEMENTS**

The work was supported by INSERM, Ligue contre le Cancer, Ministère des affaires Etrangères, Partenariat Curien Franco-Ukrainien «Dnipro», The State Fund for Fundamental Researches of Ukraine, National Academy of Sciences of Ukraine, FEBS Summer and Collaborative Fellowships, Bourse de Gouvernement Français and “AccueilDoc from Region Rhone Alpes. Authors thank to Dr. D. Gerlier and members of the INSERM-U758 group “Immunobiology of viral infections” for their help in the achievement of this study.

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## FIGURE LEGENDS

**Figure 1.** Expression of CD150 in human central nervous system (CNS) tissue. **A.** Immunohistochemical analysis of CD150 expression in human primary CNS tumors. Tumor paraffin sections of diffuse astrocytoma, anaplastic astrocytoma, glioblastoma, anaplastic ependymoma and anaplastic oligodendroglioma were stained with antibodies to CD150 (left panels), glial fibrillary acidic protein, GFAP, astrocyte marker (central panels) and nestin, neural stem/progenitor cell marker (right panels). **B.** Expression of CD150 in different regions of human normal brain: cerebellum (left panel), thalamus (central panel), subventricular zone ependyma (right panel). Microscope magnification -  $\times 400$ . DAB staining shows specific reactions in brown. Cell nuclei are lightly counterstained with hematoxylin.

**Figure 2.** Expression of CD150 in human glioma cell lines. **A.** Immunofluorescent analysis of CD150 expression in U87, U343, TE671 and A172 glioma cell lines. The cells were fixed, stained with anti-CD150 antibodies followed by Alexa 568. Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole. **B.** Measles cytopathic effect in glioma and control cell lines. U87 glioma cell line and VeroSLAM cells as a positive control were infected with wild type measles virus strain G954 and Edmonston laboratory measles strain at MOI of 1. Pictures are showing syncytia formation at 24h (for Vero SLAM cells) or 96h (for U87 cells) post-infection. **C.** Measles viral particles production in glioma and control cell lines. The titres of wild type (upper graph) and Edmonston (lower graph) infectious virus were determined daily by plaque assay on VeroSLAM cells, 24h (1), 48h (2), 72h (3) and 96h (4) post-infection. VeroSLAM cells were used as positive control.

**Figure 3.** CD150 splice isoforms expression in glioma cell lines. **A.** RT-PCR analysis of expression of extracellular (ExtraCD150), cytoplasmic (CytoCD150) and transmembrane (TM CD150) parts of the receptor in glioma cell lines and primary glioma cells. LCL and MP-1 cells were taken for positive control. The quality and quantity of cDNA was controlled by following GAPDH expression. **B.** Table representing RT-PCR results on ExtraCD150, CytoCD150 and TM CD150 expression in glioma cell lines and primary glioma cells.

**Figure 4.** The expression of novel CD150 splice isoforms (nCD150). **A.** Schematic representation of the CD150/SLAM gene structure and alternative splicing of mRNA. A novel 83 bp



exon located 509 bp downstream of the exon for the transmembrane region is used in glioma CD150 transcripts. The exon designated Cyt-new is flanked with canonical splice sites (AG/GT marked in grey in the above genomic sequence). Exons are shown by filled rectangles, noncoding sequences - by solid line. Abbreviations: LS – leader sequence, V and C2 – extracellular domains, TM – transmembrane domain, Cyt – cytoplasmic tail. **B.** Expression of nCD150 splice isoform was examined in glioma cell lines (upper part), human tonsillar B cells (TBC) and cell lines of B cell origin (middle part), human acute monocytic leukemia cell line THP-1 and dendritic cells (DC), T cells (CD3<sup>+</sup>), monocytes (Mon) and macrophages (Mac) (lower part). Primer set for nCD150 and appropriate program were applied. The quality and quantity of cDNA was controlled by following GAPDH expression.

**Figure 5.** Expression of membrane (mCD150) and novel CD150 (nCD150) isoforms in HEK293T and U87 cell lines, transfected with respective plasmids. Surface expression of mCD150 and nCD150 in transfectants. HEK293T and U87 cell lines were transfected with the plasmids expressing mCD150 and nCD150 constructs and harvested 72h after. Live cells were stained with anti-CD150 mAbs and analysed with flow cytometry.

**Figure 6.** Colocalisation of CD150 with the markers of endoplasmic reticulum and Golgi apparatus. U87 or MP-1 cells were stained for CD150 (green) and the markers of endoplasmic reticulum (A) or Golgi apparatus (B), (red). Markers for endoplasmic reticulum - kinectin-1 (A, rows 1-2) and GRP78 (A, rows 3-4), for Golgi – furin (B, rows 1-2). Nuclei were visualized by staining with DAPI (4',6-diamidino-2-phenylindole), (blue). Colocalization coefficients were determined using the Manders algorithm (which ranges from 0 to 1, where 0 is defined as no colocalization and 1 as perfect colocalization) and are indicated within the panels.

**Table 1.** CD150 expression in CNS tumors

Tumor histological type	Number of cases	CD150-positive cases, %	Tumor grade
Diffuse astrocytoma	12	66,7	II
Anaplastic astrocytoma	16	81,3	III
Glioblastoma	18	88,9	IV
Ependymoma	4	66,7	II
Anaplastic ependymoma	6	75	III
Anaplastic oligodendroglioma	8	100	III

**Table 2.** Primers for GAPDH and CD150

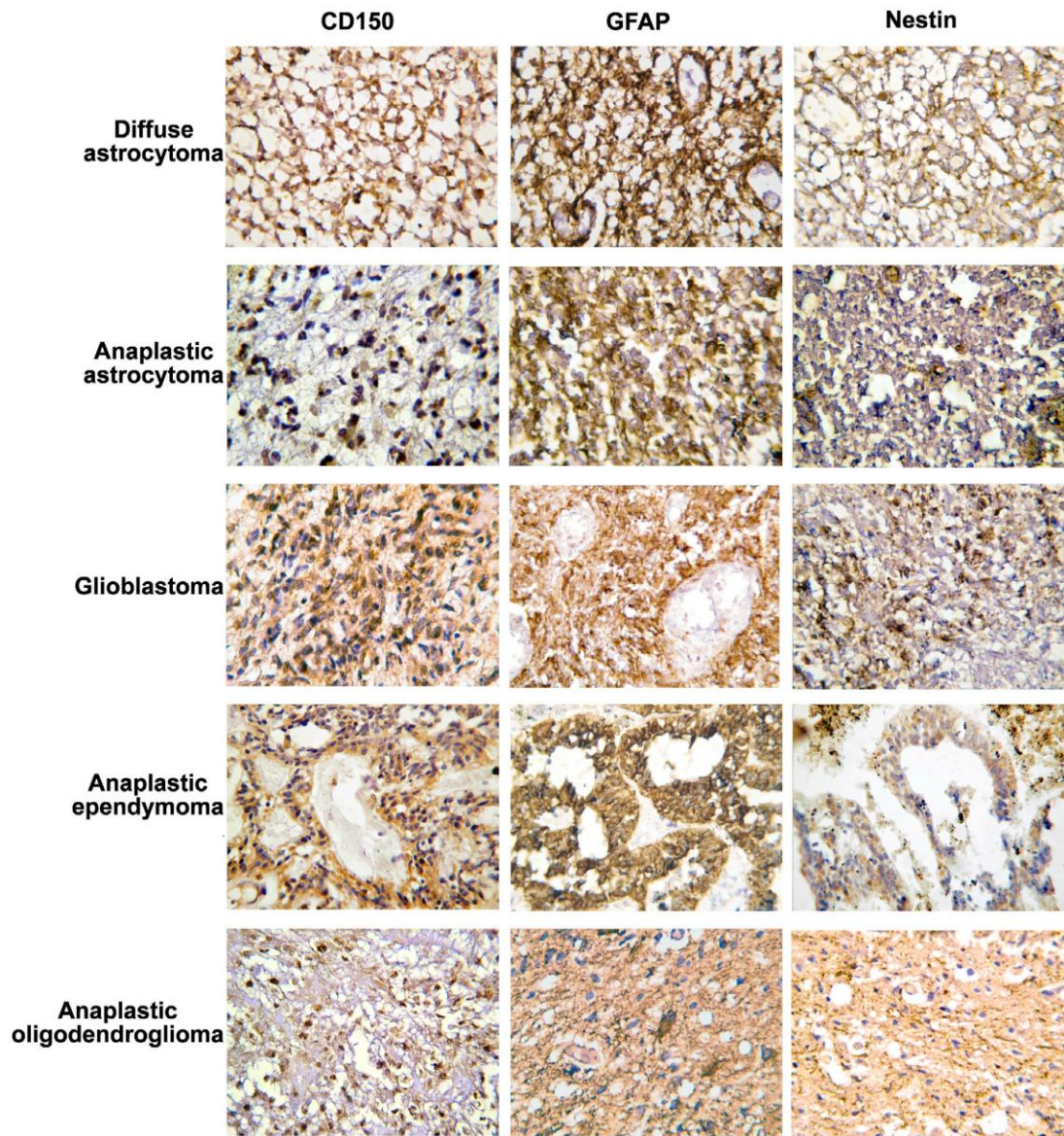
Product	Sense primer 5'→3' (position on sequence)	Antisense primer 5'→3' (position on sequence)	Product size
<b>GAPDH</b>	TCATTATGCCGAGGATTT GGA	CAGAGGGCCACAATGT GATG	500 bp
<b>ExtraCD150</b>	ATGGATCCCAAGGGGC (347-362)	CCCAGTATCAAGGTGCA GGT (815-834)	488 bp
<b>CytoCD150</b>	TTGAGAAGAAGAGGTAA AACGAAC (1124-1147)	CTGGAAGTGTCACACTA GCATAG (1324-1346)	223 bp
<b>TM CD150</b>	ACAGACCCCTCAGAAAC AAAACCAT (1034-1058)	CGTGCAGCATGTCTGCC AGAGGAAACTTG (1438- 1459)	426 bp
<b>nCD150</b>	TGCTGACAATATCTACAT CTG (952-972)	CAGTATTGGTTGGTAGT AGTC (on Cyt-new exon)	213 bp

**Table 3.** PCR programs for GAPDH and CD150

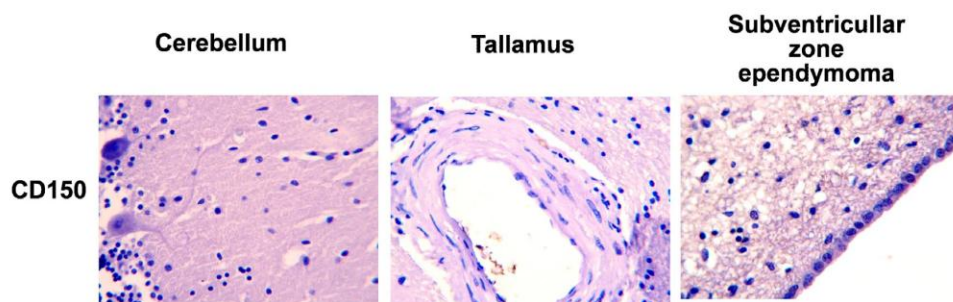
Product	PCR program
<b>GAPDH</b>	95°C, 2 min; (95°C, 15 s; 59°C, 20 s; 72°C, 30 s) x 30 cycles; 72°C, 3 min
<b>ExtraCD150</b>	95°C, 2 min; (95°C, 10 s; 60°C, 20 s; 72°C, 20 s) x 30cycles; 72°C, 3 min
<b>CytoCD150</b>	95°C, 2 min; (95°C, 5 s; 56°C, 10 s; 72°C, 10 s) x 30 cycles; 72°C, 3 min
<b>TM CD150</b>	95°C, 2 min; (95°C, 15 s; 65°C, 45 s; 72°C, 45 s) x 20 cycles; (95°C, 60 s; 55°C, 45 s; 72°C, 45 s) x 15 cycles; 72°C, 3 min
<b>nCD150</b>	95°C, 2 min; (95°C, 10 s; 54°C, 10 s; 72°C, 10 s) x 30 cycles; 72°C, 3 min

**Figure 1.**

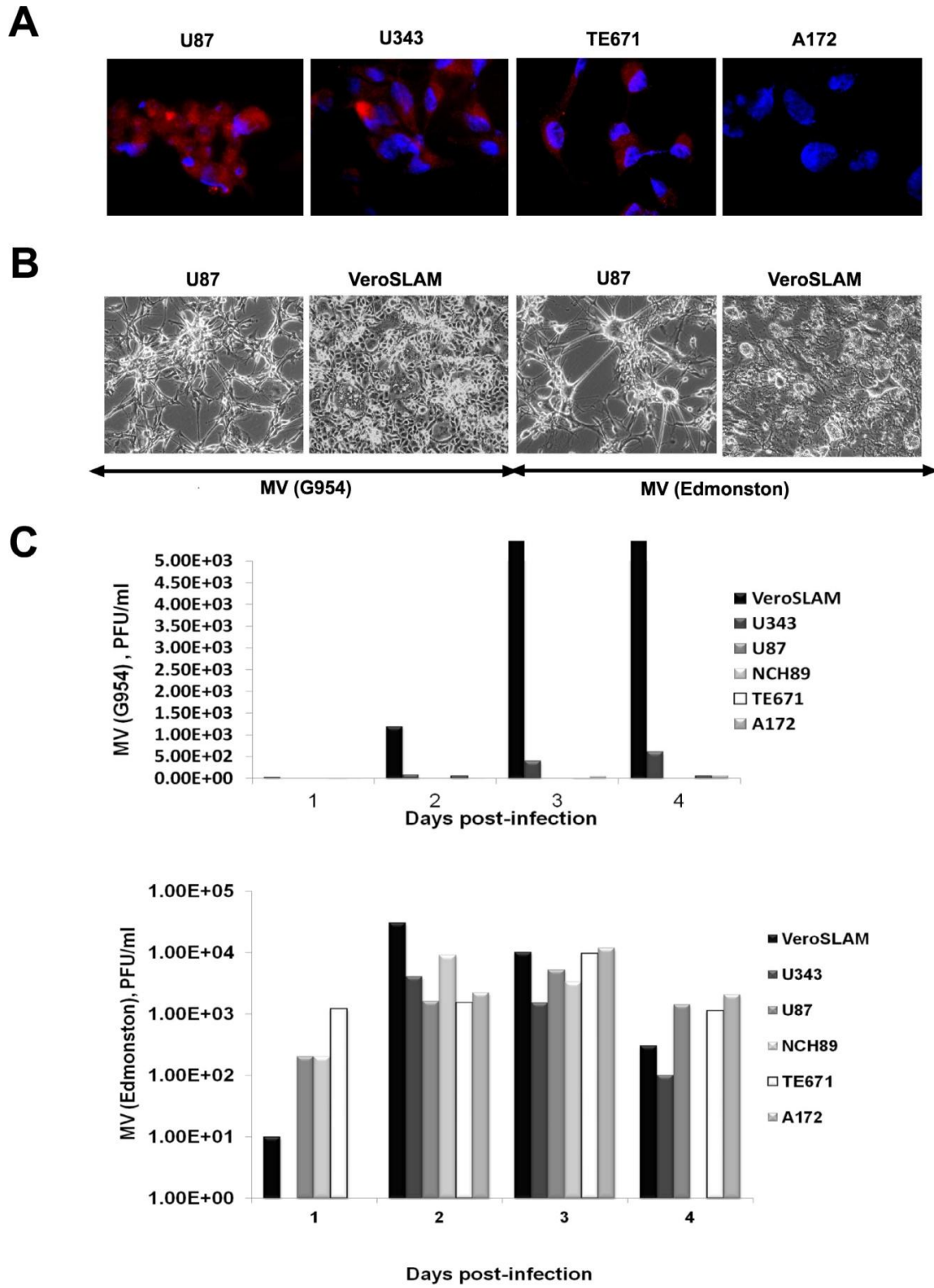
**A**



**B**

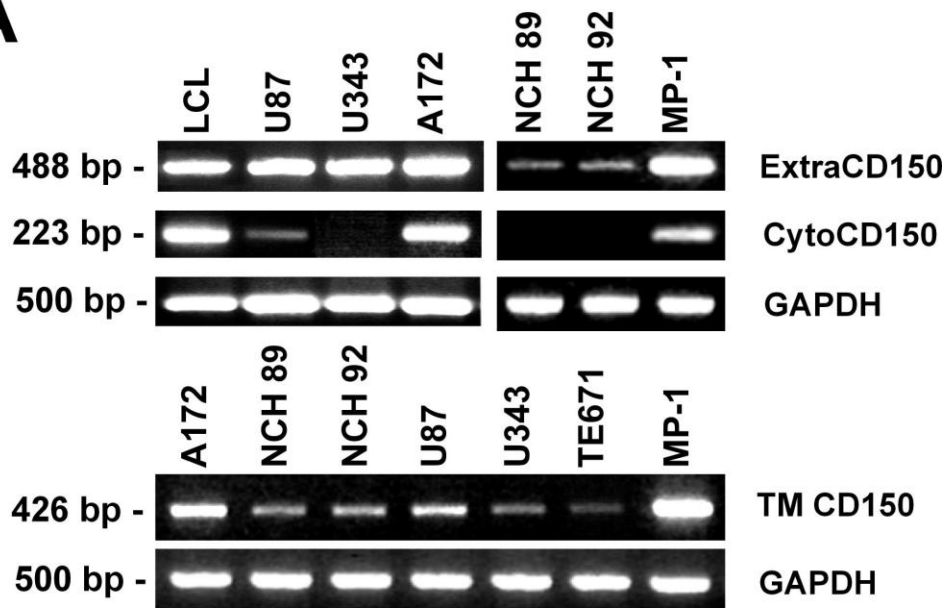


**Figure 2.**



**Figure 3.**

**A**

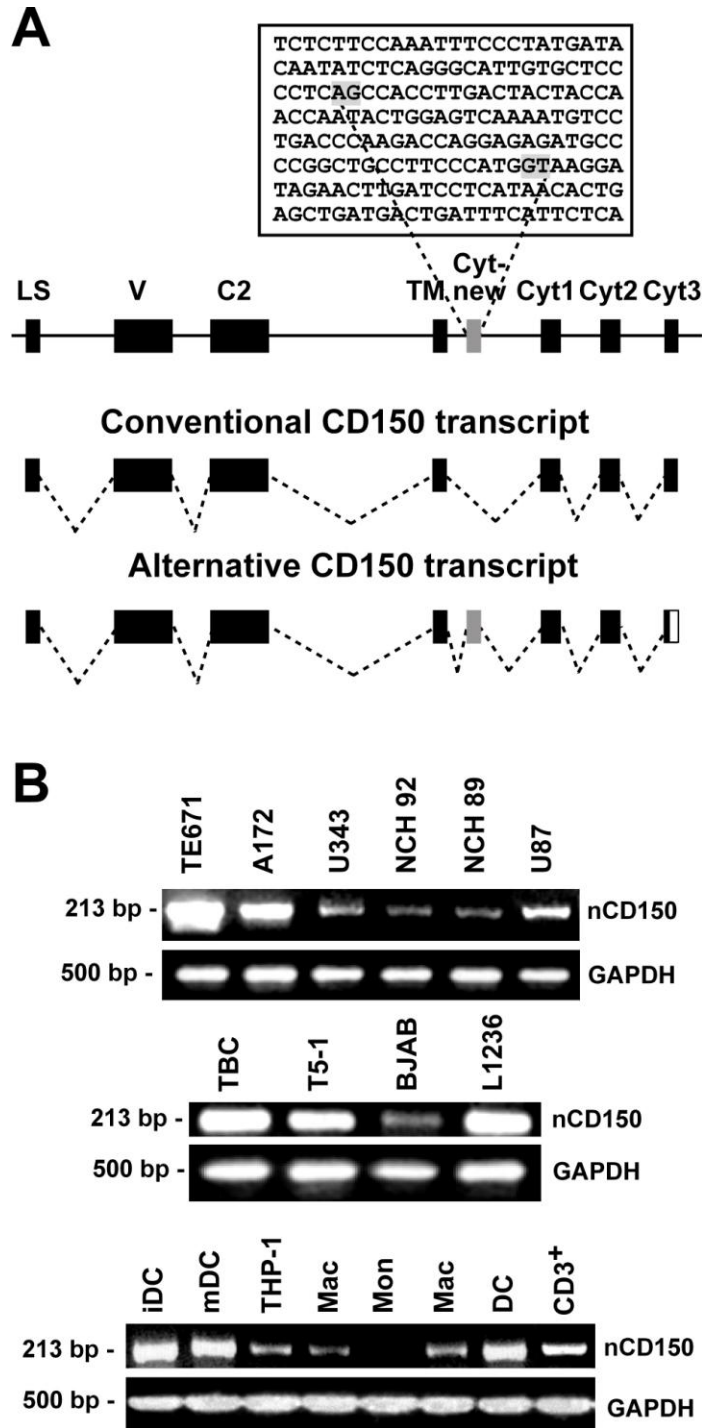


**B**

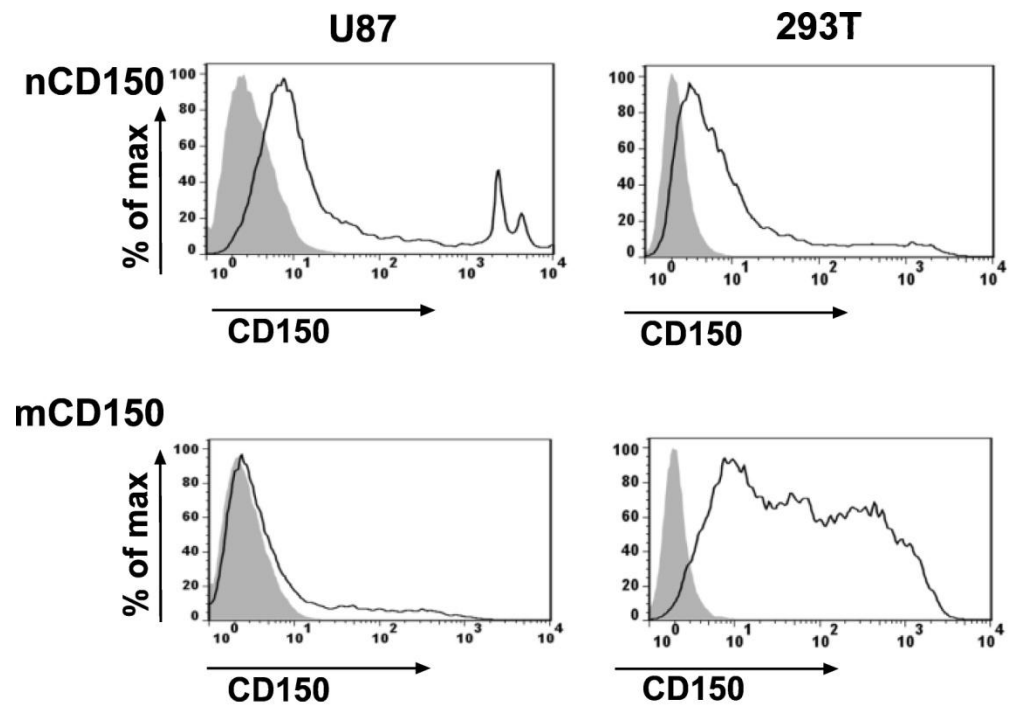
Glioma cell line	mRNA			Protein
	Extra	TM	Cyt	
A172	++	++	++	-
U87	++	+	+	++
U343	++	+	-	++
TE671	++	+	-	+
NCH 89	+	+	-	+
NCH 92	+	+	-	+



