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Immunostimulatory and Oncolytic Properties of Rotavirus Can Overcome Resistance to Immune Checkpoint Blockade Therapy

Tala Shekarian

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Immunostimulatory and Oncolytic Properties of Rotavirus Can Overcome Resistance to Immune Checkpoint Blockade Therapy

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Abbreviations:

Ab	antibody
ADCC	Antibody dependent cellular cytotoxicity
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Ag	antigen
ALK	anaplastic lymphoma kinase
APC	antigen-presenting cell
ATP	adenosine triphosphate
CAR	coxsackie- and adenovirus receptor or chimeric antigen receptor
CEA	carcinoembryonic antigen
CD	cluster of differentiation
CDC	complements dependent cytotoxicity
CLR	C-type lectin receptor
COX2	Cyclooxygenase
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DAMP	Danger associated molecular pattern
DC	dendritic cell
DLP	double layer virus particle
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	endoplasmic reticulum
Ev1	Echovirus
FACS	Fluorescent-activated cell sorting
GD2	disialoganglioside 2
GFP	green fluorescent protein
GMCSF	granulocyte-macrophage colony-stimulating factor
GrB	granzyme B

HLA	human leukocyte antigen
HMEC	Human mammary epithelial cell
HSV	herpes-simplex virus
IC	immune checkpoint
ICB	immune checkpoint blockade
ICD	immunogenic cell death
ICT	immune checkpoint therapy
IDO	indoleamine-pyrrole 2, 3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KIR	killer-cell immunoglobulin-like receptors
Luc	luciferase
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-csf	macrophage colony stimulating factor
MDSC	Myeloid-derived suppressor cells
MHC	major histocompatibility complex
MOI	multiplicity of infection
NB	neuroblastoma
NDV	Newcastle disease virus
NF- κ B	Nuclear factor κ B
NK	natural killer cells
NOD	nucleotide-binding oligomerization domain
NLR	nod-like receptor
NSG	nod scid gamma
NSP	non-structural protein
OV	oncolytic virus
PAMP	Pathogen associated molecular pattern
PBS	phosphate buffered saline

PD-I	programmed cell death protein I
PDL-I	programmed death ligand I
PG	prostaglandins
PRR	pathogen recognition receptor
RIG-I	retinoic acid-inducible gene I
RLR	rig-I like receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RVs	Rotavirus
PBMC	Peripheral blood mononuclear cell
SCF	stem cell factor
SCID	severe combined immune deficiency
SLP	single layer virus particle
TAA	tumor associated antigen
TAM	tumor associated macrophage
TCR	T-cell receptor
TIL	tumor-infiltrating lymphocyte
TGF- β	Transforming growth factor β
TK	thymidine kinase
TNF	tumor necrosis factor
TLR	toll-like receptor
TLP	triple layer virus particle
Treg	T regulatory cells
T-vec	Talimogene laherparepvec
VEGF	Vascular endothelial growth factor
VP	viral protein
VV	vaccinia virus

Introduction:

1) CANCER IMMUNOLOGY:

Many genetic and environmental factors can cause tumor formation but the cells of our immune system are continuously monitoring our tissues and recognize the abnormal cancer cells and kill them. The immune cells arise from hematopoietic stem cells inside the bone marrow that give birth to two different lineages: the myeloid and lymphoid progenitor cells (see Figure 1). The different populations derived from myeloid progenitor cells are monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells (DC), and megakaryocytes (or platelets) and the population driven from lymphoid progenitor cells are T and B lymphocytes, Natural killer cells (NK) and other innate lymphoid cells. These different populations are the principal actors of innate and adaptive immune system. The innate immunity populations include the natural killer cells, granulocytic cells such as neutrophil and antigen presenting cells (APC) such as DC and macrophages. These cells provide the first line of self-defense against foreign pathogens as well as cellular damages and cancers. The innate immune response is very rapid but has no antigen-specificity and no immunological memory. In contrast to the innate immune response, adaptive immune responses are highly specific to the particular pathogen/antigen and provide a long-lasting protection through induction of memory. The two populations of adaptive immunity are T lymphocyte populations (T helper and Cytotoxic T cells) and B lymphocytes (plasma cells which are capable to secrete the antibodies).