**Figure 4.**

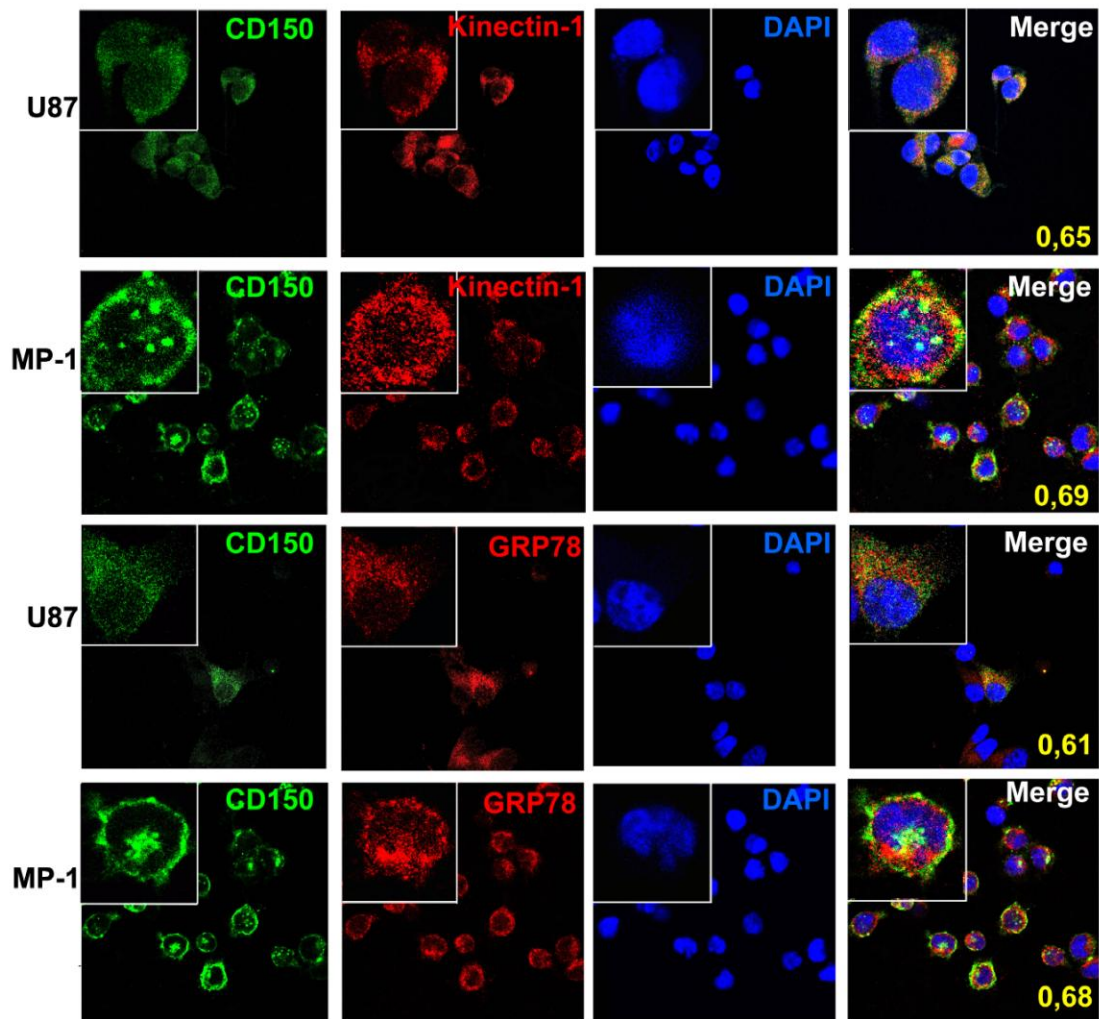


**Figure 5.**

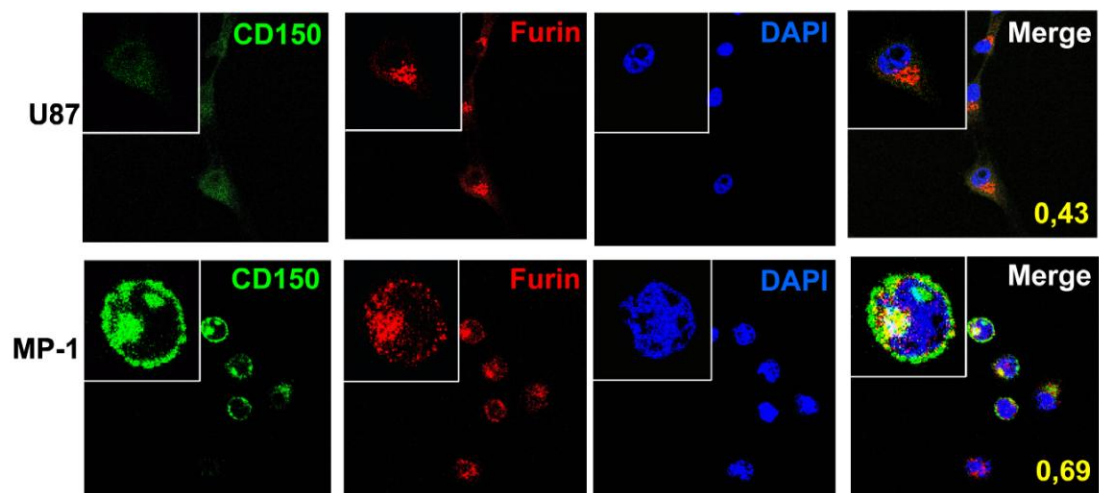


**Figure 6.**

**A**



**B**





## **IV. DISCUSSION**

Measles virus (MV) is a highly contagious pathogen, responsible for an acute childhood disease, which is accompanied with fever, cough, conjunctivitis, and skin rash. Despite the existence of effective live vaccine, measles still represents the potentially fatal disease with the high mortality rate. The principal reason of measles danger lays in its complications. Particularly, MV infection causes a profound immunosuppression, resulting in lymphopenia, cytokine imbalance and silencing of lymphocyte functions. This measles-virus-induced immune pathogenesis renders individuals more susceptible to secondary bacterial and viral infections, which can cause pneumonia and diarrhea and is responsible for much measles-related morbidity and mortality.

Measles virus interaction with human immune system results with an immunological paradox, as MV causes an active innate and adaptive immune response resulting in lifelong immunity, but at the same time MV often depresses the immune reactions to virus irrelevant antigens up to several months (Griffin, 2010; Kerdiles et al., 2006).

Several reasons could account for such dramatic immunosuppression induced by measles virus infection. First of all, the major targets for measles infection are cells of the immune system, and their infection influences the state of immune system. Moreover, the viral proteins could induce different inhibitory mechanisms to avoid or suppress a rapid viral clearance from the body. Among potential effects of viral proteins, the interplay with immune cell functions via virus interaction with the natural entry receptor CD150 remains largely unknown and has presented the major objective of our studies.

CD150 is widely expressed within the hematopoietic cell lineage and modulates intracellular signaling triggered by the antigen receptors in B and T lymphocytes. CD150 ligation itself may also induce the activation of several signal transduction pathways (Cannons et al., 2011; Sidorenko & Clark, 2003; Veillette et al., 2007). It is highly expressed in activated T and B cells, and also DCs – the cells which have a crucial role in inducing of both, innate and especially specific adaptive immune responses against viruses. So, measles virus, particularly its membrane glycoprotein hemagglutinin, may modulate immune cell differentiation, maturation and function via ligation CD150 receptor. Therefore, in **the first part of my thesis** project we aimed to study the role of CD150-MV-H interaction in measles immunopathogenesis.

DCs are potent antigen-presenting cells, essential for the generation of the primary immune response (Banchereau & Steinman, 1998). Their dysfunction may have an important role in MV pathogenesis. MV infection of human DCs *in vitro* was shown to affect their phenotype and functions. Due to the crucial role of DCs in the immunoregulation, MV interference with DC

function may represent an important mechanism of immunosuppression. Therefore, the MV-H antigen on the surface of cells could contribute to immunosuppression by binding CD150 on dendritic cell surface. Although the infection of DCs is an attractive hypothesis to explain MV-induced immunosuppression, implication of viral proteins interacting with DCs may play a role that has not been clearly elucidated so far. We thus concentrated our studies on dendritic cells. To better understand the cellular and molecular basis of MV-induced modulation of DC function we used a model that allowed us to stimulate CD150 with MV-H in the absence of infectious context. We demonstrated that MV-H is sufficient to downregulate expression on DC surface the CD150 receptor, but not CD46, as well as CD80, CD83, CD86 and HLA-DR (Article 1 Fig. 1A), suggesting that the CD150-MV-H interaction may affect the phenotype of DCs, decreasing the expression level of different co-stimulatory molecules, which are important for the immunostimulatory role of DCs. Similarly to our results, MV downregulated co-stimulatory molecules and MHC proteins on splenic DCs from mice expressing human SLAM receptor (Hahn et al., 2004). Maturation of DCs followed by the up-regulation of CD80, CD86, etc and expression of proinflammatory cytokines, both were shown to be impaired during measles infection (Servet-Delprat et al., 2000; Trifilo et al., 2006).

Since production of immunoregulatory cytokine IL-12 is an important function of DCs, orienting the immune response towards Th1, we analyzed whether MV-H may influence the production of this cytokine. For the first time we demonstrated that interaction between MV-H and human DCs may also lead to the inhibition of IL-12 production during measles (Article 1 Fig. 1B) and thus participate in the generation of the immunosuppressive state. MV infection was shown to skew host immune response towards Th2 phenotype (Griffin & Ward, 1993) and suppression of IL-12 was reported in MV-infected children (Atabani et al., 2001), in rhesus macaques during measles (Polack et al., 2002) and in DCs generated from human PBMC when infected by MV (Fugier-Vivier et al., 1997; Servet-Delprat et al., 2000). Although the engagement of CD150 with mAb IPO3 was shown to enhance DC production of IL-12 (Bleharski et al., 2001), the homotypic CD150-CD150 interaction strongly inhibited IL-12 production (Rethi et al., 2006), suggesting that different CD150 ligands may have the opposite effect on DC function.

Finally, we observed significant suppression of DC-induced T-cell proliferation in MLR (mixed lymphocyte reaction) with DCs, pre-exposed to MV-H (Article 1 Fig. 1C). Several studies have shown decreased lymphocyte proliferative responses in children with measles (Hirsch et al., 1984), as well as with *in vitro* infected human DCs (Fugier-Vivier et al., 1997; Grosjean et al.,

1997) and murine CD150 transgenic DCs (Hahm et al., 2003). Our results point to the importance of MV-H-CD150 contact in this effect.

Thus, CD150 interaction with MV-H impairs the dendritic cell maturation, shown in phenotypic changes, and function, demonstrated by decreased IL-12 production and DC failure in stimulation of T cell proliferative responses. To find out the molecular mechanisms of MV-H interaction with CD150 in DCs, we studied the activation of MAPK and Akt signaling pathways via CD150 receptor. Maturation of human DCs is regulated by the modulation of the balance of three mitogen-activated protein kinases (MAPK): JNK (c-Jun N-terminal kinases), ERK (extracellular signal-regulated kinases) and p38 MAPK (Nakahara et al., 2006). We have thus explored the effect of MV-H on the activation of JNK and ERK, since previously was shown CD150-mediated activation of these kinases in B cells (Mikhalap et al., 2004; M. Yurchenko et al., 2011; M. Y. Yurchenko et al., 2010).

We used in our studies several ways to stimulate cells: namely stimulation of CD150 on DCs with either MV-H or anti-CD150 antibodies only, and pre-incubation of DCs in the presence of MV-H followed by anti-CD150 antibody stimulation. These strategies revealed that neither in dendritic cells, nor in B cells CD150 ligation with MV-H itself does not trigger JNK or ERK phosphorylation (Article 1 Fig. 1A,C,E,F), however it does promote Akt phosphorylation/activation in both cell types (Article 1 Fig. 3). In contrast, CD150 crosslinking with monoclonal anti-CD150 antibodies IPO-3 in dendritic cells promotes the activation of JNK1/2 kinase already 5 minutes after stimulation (Article 1 Fig. 2C), which repeats the observations made on primary B cells, B lymphoblastoid and Hodgkin's lymphoma cell lines (Yurchenko et al., 2010; Article 1 Fig. 2A). However, differently to B cells (Article 1 Fig. 3A), in DCs IPO-3 mAbs did not induce Akt phosphorylation (Article 1 Fig. 3B). Similarly to B cells, pre-incubation with CHO cells slightly enhanced basal level of pAkt, and crosslinking with CD150 antibodies afterwards up-regulated Akt phosphorylation (Article 1 Fig. 3B), suggesting that for CD150-mediated Akt phosphorylation dendritic cells require pre-activation with some additional stimuli. This is in accord with our flow cytometry data on surface CD150 elevation on DCs after pre-incubation with CHO (Article 1 Fig. 1A). Upon pre-incubation of B cells and DCs with MV-H, the JNK1/2 phosphorylation level, mediated by CD150 ligation with mAb, was dramatically decreased (Article 1 Fig. 2A,C). From one side, this effect of MV-H on CD150-mediated JNK phosphorylation could be linked to reduction of CD150 surface levels and not so efficient ligation of CD150. This could be true for B cells, however for dendritic cells it is clear that MV-H inhibits JNK phosphorylation even

in the absence of mAb-induced CD150 ligation. So, MV-H by itself could reduce the level of JNK1/2 p54 phosphorylation as well as abolish CD150-initiated JNK1/2 activation (Fig. 19).

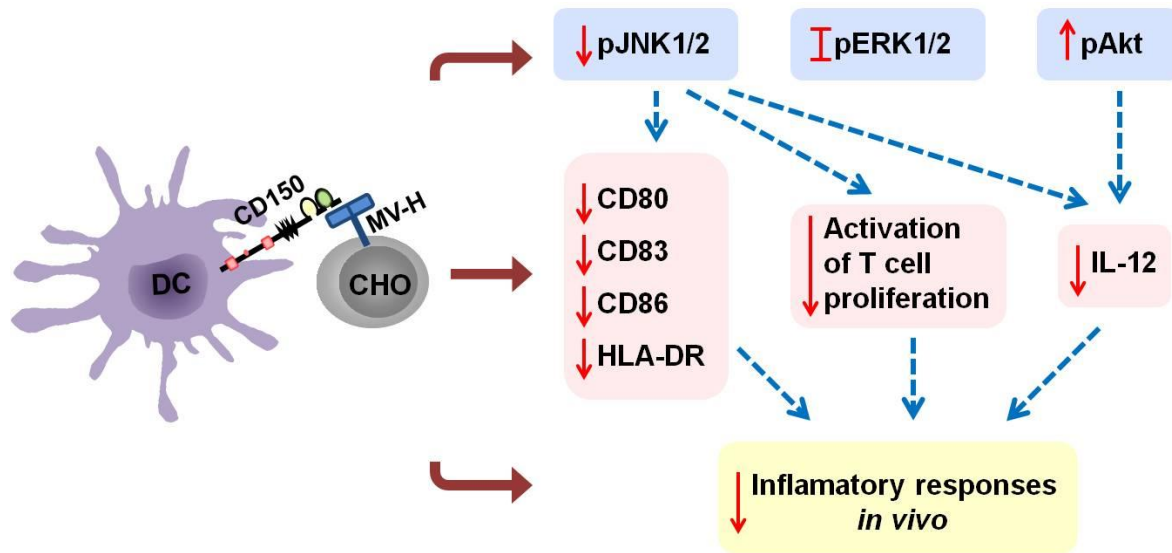
In contrast to JNK1/2, we did not reveal any significant changes in ERK1/2 activation in any of tested cells either after CD150 or MV-H ligation or CD150 stimulation after pre-incubation with MV-H, since the initial level of ERK phosphorylation was very high (Article 1 Fig. 2E,F).

The studies using specific inhibitors for different MAPK families suggest that three MAPK signaling pathways differently regulate all aspects of phenotypic and functional maturation of monocyte-derived dendritic cells (Nakahara et al., 2006). JNK and p38 MAPK kinases positively regulate DC phenotypic maturation and cytokine production which is seen in the downregulation of CD80, CD86, CD83 and HLA-DR surface expression and inhibition of IL-12 production after DC treatment with specific inhibitors of any of these kinases (Arrighi et al., 2001; Nakahara et al., 2004; Yu et al., 2004). On the contrary, the inhibition of ERK kinase does not reduce, but rather slightly increase the expression of DC markers and IL-12 production (Aiba et al., 2003; Hoarau et al., 2008; Waggoner et al., 2005). Thus, various stimuli mediate DC responses via differential regulation of three MAPKs. Therefore, the immunosuppressive effect of MV-H in lymphoid tissue, which is manifested as the downregulation of DC markers, cytokine imbalance, skewing of immune response towards Th2, may be attributed, at least in part, to the inhibition of JNK pathway (Fig. 19). We did not test the activation of the third MAPK kinase, p38 MAPK in dendritic cells, as its phosphorylation was not much changed upon CD150 crosslinking in B cells. But in the studies using selective inhibitors it appears as a major regulator of DC maturation and function (Nakahara et al., 2006), so it will be very interesting to address the role of p38 MAPK in MV-induced DC silencing.

Akt (v-Akt Murine Thymoma Viral Oncogen)/PKB (Protein Kinase B) is a serine /threonine kinase that is involved in various biological processes, including cell proliferation and apoptosis, and it was shown to be implicated in MV-induced suppression of lymphocyte function (Avota et al., 2001). In addition, CD150 crosslinking on T and B lymphocytes induces Akt phosphorylation (Howie et al., 2002; Mikhalap et al., 1999). We revealed that, in contrary to B cells, anti-CD150 mAbs do not mediate Akt phosphorylation in DCs (Article 1 Fig. 3A,B). However, both, in DCs and B cells, Akt is activated via interaction with MV-H, and its phosphorylation is significantly enhanced after stimulation with anti-CD150 mAb (Article 1 Fig. 3A,B). CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction, followed by phosphorylation of CD150 and attraction of p85 $\alpha$  regulatory subunit of PI3K. It was demonstrated by SPR that p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150 cytoplasmic tail, and that

this interaction takes place in normal tonsillar B cells and lymphoblastoid cell line MP-1 (M. Yurchenko et al., 2011). One of the downstream targets of Akt kinase in B cells is transcription factor FoxO1. Despite the high basal level of pFoxO1, CD150 mediates FoxO1 phosphorylation in normal tonsillar B cells and lymphoblastoid cell line (LCL) MP-1 (M. Yurchenko et al., 2011). FoxO1 transcriptional factor is not expressed in dendritic cells, however the member of the same family, FoxO3, is one of the major regulators of DC tolerogenicity. FoxO3 was previously reported to play a role in the regulation of DC function through a “reverse signaling” process mediated by CTLA-4 interaction with B7 molecules. FoxO3 silencing leads to the reduced DC tolerogenicity, concomitant increase in expression of the co-stimulatory molecule CD80 and the pro-inflammatory cytokine IL-6. This transcriptional factor could be phosphorylated by Akt kinase at three sites, Thr32, Ser253 and Ser315. The phosphorylation of FoxO3a results in its association with 14-3-3 proteins, nuclear export and inhibition of transcription factor activity. Whether it could be implicated in the Akt cascade, activated after CD150 ligation with MV-H in DCs, remains to be determined. The regulatory subunit of PI3K, p85 $\alpha$ , binds only to tyrosine phosphorylated cytoplasmic tail of CD150, promoting the downstream Akt activation, while CD150-mediated JNK phosphorylation does not depend on tyrosine phosphorylation of the receptor. Therefore, it would be also interesting to follow the presence or absence of tyrosine phosphorylation of CD150ct upon MV-H ligation.

The studies of the role of PI3K/Akt pathway in dendritic cell maturation were performed in different systems, and showed differential results. Particularly, it was shown that in contrast to p38 MAPK, PI3K kinase, which is an upstream kinase required for activation of Akt, induced by CD40L (Yu, Kovacs, Yue, & Ostrowski, 2004) or extracellular acidosis (Martinez et al., 2007) does not play a noticeable role in regulation of DC maturation and function as activator of T cell proliferation and CTL responses. In addition, PI3K/Akt pathway is activated upon DC infection with *Leishmania infantum*, that is accompanied with the downregulation of CD40 and CD86 surface expression while slight upregulation of IL-12 (Neves et al., 2010). However, siRNA inhibition of PTEN, phosphatase that dephosphorylates Akt, causes in dendritic cells the upregulation of CD40, CD80 and CD86, but not MHCII, and activation of antigen-specific CD8<sup>+</sup> T cells (Kim et al., 2010), that shows the positive regulation of DC maturation and function by PI3K/Akt signaling pathway. Similar role of this pathway was also found upon stimulation of human DCs with activated apoptotic peripheral monocytes that provide activation/maturation signal to dendritic cells (Pathak et al., 2012).



**Figure 19.** The interaction of MV-H with CD150 on DCs surface is sufficient to suppress immune responses in the absence of any infection. CD150-MV-H interaction was shown in our studies (solid dark red arrows) to cause the changes in dendritic cell maturation and function on different levels: molecular (changes in 2 key signalling pathways), cellular (suppression of surface markers expression, IL-12 production and induction of T cell proliferation) and systemic (inhibition of *in vivo* inflammatory responses in humanized CD150tg mice). Effects on these 3 different levels are probably inter-connected (shown in dashed blue arrows) that is justified with the numerous already published observations.

At the same time, Akt pathway is involved in the regulation of IL-12 production by DCs (Fig. 19). Enhanced production of IL-12 was observed in dendritic cells of PI3K-deficient mice (Fukao et al., 2002). IL-12 production was significantly inhibited in the presence of p38 MAPK inhibitor while increased in the presence of PI3K/Akt and ERK1/2 pathway inhibitors in response to extracellular acidosis (Martinez et al., 2007) and LPS or Bifidobacterium fermentation product (Hoarau et al., 2008). PI3K/Akt pathway also regulates suppression of IL-12 mediated by gC1qR, a complement receptor for C1q that plays role in regulation of inflammatory and antiviral T cell responses (Waggoner, Cruise, Kassel, & Hahn, 2005). It was shown using specific pharmacological inhibitors of PI3K/Akt pathway, wortmannin and LY294002.

In dendritic cells, apart to CD150, measles virus hemagglutinin also may bind to DC-SIGN and TLR2. DC-SIGN was reported to be linked to the activation of MAPK and Akt kinases upon its ligation with specific antibodies (Caparrós et al., 2006; Mittal et al., 2009; Svajger et al., 2011). However, it did not promote DC maturation shown in the absence of CD83 upregulation (Caparrós et al., 2006). Several bacterial antigens were also shown to modulate MAPK and/or Akt signaling, interacting with TLR2 (Hoarau et al., 2008). Yet TLR2 ligation induced mainly ERK1/2 activation (Dillon et al., 2004) that was not observed in our studies. The contribution of DC-SIGN and TLR2 in JNK1/2 activation can be excluded in our experiments as it was mediated after specific ligation with anti-CD150 antibodies. This is not the case for Akt phosphorylation, which was triggered after stimulation with MV-H that potentially could be linked to DC-SIGN or TLR2. However, it is currently not known if MV-H may promote any signaling via DC-SIGN. Moreover, MV-H-triggered Akt phosphorylation was up-regulated upon CD150 crosslinking with the specific antibodies that allows speculating that CD150, rather than DC-SIGN or TLR2, is implicated in the activation of this signaling pathway. However, the effect of DC-SIGN-MV-H interaction in DCs intracellular signaling will be interesting to address, and the studies using DC-SIGN ligands, like mannane, will give the final answer about the DC receptors interacting with MV-H to cause immune suppression. The implication of PVRL4 (nectin-4) in the effects mediated by MV-H in DCs is likely not possible as this antigen is mainly expressed on epithelial cells and so far was not found on cells of lymphoid origin.

After measles virus infection delayed-type hypersensitivity responses to recall antigens, such as tuberculin, are suppressed (Tamashiro et al., 1987). We have thus analyzed whether interaction between MV-H and human CD150 may be involved in the inhibition of inflammatory response in transgenic mice expressing human CD150 in the similar pattern as humans (Shingai et al., 2005).



For CD150 ligation, mice were injected with UV-inactivated vesicular stomatitis virus (VSV) expressing or not MV-H (Schnell et al., 1996). While mice injected with VSV developed good inflammatory response, the reaction was significantly reduced when they were injected with VSV-H, that strongly suggest that MV-H-CD150 interaction may play an important role in the inhibition of cellular immune response, characteristic for measles (Fig. 19). MV-H from wild type strains was reported to activate TLR2 signaling (Bieback et al., 2002). It has been also demonstrated that MV-H, as well as MV fusion protein F, interacts with DC-SIGN (de Witte et al., 2008), but the absence of the effect of VSV-H in non-transgenic mice, suggests that anti-inflammatory effect of MV-H passes via the interaction with CD150, rather than DC-SIGN or TLR2. It would be interesting to follow the maturation and function of dendritic cells isolated from hCD150 transgenic mice after VSV/VSV-H injection. It will answer the question if CD150-MV-H interaction has the same inhibitory effect on DC immune response *in vivo*, as we observed it *in vitro*.

Altogether, these results present an evidence for a novel mechanism of MV-induced immunosuppression, where direct contact between MV-H and CD150<sup>+</sup> DCs is sufficient to transmit intracellular signals via MAPKs and Akt pathways to alter DC functions in the absence of any infection (Fig. 19). As MV-H is expressed on all infected cells in addition to free viral particles and therefore can easily come in contact with circulating DCs, this interaction could thus play an important role in measles immunosuppression and amplify the effect of viral replication in the inhibition of the immune response. Furthermore, obtained results revealed the immunosuppressive capacity of CD150, known largely as an activating molecule, when it is engaged with a viral ligand, therefore expanding the immunomodulatory properties of this molecule.

Besides its important role in modulation of immune cell function, CD150 is described as potential marker for several histological types of tumors (Mikhalap et al., 2004). In the normal tissue, it is mainly expressed in hematopoietic system, so it is not surprisingly that it is also present in leukemias and lymphomas. But its expression is significantly up-regulated at several malignancies that allow considering CD150 as a new diagnostic and/or prognostic marker for particular types of lymphoid tumors (Mikhalap et al., 2004; Yurchenko et al., 2005). CD150 is also defined as a marker for Hodgkin's lymphoma Reed-Sternberg cells that loose their antigen receptor and most of other B cell surface markers, but express CD150 on extremely high level. However, the most interesting is that CD150 expression was found in tumors of ectodermal origin, namely basalioma, squamous cell carcinoma of uterine cervix, rectum and oral cavity, but not their normal

counterparts (Yurchenko et al., 2003). Therefore, CD150 could appear as a good marker for tumors of different to hematopoietic cell origin. As human central nervous system also derives from ectoderma, we hypothesized that CD150 could be expressed in CNS tumors. On the other side, measles virus complications in the brain cannot be explained in the absence of viral entry receptor in CNS, and CD150 expression was an attractive explanation.

Moreover, it has been a while, since MV attracts a lot of interest as potential oncolytic virus, as (1) it is a cytopathic virus, (2) a safe attenuated anti-measles vaccine is available and (3) it showed a potent antitumor activity against multiple tumor lines (Fielding, 2005). One of the objects of that kind of studies are CNS tumors, including gliomas, which constitute an important health problem because of infiltrative, aggressive and recurrent nature with only 5 to 15% of 5 year survival. Current therapy includes surgery plus radiotherapy, although the high invasiveness and intracranial localization of these tumors largely limits the effectiveness and even safety of surgery. Oncolytic viruses can replicate preferentially in cancer cells, thereby leading to oncolysis, such that no therapeutic transgene is need as the virus infection itself destroys the tumor mass. Only vaccine strains of MV were used in glioma oncolytic therapy studies as wild type MV receptor has not been found on the cells of nervous system yet (Allen et al., 2008; Galanis, 2010; Paraskevovou et al., 2007). Therefore, **the second objective of the thesis**, to study the CD150 expression in human central nervous system, has several points of interest in terms of potential diagnostical application and targeting.

Our immunohistochemistry studies revealed CD150 expression in malignant cells of several tumors of central nervous system (Article 2 Fig. 1A). Since CD150 was not detected in normal brain tissues (Article 2 Fig. 1B), expression of this antigen could be considerate as a potential diagnostic marker for CNS tumors. The number of CD150-positive cases varied for different histological types of CNS tumors, nevertheless, a clear tendency persisted: the number of CD150-positive cases directly correlated with the tumor grade. Therefore, further broad studies of CD150 expression in CNS tumors in conjunction with evaluation of clinical outcome will give the answer whether CD150 expression can be used as a prognostic marker.

Our immunohistochemical studies clearly showed CD150 expression in cytoplasm of tumor cells, however, were not able to clarify whether this antigen is expressed on the cell surface of malignant cells. To answer this question we used two approaches: first – immunofluorescent microscopy and flow cytometry, second – RT-PCR analysis. Both flow cytometry and immunofluorescent microscopy did not reveal CD150 expression on the cell surface of glioma cell lines, while confirm

CD150 cytoplasmic expression (Article 2 Fig. 2A). We examined the CD150 splice isoforms expression in glioma cells. However, mCD150 expression was limited to A172 and U87 cells, since other glioma cell lines and primary glioma cells expressed only extracellular and transmembrane parts of the receptor, and conventional cytoplasmic part was detected only in A172 and at low level in U87 cells (Article 2 Fig. 3A,B). This allowed us to suppose that in glioma cells CD150 could be aberrantly transcribed or a novel splice isoform with alternative cytoplasmic tail is present in these cells (Fig. 20). Indeed, sequencing of 500 bp PCR product from U87 cells obtained with TM primers revealed the expression of a novel splice isoform nCD150 with 83 bp insert between transmembrane and cytoplasmic parts (Article 2 Fig. 4A). Bioinformatic analysis showed that nCD150 isoform was formed due to alternative splicing, which intergrated an additional previously unrecognized exon Cyt-new. This exon is an exclusive feature of CD150 in primates and was not found in other mammals. It should be emphasized that nCD150 is expressed in all tested glioma cells, as well as in majority of hematopoietic cells tested (Article 2 Fig. 4B). It would be also interesting to examine the nCD150 expression in primary glioma tumors that will need to use the primary tumor cultures, as the tumor tissue contains blood vessels full of lymphocytes that do express nCD150 according to our studies.

The absence/modification of leader sequence in glioma cells could potentially lead to the cytoplasmic expression of CD150 splice isoforms in these cells. We obtained the full sequences of mCD150 and nCD150 from U87 cells. However, besides the additional exon, there were no other differences in the sequences of mCD150 and nCD150. Both mCD150 and nCD150 splice isoforms did not contain mutations in the extracellular part and had original leader sequence (Fig. 20). Thus, these isoforms should be able to be exposed on the cell surface. Indeed, transfection of mCD150 and nCD150 to HEK293T or U87 cells resulted in surface expression of CD150 receptor (Article 2 Fig. 5). So, there should be an intrinsic mechanism for regulation of CD150 surface recruitment in glial cells.

Confocal microscopy studies of CD150 compartmentalization in glial cells found CD150 both in endoplasmic reticulum (ER) and Golgi complex, however the colocalization coefficient for Golgi was lower than for ER (Article 2 Fig. 6). Moreover, CD150 colocalization coefficient for Golgi in glial cells was much lower than in B cell line with high level of surface CD150 expression. It was shown recently that in dendritic cells CD150 is colocalized with Golgi complex and Lamp-1 positive lysosomal compartment (Avota et al., 2011). Moreover, CD150 shares an intracytoplasmic

### mCD150

MDPKGLLSLTFVLFSLAFGASYGTGGRMMNCPKILRQLGSKVLLPLTYERI  
NKSMNKSIHIVVTMAKSLNSVENKIVSLDPSEAGPPRYLGDRYKFYLENLT  
LGIRESRKEDEGWYLMTLEKNVSVQRFCLQLRQVSTPEIKVLNKTQEN  
GTCTLILGCTVEKGDHVAYSWSEKAGTHPLNPANSSHLLSLTLGPQHADNI  
YICTVSNPISNNSQTFSPWPGCRTDPSETKPWAVYAGLLGGVIMILIMVVIL  
QLRRRGKTNHYQTTVEKKSLTTIYAQVQKPGPLQKKLDSFPAQDPCTTIYVA  
ATEPVPESVQETNSITVYASVTLPE

### nCD150

MDPKGLLSLTFVLFSLAFGASYGTGGRMMNCPKILRQLGSKVLLPLTYERI  
NKSMNKSIHIVVTMAKSLNSVENKIVSLDPSEAGPPRYLGDRYKFYLENLT  
LGIRESRKEDEGWYLMTLEKNVSVQRFCLQLRQVSTPEIKVLNKTQEN  
GTCTLILGCTVEKGDHVAYSWSEKAGTHPLNPANSSHLLSLTLGPQHADNI  
YICTVSNPISNNSQTFSPWPGCRTDPSETKPWAVYAGLLGGVIMILIMVVIL  
QLRRRATLTTTNQYWSQNVLTQDQERCPGCLPMVKRTITRQQWKKKALR  
SMPKSRNQVLFRRNLTPSQLRQLAPPYMLLPQSLSQSLSRKQIPSQSMLV

Signal peptide Extracellular region Transmembrane region Cytoplasmic region

Part of sequence different for mCD10 and nCD150 ITSM motifs

**Figure 20.** The comparison of amino acid sequences of mCD150 and nCD150 isoforms. Leader sequence is shown in blue font, extracellular region – in black, transmembrane – in green, cytoplasmic region – in violet with underlined amino acids that differ between two isoforms. The ITSM motifs in mCD150 are shown in red.

lysosomal compartment with acid sphingomyelinase and they are co-transported with CD150 from lysosomal compartments on cell surface upon DC-SIGN ligation (Avota et al., 2011). DC-SIGN ligation led to rapid activation of neutral and acid sphingomyelinases followed by accumulation of ceramides in the outer membrane leaflet. This initiates formation of ceramide-enriched membrane microdomains which promote vertical segregation of CD150 from intracellular storage compartments (Avota et al., 2011). Thus, ceramide is involved in upregulation of CD150 expression on cell surface. Glial tumors are characterized by significantly lower levels of ceramide, compared with peritumoral tissue (Riboni et al., 2002). Furthermore, ceramide is inversely associated with malignant progression of human astrocytomas and poor prognosis, and also determines glioma cell resistance to chemotherapy (Dumitru et al., 2009; Riboni et al., 2002). PI3K-Akt-PTEN pathway is among the dysregulated oncogenic pathways in gliomas (Knobbe et al., 2002; Riboni et al., 2002). In these tumors, the activation of the PI3K/Akt pathway is crucial to control ER to Golgi vesicular transport of ceramide, coordinates the biosynthesis of membrane complex sphingolipids with cell proliferation and growth and/or maintains low ceramide levels. So, the PI3K/Akt pathway could contribute to ceramide downregulation, as a means of avoiding cell growth arrest and cell death in gliomas (Giussani et al., 2009). It is possible that downregulation of ceramide levels in glial tumors could contribute to the inability of CD150 surface relocalization. Membrane ceramides are generated by activated neutral and acid sphingomyelinases (SMases). Multiple stress stimuli, such as ultraviolet and ionizing radiation, ligation of death receptors and chemotherapeutic agents (including platinum, paclitaxel and histone deacetylase inhibitors) have been shown to activate acid SMase, usually within a few minutes of cell stimulation (Hannun & Obeid, 2008; Rotolo et al., 2005). Presumably, if CD150 expression could be induced on the surface of tumor cells, this receptor may be considered as a potential target for measles virus based oncolytic therapy of human central nervous system tumors.

Altogether, these data suggest that CD150 may represent a new diagnostic and possible prognostic marker for human brain tumors. The functional relevance of the expression of new CD150 isoform in both immune cells and tumors remains to be determined. Novel mechanism of CD150-induced immunosuppression and new CD150 isoform identified in these studies shed new light on the immunoregulatory role of this molecule and open perspectives for its clinical applications.

## CONCLUSIONS:

The study performed during this thesis allowed us to answer several questions defined in our initial objectives and to formulate following conclusions and hypothesis.

### **First objective**

- The interaction of CD150 on DCs with MV-H alters the maturation of dendritic cells that is accompanied with the downregulation of CD150, CD80, CD83, CD86 and HLA-DR surface expression level.
- MV-H in the absence of infectious context is sufficient to inhibit IL-12 production by DCs and suppress DC-mediated T cell alloproliferation.
- Interaction with MV-H inhibits CD150-induced JNK1/2 but not ERK1/2 phosphorylation in DCs and B cells.
- MV-H stimulates Akt phosphorylation on S473 in CD150<sup>+</sup> DCs and B cells.
- Engagement of CD150 by MV-H in mice transgenic for human CD150 decreases the inflammatory response *in vivo*.

***Taken together, our results reveal a novel mechanism of MV-induced immunosuppression that may open perspectives towards the development of new therapeutic approaches to reduce measles complications and shed new light on the immunoregulatory role of CD150.***

### **Second objective**

- CD150 is expressed in several diagnostic types of primary human CNS tumors.
- Expression of CD150 in human normal brain was not observed.
- In glioma cell lines CD150 is not expressed on the cell surface, however it was detected in cytoplasm and colocalized with endoplasmic reticulum and Golgi complex.
- Besides full-length mCD150 splice isoform, in glioma cells we found a novel CD150 transcript (nCD150) containing 83 bp insert, derived from a previously unrecognized Cyt-new exon located 510 bp downstream of the exon for the transmembrane region.
- The novel nCD150 isoform is expressed on mRNA level in glioma cells, normal and malignant B lymphocytes, primary T cells, dendritic cells and macrophages.

***Overall, this study revealed CD150 expression in the tumors of Central Nervous System and identified a novel CD150 isoform. These results suggest that CD150 could be a potential diagnostic marker for CNS tumors and may be considered as a new target for glioma treatment.***

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## **V. ANNEXES**

## **Article 3 (review).**

# **The role of measles virus receptor CD150 in measles immunopathogenesis**

Olga Romanets

*INSERM U758-Ecole Normale Supérieure de Lyon, Lyon, France*

*Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Kyiv, Ukraine*

Svetlana P. Sidorenko

*Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine, Kyiv, Ukraine*

Branka Horvat

*INSERM U758-Ecole Normale Supérieure de Lyon, Lyon, France*

## **1 Introduction**

Measles virus (MV) is one of the most contagious pathogens for humans. Despite the existence of an efficient vaccine, measles outbreaks still occur all around the world. Three different cell surface receptors have been identified to serve for MV entry. The major viral receptor CD150 that is mainly expressed on hematopoietic cells, mediates viral entry in respiratory tract and systemic propagation in lymphoid tissues. Recently identified MV receptor poliovirus receptor-related 4 (PVRL4) or nectin-4 is expressed on the basal side of the respiratory epithelium and facilitates virus release from the airways. The third MV receptor, CD46, though expressed on all nucleated human cells, serves as the entry receptor for only laboratory adapted MV strains. Binding to cell receptors is achieved via viral envelope protein hemagglutinin. Interaction between measles virus and CD150 could play a significant role in the modulation of the immune response, seen during measles. The immediate effect of this interaction results with virus-cell fusion, cytolytic viral replication and consequent elimination of CD150-positive immunocompetent cells. Among them are T and B lymphocytes, dendritic cells, activated monocytes and macrophages. This instant effect along with the MV-induced modulation of immunocompetent cell functions leads to the development of profound immunosuppression.

Since CD150 is a potent immunosignaling receptor and its ligation on cell surface can change the cytokine profile and functions of dendritic cells, T cells and macrophages, it is thus likely that its interaction with MV hemagglutinin could be also implicated in measles immunopathogenesis. CD150 is linked to signal transduction pathways that are involved in regulation of immune response and cell differentiation: Akt/PKB, ERK1/2, p38 MAPK, and JNK1/2. Understanding the signaling events upon CD150 ligation with measles virus hemagglutinin may answer the multiple questions concerning the molecular mechanism of measles virus mediated immunosuppression.

## **2 Measles virus**

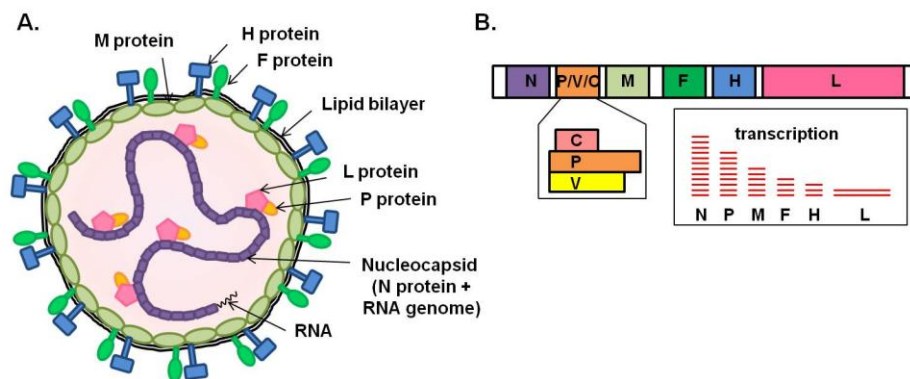
Measles is a highly infectious and potentially fatal disease. The causative agent for this infection is measles virus (MV), a member of the family *Paramyxoviridae*, genus *Morbilliviruses*. *Morbilliviruses* also include rinderpest virus (RPV), canine distemper virus (CDV), peste des petits ruminants virus (PPRV), phocine distemper virus, cetacean morbillivirus and seal distemper virus (Lamb & Parks, 2007). MV is one of the most highly contagious infectious agents and outbreaks can occur in populations in which less than 10% of

individuals are susceptible to infection. Live attenuated and effective vaccines were developed more than 40 years ago to limit this severe and fatal disease. After a global campaign for vaccination, estimated global measles mortality decreased by 74% from 535,300 deaths in 2000 to 139,300 in 2010. Measles mortality was reduced by more than three-quarters in all WHO regions except the WHO Southeast Asia region. India accounted for 47% of estimated measles mortality in 2010, and the WHO African region accounted for 36% (Simons et al., 2012). Despite all efforts to eradicate measles, it has still not been achieved because of the limitation of the licensed vaccines, including their instability at room temperature, the requirement of two-dose application and necessity of vaccine coverage of greater than 95% to prevent further outbreaks. In addition, the vaccination cannot be provided in early infancy as maternal antibodies reduce its efficiency and to immunocompromised persons as it could potentially be dangerous. All the factors mentioned above together with anti-vaccine opposition on religious or philosophical grounds in some communities limit the possibility of global measles elimination. Measles virus is a close relative of and shares a lot of biological properties with another member of the genus *Morbillivirus*, RPV. As in 2011 the Food and Agriculture Organization officially announced rinderpest to be eradicated from the

globe. This experience may help in the future eradication of measles (OIE, 25 May 2011).

Clinically apparent measles begins with a prodrome characterized by fever, cough, coryza (runny nose) and conjunctivitis and ends with the characteristic erythematous and maculopapular rash. Though pneumonia appears to be the most common fatal complication of measles, complications have been described in almost every organ system including the central nervous system (CNS). The CNS involvement accounts for acute postinfectious measles encephalomyelitis with either infectious or autoimmune etiology, measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE) (Schneider-Schaulies et al., 2003).

MV is a spherical enveloped virus with a single stranded RNA genome of negative polarity that contains 6 genes encoding eight proteins. V and C proteins are encoded by the P gene in addition to phosphoprotein (P) via RNA editing process and use of alternative translational frame respectively. Of the six structural proteins, P, large protein (L) and nucleoprotein (N) form the nucleocapsid that encloses the viral RNA. The hemagglutinin protein (H), fusion protein (F) and matrix protein (M), together with lipids from the host cell membrane, form the viral envelope (Griffin, 2007; Fig. 1).



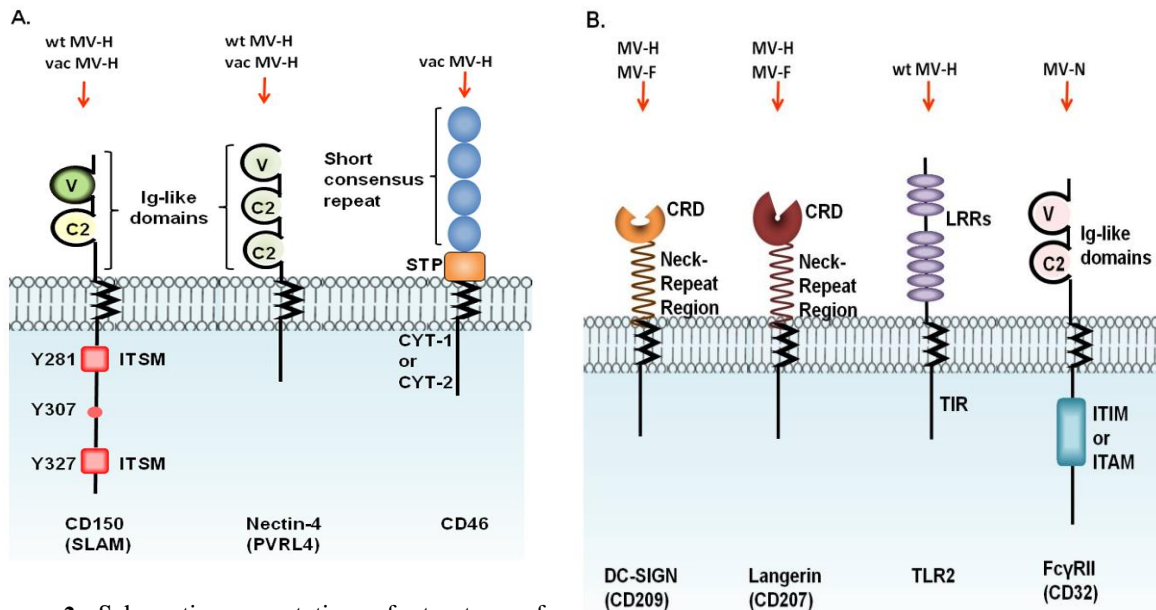
**Figure 1.** Measles virus particle and genome. A. The structure of measles virus particle. B. Schematic representation of measles virus genome.

Unlike some other members of the *Paramyxoviridae*, which utilize sialic acid moieties on glycoproteins and glycolipids to

enter cells, *Morbilliviruses* use specific cellular receptors. In case of MV they are: CD46 for laboratory MV strains (Dorig et al., 1993;

Naniche et al., 1993), CD150, for both, wild-type and laboratory MV strains (Tatsuo et al., 2000; de Vries, Lemon et al., 2010), and recently discovered PVRL4 (nectin-4), allowing viral egress from the respiratory tract (Muhlebach et al., 2011; Noyce et al., 2011; **Fig. 2A**). Although they do not serve as entry receptors, the C-type lectins, DC-SIGN (CD209) and Langerin (CD207) facilitate MV attachment

to the myeloid dendritic cells (de Witte et al., 2008) and Langerhans cells (van der Vlist et al., 2011) respectively (**Fig. 2B**). CD150 and PVRL4 account for the lymphotropic and epitheliotropic properties of natural MV. MV is also endotheliotropic and neurotropic, however the entry receptors for MV on the surface of these types of cells have not yet been identified.



**Figure 2.** Schematic presentation of structure of measles virus receptors. **A.** Three entry MV receptors, able to interact with MV hemagglutinin have been identified: CD46 for laboratory MV strains, CD150 for both, wild type and laboratory MV strains in lymphoid cells, and nectin-4, for both MV strains again, but in epithelial cells. **CD46** consists of four short consensus repeats (SCR) on N-terminus, followed by alternatively spliced serine, threonine and proline-rich region (STP), transmembrane domain and one of two cytoplasmic tails (CYT-1 or CYT-2). **CD150** comprises two immunoglobulin(Ig)-like domains (V and C2) on N-terminus, transmembrane region and cytoplasmic tail containing three tyrosine residues which could be phosphorylated, two of which form the core of immunoreceptor tyrosine-based switch motif (ITSM). The structure of **PVRL4** (nectin-4) is defined by three extracellular Ig-like domains (V and two C2-type domains), a single transmembrane helix, and an intracellular domain. **B.** Cell membrane molecules interacting with MV, but not allowing virus fusion. Four other surface molecules have been found to interact with MV proteins: DC-SIGN, Langerin, TLR2 and FcγRII. **DC-SIGN** (CD209) consists of carbohydrate recognition domain (CRD) and neck-repeat region in the extracellular region, after that transmembrane and cytoplasmic domains. It interacts with both MV membrane glycoproteins: H and F. **Langerin** (CD207) and DC-SIGN are highly homologous, share some ligands, but are expressed by different cell types. While DC-SIGN represents a trimer, Langerin is present as tetramer on cell surface. Langerin also has an additional carbohydrate-binding  $Ca^{2+}$ -independent site in its CRD domain. **TLR2** is composed of extracellular multiple leucine-rich repeats (LRRs), transmembrane domain and cytoplasmic region with Toll/IL-1R (TIR) domain and interacts only with wt MV-H. **FcγRII** (CD32) has two Ig-like domains on N-terminus, followed by transmembrane and cytoplasmic regions. FcγRII receptors represent two subfamilies: activatory FcγRII with immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic tail, and inhibitory FcγRII containing immunoreceptor tyrosine-based inhibitory motif (ITIM) instead of ITAM. In contrast to other described receptors, FcγRII binds measles virus N protein.



### 3 CD150 receptor

#### 3.1 CD150 structure and expression

The CD150 cell surface receptor was first described as 'IPO-3 antigen' (Pinchouk et al., 1988; Pinchouk et al., 1986; Sidorenko & Clark, 1993) on activated human lymphocytes. Its preferential expression on cells of hematopoietic tissues, biochemical and functional features have been characterized (Sidorenko & Clark, 1993). In 1995 the cDNA for this receptor was cloned under the name signaling lymphocyte activation molecule (SLAM) (Cocks et al., 1995). The monoclonal antibody IPO-3 was submitted for the international collaborative studies in the frame of 5-7 workshops on Human Leukocyte Differentiation Antigens (HLDA). In 1996 on the 6th workshop on HLDA (Kobe, Japan) the IPO-3 antigen received international nomenclature CDw150 (Sidorenko, 1997) that was transformed into CD150 at the 7th HLDA workshop (Harrogate, Great Britain, 2000) (Sidorenko, 2002).