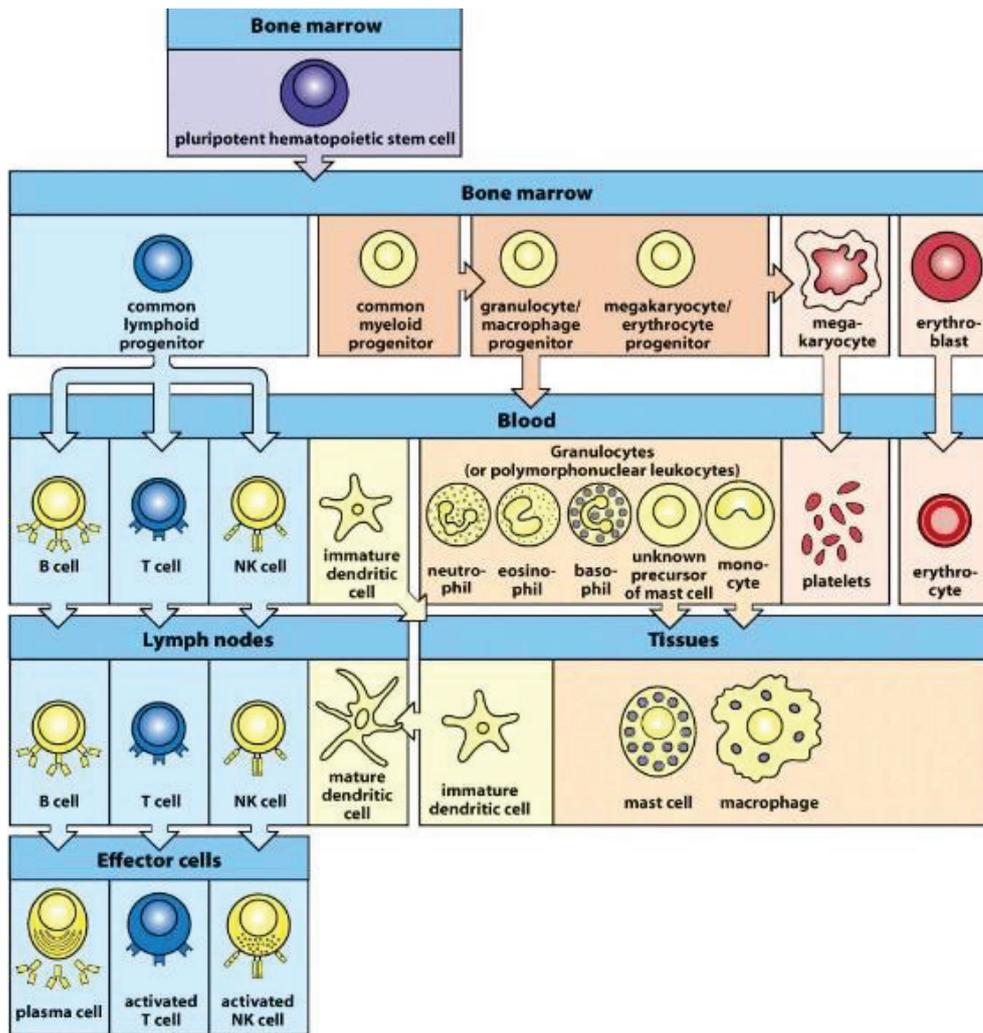


Figure 1: All of the cellular elements of blood, including the lymphocytes of the adaptive immune system, arise from hematopoietic stem cells in the bone marrow. (Janeway's immunobiology, 8ed, Garland science 2012)