CD150 (IPO-3/SLAM F1) is a member of SLAM family within the immunoglobulin superfamily of surface receptors. According to the nomenclature accepted by 9th international Workshop on Human Leukocyte Differentiation Antigens (Barcelona, 2010) SLAM family includes nine members: SLAMF1 (CD150 or IPO-3, SLAM), SLAMF2 (CD48 or BCM1, Blast-1, OX-45), SLAMF3 (CD229 or Ly9), SLAMF4 (CD244 or 2B4, NAIL), SLAMF5 (CD84 or Ly9B), SLAMF6 (CD352 or NTB-A, SF2000, Ly108), SLAMF7 (CD319 or CRACC, CS1, 19A24), SLAMF8 (CD353 or BLAME) and SLAMF9 (SF2001 or CD84-H1). The characteristic signature of CD150 family is within the extracellular region: an N-terminal variable (V) Immunoglobulin (Ig) domain lacking disulfide bonds is followed by a truncated Ig constant 2 (C2) domain with two intradomain disulfide bonds (**Fig. 2A**). The genes of the SLAM family are clustered close together on the long arm of chromosome 1 at bands 1q21-24 (Sidorenko & Clark, 2003). The gene structure for all CD150/SLAM family receptors is similar with the signal peptides and each of the Ig domains encoded by separate exons. All these receptors are type I

transmembrane proteins with the exception of CD48 (Cannons et al., 2011).

CD150 is a single chain transmembrane phosphoglycoprotein of 75-95 kDa in size with a 42 kDa protein core. It is heavily N-glycosylated with terminal sialic acid residues on oligosaccharide chains. Moreover, it was shown that this receptor is associated with tyrosine (Y) and serine/threonine (S/T) kinases and is phosphorylated at tyrosine and serine residues (Sidorenko & Clark, 1993).

In addition to transmembrane form of CD150 (mCD150), activated T and B lymphocytes, CD40 ligand-activated dendritic cells and also lymphoblastoid and Hodgkin's lymphoma cell lines express mRNA encoding secreted form of CD150 (sCD150) that lacks 30 amino acids – the entire transmembrane region (TM), mRNAs for cytoplasmic form (cCD150) lacking the leader sequence, and a variant membrane CD150 (vmCD150) with truncated cytoplasmic tail (CY). However, expression of vmCD150 (tCD150) isoform was not confirmed at the protein level (Bleharski et al., 2001; Cocks et al., 1995; Punnonen et al., 1997; Sidorenko & Clark, 2003; Yurchenko et al., 2005).

CD150 receptor is mainly expressed within hematopoietic cell lineage. Its expression was detected on immature thymocytes, activated and memory T cells, CD4<sup>+</sup> T cells with much higher expression level on Th1 cells, CD4<sup>+</sup>CD25<sup>+</sup> regulatory and follicular helper T cells, peripheral blood and tonsillar B cells, mature dendritic cells and macrophages. The level of CD150 expression on subpopulations of tonsillar B cells differs with its maximum levels on plasma cells. The activation of T and B cells leads to the rapid upregulation of CD150 expression. Low level of CD150 expression was also found on NKT cells, platelets and eosinophiles. The expression of CD150 on peripheral blood monocytes could be induced by mitogen and cytokines stimulation, and also by measles virus particles (Browning et al., 2004; Cocks et al., 1995; Kruse et al., 2001; Minagawa et al., 2001; Sidorenko & Clark, 1993, 2003; Veillette et al., 2007; Yurchenko et al.,

2010). CD150 expression is a distinguishing feature of hematopoietic stem cells (HSC) in mice (Hock, 2010). CD150 was also found on

### 3.2 CD150 ligands

With the exception of CD48 and CD244/2B4 (receptor-ligand pair), all SLAM family receptors are self-ligands. Intriguingly, there is a marked variation in the apparent affinity of these self-associations ( $K_d=0.5\text{--}200\ \mu\text{M}$ ), suggesting that the various members of the CD150 family may modulate immune cell functions to a different extent. Crystallographic determination of the structures of SLAM family members homodimers indicated that self-association is mediated by the N-terminal variable (V)-type Ig-like (IgV) domain, which enables head-to-head contact between two monomers (Mavaddat et al., 2000; Sintes & Engel, 2011; Veillette et al., 2007). CD150 binds to itself with low affinity ( $K_d\Rightarrow 200\mu\text{M}$ ). Although the affinity of this receptor is low, the binding avidity increases due to the redistribution or clustering of molecules after cell activation. It is probable that the binding is affected by other receptor–ligand interactions that help to bring the membranes into proximity and induce changes in the lateral mobility of receptors (Engel et al., 2003; Mavaddat et al., 2000).

CD150 was found to be the major receptor for several *Morbilliviruses* (Tatsuo & Yanagi, 2002). In case of MV the role of attachment protein is played by hemagglutinin (MV-H), which directly binds the cellular receptors of the virus (CD150, CD46 and nectin-4), mediating virus entry into the cell (**Fig. 2A**). Human CD150 (hCD150) was reported as a cellular receptor for wild type (wt) measles virus (Tatsuo et al., 2000). The hCD150 V domain is necessary and sufficient for its function as a MV receptor; however, the other regions of hCD150, including the C2 domain, TM, and CY are not required for this function (Ono et al., 2001). Histidine (H) at position 61 appears to be critical for CD150 to act as a receptor for MV. Isoleucine (I) at position 60, Valine (V) 63, lysine (K) at position 58 and S59 also appear to contribute to receptor function of hCD150 (Ohno et al., 2003; Xu et al., 2006). The mutagenesis of the H protein based on its ability to induce SLAM-dependent cell–cell fusion has

malignant cells of leukemias and lymphomas mainly with an activated cell phenotype (Mikhalap et al., 2004).

revealed that residues important for interaction with SLAM are I194, D505, D507, D530, R533, F552 and P554 (Masse et al., 2004; Navaratnarajah et al., 2008; Vongpunsawad et al., 2004).

CD150 receptors from human, mouse, dog, cow and marmoset have about 60–70% identity at the amino acid level, except human and marmoset CD150, which have 86% identity. The sequences at positions 58–67 are well conserved among these species, especially I60 and H61 which are conserved in human, marmoset, dog and cow CD150 (Ohno et al., 2003). Mouse CD150 has functional and structural similarity to human CD150 (60% identity at the amino acid level), but it cannot act as a receptor for MV, partly explaining why mice are not susceptible to MV. It has been proposed that amino acid substitutions at the positions 60, 61, 63 to human-type residues may make mouse CD150 act as an MV receptor, as introduction of changes at these positions compromises the receptor function of human CD150., while introduction of changes at these positions compromises the receptor function of human CD150 (Yanagi et al., 2009). CDV and RPV use dog and bovine CD150 respectively as cellular receptors. Furthermore, it was found that MV, CDV and RPV can use CD150 of non-host species as receptors but with lower efficiency (Yanagi et al., 2009).

CD150 is not only a self ligand and a receptor for Morbilliviruses, but also a bacterial sensor that regulates intracellular enzyme activities involved in the removal of Gram-negative bacteria (Berger et al., 2010; Sintes & Engel, 2011).

### 3.3 CD150-mediated signaling

Engagement of CD150 on cell membrane results in numerous intracellular signaling events. CD150-mediated signals can be divided into receptor-proximal, including immediate binding partners (**Fig. 3A**), and distal – signal transduction pathways (**Fig. 3B**). CD150

receptor interacts with intracellular partners through its cytoplasmic tail (CD150ct), specifically via three key tyrosine residues which could be phosphorylated: Y281, Y307 and Y327 (Y288, Y315 and Y335 in mice respectively). The first and the last tyrosine residues form the core for the unique immunoreceptor tyrosine-based switch motifs (ITSMs) (Shlapatska et al., 2001; **Fig. 2A**). Six members of SLAM family receptors contain 1 to 4 ITSMs. The cytoplasmic tail of CD150 has 2 ITSMs. Upon the tyrosine phosphorylation by Src family kinases ITSMs bind key SH2-containing components of signal transduction pathways, including the adaptor proteins SH2D1A, SH2D1B (EAT-2) and SH2D1C (ERT) (Shlapatska et al., 2001; Sidorenko & Clark, 2003).

Upon antigen receptor cross-linking the cytoplasmic tail of CD150 becomes phosphorylated, while after CD150 ligation with the IPO-3 antibodies – the opposite effect is observed (Mikhalap et al., 1999). It was suggested that A12 antibody is antagonistic, while the outcome of IPO-3 cross-linking is similar to the effect of hemophilic stimulation with CD150 recombinant protein (Punnonen et al., 1997; Veillette, 2004). Several Src-family kinases, namely Lck phosphorylates Y307, Lyn – Y327 and Fyn – Y281, were found to be able to phosphorylate key tyrosine residues of CD150 (Howie et al., 2002; Mikhalap et al., 2004; **Fig. 3A**).

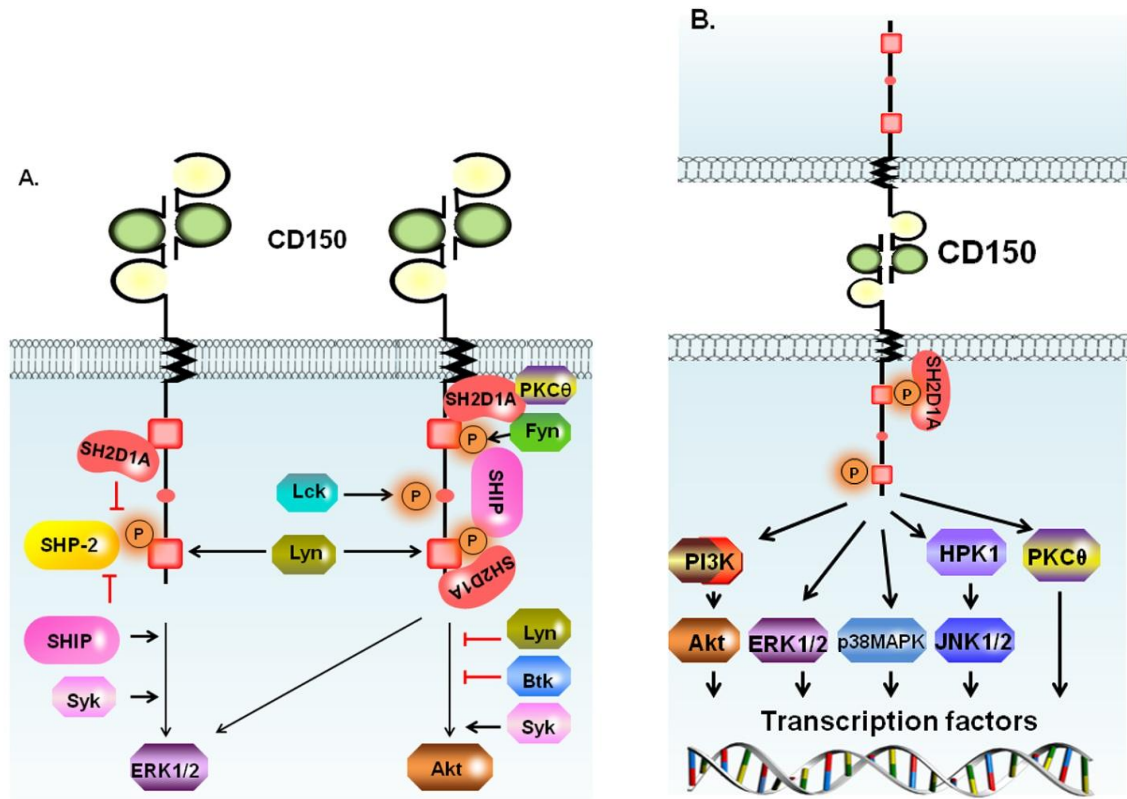
The signaling events downstream of CD150 could be divided on those that are dependent on CD150-SH2D1A interaction and those which are not. SH2D1A binds Y281 of CD150 cytoplasmic tail independently of phosphorylation, does not bind Y307, and binds Y327 only after phosphorylation (Howie et al., 2002). The SH2D1A interaction with Y281 of SLAM is mediated via R32 of SH2D1A (Sayos et al., 1998).

The coupling of CD150 to signal transduction pathways is provided by two SH2D1A functions: recruitment of enzymes or, alternatively, blocking binding of SH2-containing proteins. SH2D1A SH2 domain binds to the SH3 domain of FynT and indirectly couples FynT to CD150. The crystal structure of a ternary CD150-SH2D1A-Fyn-SH3 complex

reveals that SH2D1A SH2 domain binds the FynT SH3 domain through a R78-based motif which does not involve canonical SH3 or SH2 binding interactions (Chan et al., 2003; Latour et al., 2001; Latour et al., 2003). The observed mode of binding to the Fyn-SH3 domain is expected to preclude the auto-inhibited conformation of Fyn, thereby promoting activation of the kinase after recruitment (Chan et al., 2003). SHIP also is recruited in CD150-SH2D1A signaling complex via binding of its SH2 domain to phosphorylated Y315 and/or Y335 of mouse CD150 and appears to be the intermediate molecule that links CD150-SH2D1A to Dok-related adaptors and RasGAP (Latour et al., 2001). This signaling cascade is dependent on the generation of a ternary CD150-SH2D1A-Fyn complex for CD150 phosphorylation. The presence of SH2D1A facilitates binding of SHIP to CD150 (Latour et al., 2001; Shlapatska et al., 2001). Although SHIP possesses only one SH2 domain, both Y281 and Y327 in CD150 are important for its association with SHIP (Shlapatska et al., 2001).

R78 residue of SH2D1A is also critical for its constitutive association with PKC $\theta$  in T cells. This SH2D1A-PKC $\theta$  interaction is Fyn independent. Moreover, CD150 engagement increases TCR-induced PKC $\theta$  recruitment to the site of T cell stimulation (Cannons et al., 2010). As PKC $\theta$  could be detected as part of the CD150-SH2D1A complex following TCR ligation, CD150 engagement can facilitate PKC $\theta$  recruitment, nuclear p50 NF- $\kappa$ B levels, and IL-4 production via a ternary CD150-SH2D1A-PKC $\theta$  complex (Cannons et al., 2004; Cannons et al., 2010).

SH2D1A is capable of not only bridging the interaction of FynT, PKC $\theta$  or SHIP with CD150 but also inhibiting the binding of SHP-2 to phosphorylated CD150 (Sayos et al., 1998; Shlapatska et al., 2001). SH2 domains of SHIP and SHP-2 bind to the same sites in the receptors of SLAM family as does SH2D1A strongly suggesting that these three proteins are competing binding partners. Apparently, displacement of SHP-2 by SH2D1A makes Y281 and Y327 available for binding by SHIP, which also requires Y281 and Y327 (Liet et al., 2003; Shlapatska et al., 2001).



**Figure 3.** CD150-mediated signaling. A. Receptor-proximal signaling events triggered by CD150 ligation could be divided on those which are dependent on CD150-SH2D1A interaction and those which are not. Upon ligation, three tyrosine residues in cytoplasmic tail could be phosphorylated. Lck, Lyn and Fyn kinases were shown to be able to do it. Afterward several SH2- and SH3-containing molecules were shown to be able to bind to these tyrosines either via SH2D1A (Fyn, SHIP, PKC $\theta$ ) or not (SHP-2). CD150 ligation mediates the activation of Akt signaling pathway (requires Syk and SH2D1A and is negatively regulated by Lyn and Btk) and ERK signaling pathway (requires SHIP and Syk, but not SH2D1A). B. Receptor-distal signaling events after CD150 ligation: activation of Akt, ERK1/2, JNK1/2, p38MAPK signaling pathways that lead to the activation of transcription factors, e.g. FoxO1, c-Jun and NF- $\kappa$ B.

CD150 also associates with CD45 in human B lymphoblastoid cell lines MP-1 and CESS that express SH2D1A, however it is not clear whether CD150-CD45 association is direct, or is mediated via SH2D1A (Mikhalap et al., 1999). It should be noted that in SH2D1A negative B cell lines CD150 is strongly associated with SHP-2 that binds only tyrosine phosphorylated ITSMs (Shlapatska et al., 2001). Since BCR engagement induces CD150 tyrosine phosphorylation (Mikhalap et al., 1999) it could be that activation of B cells is required for SHP-2 binding to CD150. In B cells CD150 is associated with Src-family kinases Fgr and Lyn (Mikhalap et al., 1999), however only Lyn is

able to phosphorylate CD150 *in vitro* (Mikhalap et al., 2004).

CD150-binding partners link it to several signal transduction pathways, among them are MAPK and Akt/PKB signaling networks (**Fig. 3B**). In T and B cells CD150 crosslinking alone results in an increase in serine phosphorylation of Akt/PKB on S473, which is the hallmark of kinase activation state (Mikhalap et al., 1999; Howie et al., 2002). CD150-mediated Akt phosphorylation requires Syk and SH2D1A, is negatively regulated by Lyn and Btk, but is SHIP independent as was shown in the model of DT40 chicken knockout cell sublines transfected with SH2D1A and/or CD150 (Mikhalap et al.

2004). In B cells CD150-induced ERK phosphorylation requires SHIP and Syk but not SH2D1A. Lyn, Btk, and SHP-2 appear to be involved in the regulation of constitutive levels of ERK1/2-phosphorylation. These data allow proposing of the hypothesis that CD150 and SH2D1A are co-expressed during a narrow window of B-cell maturation and SH2D1A may be involved in regulation of B-cell differentiation via switching the CD150-mediated signaling pathways (Mikhalap et al., 2004; **Fig. 3A**).

It was also shown that Akt kinase could be phosphorylated via CD150 both in naïve and activated primary tonsillar B cells. CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction followed by phosphorylation of CD150ct and attraction of p85 $\alpha$  regulatory subunit of phosphatidylinositol-3-kinase (PI3K). As was demonstrated by surface plasmon resonance, p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150ct, and this interaction takes place in normal tonsillar B cells and lymphoblastoid cell line MP-1. One of the downstream targets of Akt kinase in B cells is

### 3.4 CD150 functions

Several models were used to study CD150 functions: ligation of CD150 with monoclonal antibodies or CD150 recombinant protein in human and murine *in vitro* system and CD150 knockout mice, where the expression of murine CD150 was genetically deleted. These experimental approaches revealed numerous roles of CD150 in the functions of different immune cell subsets.

#### 3.4.1 CD150 functions in lymphocytes

CD150 is involved in the regulation of interferon-gamma (IFN- $\gamma$ ) response. Initial studies showed that the engagement of human CD150 with mAb A12 enhances the antigen-specific proliferation, cytokine production by CD4<sup>+</sup> T cells, particularly IFN- $\gamma$ , and direct the proliferating cells to a T0/Th1 phenotype (Aversa et al., 1997; Cocks et al., 1995). Similarly, antibodies against mouse CD150 also enhanced IFN- $\gamma$  production (Howie et al., 2002; Castro et al., 1999). However, it seems that

transcription factor FoxO1. Despite the high basal level of pFoxO1, CD150 mediates FoxO1 phosphorylation in normal tonsillar B cells and MP-1 cell line (Yurchenko et al., 2011).

In primary tonsillar B cells and Hodgkin's lymphoma cell lines CD150 signaling not only induces the activation of ERK1/2 kinases but also regulates the activation of two other families of MAPK kinases – p38 MAPK and JNK1/2. CD150 mediates the activation of JNK1/2 p54 and JNK2- $\gamma$  isoforms in all tested human B cells. Furthermore, the CD150-mediated JNK activation is significantly enhanced after HPK1 overexpression and HPK1 is co-precipitated with CD150, suggesting that HPK1 is downstream of CD150 in this signaling pathway (Yurchenko et al., 2010).

All these facts demonstrate that signaling properties of CD150 depend on the cell type and availability of other components of signal transduction networks. However, it is clear that downstream of CD150-mediated signaling pathways are transcription factors that regulate cell biology and could modulate immune response.

some anti-CD150 antibodies, including A12 mAb, are antagonistic, since CD150 ligation on T cells inhibits IFN- $\gamma$  production rather than stimulate it. It was shown that expression of full-length CD150 and SH2D1A in BI-141 T cell transfectants, activated with anti-CD3, abolished the production of IFN- $\gamma$  (Latour et al., 2001). In addition, homophilic CD150-CD150 interactions between T cells and artificial CD150 expressing APC (antigen-presenting cell) line reduced IFN- $\gamma$  expression (Cannons et al., 2004, **Fig. 4**). It was also shown that SH2D1A deficient T cells produce more IFN- $\gamma$  in response to CD3 triggering (Cannons et al., 2004; Howie et al., 2002).

Studies of CD150-knockout mice revealed an important role for CD150 in regulation of Th2 development. In CD150-deficient CD4<sup>+</sup> T cells a significant decrease in TCR-mediated IL-4 production and slight upregulation in IFN- $\gamma$  secretion was observed (Wang et al., 2004). Moreover, CD150 receptor positively regulates TCR-induced IL-4 production by naive and

activated CD4<sup>+</sup> T cells, but negatively regulates IFN- $\gamma$  secretion by CD8<sup>+</sup> and to a lesser extent by CD4<sup>+</sup> cells (Wang et al., 2004). SH2D1A expression may be a limiting factor coordinating the TCR and CD150-mediated signaling pathways contributing to IL-4 regulation, as overexpression of SH2D1A potentiates the effects of CD150 on IL-4 production (Cannons et al., 2010).

CD150 is also specifically required for IL-4 production by germinal center (GC) T follicular helper (TFH) cells, CD4<sup>+</sup> T cells, specialized in B cell help. On the model of CD150 knockout mice it was shown, that for optimal help to B cells TFH cells require CD150 signaling to produce IL-4 (Yusuf et al., 2010; **Fig. 4**).

Studies with knockout mice revealed that CD150-SH2D1A mediated signaling may contribute to some functions of innate-like lymphocytes (Griewank et al., 2007; Jordan et al., 2007; Nichols et al., 2005; Veillette et al., 2007). Innate-like lymphocytes are subsets of lymphocytes which, when activated, exhibit faster and more robust effector functions. The prototypical innate-like lymphocytes are the NKT cells (Godfrey & Berzins, 2007; Veillette et al., 2007). It was demonstrated that SH2D1A-driven signals may be necessary for the positive selection, proliferation, and/or prevention of negative selection of immature NKT cells. One possibility is that SH2D1A-mediated signals are triggered by homotypic interactions between SLAM family receptors expressed on double-positive thymocytes. CD150 and Ly108 seem to be the predominant SLAM family receptors triggering the function of SH2D1A during NKT cell development (Griewank et al., 2007). Another indication of CD150 role in NKT differentiation comes from the characterization of the *Nkt1* locus on mouse chromosome 1. The differential expression of *Slamf1* and *Slamf6* genes mediates the control of NKT cell numbers attributed to the *Nkt1* gene (Jordan et al., 2007; **Fig. 4**).

CD150 is also involved in the regulation of cell proliferation and survival. Ligation of CD150 with monoclonal antibody IPO-3, signaling properties of which are opposite to A12 mAb, as well as homotypic CD150-CD150 interactions augment proliferation of resting human B lymphocytes, induced by BCR or

CD40 ligation, mitogens or cytokines, especially IL-4. Ligation of CD150 also enhances immunoglobulin production by CD40-activated B cells (Punnonen et al., 1997; Sidorenko & Clark, 1993). CD150-induced signals can both synergize with and augment CD95-mediated apoptosis in human B cells and T cells (Mikhalap et al., 1999). In human B cell lines MP-1, RPMI-1788, and Raji, signaling via CD40 rescues cells from CD95-mediated apoptosis, but ligation of CD150 can block CD40-mediated protection. Conversely, CD40 ligation cancels out the synergistic effect of CD95 and CD150 (Mikhalap et al., 1999; **Fig. 4**). Besides, CD150 could inhibit cell proliferation of Hodgkin's lymphoma cell lines and induce apoptosis in one of them (Yurchenko et al., 2010).

### 3.4.2 CD150 functions in antigen-presenting cells

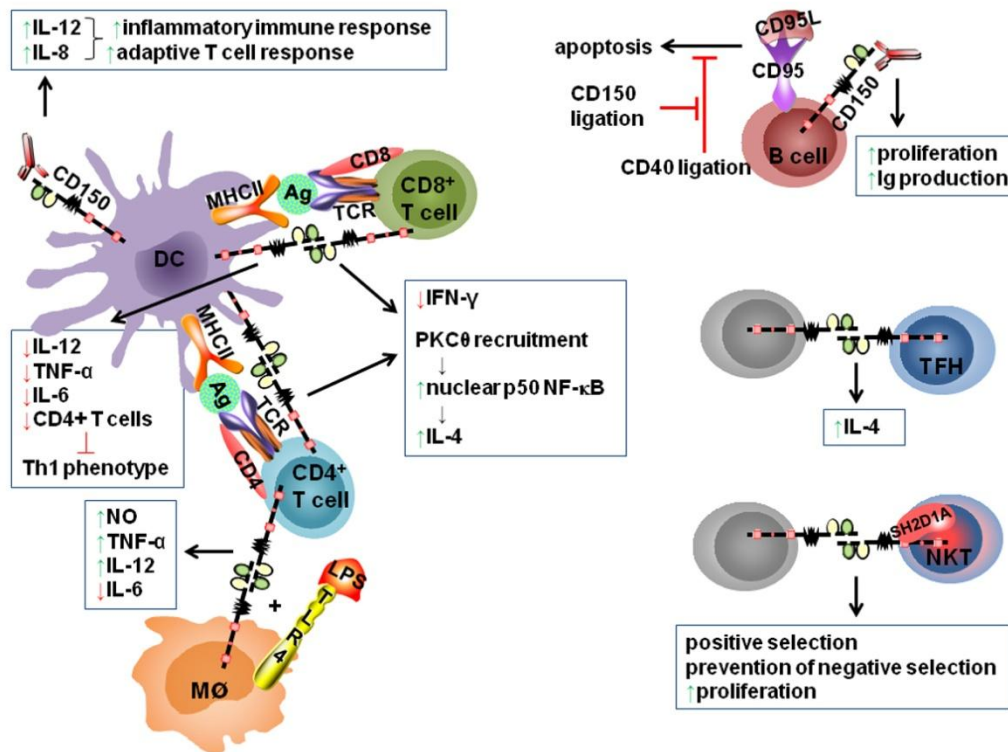
CD150 also plays an important role in immune responses of macrophages. CD150 acts as a co-receptor that regulates signals transduced by Toll-like receptor (TLR) 4, the major lipopolysaccharide (LPS) receptor on the surface of mouse macrophages (Wang et al., 2004). In CD150<sup>-/-</sup> macrophages LPS-induced IL-12 and TNF secretion is impaired, while IL-6 is overproduced even in the absence of any extraneous stimuli. Moreover, CD150<sup>-/-</sup> macrophages produce reduced amounts of nitric oxide (NO) upon stimulation with LPS (**Fig. 4**). However, CD150 does not regulate phagocytosis or responses to peptidoglycan or CpG (Wang et al., 2004). In SLAM-deficient (*Slamf1*<sup>-/-</sup>) mice macrophages also exhibit impaired LPS-induced production of TNF- $\alpha$  and nitric oxide. These experiments allow to conclude that CD150 acts as a vital regulator in the innate immune defense against Gram-negative bacteria in macrophages controlling two main independent bactericidal processes: phagosome maturation and the production of free radical species by the NOX2 complex (Berger et al., 2010).

Macrophages of CD150<sup>-/-</sup> C57Bl/6 mice are defective at clearing the parasite *Leishmania major* (Wang et al., 2004). The activation of CD150 may also promote the cell-mediated immune response mediated by IFN- $\gamma$  to

intracellular bacterial pathogens, namely to *Mycobacterium leprae* (Garcia et al., 2001). Finally, CD150 is required for the replication of *Trypanosoma cruzi* (*T.cruzi*) in macrophages and DCs (Calderon et al., 2012). *T. cruzi*, the protozoan parasite responsible for Chagas' disease, causes severe myocarditis. In the absence of CD150, macrophages and DCs are less susceptible to *T. cruzi* infection and produce

less myeloid cell specific factors that are keys in influencing the host response to parasite and the outcome of the infection. Consequently, CD150 deficient mice are resistant to *T. cruzi* infection.

These findings clearly define CD150 as a novel bacterial sensor (Sintes & Engel, 2011) and suggest its role in the regulation of some parasite infections.



**Figure 4.** CD150 functions. CD150 is expressed on several cell populations in immune system: T and B lymphocytes, dendritic cells, macrophages, follicular helper T cells and NKT lymphocytes. It has been shown that CD150 ligation mediates the functional changes in these cell types. In **CD4<sup>+</sup> and CD8<sup>+</sup> T cells** CD150 engagement mediates the inhibition of IFN- $\gamma$ , but activation of IL-4 production via increase of nuclear p50 NF- $\kappa$ B levels in PKC $\theta$ -dependent manner. In **DCs**, CD150 ligation with different ligands had (exerted) the opposite effects. Stimulation with anti-CD150 antibodies resulted in activation of inflammatory and adaptive T cell responses due to the increase of IL-12 and IL-18 production, while CD150 self-ligation mediated inhibition of IL-12, TNF- $\alpha$  and IL-6 production by DCs, leading to the inhibition of naïve CD4<sup>+</sup> T cell differentiation towards Th1 phenotype. In **macrophages** CD150 acts as a co-receptor that regulates signals transduced by the major LPS receptor TLR4, controlling the production of TNF- $\alpha$ , NO, IL-12 and IL-6. CD150 ligation blocks the rescue of **B cells** by CD40 ligation from CD95-mediated apoptosis. Besides, it augments B cell proliferation and enhances Ig production by CD40-activated B cells. In **follicular helper T cells (TFH)** CD150 engagement leads to upregulation of IL-4 production, and finally in **NKT cells** it promotes positive selection and enhances their proliferation. Ag - antigen; M $\phi$  - macrophage; MHCII – major histocompatibility complex class II; TCR – T cell receptor.

CD150 is highly expressed on mature dendritic cells (DCs) but only at a low level on immature DCs (Polacino et al., 1996). CD150

surface expression is strongly up-regulated by IL-1 $\beta$  that leads to an increased DC-induced T cell response (Kruse et al., 2001). CD150



expression is rapidly increased on DCs during the maturation process (Bleharski et al., 2001; Polacino et al., 1996). CD150 could be upregulated on DCs with CD40L, poly(I:C) (polyinosinic polycytidylic acid) or LPS. mRNA encoding both membrane-bound and soluble secreted isoforms of CD150 was detected in CD40 ligand-activated DCs. The functional outcome of CD150 ligation on DCs surface depends on the nature of its ligands. Particularly, engagement of CD150 with anti-CD150 mAbs IPO-3 enhances production of IL-12 and IL-8 by DC, but has no effect on the production of IL-10 (Bleharski et al., 2001). At the same time, another group of scientists has obtained the opposite results with the stimulation of CD150 on the surface of DCs with its self-ligand, CD150, expressed on transfected cells (Rethi et al., 2006). This may suggest that the differential functional outcome of CD150 signaling in DCs depends on the receptor region, engaged by its ligand. CD150/CD150 interactions did not interfere with sCD40L induced DC maturation. CD150/CD150 associations inhibited IL-12, TNF- $\alpha$ , and IL-6 secretion by CD40L-activated DCs. CD150 signaling also modulated the function of CD40L-activated DCs by reducing their ability to promote naïve T-cell differentiation toward IFN- $\gamma$ -producing effector

cells (**Fig. 4**). CD150 inhibited the differentiation of CD4<sup>+</sup> naïve T cells into Th1-type effector cells without promoting their differentiation into Th2 cells which was shown by the lack of IL-4 production and the low levels of IL-13 in the presence of high concentration of IFN- $\gamma$  (Rethi et al., 2006).

Thus, CD150 isoforms are expressed on a wide range of lymphoid cell types. With its extracellular part, CD150 binds to several types of ligands, including CD150 by itself, glycoproteins of *Morbilliviruses* and bacterial antigens, while its cytoplasmic tail may interact with key signaling molecules that connect CD150 to several intracellular signal transduction pathways. This allows CD150 to be involved in the regulation of cell transcription programs and gene expression and therefore control cell proliferation, differentiation and survival. In the context of all these findings CD150 has attracted a lot of interest in terms of its involvement in the measles virus induced immunosuppression. As the major measles virus receptor and a dual-function signaling lymphocyte receptor, CD150 may play an important role in mediating the inhibition of immune response by measles virus and its proteins.

## 4 Measles immunopathogenesis

### 4.1 Measles virus major cell targets and spread in the host

Measles represents a typical childhood disease, which is contracted only once in the lifetime of an individual because the anti-viral immune responses are efficiently induced and persist (Avota et al., 2010). But paradoxically measles virus infection induces both an efficient MV-specific immune response and a transient but profound immunosuppression. This depression of host immune response leads to an increased susceptibility to secondary infections following measles, which are responsible for its high mortality rate.

It is currently postulated that the primary cell targets for MV are CD150-positive alveolar macrophages, dendritic cells and lymphocytes in the respiratory tract. This concept is also supported with the fact that almost all CD14<sup>+</sup>

monocytes in human tonsils express CD150. DC infection has been observed in experimentally infected cotton rats (Niewiesk et al., 1997), transgenic mice (Shingai et al., 2005; Ferreira et al., 2010), and macaques (de Swart et al., 2007; Lemon et al., 2011). Both cultured CD1a<sup>+</sup> DCs and epidermal Langerhans cells can be infected *in vitro* by both vaccine and wild type strains of MV. Both vaccine and wild-type strains of MV undergo a complete replication cycle in DCs (Grosjean et al., 1997).

In peripheral tissues DCs are found in immature state but in response to danger signals they undergo maturation and upregulate co-stimulatory molecules as well as MHC class I and II. MV replication induces maturation of immature DCs, causing down-regulation of



CD1a, CD11c, and CD32 expression; up-regulation of MHCI, MHCII, CD80, and CD40 expression; and induction of CD25, CD69, CD71, CD86, and CD83 expression, alteration in the expression of chemokines, chemokine receptors, and chemotaxis (Abt et al., 2009; Schnorr et al., 1997; Servet-Delprat et al., 2000; Zilliox et al., 2006). Such dramatic changes allow DCs to increase their migratory potential and effectively realize their function – antigen presentation and activation of effector cells. All these events are also supported with the production of respective cytokines by DCs. Infection of DCs results in rapid induction of IFN- $\beta$  mRNA and protein and multiple IFN- $\alpha$  mRNAs, as well as upregulation of IL-10 and IL-12 secretion and acquisition of MIP-3 $\beta$  responsiveness, which provides the migratory capacity to DCs (Dubois et al., 2001; Zilliox et al., 2006). DC infection with vaccine strains of MV also induces their functional maturation (Schnorr et al., 1997).

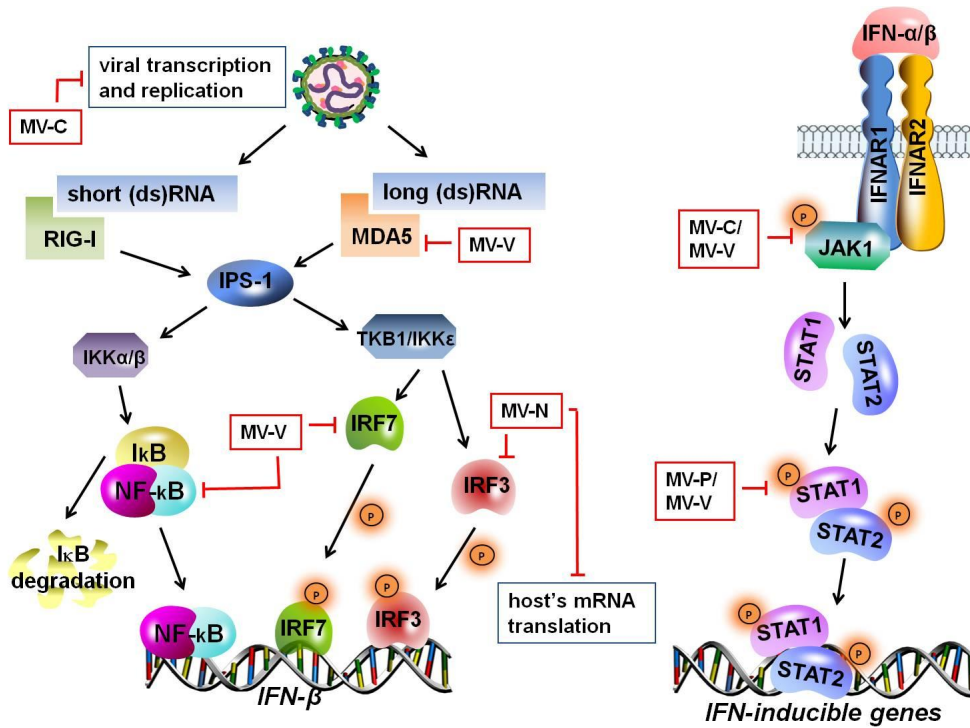
Infection spreads in DC cultures, but release of infectious virus is minimal unless cells are activated (Fugier-Vivier et al., 1997; Grosjean et al., 1997). Immature DCs do not express CD150 receptor at a high level. However, DC maturation is followed by the upregulation of CD150 expression level on their surface which could be used for viral spreading. Immature DCs form a network within all epithelia and mucosa, including the respiratory tract (Servet-Delprat et al., 2003). MV may exploit the DC network to travel to secondary lymphoid organs by infecting immature DCs, inducing their maturation and migration to these organs (Hahm et al., 2004). Moreover, the virus could be transported attached to DC-SIGN molecule on the surface of uninfected dendritic cells. DCs expressing either CD150 or DC-SIGN can transmit MV to co-cultured T cells (de Witte et al., 2008). The interaction of lymphocytes with MV-infected DCs results in lymphocyte infection, DC activation, and enhanced MV replication, leading to DC death (Fugier-Vivier et al., 1997; Murabayashi et al., 2002; Servet-Delprat et al., 2000). Thus, some of dendritic cells get infected but some carry the virus to the lymph nodes where MV meets its main lymphoid targets – B and T cells (Condock et al., 2007; de Swart et al., 2007). However, MV

transmission to T cells occurs most efficiently from cis-infected DCs, and this involves the formation of polyconjugates and an organized virological synapse (Koethe et al., 2012).

#### 4.2 Induction of innate and adaptive immune response by measles virus

MV infection activates the innate immunity with the induction of a type I interferon (IFN) response *in vitro* and *in vivo* (Devaux et al., 2008; Gerlier & Valentin, 2009; Herschke et al., 2007; Plumet et al., 2007). Type I interferons (IFN- $\alpha/\beta$ ) are an important part of innate immunity to viral infections because they induce an antiviral response and limit viral replication until the adaptive response clears the infection. Type I IFNs are induced upon pathogen recognition by the pattern recognition receptors which include TLRs, RIG-I like receptors (RLRs) and Nod-like receptors (NLRs). RLRs are RNA helicases, expressed by all nucleated cells, that recognize RNA molecules, which are absent in cell cytoplasm in normal conditions. Three types of RLRs, namely RIG-I, MDA5 and LGP2, could be distinguished depending on their ligands. MDA5 recognizes long double-stranded RNAs, RIG-I detects short double-stranded RNAs with a 5'-triphosphate residue, while LGP2 has greater affinity for dsRNA and regulates function of RIG-I and MDA5. This RNA detection induces IFN- $\beta$  gene transcription and activation of the transcription factors: IFN regulatory factor 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Griffin, 2010; **Fig. 5**).

During MV replication MV RNA interacts with RIG-I and less with MDA5 and thus stimulates IFN- $\beta$  transcription in responsive cells. Moreover, measles N protein can interact with and activate IRF3 by itself (Herschke et al., 2007; Plumet et al., 2007; **Fig. 5**). However, MV infection usually suppresses type I IFN production in PBMCs (Naniche et al., 2000), CD4<sup>+</sup> T cells (Sato et al., 2008; **Fig. 5**) and has a variable effect in plasmacytoid DCs (Druelle et al., 2008; Schlender et al., 2005). In addition wild type MV is less able to induce IFN, than MV vaccine strains most of the time (Naniche et al., 2000).



**Figure 5.** Innate anti-measles immune response and viral escape from its detection. MV leader RNA interacts with and activates RIG-I and to a lesser extent MDA5. Both pathways lead to the induction of signal transduction pathways with the activation of several transcription factors that regulate the expression of IFN genes. MV has developed several strategies to escape the induction of IFN response, some of which do not depend on viral replication but only the presence of viral proteins and their interaction with host cell proteins.

Besides of RNA helicases, measles viral components or replication intermediates could also be recognized by pattern-recognizing host machinery of TLRs. Pattern recognition by antigen-presenting cells via TLRs is an important element of innate immunity (Beutler, 2004). TLR2 was found to interact with the hemagglutinin of wild-type measles virus (**Fig. 2B**). This effect of MV-H could be abolished by mutation of a single amino acid, asparagine at position 481 to tyrosine, which is found in attenuated strains and is important for interaction with CD46. TLR2 activation by MV wild-type H protein induces surface CD150 expression in human monocytes and stimulates IL-6 production (Bieback et al., 2002).

The induction of adaptive immune response during measles infection coincides with the appearance of its clinical manifestations, including skin rash. At the time of the rash, both MV-specific antibody and activated T cells are detectable in circulation, however in few days

after its appearance the virus is cleared from peripheral blood cells. The central role in viral clearance belongs to CD8<sup>+</sup> T cells that is supported by studies in infected macaques (Permar et al., 2003; de Vries, Yuksel et al., 2010). In early immune response, CD4<sup>+</sup> T cells produce type 1 cytokines, including IFN- $\gamma$  and IL-2, while after clearance of infectious virus they switch to the production of type 2 cytokines, like IL-4, IL-10, and IL-13 (Moss et al., 2002; **Fig. 6**). Th2 cytokine skewing after acute measles infection facilitates the development of humoral responses important for lifelong memory protection against any new measles infection, but depresses the induction of type 1 responses that is necessary for fighting new pathogens. However, recent studies on macaque model showed that humoral responses could also be abrogated upon MV infection. MV efficiently replicates in B lymphocytes, resulting in follicular exhaustion and disorganization of the germinal centers, which are essential in

actively ongoing humoral immune responses (de Vries et al., 2012; Fig. 6).

### 4.3 Measles virus strategies for suppression of immune response

The immunosuppressive effects of measles were first recognized and quantified by von Pirquet in the 19th century, in his study of delayed type hypersensitivity (DTH) skin test responses during a measles outbreak in a tuberculosis sanitarium (Von Pirquet, 1908). In addition to depressed immune responses to recall antigens (such as tuberculin), the cellular immune responses and antibody production to new antigens are impaired during and after the acute measles (Moss et al., 2004). The fact that just a small proportion of peripheral blood cells are infected during measles allows us to speculate about alternative mechanisms of immunosuppression that do not involve direct viral infection of lymphoid cells. During several weeks after resolution of the rash and visible recovery, infectious virus is no longer detectable, but viral RNA continues to be present in PBMCs, as well as in respiratory secretions and urine (Permar et al., 2001; Riddell et al., 2007). A number of different potential mechanisms for MV-induced immunosuppression have been proposed, including lymphopenia, prolonged cytokine imbalance, leading to inhibition of cellular immunity, suppression of lymphocyte proliferation and compromised DC functions (Avota et al., 2010; Griffin, 2010). The interaction of measles viral proteins with cellular receptors and other intracellular elements involved in the induction of immune response may play an important role in MV-induced immunosuppression.

#### 4.3.1 The role of viral glycoproteins in measles immunosuppression

Measles virus glycoproteins are highly implicated in the suppression of lymphocyte proliferation during and after measles acute infection even in the absence of MV replication. In cotton rat model transiently

transfected cells expressing the viral glycoproteins (hemagglutinin and fusion protein) inhibit lymphocyte proliferation after intraperitoneal inoculation (Niewiesk et al., 1997). This is not connected to cell fusion or unresponsiveness to IL-2, but rather to the retardation of the cell cycle that correlates with inhibition of proliferation. This inhibitory signal prevents entry of T cells into S phase and results in the reduction of cyclin-dependent kinase complexes and delayed degradation of p27Kip leading to cell cycle retardation and accumulation of cells in the G0/G1 phase (Engelking et al., 1999; Niewiesk et al., 1999; Schnorr et al., 1997). Similarly, the complex of both MV glycoproteins, F and H, is critical in triggering MV-induced suppression of mitogen-dependent proliferation of human PBLs *in vitro* (Dubois et al., 2001; Schlender et al., 1996). It is also supported by the data that human DCs expressing MV glycoproteins *in vitro* fail to deliver a stimulation signal for proliferation to T cells, despite the presence of co-stimulatory molecules on their surface (Klagge et al., 2000). However, the nature of the receptor responsible for mediating MV glycoprotein-induced immunosuppression has not been identified so far.

Some immunosuppressive effects of measles virus were shown to be caused by direct interaction of its glycoproteins with CD46 receptor. Thus, upon the intraperitoneal injection of UV-inactivated MV in hapten-sensitized CD46-transgenic mice a significant decrease in dendritic cell IL-12 production from draining lymph nodes was observed, providing *in vivo* evidence of the systemic modulation of IL-12 production by MV proteins (Marie et al., 2001). Down-regulation of IL-12 production in primary human monocytes, could be induced in the absence of viral replication by cross-linking of CD46 with an antibody or with the complement activation product C3b (Karp et al., 1996). Moreover, the engagement of distinct CD46 isoforms (CD46-1 and CD46-2) induces different effects in immune response. CD46-2 induces increased generation of specific CD8<sup>+</sup> T cells and decreases proliferation of CD4<sup>+</sup> T cells, which is associated with strongly suppressed IL-10 secretion. In contrast, CD46-1 engagement decreases the inflammatory response as well as

the generation of specific CD8<sup>+</sup> T cells. Moreover, it markedly increases the proliferation of CD4<sup>+</sup> T cells which are capable of producing the anti-inflammatory cytokine IL-10 (Marie et al., 2002).

CD46 downregulation can be rapidly induced in uninfected cells after surface contact with MV particles or MV-infected cells. Contact-mediated CD46 modulation can lead to complement-mediated lysis of uninfected cells that also may cause lymphopenia (Schneider-Schaulies et al., 1996; Schnorr et al., 1995). Recent discovery of Notch ligand Jagged1, as a natural ligand for CD46, which induces Th1 differentiation (Le Friec et al., 2012), opens new possibilities to explain the importance of CD46-pathogen interaction in the modulation of the immune response. However, CD46 is most probably involved in the pathogenesis of only vaccine strains of measles virus, while most of wild type MV strains do not use this receptor.

#### **4.3.2 The impact of measles non-structural proteins on antiviral immune response**

MV non-structural proteins can block the induction of IFN response using multiple strategies. The MV V protein interacts with RNA helicase MDA5 and inhibits the IFN response (Andrejeva et al., 2004; Childs et al., 2007; **Fig. 5**). Moreover, in plasmacytoid dendritic cells MV V can be a substrate for IKK $\alpha$ , which results in competition between MV-V and IRF7 for the phosphorylation by IKK $\alpha$ . In addition MV V can bind to IRF7 and inhibit its transcriptional activity. Binding to both IKK $\alpha$  and IRF7 requires the 68-amino-acid unique C-terminal domain of V (Pfaller & Conzelmann, 2008; **Fig. 5**). Both P and V proteins can inhibit the phosphorylation of STAT1 and the signal cascade downstream from the IFNAR (Caignard et al., 2007; Devaux et al., 2007). In MV-infected cells, C and V proteins of measles virus form a complex containing IFNAR1, RACK1, and STAT1 and inhibit the Jak1 phosphorylation (Yokota et al., 2003). Moreover, MV V protein could act as an inhibitor of cell death by preventing the activation of the apoptosis regulator PUMA (Cruz et al., 2006). The nonstructural C protein

inhibits type I IFN induction and signaling, in part by decreasing viral RNA synthesis, and has been implicated in prevention of cell death (Escoffier et al., 1999; Reutter et al., 2001; Shaffer et al., 2003; **Fig. 5**). Recombinant MV lacking the C protein grows poorly in cells possessing the intact IFN pathway and this growth defect is associated with reduced viral translation and genome replication. The translational inhibition correlates with phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (Nakatsu et al., 2006). In addition, the protein kinase PKR plays an important role as an enhancer of IFN- $\beta$  induction during measles virus infection via activation of mitogen-activated protein kinase and NF- $\kappa$ B (McAllister et al., 2010). Besides the anti-IFN- $\alpha/\beta$  properties of V and C proteins *in vivo*, both C and V proteins are required for MV to strongly inhibit the inflammatory response in the peripheral blood monocyctic cells of infected monkeys. In the absence of V or C protein, virus-induced TNF- $\alpha$  downregulation is alleviated and IL-6 is upregulated (Devaux et al., 2008).