The idea that the immune system plays a role in controlling cancer development has emerged in the 19th century. In 1909, Paul Ehrlich, a German physician and scientist, predicted that cancer can occur at “incredible frequency “ if host defenses did not prevent the outgrowth of continuously arising cancer cells (Ehrlich, 1909) (Dunn, Old, & Schreiber, 2004a). Fifty years later the idea of cancer immune surveillance re-emerged with the works of Sir Macfarlane Burnet and Lewis Thomas. They proposed that the immune system, and especially the adaptive immune system, plays an important role in the control of cancer development. Thomas and Burnet proposed that accumulating tumor cells express new tumor antigens eliciting an effective immune response through the lymphocytes and leading to the regression of the tumor. However no experimental or clinical hint validating this

concept was provided (Dunn, Old, & Schreiber, 2004b)(Dunn et al., 2004a). The cancer immune surveillance hypothesis was latter demonstrated through many different experiments showing that hosts with impaired or non-functional immune system are more prone to develop spontaneous or chemically induced tumors (Kaplan, 1971)(Kaplan et al., 1998)(Street, Trapani, MacGregor, & Smyth, 2002). However, the cancer immune surveillance hypothesis was challenged, based on the fact that tumor cells would not present danger signals allowing the immune system to recognize them as cancer cells (Outzen, Custer, Eaton, & Prehn, 1975)(Penn, 1988). The fact that tumors arise from self-tissues expressing antigens which induced immune tolerance implies the lack of immunogenicity and of immune control of the tumor. Latter based on different studies (Shankaran et al., 2001)(Naito et al., 1998)(Mihm, Clemente, & Cascinelli, 1996), Robert Schreiber completed the cancer immune surveillance hypothesis through the concept of immune editing (Figure 2) (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). This process contains three phases: elimination, equilibrium and escape. Elimination is the classical concept of immune surveillance, whereas the Darwinian natural selection of tumor variant developing the mutations which make them resistant to immune attacks occurs in the equilibrium phase. This process can lead to immune escape of immune resistant tumor variants and the formation of clinically apparent tumors (Dunn et al., 2004a).

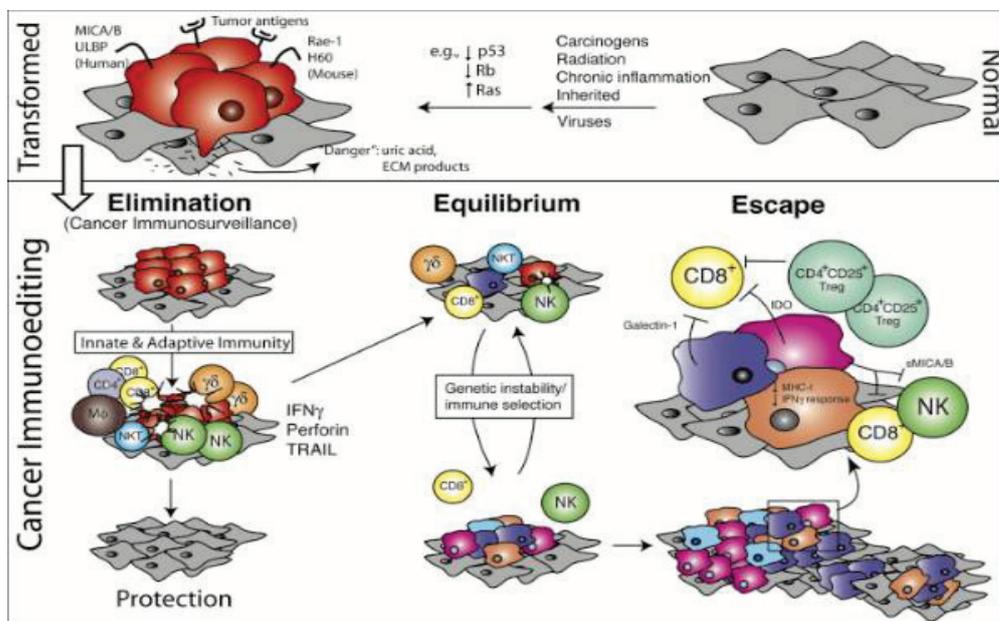


Figure 2: The three phases of the cancer Immunoeediting: Elimination, equilibrium and escape. developing tumor is detected by the immune system and its ultimate fate is determined by whether or not it is eliminated by the host protective actions of immunity (Elimination phase), maintained in a dormant or equilibrium state (Equilibrium phase) or escapes the extrinsic tumor suppressor actions of immunity by either becoming non-immunogenic or through the elaboration of immunosuppressive molecules and cells (Escape phase). (Gavin p Dunn and al. *Immunity* Volume 21, Issue 2, August 2004, Pages 137–148)

Cancer immune surveillance involves both innate and adaptive immune cells and mediators. The cells mediating anticancer responses include innate lymphocyte (NK, NKT, $\gamma\delta$ T cells), APC (DCs, macrophages) and adaptive lymphocytes (CD4, CD8 T and B cells). The mediators include molecules such as: cytokines (IFN- γ , IFN- α/β , IL-2, TNF α ...), Recognition receptors (NKG2D, CD1d, MHC1) and cytotoxic molecules such as Perforin and Trail. It is now accepted that the immune system has a primary role in the prevention of tumors. The immune system also protects us from the formation of Cancer through additional mechanisms: i) the elimination of viral infections in the case of oncogenic viruses (such as Human papilloma virus which contributes to tumor formation); ii) the control of pathogens and the resolution of inflammatory responses which can contribute to cancer transformation (Garaude & Blander, 2012)(Balkwill & Mantovani, 2001). The immune system is not always the winner of this battle because of the capacity of cancer cells to develop strategies to escape from the immune system.

2) TUMOR ESCAPE MECHANISMS FROM ANTITUMOR IMMUNE RESPONSES:

Our immune system protects us from invading microbes such as bacteria, viruses and parasites by discriminating self /non-self-antigens. Cancer cells accumulate somatic mutations leading to abnormal and altered protein expression recognized by the immune system allowing the elimination of cancer cells (Sadelain, Rivière, & Brentjens, 2003). However this is not always the case, and when a tumor is clinically detectable, it has acquired tumor escape mechanisms. The strategy of tumor immune escape can be summarized as follow (Figure 3):

2.1) Loss of tumor antigen or tumor antigen presentation machinery (during the equilibrium phase):

Tumor cells can downregulate the expression of antigens recognized by immune cells leading to antitumor immune response blockade. This has been shown for example, for viral induced lymphomas where downregulation of viral antigens expression permits tumor cells to escape T cells immune response (Vasmel, Sijts, Leupers, Matthews, & Melief, 1989). HLA class I downregulation is a frequent strategy associated with tumor invasion due to the role of MHC molecule in antigen presentation to T-lymphocytes (Townsend et al., 2006) and the regulation of NK cell function (Ljunggren & Kärre, 1990). The downregulation of HLA class I presentation machinery can be a consequence of different mechanisms such as proteasome defects, downregulation of TAP, TAP2 and chaperon proteins such as calnexin, tapasin and ERp57 and finally defects of the HLA class I molecule itself (Ritz et al., 2001)(Concha-Benavente, Srivastava, Ferrone, & Ferris, 2016). The downregulation of HLA-I by cancer cells can make them sensitive to NK cells, since NK effector cells

monitor MHC class I cell surface expression through their specific receptor (KIRs), and eliminate those cells with downregulated HLA class I molecules (Moretta et al., 1996).

2.2) Production of immunosuppressive mediators:

Tumors also mediate immunosuppression through secretion of various suppressive factors, such as transforming growth factor β -1 (TGF- β 1) and - β 2 (TGF- β 2)(Kaklamani & Pasche, 2004), IL-10 (Gerlini et al., 2004) or suppressive metabolites produced by intracellular enzymes such as indoleamine 2,3-dioxygenase (IDO) (Uyttenhove et al., 2003) and Cyclooxygenase2 (COX2) (Droller, Lindgren, Claessen, & Perlmann, 1979) (Akasaki et al., 2004).

2.3) Recruitment and induction of immunosuppressive cells:

Tumors subvert tumor immunity by promoting the expansion, recruitment and activation of different immunosuppressive populations such as regulatory T-cell (Tregs), myeloid derived suppressor cells (MDSC) and tumor associated macrophages (TAM)(Sakaguchi, 2004). Tregs migrate into the tumor site under the influence of the C-C motif chemokine 22 (CCL22) secreted by tumor cells or/and the TAMs, providing convincing evidence that Tregs create a favorable environment for tumor escape (Curiel et al., 2004). An expansion of MDSC has also been detected in practically all studied tumor models and in patients with many types of cancer (Ostrand-Rosenberg & Sinha, 2009).