In addition, MV P efficiently stabilizes the expression and prevents the ubiquitination of the human p53-induced-RING-H2 (PIRH2), an ubiquitin E3 ligase belonging to the RING-like family. PIRH2 is involved in the ubiquitination of p53 and the  $\epsilon$ -subunit of the coatmer complex,  $\epsilon$ -COP, which is part of the COP-I secretion complex. As such, PIRH2 can regulate cell proliferation and the secretion machinery (Chen et al., 2005).

#### **4.3.3 The effect of MV nucleoprotein in the regulation of immune response**

Measles virus nucleoprotein (N) interacts with Fc $\gamma$ R receptor on dendritic cells via its C-terminal part (Laine et al., 2003; Ravelle et al., 1997; **Fig. 2B**). This interaction is critical for MV-induced immunosuppression of inflammatory responses *in vivo* that was shown by the inhibition of hypersensitivity responses in CD46-transgenic mice. MV N protein impairs the APC function affecting the priming of naive CD8<sup>+</sup> lymphocytes during sensitization as well as the development of the effector phase following secondary antigen contact (Marie et

al., 2001). It has been difficult to understand how this cytosolic viral protein could leave an infected cell and then perturb the immune response. Finally it was shown that intracellularly synthesized nucleoprotein enters the late endocytic compartment, where it recruits its cellular ligand, the FcγR. Nucleoprotein is then expressed at the surfaces of infected leukocytes associated with the FcγR and is secreted into the extracellular compartment, allowing its interaction with uninfected cells. Cell-derived nucleoprotein inhibits the secretion of IL-12 and the generation of the inflammatory reaction, both shown to be impaired during measles (Marie et al., 2004). MV N was also shown to induce an anti-inflammatory regulatory immune response and its repetitive administration into apolipoprotein E-deficient mice resulted in the reduction of atherosclerotic lesions (Ait-Oufella et al., 2007). Finally, the immunosuppressive properties of MV N seem to be shared with the other members of *Morbilliviruses*, since both PPRV and CDV nucleoproteins bind FcγR and suppress delayed hypersensitivity responses (Kerdiles et al., 2006).

Interestingly, N protein also binds to the eukaryotic initiation factor 3 (eIF3-p40). The interaction between MV-N and eIF3-p40 *in vitro* inhibits the translation of a reporter mRNAs (Sato et al., 2007). This is the first observation indicating that MV can repress the translation in host cells. Therefore, viral mRNAs may selectively escape requirement for eIF3-p40 initiation factor for translation of viral proteins (Gerlier & Valentin, 2009).

#### **4.3.4 The effect of measles virus infection on host pathophysiology**

MV infection is associated with lymphopenia with decreased numbers of T cells and B cells in circulation during the rash. Increased surface expression of CD95 (Fas) and Annexin V staining on both CD4<sup>+</sup> and CD8<sup>+</sup>T cells during acute measles suggests that apoptosis of uninfected lymphocytes may account for some of the lymphopenia. While B cell numbers may remain low for weeks, T cell counts return to normal within few days (Ryonet al., 2002). MV-induced lymphopenia could be due to loss of

precursors, albeit MV interaction with CD34<sup>+</sup> human hematopoietic stem cells (HSCs) or thymocytes did not affect viability and/or differentiation *in vitro* (Avota et al., 2010).

Recent studies on macaque model proposed the alternative explanation for MV-induced lymphopenia and its relation to immunosuppression. It showed that MV preferentially infects CD45RA<sup>-</sup> memory T lymphocytes and follicular B lymphocytes, resulting in high infection levels in these populations, which causes temporary immunological amnesia thus explaining the short duration of measles lymphopenia yet long duration of immune suppression. Therefore, MV infection wipes immunological memory, resulting in increased susceptibility to opportunistic infections (de Vries et al., 2012).

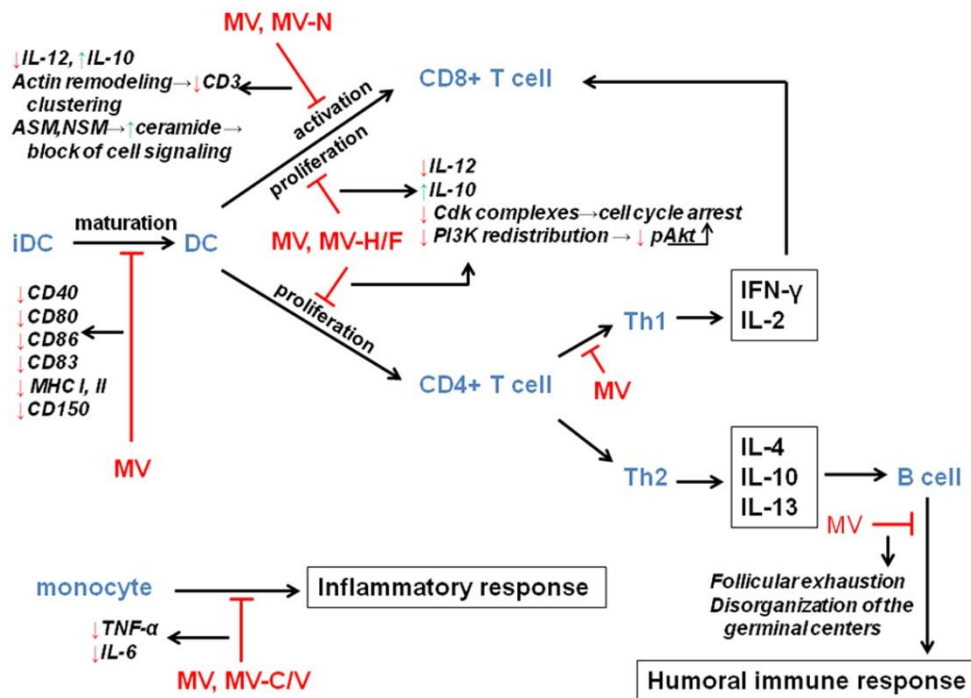
The influence of measles virus infection on gene expression by human peripheral blood mononuclear cells (PBMCs) was examined with cDNA microarrays. MV infection up-regulated the NF-κB p52 subunit and B cell lymphoma protein-3 (Bcl-3). This finding suggests the modulation of the activity of NF-κB, transcription factor that regulates the expression of a wide range of genes involved in inducing and controlling immune responses (Bolt et al., 2002). MV also upregulated the activating transcription factor 4 (ATF-4) and the pro-apoptotic and growth arrest-inducing CHOP/GADD153 genes indicated that MV infection induces the endoplasmic reticulum (ER) stress response in PBMCs. It is possible that the ER stress response is involved in the MV-induced reduction of lymphocyte proliferation through the induction of CHOP/GADD153 (Bolt et al., 2002).

It was shown that MV infection induces downregulation of human receptor CD150 (hCD150) in transgenic mice, inhibits cell division and proliferation of hCD150<sup>+</sup> but not hCD150<sup>-</sup> T cells, and makes them unresponsive to mitogenic stimulation. The role of CD150 in this process was studied on the transgenic mice expressing hCD150 under the control of the *lck* proximal promoter. The use of the *lck* proximal promoter in producing transgenic mice results in specific expression of CD150 protein on immature and mature T lymphocytes in blood, spleen, and thymus that partially mimics the

CD150 expression in human T cells (Hahm et al., 2003).

Activation of the PI3K/Akt kinase pathway is an important target for MV interference in T cells. Shortly after MV exposure *in vivo* and *in vitro* this pathway was shown to be efficiently inhibited after IL-2R or CD3/CD28 ligation in T cells (Avota et al., 2001; Avota et al., 2004). In the absence of MV replication, MV H/F can inhibit T cell proliferation *in vitro* and the intracellular pathway involves the impairment of the Akt kinase activation (Avota et al., 2001). Downstream effectors of Akt kinase include molecules essentially involved in S-phase entry such as subunits of cyclin-dependent kinases, and consequently, these were found deregulated in T-cell cultures exposed to MV (Engelking et al., 1999; Fig. 6). Active Akt kinase largely abolished MV-induced proliferation arrest

(Avota et al., 2001). The regulatory subunit of the PI3K, p85, which acts upstream Akt kinase activation, was tyrosine phosphorylated shortly after TCR ligation in MV-exposed T cells, yet failed to redistribute to cholesterol-rich detergent-resistant membranes (DRMs) and this correlated with a lack of TCR-stimulated degradation of Cbl-b protein (Avota et al., 2004). This signal also impairs actin cytoskeletal remodeling in T cells, which lose their ability to adhere to and promote microvilli formation (Muller et al., 2006). The role of Akt pathway in the MV infection may be cell dependent, as its specific inhibitor strongly reduces the replication of several *Paramyxoviruses*, including MV (Sun et al., 2008). In this study Akt signaling was shown to support the viral replication either directly or by promoting host cell survival and cell cycle entry in nonlymphoid cell types.



**Figure 6.** MV evasion strategies to avoid host adaptive immune response. Dendritic cells lay in the center of regulation of antiviral immune responses. By abrogation of DC maturation via down-regulation of surface markers like CD40, CD80, CD86, CD83, MHC I/II, CD150, and deregulation of cytokine production (IL-12 and IL-10), MV influences different stages of DC life cycle, and inhibits not only DC function, but also other immune cell responses. The mechanisms of such action include the disruption of cell signaling (PI3K/Akt pathway), cytoskeletal remodeling that crashes the stable immunological synapses, ceramide accumulation, etc.

Another measles strategy to influence the intracellular signaling in immunocompetent cells

is to cause ceramide accumulation in human T cells in a neutral (NSM) and acid (ASM)

sphingomyelinase-dependent manner (Avota et al., 2011; **Fig. 6**). Ceramides promote formation of membrane microdomains and are essential for clustering of receptors and signaling platforms in the plasma membrane. They also act as signaling modulators, namely by regulating relay of PI3K signaling. In T cells ceramides, induced by MV, downmodulate chemokine-induced T cell motility on fibronectin that mimicks extracellular matrix interaction (Avota et al., 2011; Gassert et al., 2009). In human DCs SMase activation and subsequent ceramide accumulation are directly linked to c-Raf-1 and ERK activation in response to DC-SIGN ligation. DC-SIGN signaling in turn may weaken TLR4 signaling, thereby downregulating inflammatory responses. SMase activation also promotes surface translocation and compartmentalization of CD150 receptor, promoting MV fusion (Avota et al., 2011).

MV exposure also results in an almost complete collapse of membrane protrusions associated with reduced phosphorylation levels of cofilin and ezrin/radixin/moesin (ERM) proteins. The signal elicited by MV prevents efficient clustering and redistribution of CD3 to the central region of the immunological synapse in T cells. Thus, by inducing microvillar collapse and interfering with cytoskeletal actin remodeling, MV signaling disturbs the ability of T cells to adhere, spread, and cluster receptors essential for sustained T-cell activation (Muller et al., 2006). Together, these findings highlight an as yet unrecognized concept of pathogenesis where a virus causes membrane ceramide accumulation or cytoskeletal remodeling to reorganize the immunological synapses and to target essential processes in T cell activation.

Different murine models have been generated to analyze the effect of MV infection *in vivo*. Suckling mice ubiquitously expressing CD150 were highly susceptible to the intranasal MV infection (Sellin et al., 2006) and generated FoxP3<sup>+</sup> T regulatory cells. After being crossed to IFN-I deficient background, adult mice developed generalized immunosuppression with strong reduction of hypersensitivity responses (Sellin et al., 2009). In double transgenic mice, expressing both CD150 and CD46 in the IFN-I deficient background, wild-type MV induced infection of DCs, but functional immunological

defect was not further analyzed (Shingai et al., 2005). Finally, the transgenic murine model, capable of reproducing several aspects of measles-induced immunosuppression was made by introducing the V domain of human CD150 to mouse CD150 that allows MV infection in mice. The infected mice developed lymphopenia, impaired T cell proliferation and cytokine imbalance (Koga et al., 2010) illustrating potential *in vivo* role of CD150 in measles immunopathogenesis, although the effect on MV replication and its interaction with CD150 on the modulation of immune responses was not clearly dissociated in any of these transgenic models.

#### **4.3.5 MV interference with the function of dendritic cells**

The initiation of adaptive immune responses to viruses and bacterial infections relies on the activation of T and B cells by professional antigen-presenting cells, dendritic cells (Trifilo et al., 2006). Maturation of DCs includes the up-regulation of co-stimulatory molecules and expression of proinflammatory cytokines, shown to be impaired during measles infection (Trifilo et al., 2006). At initial stages of infection, MV replication induces maturation of immature DCs. However, CD40-dependent maturation of DCs is inhibited by MV replication, which is demonstrated by repressed induction of co-stimulatory membrane molecules (CD40, CD80, CD86) and a lack of activation (CD25, CD69, CD71) and maturation (CD83) marker expressions (**Fig. 6**). The inhibition of CD80 and CD86 expression could be related to the impairment of CD40 signaling, which is demonstrated by inhibition of tyrosine-phosphorylation in MV-infected CD40-activated DCs (Servet-Delprat et al., 2000). The same effect of MV infection was observed in transgenic mice expressing hCD150 on dendritic cells under the *CD11c* promoter. After infection with wt MV, murine splenic hCD150<sup>+</sup> DCs decreased the expression level of B7-1 (CD80), B7-2 (CD86), CD40, MHC class I, and MHC class II molecules and increased the rate of apoptosis (**Fig. 6**). Furthermore, MV-infected DCs failed to stimulate allogeneic T cells. Analysis of CD8<sup>+</sup> T cells showed that they were

poorly or even not activated, as judged by their low expression levels of CD44, CD25, and CD69 molecules and by their inability to proliferate (Hahm et al., 2004).

After the first contact with pathogen antigens DCs undergo morphological changes and increase migratory capacities. Upon measles virus infection antigen-presenting function of DCs is partly damaged. Antigen uptake, as measured by mannose receptor-mediated endocytosis, is not affected (Grosjean et al., 1997). However, the ability of MV-infected DCs to stimulate proliferation of heterologous CD4<sup>+</sup> T cells is affected that was shown in mixed leukocyte reaction (MLR) (Dubois et al., 2001; Fugier-Vivier et al., 1997; Grosjean et al., 1997; Schnorr et al., 1997). MV infection of DCs also prevents CD40L-dependent CD8<sup>+</sup> T cell proliferation (Servet-Delprat et al., 2000). The full T-cell activation does not occur probably because T-cell conjugation with DCs is unstable and short (Shishkova et al., 2007). MV-exposed T cells are recruited into conjugates with DCs *in vitro* but have impaired clustering of receptors needed for sustained T-cell activation, in part due to accumulation of ceramide and preventing stimulated actin cytoskeletal dynamics and recruitment of surface receptors and membrane-proximal signaling complexes (Gassert et al., 2009).

Measles virus attached to DC-SIGN (de Witte et al., 2008) or Langerin (Van der Vlist et al., 2011) probably uses DCs as the vehicle to the secondary lymphoid organs. However DCs are not found in efferent lymphatic vessels as they probably undergo apoptosis in the lymph nodes. MV-infected DCs develop increased sensitivity to CD95L and undergo CD95-mediated apoptosis when co-cultured with activated T cells. Moreover, CD95-dependent DC apoptosis participates in MV release (Servet-Delprat et al., 2000). IFN- $\alpha/\beta$  production induced by MV in human DCs, triggers the synthesis of functional TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) on their surface. TRAIL belongs to TNF family receptors and was shown to be involved in apoptosis of several tumor cell lines. Moreover, MV was found to induce functional TRAIL expression also in monocytes after activation of IFN- $\alpha/\beta$  expression (Vidalain

et al., 2000). Thus, IFN- $\alpha/\beta$ -activated DCs and monocytes may exert an innate TRAIL-mediated cytotoxic activity towards surrounding MV-infected cells (Servet-Delprat et al., 2003).

Prolonged suppression of IL-12 production has been observed in children with measles that is critical for the orchestration of cellular immunity (Atabani et al., 2001). CD40-induced cytokine pattern in DCs is also modified *in vitro* by MV replication: IL-12 and IL-1 $\alpha/\beta$  mRNA are decreased, whereas IL-10 mRNA is induced (Fugier-Vivier et al., 1997; Servet-Delprat et al., 2000; **Fig. 6**).

When infected with MV, transgenic mice, which express human CD150 receptor on DCs are defective in the selective synthesis of IL-12 in response to stimulation of TLR4 signaling, but not to engagements of TLR2, 3, 7 or 9. MV suppressed the TLR4-mediated IL-12 induction in DCs even in the presence of co-stimulation with other ligands for TLR2, 3, 7 or 9. MV V and C proteins are not responsible for IL-12 suppression mediated TLR4 signaling, while the interaction of MV-H with human CD150 facilitates this suppression. IL-12 is a key cytokine inducing cellular immune responses to protect host from the pathogenic infections. It is possible that expressed on MV-infected cells MV-H, interacts with CD150 receptor on uninfected cells to cause the inhibition of TLR4-mediated IL-12 synthesis. Similarly, MV abrogates the function of DCs in detecting and delivering of danger signals by pathogenic components via TLRs to the host immune system (Hahm et al., 2007). Modulation of TLR signaling by MV also occurs upon co-ligation of DC-SIGN, as reflected by induction of DC-SIGN-dependent Raf-1 activation, which in turn promotes IL-10 transcription in immature DCs by inducing acetylation of the NF- $\kappa$ B subunit p65. Measles virus induces IL-10 in the absence of LPS, because it binds to both TLR2 and DC-SIGN (Gringhuis et al., 2007).

IL-10, an immunoregulatory and immunosuppressive cytokine, is also elevated for weeks in the plasma of children with measles (Moss et al., 2002). It downregulates the synthesis of cytokines, suppresses macrophage activation and T cell proliferation, and inhibits delayed-type hypersensitivity responses, which could contribute to the increased susceptibility



to the secondary infections), often observed

## 5 Conclusions

CD150 surface receptor is expressed on the main measles virus cell targets: DCs, T and B cells, activated monocytes and macrophages. Its interaction with MV hemagglutinin mediates the viral entry and therefore plays an important role in the primary steps of the infection. CD150 is described as a dual-function receptor, involved in the regulation of immune cell functions. While in B cells CD150 stimulation promotes cell activation, proliferation and Ig production, in T cells its ligation has mostly inhibitory effect (particularly, the inhibition of IFN- $\gamma$  production). Moreover, by mediating the inhibition of IL-12, IL-6 and TNF- $\alpha$  secretion by DCs and induction of IL-4 production by NKT cells, CD150 shifts the differentiation of CD4<sup>+</sup> T cells towards Th2 phenotype, thus inhibiting the generation of IFN- $\gamma$  producing Th1 cells. Engagement of CD150 was shown to regulate the activation of several signaling pathways, including MAPK and Akt/PKB. Measles virus infection is associated with dramatic suppression of cellular immune response, which is accountable for the risk of secondary infections and high mortality rate after measles infection. MV-induced immunopathogenesis is linked to the aberrant DC maturation, modulation of IL-12 production and inhibition of T cell proliferation. At the same time, MV induces virus-specific lifelong immunity as humoral immune responses

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during measles.

remain almost intact, presenting an immunological paradox associated with this infectious disease. It is possible, that measles virus uses CD150-mediated signaling machinery to inhibit cellular immune response allowing successful viral propagation. This consequence of CD150 engagement, which remains to be experimentally determined, may contribute to the establishment of the measles paradox. More complete understanding the role of CD150-measles interaction in viral immunopathogenesis may open novel avenues in the development new therapeutic approaches aiming to reduce complications of measles-induced immunosuppression and will shed new light on the immunoregulatory role of CD150.

## 6 Acknowledgments

The work was supported by INSERM, Ligue contre le Cancer and Ministère des affaires Etrangères, Partenariat Curien franco-ukrainien “Dnipro”, The State Fund for Fundamental Researches of Ukraine, National Academy of Sciences of Ukraine, FEBS Summer and Collaborative Fellowships, Bourse de Gouvernement Français and “AccueilDoc” from Region Rhone Alpes. Authors thank to A. Talekar for reading the review and English editing.

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## **Article 4.**



## CD150-MEDIATED AKT SIGNALLING PATHWAY IN NORMAL AND MALIGNANT B CELLS

M. Yurchenko<sup>1,\*</sup>, L.M. Shlapatska<sup>1</sup>, O.L. Romanets<sup>1</sup>, D. Ganshevskiy<sup>1</sup>, E. Kashuba<sup>1,2</sup>,  
A. Zamoshnikova<sup>1</sup>, Yu.V. Ushenin<sup>3</sup>, B.A. Snopok<sup>3</sup>, S.P. Sidorenko<sup>1</sup>

<sup>1</sup>R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine,  
Kyiv 03022, Ukraine

<sup>2</sup>Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm 171 77, Sweden

<sup>3</sup>V.E. Lashkarev Institute of Semiconductors Physics NAS of Ukraine, Kyiv 03028, Ukraine

**Aim:** To study upstream and downstream events in CD150-mediated Akt signaling pathway in normal human B cells, EBV-transformed lymphoblastoid (LCL) and malignant Hodgkin's lymphoma (HL) B cell lines. **Methods:** To access protein-protein interaction we applied immunoprecipitation, Western blot analysis and surface plasmon resonance (SPR) technique. A novel modification of SPR technique using reduced glutathione bound to golden surface was proposed. Immunostaining and isolation of cytoplasmic fractions and nuclear extracts were performed to detect proteins' localization in cells. Western blot analysis was performed to follow up the phosphorylation of proteins on specific sites and proteins' expression level. **Results:** It was shown that CD150 ligation induced Akt activation in normal tonsillar B cells (TBC), SH2D1A positive LCL and HL B cell lines. The p85 $\alpha$  subunit of PI3K co-precipitated with CD150 cytoplasmic tail. This direct association depends on tyrosine phosphorylation and is mediated by N terminal SH2 domain of p85 $\alpha$ . CD150 initiated phosphorylation of FoxO1 transcription factor in normal B cells as well as in LCL MP-1 and HL cell line L1236. At the same time, CD150 ligation triggered GSK-3 $\beta$  kinase phosphorylation only in immortalized LCL MP-1 and HL cell line L1236. **Conclusions:** We have demonstrated that CD150 receptor could trigger PI3K-mediated Akt signaling pathway in normal, EBV-transformed and malignant B cells. CD150-mediated phosphorylation of Akt downstream targets GSK-3 $\beta$  and FoxO1 in EBV-transformed and HL cells could be one of the mechanisms to avoid apoptosis and support survival program in these immortalized B cells.

**Key Words:** CD150 receptor, PI3 kinase, Akt, GSK-3 $\beta$ , FoxO1, immortalized B cells, Hodgkin's lymphoma.

CD150 (IPO-3/SLAM) is a member of CD2 family of the immunoglobulin (Ig) superfamily of surface receptors [1–5]. It is expressed on activated T and B lymphocytes, dendritic cells and monocytes [3, 6]. Low level of CD150 expression was also found on natural killer T cells, platelets, basophiles and mature dendritic cells [7, 8]. This receptor is upregulated via antigen receptors, CD40, Toll-like receptors and by mitogenes or cytokines (reviewed in [9]). There are experimental evidences that CD150 and SH2D1A adaptor protein are co-expressed during a narrow window of B cell maturation, and SH2D1A may be involved in regulation of B cell differentiation via switching of CD150-mediated signaling pathways [10, 11]. CD150 is co-expressed with SH2D1A adaptor protein in some B-lymphoblastoid cell lines and lymphoma cells with activated B cell phenotype, such as Hodgkin's lymphoma (HL) and ABC-type diffuse large B cell lymphoma [10, 12].

Tumor cells of classical HL are known as Hodgkin and Reed-Sternberg cells (HRS), and originate from preapoptotic germinal center B cells that do not express functional B cell receptor (BCR) [13, 14]. HRS cells are able to escape the regulation mechanisms aimed to eliminate B cells lacking functional BCR. Several aberrantly activated signaling pathways have been identified that contribute to the rescue of HRS cells from apoptosis: JAK-STAT [15, 16], MAPK/ERK initiated by CD30-, CD40-, RANK [17] and CD150 [18] signaling, PI3K/AKT linked to CD40 [19], LMP1 and LMP2a in EBV-positive cases [20–22]; NF- $\kappa$ B due to aberrant expression, mutations, CD40, CD30 stimulation (reviewed in [13, 14, 23]).

Activation of the phosphatidylinositol 3-kinase (PI3K) pathway has been linked with tumor cell growth, survival and resistance to therapy in several cancer types [24]. The main downstream PI3K effector, which controls the survival of normal and malignant cells, is Akt/PKB [25]. Ligation of tumor necrosis factor (TNF) family receptors could induce Akt phosphorylation/activation in normal [26] and HRS cells [19]. Previously, we have demonstrated that Akt could be activated via CD150 in DT40 model system, lymphoblastoid cell line MP-1 [10, 27] and HL cells L1236 [28]. The hypothesis was proposed that CD150-SH2D1A association could play decisive role in CD150-mediated Akt signaling. In current study we further explored the upstream and downstream events in CD150-mediated Akt pathway in normal and malignant B cells.

Received: November 15, 2010.

\*Correspondence: Fax: +380442581656;

E-mail: myurchenko@hotmail.com

**Abbreviations used:** BCR – B cell receptor; CD150ct – CD150 cytoplasmic tail; Cyt – cytoplasmic fraction; HL – Hodgkin's lymphoma; HRS – Hodgkin's and Reed-Sternberg; ITSM – immunotyrosine based switch motif; LCL - lymphoblastoid cell line; NE – nuclear extract; NSH2 – N-terminal SH2 domain; PI3K - phosphatidylinositol 3-kinase; SH2D1A – SH2 domain containing protein 1A; SPR – surface plasmon resonance; TBC – tonsillar B cells; TCL – total cell lysate; TNF – tumor necrosis factor.

## MATERIALS AND METHODS

**Cell lines.** The B lymphoblastoid cell lines (B-LCL) MP-1, CESS, T5-1, freshly infected LCLs from peripheral blood B cells and HL cell line of B cell origin L1236 were maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, and antibiotics.

**Human tissue specimens.** After receiving informed consent in accordance with the Declaration of Helsinki, fresh tonsils were obtained from 23 patients undergoing tonsillectomy. Tonsillar B cells were isolated by density fractionation on discontinuous Lymphoprep (Axis-Shield PoC AS, Norway) and Percoll (Sigma, USA) gradients as described [29]. 96.5–99.6% of cells in CD2<sup>-</sup> populations were of B cell lineage since expressed CD19 and/or CD138 antigens.

**GST proteins preparation.** GST-fusion constructs of CD150 cytoplasmic tail (CD150ct) were prepared earlier and described in [10, 27]. cDNA of p85 $\alpha$  regulatory subunit was a kind gift of Dr. I. Gout (University College, London, UK). N-terminal SH2 domain of p85 $\alpha$  regulatory subunit was cloned into pGEX-2T vector using specific primers, and sequenced (we thank for the help Dr. Vladimir Kashuba, MTC, Karolinska institutet, Sweden). The plasmid encoding GST-SH2D1A was kindly provided by Dr. Kim Nichols (The Children's Hospital of Philadelphia, Philadelphia, PA, USA). Plasmids containing GST-CD150ct, GST-SH2D1A and GST-NSH2-p85 $\alpha$  were transformed into the *Escherichia coli* strains BL21DE3 (Invitrogen, USA) to produce non-phosphorylated fusion proteins, and GST-CD150ct with and without point mutations were transformed to TKX1 strain (Stratagene, USA) for production of tyrosine-phosphorylated fusion proteins.

**Surface plasmon resonance (SPR) instrumentation and SPR analysis.** The substrates and cell design of scanning SPR spectrometer "BioSuplar" ("PLASMON-005") (V. Lashkarev Institute of Semiconductor Physics, NAS of Ukraine, Kyiv, Ukraine) as well as its other characteristics are described in [30]. This spectrometer, which has open measurement architecture, was used to study protein–protein interactions. The peristaltic pump provided a constant solution flow (> 50  $\mu$ l/min) through the experimental cell. SPR kinetics was analyzed with a model that takes into account the heterogeneous processes at the interface using a stretched exponential function [31].

**Surface modification and protein immobilization.** The surface cleaning and activation procedure (for planar metal electrodes of SPR chips, each with a metal layer 500 Å thick consisting of a 450-Å layer of gold on a 50-Å chromium adhesive layer) was carried out at room temperature as follows. To remove organic contamination, the chips were washed in a freshly prepared mixture of aqueous (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (0.12 M) and H<sub>2</sub>O<sub>2</sub> (30%) (15:6 vol:vol) for 10 min. Traces of heavy metal ions were removed with a freshly prepared mixture H<sub>2</sub>O<sub>2</sub> (30%) and HCl (37%) in water (1:1:3, vol:vol:vol) for 10 min. The surface was modified by thiols COOH-(CH<sub>2</sub>)<sub>11</sub>-SH (3.3  $\mu$ M) and CH<sub>3</sub>-(CH<sub>2</sub>)<sub>8</sub>-SH (1  $\mu$ M), (Sigma, USA) in ratio 1:300 (12 h, 37 °C) [32]. Chips were washed

by ethanol. Cadmium acetate (10 mM solution, room temperature) was used for activation of carboxyl groups at least for 2 h prior experiment. Cadmium acetate was used as an immobilization bridge, which has selectivity to thiogroup of glutathione (as ligand) and interacts with carboxyl groups on charged thiols. Glutathione immobilization: 1 mM solution of reduced glutathione (Sigma, USA) in TBE buffer (pH 8.0) was applied on chips' surface for 1 h at room temperature. Proteins were immobilized in PBS buffer.

**Biochemical methods and antibodies.** Cell lysis, SDS-PAGE, Western blotting, stimulation of cells by anti-CD40 (G28-5, kind gift of Prof. Edward Clark, University of Washington, Seattle, WA, USA), anti-IgM (AffiniPure F(ab')<sub>2</sub> fragment goat anti-human IgM Fc<sub>5b1</sub> fragment specific, Jackson ImmunoResearch laboratories, West Grove, PA, USA), anti-CD150 mAb (IPO-3, produced in IEPOR NASU, Kiev, Ukraine), CD150 immunoprecipitations were performed as described earlier [10, 27]. Western blot results were visualized using a 20x LumiGLO<sup>®</sup> Reagent and 20x Peroxide (Cell Signaling Technology, Beverly, MA, USA). Goat antisera against actin, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-SH2D1A serum was a kind gift of Dr. Kim Nichols (The Children's Hospital of Philadelphia, Philadelphia, PA, USA). Rabbit anti-pAkt (Ser473) and (Thr308), anti-Akt, anti-pFoxO1(Thr24)/FoxO3a(Thr32), anti-FoxO1, anti-FoxO3a, anti-pGSK-3 $\beta$  (Ser9), anti-PI3K p85 $\alpha$  antibody, anti-PARP, anti-Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA, USA).

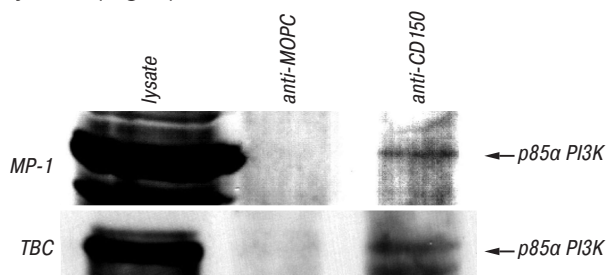
**Immunostaining.** The double staining for CD150 receptor and SH2D1A adaptor protein was done as follows: cells were spun on glass slides (10 x 10<sup>4</sup> per slide); fixed in cold methanol-acetone (1:1) at -20 °C for 1 h; rehydrated with PBS for 1 h; stained with anti-CD150 (IPO-3) mAb (5  $\mu$ g/ml) for 1 h, followed by 3 washes in PBS; incubated with horse anti-mouse Texas Red-conjugated antibodies (VectorLab, CA, USA), and washed 3 times. Cells were stained with anti-SH2D1A antibodies (dilution 1:200) for 1 h, three washes in PBS, incubated with mouse anti-rabbit FITC-conjugated antibodies (DAKO, Denmark), and mounted with 80% glycerol solution in PBS that contained 2.5% 1,4-diazabicyclo-(2.2.2)octane (Sigma). Bisbenzimidazole (Hoechst 33258) was added at a concentration of 0.4  $\mu$ g/ml to the last secondary antibody for DNA staining. The images were recorded on a DAS microscope Leitz DM RB (Leica Inc., Deerfield, IL, USA) with a Hamamatsu dual mode cooled CCD camera C4880 (Hamamatsu City, Japan).

**Isolation of cytoplasmic fraction and nuclear extracts.** Total cell lysates (TCL) were prepared from 5 x 10<sup>6</sup> of cells in RIPA lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA (pH 8.0), 1% Triton X-100, protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), and sonicated on ice for 15 s (Sonicator MSE Soniprep 150 Plus, MSE (UK) Limited, UK), spun for 15 min, 17000 g, +4 °C, supernatants transferred to fresh tubes. For subcellular

fractionation, control and stimulated cells ( $20 \times 10^6$ ) were lysed in buffer A (50 mM NaCl, 10 mM HEPES (pH 8.0), 500 mM sucrose, 1 mM EDTA (pH 8.0), 0.2% Tryton X-100, protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), vortexed and spun for 2 min (875 g, +4 °C). Cytoplasmic fractions (Cyt) of supernatant were collected to fresh tubes. Pellets (nuclei) were washed twice by 1.5 ml of cold buffer B (50 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA (pH 8.0)) and spun for 2 min (875 g, +4 °C). Pellets were resuspended in 200  $\mu$ l of buffer C (350 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA (pH 8.0), protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), and sonicated on ice for 15 s, spun for 15 min, 17000 g, +4 °C, and supernatants were transferred to fresh tubes. TCL, NE and Cyt fractions were stored at -80 °C. Protein concentration was measured in all samples (TCL, NE, Cyt), and total amount of 20  $\mu$ g of protein was loaded to each well for Western blot analysis.

## RESULTS

**p85 $\alpha$  subunit of PI3K co-precipitates with CD150 cytoplasmic tail.** Previously, we have shown that CD150-mediated signals lead to Akt S473 phosphorylation in human B-lymphoblastoid cell line MP-1 [27] and chicken B cell line DT40 [10]. Moreover, using DT40 model system it was shown that CD150-mediated Akt pathway was dependent on SH2D1A binding to CD150, was negatively regulated by Lyn and Btk, and positively - by Syk [10]. Also, another member of CD2 family, CD244 (2B4), upon tyrosine phosphorylation was shown to recruit not only SH2D1A, but also associate with the p85 $\alpha$  regulatory subunit of PI3K [33]. To answer the question whether CD150 could form a complex with PI3K in B cells, we performed immunoprecipitations of CD150 by specific mAb (IPO-3) from tonsillar B cell (TBC) and MP-1 lysates. Western blot analysis of these immunoprecipitates demonstrated that p85 $\alpha$  regulatory subunit of PI3K co-precipitated with CD150 from both examined lysates (Fig. 1).



**Fig. 1.** PI3K p85 $\alpha$  regulatory subunit precipitated with CD150 receptor both from MP-1 and tonsillar B cells (TBC). Western blot analysis with anti-p85 $\alpha$  of CD150 immunoprecipitates. Ig isotype matched MOPC21 antibody served as negative control

**CD150ct directly interacts with p85 $\alpha$  subunit N terminal SH2 domain of PI3K.** To test whether p85 $\alpha$  regulatory subunit of PI3K directly binds CD150 cytoplasmic tail we applied surface plasmon resonance (SPR) approach. For SPR-based analysis of protein-protein interactions we used tyrosine

phosphorylated and non-phosphorylated GST-fused cytoplasmic tail of CD150 (GST-CD150ct) with point mutations (Y/F substitution) in ITSM motifs, described earlier [11]. Also, fusion proteins of GST-SH2D1A, GST-fused N-terminal SH2 domain of p85 $\alpha$  regulatory subunit of PI3K (GST-NSH2-p85 $\alpha$ ) were used in this study. GST-NSH2-p85 $\alpha$  was chosen since it was demonstrated for p85-associated receptors EGF and PDGF that the affinity of the N-SH2 domain of p85 $\alpha$  for the receptors correlated with the steady-state level of p85-receptor complex [34]. The quantitative ratio of protein partners was calculated based on molecular weight of interacting proteins.

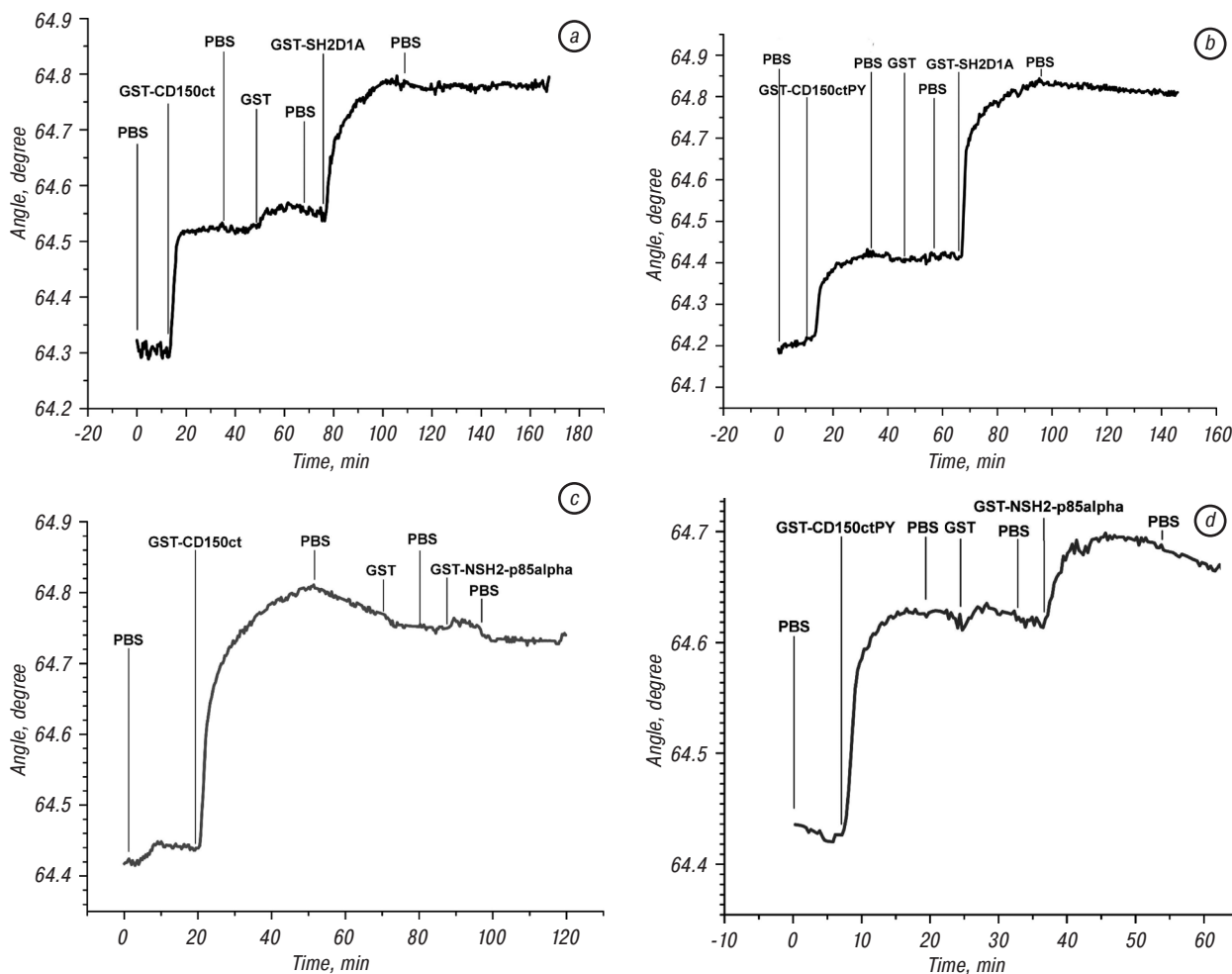
Interaction between GST-fusion proteins of CD150ct (GST-CD150ct) and SH2D1A (GST-SH2D1A) was shown to be 1:1 for non-phosphorylated GST-CD150ct (Fig. 2, a), and 1:2 for tyrosine phosphorylated GST-CD150ctPY and GST-SH2D1A (Fig. 2, b). This ratio corresponds to the data obtained by biochemical studies, showing that immunotyrosine based switch motif (ITSM) with non-phosphorylated tyrosine Y281 could bind SH2D1A, and that both ITSMs with Y281 and Y327 could bind adaptor protein SH2D1A when phosphorylated [11, 35]. Thus, our results obtained by SPR approach with glutathione immobilized on golden chips, completely match with previously obtained results for CD150-SH2D1A interaction, and, therefore, this approach could be used for studies of GST-CD150ct direct interaction with other SH2-domain containing proteins.

Using this approach we examined GST-NSH2-p85 $\alpha$  interaction with non-phosphorylated (Fig. 2, c) and tyrosine phosphorylated GST-CD150ct (Fig. 2, d). It was found that NSH2 domain of p85 $\alpha$  subunit interacts only with tyrosine phosphorylated receptor (see Fig. 2, d). To determine, which tyrosines (Y281, Y327) in CD150ct are essential for CD150 - NSH2 p85 $\alpha$  interaction we used fusion proteins of CD150ct with point mutations Y/F. It was shown that mutation Y327F did not alter CD150ct-p85 $\alpha$  SH2 interaction. However, double mutant Y281F/Y327 did not bind p85 $\alpha$  SH2 domain (data not shown). Thus, the first ITSM motif containing Y281 should be available and phosphorylated for NSH2-p85 $\alpha$  interaction with CD150ct.

Thus, we demonstrated by SPR that p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150ct, and that this interaction takes place in normal TBC and lymphoblastoid cell line MP-1. The direct interaction of CD150 with p85 $\alpha$  subunit of PI3K could be a starting point for regulation of PI3K activation and subsequent Akt activation via CD150.

**CD150-mediated Akt activation in lymphoblastoid cell lines.** MP-1 and CESS lymphoblastoid cell lines both express CD150 and SH2D1A proteins. Here we demonstrate that CD150 ligation led to phosphorylation of both Akt activation sites (T308 and S473) in MP-1 cells (Fig. 3). In CESS cells CD150 ligation induce only slight Akt phosphorylation on T308 and S473, and CD150 signals had no effect on Akt phosphorylation levels in T5-1 cells and freshly obtained





**Fig. 2.** Representative SPR responses of protein-protein interactions (a-d). Non-tyrosine phosphorylated GST-CD150ct interacted with GST-SH2D1A protein in ratio 1:1 (a). Tyrosine phosphorylated GST-CD150ct (GST-CD150ctPY) interacted with GST-SH2D1A protein in ratio 1:2 (b). Non-tyrosine phosphorylated GST-CD150ct did not bind to GST-NSH2-p85alpha (c). GST-CD150ctPY interacted with GST-NSH2-p85alpha in ratio 1:1 (d)

from peripheral blood B cells LCLs (see Fig. 3). These cell lines differed by adaptor protein SH2D1A expression levels: MP-1 cells expressed SH2D1A protein at high level, CESS cells and LCLs – at much lower level, and T5-1 cells did not express SH2D1A at all ([11] and data not shown).

**CD150 and SH2D1A localization in lymphoblastoid cell lines.** Studies performed on regulation of CD150-mediated Akt activation in DT40 model system [10] and Hodgkin's lymphoma cell lines [10, 28] have shown that CD150-mediated Akt activation could be regulated by SH2D1A expression and its cellular localization. Using immunofluorescent staining here we found that in MP-1 and CESS cells SH2D1A was localized in cytoplasm close to the cellular membrane (Fig. 4). Practically all MP-1 cells in contrary to 10% of CESS cells were SH2D1A positive (see Fig. 4). As CD150-SH2D1A interaction was shown to be crucial for Fyn recruitment and CD150 tyrosine phosphorylation at least in T cells [36, 37] we examined the phosphorylation of receptor in both cell lines. It was shown that CD150 had lower level of tyrosine phosphorylation in CESS cells than in MP-1 cells (data not shown). Thus, the level of SH2D1A expression directly correlated with tyrosine phosphorylation. Since p85 $\alpha$  PI3K

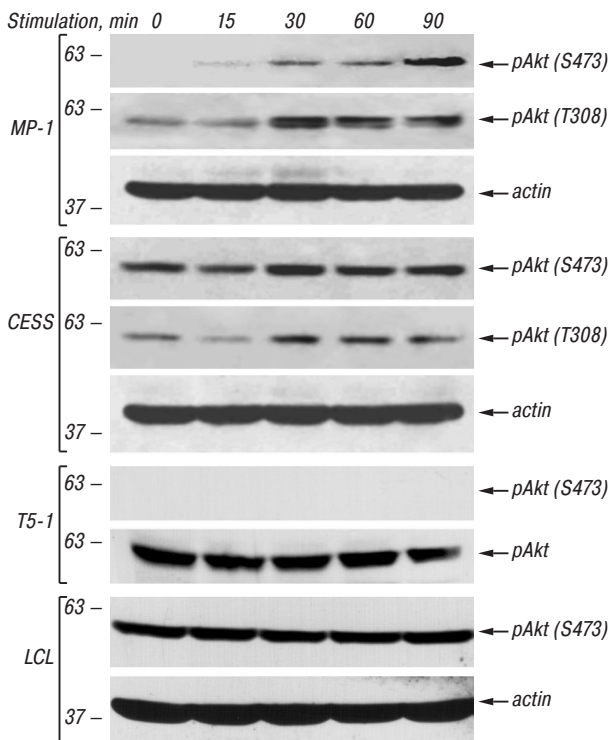
binds only to tyrosine phosphorylated CD150, obtained data could explain the lack of CD150-mediated Akt phosphorylation in cells, which do not express SH2D1A or have low expression level of this adaptor protein.

**Akt activation in primary B cells.** CD150-mediated Akt phosphorylation was studied in dense (naive and memory B cells) and buoyant fractions (activated cells) of TBC. It was shown that Akt kinase could be phosphorylated via CD150 both in naive and activated primary TBC (Fig. 5a, b). As a control, cells were stimulated via B cell receptor. It was found that stimulation of dense TBC via CD150 and IgM had additive effect on Akt phosphorylation (Fig. 5a, c, graph).

**Akt distribution between cytoplasmic and nuclear fractions in LCL and HL cell lines.** Subcellular localization of Akt kinase is very important for its function. In resting unstimulated cells Akt molecules resides both in the cytoplasm and nucleus [38]. Activation of Akt occurs in proximity of plasma membrane and has been shown to be followed by pAkt translocation to cytosol and nucleus [38]. Such translocation allows pAkt to phosphorylate its downstream targets, among which there are cytoplasmic as well as nuclear proteins.

In current study we addressed the question how Akt kinase was distributed between nucleus and cy-

toplasm in EBV-transformed cells MP-1 and HL cells (L1236) before and after stimulation via surface receptors CD40 and CD150. Before ligation of receptors, Akt kinase was mainly localized in cytoplasmic fraction in L1236 HL cells, and partially in the nuclear extract (Fig. 6, a). Upon stimulation of L1236 cells via CD150 and CD40 receptors, Akt was completely exported from the nucleus. In MP-1 cells, Akt kinase was localized exceptionally in cytoplasm (Fig. 6, b). Signals



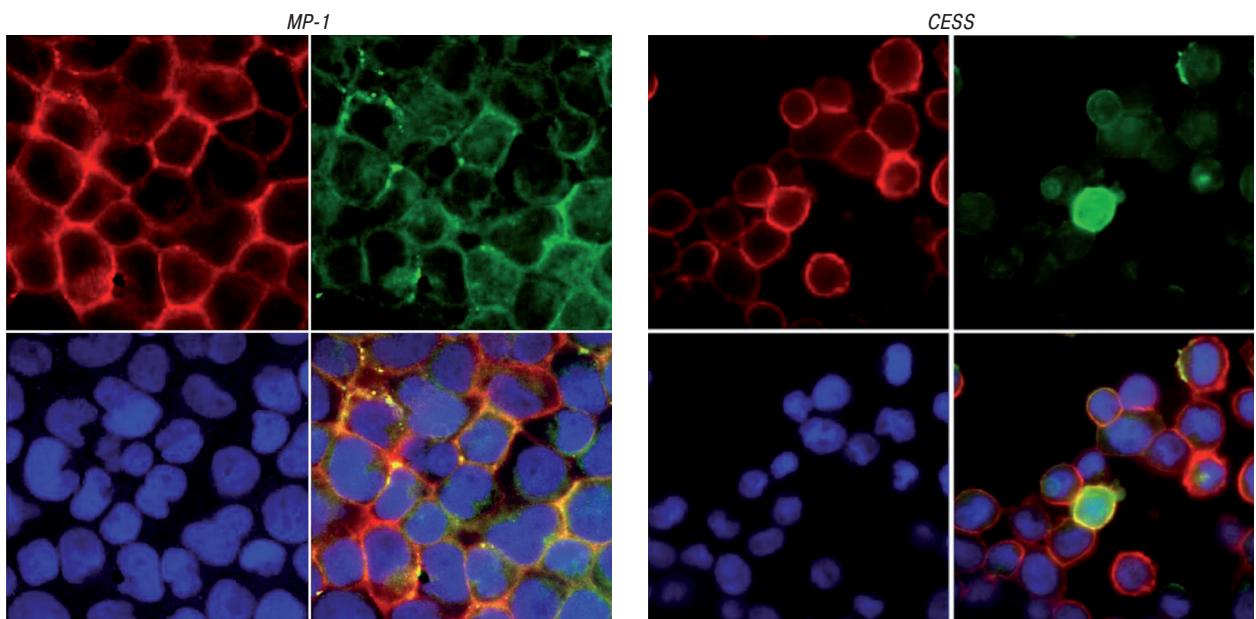
**Fig. 3.** CD150-mediated Akt phosphorylation on serine 473 (S473) and threonine 308 (T308) in lymphoblastoid cell lines (a). Akt kinase was markedly phosphorylated on both sites S473 and T308 in MP-1 cells, only slightly phosphorylated in CESS cells (a, upper panels). The level of Akt phosphorylation was not altered in T5-1 and LCL (b, lower panels). Equal loading was monitored by anti-actin or anti-Akt Western blot

via CD40 or CD150 receptor had no influence on Akt distribution between nucleus and cytoplasm (Fig. 6, b).