Tumor-derived factors such as prostaglandins, stem cell factor (SCF), Macrophage colony-stimulating factor (M-CSF), IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF) and Vascular endothelial growth factor (VEGF) have been implicated in the accumulation and induction of MDSCs in the tumor microenvironment (Gabrilovich & Nagaraj, 2009). MDSCs can induce tumor-specific T-cell tolerance and the development of Tregs (Pan et al., 2008). Tumor associated macrophages are the major leukocyte population infiltrating solid tumors (F. R. Balkwill & Mantovani, 2012). Although macrophages have the potential to attack and eliminate tumor cells, TAMs have a very weak ability to present antigens to T cells but exhibit a very potent ability to promote tumor progression through a variety of mechanisms (Y. Liu & Cao, 2015). Chemokines such as CCL2 and M-CSF can recruit monocytes to the tumor tissue, and then IL-4, IL-10, IL-13 and other cytokines in the tumor microenvironment can induce monocytes to differentiate into TAMs (Van Dyken & Locksley, 2013).

2.4) Resistance to immune effectors:

Cancer cells impair the function of T lymphocytes through an aberrant expression and engagement of receptor-ligand interactions that adversely affect the lymphocyte survival (for example, Fas ligand (FasL)) or their effector functions through the stimulation of inhibitory receptors at the surface of infiltrating lymphocytes such as Programmed cell death 1(PD-1) (G J Freeman et al., 2000) and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) (Coyle & Gutierrez-Ramos, 2001). This part

will be explored in detail in the immune checkpoints chapter. T cells in the tumor microenvironment can be suppressed, whereas similar cells at other sites can exhibit profound effector functions. T cells can induce apoptosis of cancer cells by 2 distinct mechanisms: the death receptor pathway (FasL and TNF α) and the granzyme/perforin pathway. It has been shown that in different types of tumors, cancer cells have developed the capacity to resist to cytotoxic T lymphocyte (CTL) mediated killing. This escape mechanism is happening through the expression of the serine protease inhibitor PI-9/SPI-6, which inactivates the apoptotic effector molecule GrB (Medema et al., 2001). Human tumor cells of diverse origins, including melanoma, gastric carcinoma, astrocytoma and lymphoma, express FasL and induce T cell death through the interaction with their receptor on T cells. T-cell apoptosis has been found *in situ* to be associated with FasL-expressing tumors (Igney & Krammer, 2005).