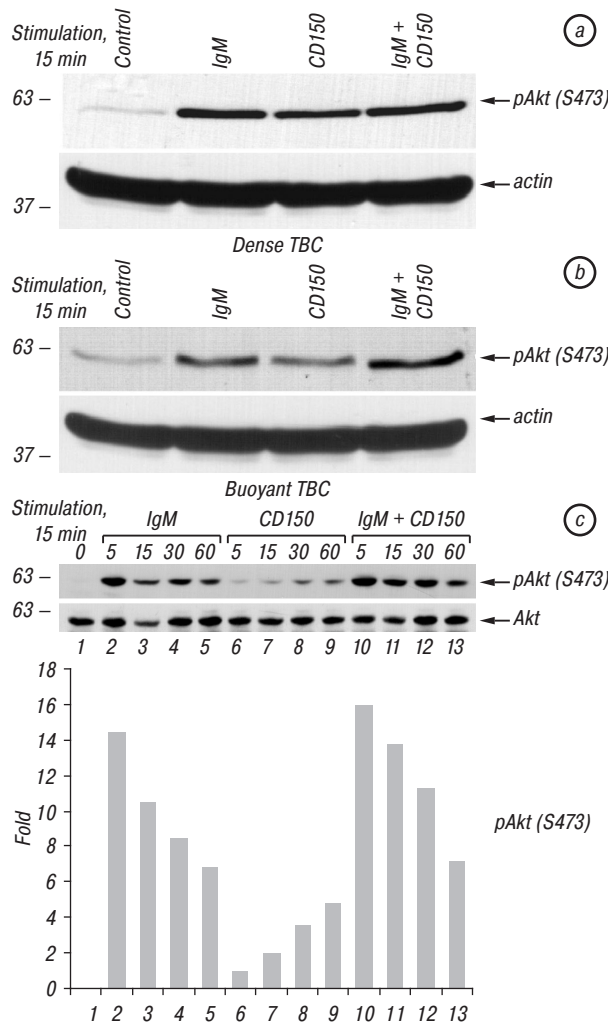
**CD150-mediated phosphorylation of Akt downstream targets in normal and malignant B cells.** Akt kinase regulates the activity of multiple target proteins in cells, which results in promotion of cell survival and inhibition of apoptosis. Among them are stress-activated kinases, BAD, Forkhead family of transcription factors, I $\kappa$ B kinase, GSK-3, mTOR, c-Raf, B-Raf, Nur77, etc (reviewed in [24]).

GSK-3 serine/threonine protein kinase is one of the crucial Akt target proteins. Two homologous mammalian GSK-3 isoforms, encoded by different genes, are known – GSK-3 $\alpha$  and GSK-3 $\beta$ . Akt could phosphorylate GSK-3 $\alpha$  on S21 and GSK-3 $\beta$  on S9, and inactivate these proteins [39]. We examined if CD150-mediated Akt activation could lead to phosphorylation of GSK-3 $\beta$  in primary TBC, LCL MP-1 and HL cell line L1236. It was found that in primary TBC, both in dense and buoyant fractions, the basal level of GSK-3 $\beta$  phosphorylation on S9 was very high, and it was not altered by stimulation via CD150 receptor (Fig. 7, a). At the same time, CD150-mediated Akt activation both in MP-1 and L1236 cells resulted in upregulation of GSK-3 $\beta$  S9 phosphorylation with kinetics similar to Akt S473 phosphorylation (Fig. 7, b, c).

FoxOs transcription factors constitute important downstream targets of the PI3K/Akt signaling pathway. FoxOs are regarded as tumor suppressor genes (reviewed in [40]). FoxOs regulate transcription of proapoptotic genes, while Akt kinase protects cells from apoptosis by phosphorylating FoxOs, which target them to 14-3-3-mediated nuclear export and/or proteasome-mediated degradation [41–43]. The sites for Akt phosphorylation in FoxO1 are T24, S256 and S319, and for FoxO3a are T32, S253 and S315 (reviewed in [40]). Here we explored CD150-mediated phosphorylation of FoxO1/FoxO3a on T24/T32 in comparison



**Fig. 4.** Double immunostaining of CD150 and SH2D1A demonstrated that these two proteins are co-expressed and co-localized close to the cellular membrane in all MP-1 cells. Only 10% of CESS cells were SH2D1A positive, and SH2D1A was also co-localized with CD150, x400

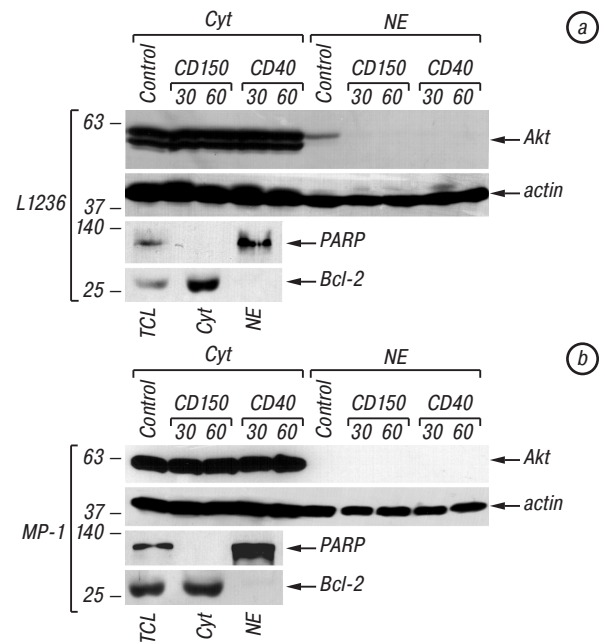


**Fig. 5.** CD150 receptor ligation induced Akt kinase phosphorylation (S473) in TBC. Crosslinking of IgM and CD150 on dense (a,c) and buoyant (b) TBC resulted in Akt phosphorylation on S473. Co-ligation of IgM with CD150 on these cells had additive effect (b and c, graph). Equal loading was monitored by anti-actin or anti-Akt Western blot. The level of pAkt was normalized against the level of Akt using TotalLab program (c, graph).

to stimulation via CD40 and IgM receptors. We found that only FoxO1, but not FoxO3a is expressed in TBC, MP-1 and HL cells L1236 on protein level (data not shown). It was shown that FoxO1 phosphorylation level on T24 was upregulated via CD150 and IgM, but not CD40 in dense fraction (naïve and memory cells) of TBC (Fig. 8, a, upper panel). In buoyant TBC the level of pFoxO1 was not altered by either CD150 or CD40 ligation, but was only enhanced by ligation of IgM (see Fig. 8, a, lower panel).

The basal phosphorylation level of FoxO1 on T24 was quite high in LCLMP-1. Nevertheless signals via CD150 and CD40 upregulated the level of pFoxO1 in 30 and 60 min upon stimulation (Fig. 8, b, graph). The effect of CD150 ligation was more pronounced than of CD40 (see Fig. 8, b, graph).

Similarly, in HL cells L1236 both signals via CD150 or CD40 receptors significantly enhanced the phosphorylation of FoxO1 T24 in 15 and 60 min upon receptors' ligation, however, effect of CD40 crosslinking was more prominent (Fig. 8, c).



**Fig. 6.** Western blot analysis of Akt kinase localization in cytoplasmic fraction (Cyt) and nuclear extracts (NE) of L1236 (a) and MP-1 (b) cells. Akt kinase was localized both in Cyt and NE of L1236 cells, and was completely exported from the nucleus upon L1236 stimulation via CD150 and CD40 receptors (a, upper panel). In MP-1 cells, Akt was localized only in cytoplasmic fraction (Cyt), (b, upper panel). Poly (ADP-ribose) polymerase (PARP) and Bcl-2 expression levels were checked to control the purity of fractions (TCL – total cell lysate). Anti-actin Western blot was performed to control the protein loading to gels

**DISCUSSION**

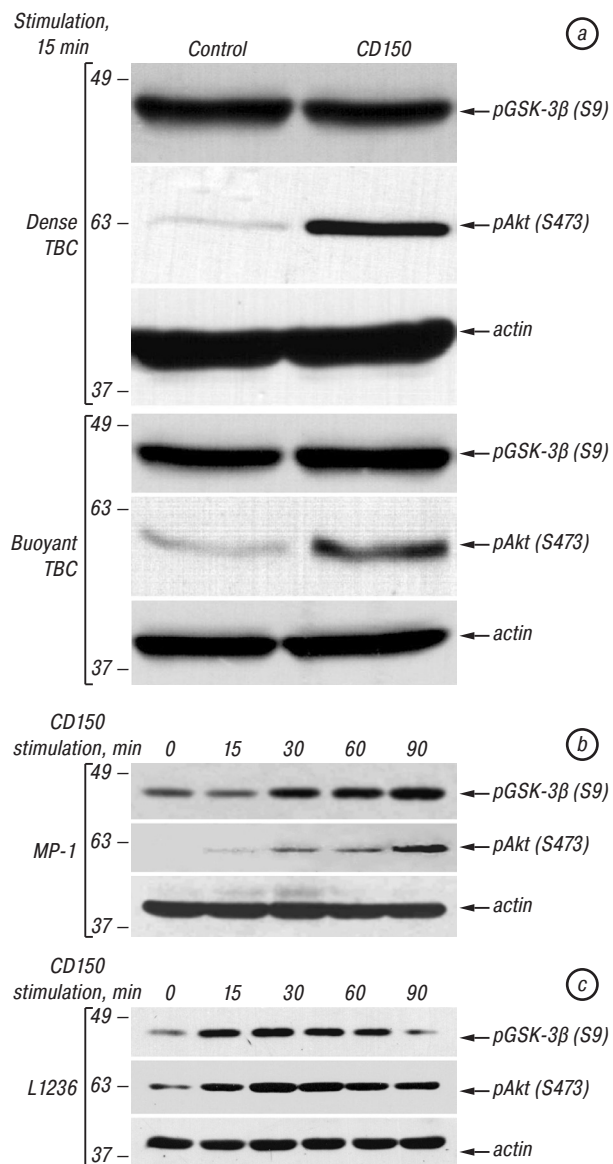
The serine/threonine kinase Akt/PKB is a crucial regulator of divergent cellular processes, including proliferation, differentiation and apoptosis [24]. Akt signaling is often deregulated in cancer, leading to constitutively active Akt kinase [24].

The phosphorylation of Akt (pAkt S473) was found to be upregulated in HL derived cell lines and in HRS cells in 64% [19] and 100% [44] of primary lymph node sections of HL. In all tested cases, Akt was detected both in HRS cells and the surrounding reactive cells, while active phosphorylated form of Akt was expressed only by the HRS cells [19]. This high level of basal Akt phosphorylation thought to be maintained by signals via tumor necrosis factor (TNF) family receptors CD40, RANK, and CD30 [19]. We have shown previously that CD150 receptor mediated signaling could also contribute to Akt activation in HL cells [10, 28]. Here we demonstrated that this signaling pathway could be activated via CD150 in primary normal B cells, as well as in LCL and HL cell lines.

Previously, it was shown that CD150ct interaction with adaptor protein SH2D1A was necessary for CD150 interaction with protein kinases and ITSMs' phosphorylation [36, 45]. Our data suggested that CD150-SH2D1A association and SH2D1A localization play decisive role in activation of Akt signaling pathway upon CD150 ligation (Fig. 3, 4).

Based on our data it could be suggested that SH2D1A adaptor protein association with CD150ct allows tyrosine phosphorylation of CD150ct by cellular

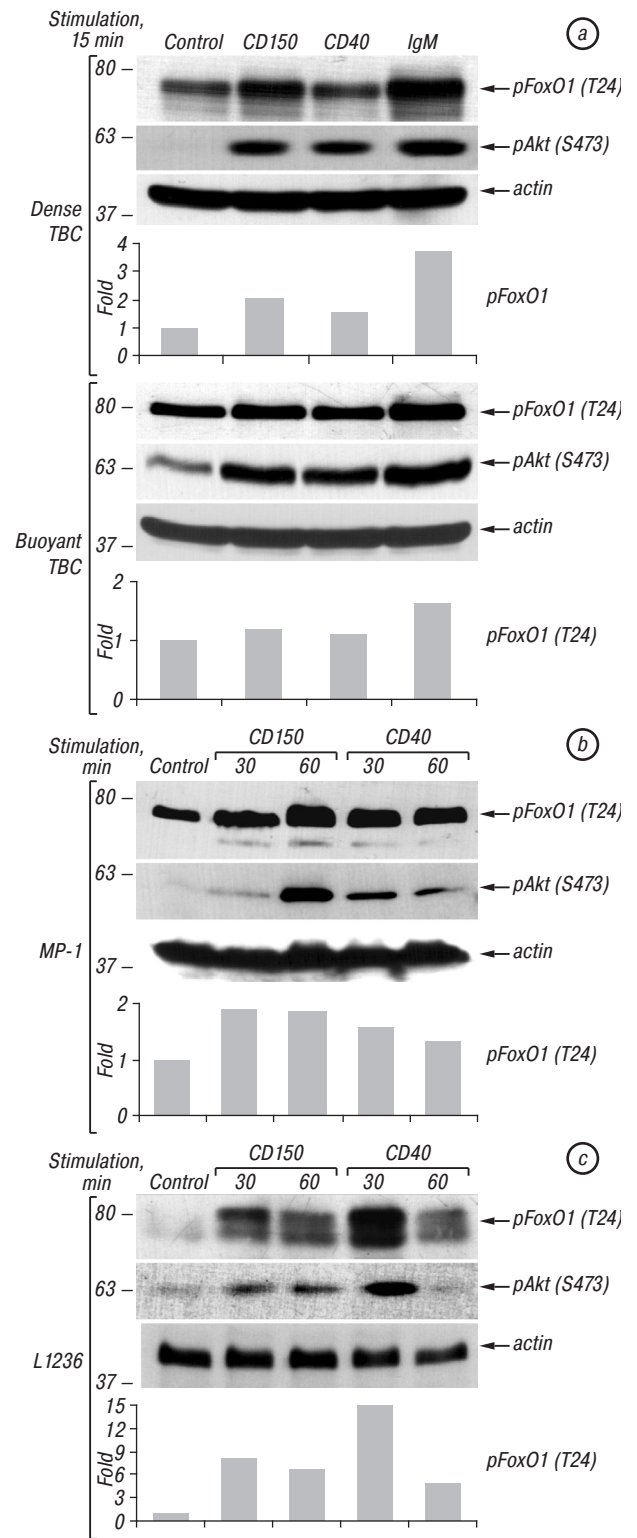




**Fig. 7.** CD150-mediated phosphorylation of Akt target protein GSK-3β kinase in TBC, MP-1 and L1236 cell lines. Both dense and buoyant TBC had high basal level of GSK-3β phosphorylation, which was not altered by CD150-initiated signaling (a). The level of pGSK-3β was upregulated in MP-1 (b) and L1236 cells (c) upon stimulation via CD150. Anti-pAkt Western blot was performed to monitor Akt phosphorylation upon ligation of CD150. Equal loading was monitored by anti-actin Western blot

tyrosine kinases (Lyn in B cells [10]), which permit direct interaction of CD150ct and p85α regulatory subunit of PI3K. Immunoprecipitation of CD150 from MP-1 cells and TBC demonstrated that CD150 co-precipitated with p85α (Fig. 1). By SPR approach, we demonstrated that this interaction was direct, dependent on CD150ct phosphorylation (Fig. 2, c, d), and ITSM motif containing Y281 was necessary for NSH2-p85α interaction with CD150ct. Thus, CD150-initiated Akt activation in B cells was mediated by direct interaction of CD150ct with p85α regulatory subunit of PI3K.

For SPR analysis we applied modification of method first proposed by Boltovets *et al.* [32], when glutathione is fixed on the golden surface of sensor chips. This low-cost and effective modification of method for GST-protein immobilization on sensor chips could



**Fig. 8.** CD150-mediated phosphorylation of Akt target protein FoxO1 transcription factor on T24 in TBC, MP-1 and L1236 cell lines. The basal level of pFoxO1 was high both in dense and buoyant TBC fractions (a, control). Ligation of receptors mediated the upregulation of pFoxO1 level in dense cells, and had almost no influence on pFoxO1 level in buoyant cells (a). LCL MP-1 was characterized by high basal level of FoxO1 phosphorylation, which was upregulated by CD150- and CD40-mediated signaling (b). CD150 and CD40 induced strong upregulation of pFoxO1 level in HL cell line L1236 (c). The level of pFoxO1 was normalized against actin or FoxO1 levels using TotalLab program (graphs). Anti-pAkt Western blot was performed to monitor Akt phosphorylation upon ligation of CD150 receptor. Equal loading was monitored by anti-actin or anti-FoxO1 Western blot

be efficiently applied to study other protein-protein interactions using GST fusion proteins as interacting partners without using specific antibodies.

Several downstream effectors of Akt signaling, including GSK-3 $\alpha/\beta$ , and mTOR substrates 4E-BP1 and p70 S6 kinase, were shown to be phosphorylated in primary HL cells [44]. GSK-3 is one of the crucial Akt target protein. GSK-3 phosphorylates a broad range of substrates and is implicated in multiple biological processes apart from its well studied function as regulator of glycogen synthesis. GSK-3 $\beta$  has been shown to regulate cyclin D1 proteolysis and subcellular localization during the cell division cycle, thereby triggering rapid cyclin D1 turnover [46]. Also GSK-3 controls transcription factor c-myc proteolysis and subnuclear localization as phosphorylation of c-Myc T58 facilitates its rapid proteolysis by the ubiquitin pathway [47].

We have demonstrated that GSK-3 $\beta$  was phosphorylated upon CD150 ligation in MP-1 and HL cell line L1236, but not in normal TBC (Fig. 7). It seems like regulation of GSK-3 $\beta$  phosphorylation level/inactivation via CD150 receptor is more critical for immortalized tumor cells (HL) and EBV-transformed cells (LCL), which use all cellular signaling machinery to avoid apoptosis and support their proliferation. In normal TBC GSK-3 $\beta$  is initially switched off (highly phosphorylated on inhibitory site), which is probably needed for further differentiation of these cells.

In mammalian cells, the class O of fork head (fkh) transcription factors is homologous to *Caenorhabditis elegans* transcription factor DAF-16 (abnormal DAuer Formation-16), and consists of four members: FoxO1, FoxO3, FoxO4 and FoxO6 [40]. Several kinases were shown to phosphorylate FoxOs in different sites: Akt, serum and glucocorticoid-inducible kinase (SGK), mammalian sterile 20-like kinase-1 (MST1), cyclin-dependent kinase-2 (CDK2), p90-kDa ribosomal S6 kinase-2 (Rsk-2), dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1a) (reviewed in [40, 48, 49]). As to the other post-translational modifications, FoxOs activity/stability could be regulated by acetylation and ubiquitination [50].

In our study we showed that despite the high basal level of pFoxO1, CD150 mediated FoxO phosphorylation in normal TBC and LCL MP-1 (Fig. 8). Moreover, CD150 as well as CD40 was involved in inactivation of pro-apoptotic FoxO1 transcription factor in HL cell line L1236, which could contribute to the survival of HRS cells. It should be emphasized that all studied B cells expressed FoxO1, but not FoxO3a on protein level. It is an important finding since it was thought that the dominant isoform expressed in lymphocytes, at least on mRNA level, is FoxO3a [49].

Recently, we have shown that CD150 mediates JNK activation in normal and HL cells [18]. JNK kinases could phosphorylate FoxO in transactivating domain [51]. Also, JNK has been shown to phosphorylate 14-3-3 proteins, which result in reduced 14-3-3 binding to partner proteins, including FoxOs [52]. Regulation of multiple posttranslational modifications of FoxOs via

CD150-mediated JNK and other signaling pathways should be further explored to get more clear understanding of the FoxOs activity regulation in normal and malignant B cells.

Subcellular localization of Akt kinase is important for downstream cytoplasmic and nuclear protein targets phosphorylation. Using model cell lines it was shown that activation of Akt results in its nuclear translocation within 20 to 30 min after stimulation [53]. Following BCR stimulation of mouse B cell line pAkt resides both in the cytosol and nucleus [38]. In HL cell lines KM-H2 and L428 pAkt was localized close to the membrane or in cytosol [44]. Here we have shown that in unstimulated L1236 cells Akt kinase was present both in cytoplasmic fraction and nuclear extract. Upon stimulation of cells via CD150 and CD40, Akt was completely exported from the nucleus (Fig. 6). It is consistent with the fact that in most studied cases of primary HL in HRS cells pAkt was shown to be preferentially cytoplasmic [44]. This could be achieved by sustained signals via TNF receptors (including CD40) and CD150. However, the biological effect of this nuclear-cytoplasmic translocation in HL cells should be studied further. We have shown previously that sustained signaling via CD150 and CD40 receptors induced inhibition of cellular proliferation and cell death of L1236 cells [18]. We may hypothesize that retaining of Akt in cytoplasm would prevent the phosphorylation of its nuclear targets in these cells, which could be the reason of inability of Akt survival pathway to inhibit CD150-induced cell death.

Although the PI3K/Akt pathway inhibits apoptosis and promotes cell cycle progression and proliferation [24], the consequences of its activation in HRS cells remain currently unclear. Though Dutton *et al.* [44] showed that HL cell lines and primary HL tumor cases were characterized by high levels of phosphorylated/activated Akt, inhibition of PI3K and mTOR had quite modest effect on cell survival. At the same time, other authors [19] demonstrated that PI3K inhibitor LY294002 had anti-proliferative and pro-apoptotic effects on some studied HL cell lines. Moreover, CD150-stimulation of L1236 HL cell line induced cell death, though this cell line was characterized by simultaneous fast and marked Akt activation via CD150 receptor [18]. Thus, outcome of PI3K/Akt signaling cascade may depend on interaction with other signaling pathways that are initiated by different stimuli from HL microenvironment.

Taken together, in current study we have shown that CD150 ligation induced Akt phosphorylation/activation in normal TBC, LCLs and HL cell lines. It could be assumed that CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction followed by phosphorylation of CD150ct and attraction of p85 $\alpha$  regulatory subunit of PI3K. Akt kinase, activated via CD150, could phosphorylate its downstream target FoxO1 transcription factor in normal B cells, as well as in immortalized HL and B-lymphoblastoid cell lines. At the same time Akt phosphorylation of its substrate



GSK-3 $\beta$  kinase was detected only in HL cell line L1236 and LCL MP-1, but not in normal TBC. CD150-mediated Akt signaling, followed by regulation of GSK-3 $\beta$  and FoxO1 activity in EBV-transformed and HL tumor cells could interplay with other CD150-initiated signaling pathways (i.e. MAPK) creating signaling network favorable for maintaining the survival program and escaping apoptosis of transformed B cells.

### ACKNOWLEDGMENTS

We thank Dr. E.A. Clark for valuable discussion and anti-CD40 antibodies, and Dr. K. Nichols for providing anti-SH2D1A antibodies and GST-SH2D1A construct. This work was supported by Ukrainian governmental grants 0110U006647 and 0107U002244, and by Swedish Institute in the frame of Visby program.

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