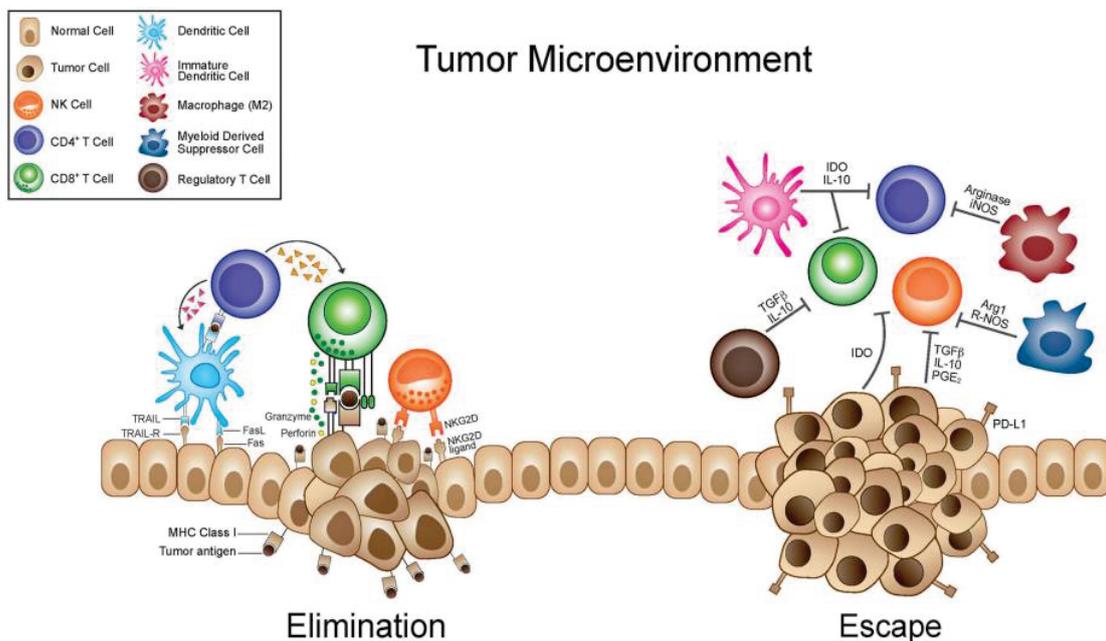


Figure 3: Tumor immunosuppressive microenvironment and antigen loss mediate tumor escape. During the elimination phase immune effector cells are able to recognize and eliminate tumor cells. The tumor cells which are less immunogenic or have up-regulated immunosuppressive factors are able to subvert the immune response and escape immune surveillance and provoke cancer appearance. (Monjazeb and al. *Front Oncol.* 2013 Jul 26; 3:197)

3) CANCER IMMUNOTHERAPY:

I would like to address this part of my thesis by quoting our review which was published in *Mutagenesis* in 2015 entitled: Paradigm shift in oncology: targeting the immune system rather than cancer cells (review *mutagenesis*, you will find the review in the Annex).

3.1) The origin of cancer immunotherapy:

The connection between cancer and the immune system was first uncovered about 100 years ago. In the early 1890s, Dr. William B. Coley, a New York surgeon, became intrigued by the disappearance of malignant tumors that he observed in cancer patients who had contracted acute streptococcal infections. Suspecting that the onset of bacterial infection was in some way responsible for the regression of the tumor, he decided to inject live streptococci into the tumor of a patient with an inoperable cancer resulting in the complete disappearance of the disease. Dr. Coley then developed a mixture of killed bacteria known as Coley's mixed bacterial toxin. He and other physicians treated over 1,000 cancer patients with this substance, with variable success. His results were unpredictable however, and neither he nor the medical community at large could explain precisely why his mixture worked in some patients. This was due to the fact that the science of immunology was in its rudimentary stages at that time. Gradually, scientists began to understand why Dr. Coley's preparation worked—the bacterial products acted as immune potentiators. In other words, they stimulated certain immune cells to kill the cancer cells directly or through cancer-killing factors. Today, cancer immunology is a rapidly advancing field and Dr. Coley has come to be regarded as the “father of cancer immunotherapy” (Mellman, Coukos, & Dranoff, 2011).

3.2) Immunotherapy strategies:

In this chapter I would like to discuss very briefly all the strategies of cancer immunotherapy, but among these strategies I will focus on three of them (PRR agonists, Oncolytic viruses and Immune checkpoint blockade) that are at the center of my PhD project.

We can divide immunotherapies into 2 classes, active and passive strategies, based on their ability to activate the host immune system against cancer cells. Active immunotherapies attempt to stimulate the immune system by presenting antigens in a way that triggers an immune response whereas passive immunotherapy directly targets the disease by the injection of immune compounds/actors that attack directly the cancer cells (Galluzzi et al., 2014)(Figure 10).

4) PASSIVE CANCER IMMUNOTHERAPY:

Passive immunotherapies can be categorized into two treatment approaches: tumor targeting monoclonal antibodies (mAbs) which rely on the administration of antibodies that may further be coupled to a toxin or the adoptive transfer of an activated immune effector cell (CTLs) component to act against cancer cells in the host (Nagasawa et al., 2012)(Figure 4).

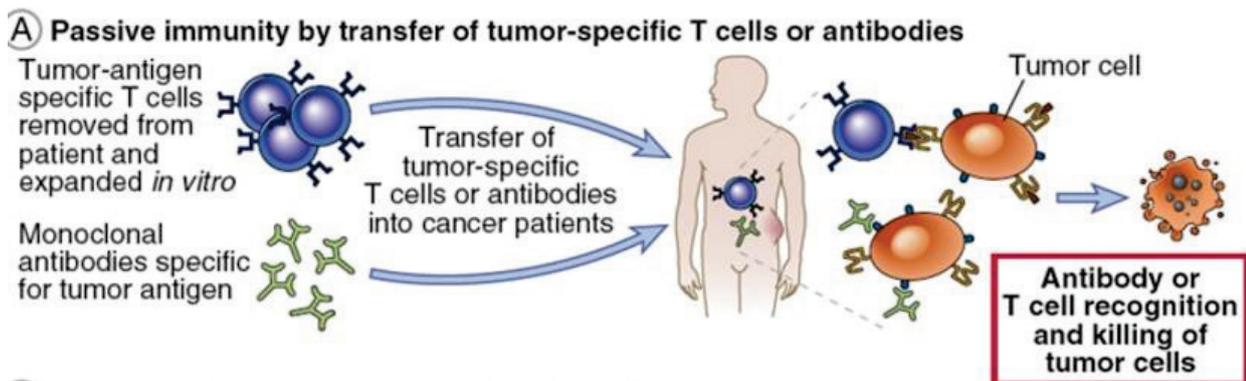


Figure 4: Passive immunotherapy strategies: tumor-targeting monoclonal antibodies and adoptive transfer of CTLs. (Image adapted from *Basic immunologic function and disorder of the immune system* book)

4.1) Tumor targeting mAbs:

The development of the hybridoma technology and the invention of monoclonal antibodies (mAbs) by Köhler and Milstein in 1975, enabled to use antibodies in diagnosis and therapy, including specific targeting of tumor cells (Margulies, 2005). In the last decade, mAbs have become one of the largest class of new agents approved for the treatment of human cancers (Reichert & Valge-Archer, 2007). These monoclonal antibodies target tumor cell-surface antigens that are overexpressed, mutated or selectively expressed compared to normal tissues (e.g cetuximab that target epithelial growth factor receptor (EGFR))(Scott et al., 2007). Also they can inhibit an over-activated pathway responsible for the proliferation of the tumor cells and, by doing so, make the disease disappear without too much toxicity for the patient. The mode of action of tumor targeted antibodies relies on several mechanisms: a direct action of the antibody through receptor blockade or agonistic activity, an induction of apoptosis, or the delivery of a cytotoxic agent or an immune-mediated cell killing mechanism through complement-dependent cytotoxicity (CDC), or antibody-dependent cellular cytotoxicity (ADCC) (Rizvi et al., 2015a). ADCC is the destruction of an antibody-coated cell by the recruitment of effector cells such as NKs, macrophages and neutrophils whereas CDC induces the target cell destruction through the activation of the complement system. ADCC occurs when antibodies bind to antigens on tumor cells and the Fc portion of the mAb engages Fcγ receptors (FcγRs) on the surface of immune effector cells. It has been shown that mice deficient in activating Fc receptors as well as antibodies engineered to disrupt Fc binding to those receptors were unable to arrest tumor growth *in vivo* (Reichert & Valge-Archer, 2007)(Clynes, Towers, Presta, & Ravetch, 2000). However, most cancers harbor multiple driver mutations and have a large mutational load. These patients would therefore require multi-targeted therapies to control the proliferation of their disease and would be highly susceptible of relapse due to an early acquired resistance (Shekarian, Valsesia-Wittmann, Caux, & Marabelle, 2015).

4.2) Adoptive T cell transfer:

Adoptive T-cell transfer is an intravenous injection of the patients' own circulating or tumor infiltrating lymphocytes that have been previously expanded and activated *ex vivo*. Shortly, peripheral blood lymphocytes are transduced by genetic engineering with a plasmid encoding a T cell receptor (TCR) recognizing a tumor specific antigen (TSA) or a chimeric antigen receptor (CAR) recognizing a tumor associated antigen (TAA). Historically, cell-based therapies have focused on cytotoxic T cells targeting MHC-restricted antigens. Although this approach remains promising, its efficacy is limited because of the tendency for tumors to downregulate MHC molecules and targeting MHC-restricted antigens is particularly challenging. For these reasons, chimeric antigen receptors modified T cells, represent a promising new therapeutic modality for cancer. CARs are engineered receptors made of the combination of an antigen binding domain of a monoclonal antibody specific for a cancer antigen (not MHC restricted) together with an intracellular domain of the CD3-zeta chain (Irving & Weiss, 1991). Chimeric antigen receptor-mediated T-cell responses have been further enhanced with the addition of a costimulatory domain such as CD28 and CD137 (4-1BB)(Carpenito et al., 2009). Over the past decade multiple tumor antigens have been targeted by CARs. They have shown clinical benefit in patients treated with CD19-directed autologous T-cells (Sadelain, Brentjens, & Rivière, 2009)(Sadelain et al., 2003). Ongoing work focuses on identifying optimal tumor targets and elucidating and manipulating both cell- and host-associated factors to support the expansion and persistence of genetically engineered cells *in vivo* (Figure 5).

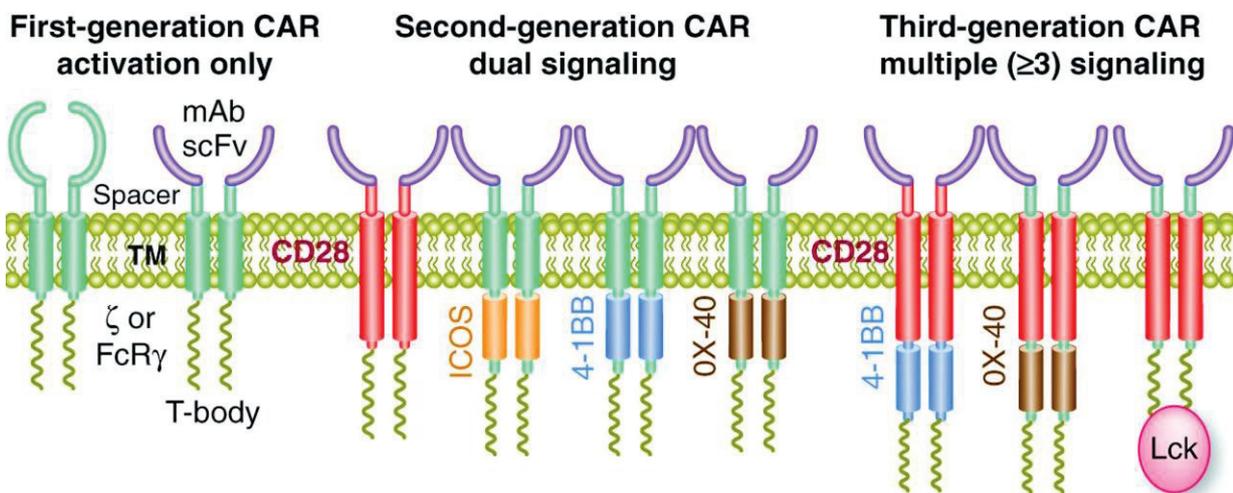


Figure 5: Structure of chimeric antigen receptors: Left, first-generation CARs, including activating receptors such as CD8/CD3- ζ fusion receptors); middle, second-generation CARs providing dual signaling to direct combined activating and costimulatory signals; right, third-generation CARs comprising more complex structures with 3 or more signaling domains. (Sadelain and al. *Cancer Discovery* 10.1158/2159-8290.CD-12-0548, April 2013)

5) ACTIVE CANCER IMMUNOTHERAPY:

5.1) Oncolytic viruses (OVs):

5.1.1) Introductions: Every virus has a cellular tropism for a specific tissue and preferentially infects and causes a disease in that tissue (e.g hepatitis B virus damages hepatocytes or HIV that damages helper T lymphocytes). Oncolytic viruses are common viruses which can selectively target, replicate and destroy cancer cells. The idea of using oncolytic viruses as a cancer therapy began in 1949-1950 by the report of the destructive effects of the Russian Far East encephalitis virus on mice sarcoma (Moore & O'Connor, 1950) and the interference of influenza and herpes simplex virus on the tumor growth in mice (Moore, 1949). Indeed, cancer cells are good hosts for viral infections and virus replication as they never shut down their protein synthesis machinery or never enter prematurely into apoptosis. Even attenuated viruses with defects in the proteins able to block immune responses, often have the ability to propagate into tumor cells. Cancer cells have high levels of metabolic activity and can resist to signals that would normally lead to the suppression of protein synthesis. As a consequence they evolved to avoid cell death and to continue uncontrolled propagation. This was defined by Weinberg as one of the hallmarks of cancer cells with self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, tissue invasion and metastasis and sustained angiogenesis (Hanahan & Weinberg, 2000). Mutations that render cancer cells susceptible to virus infection can be divided into two types: i) mutations that prevent apoptosis such as p53 in almost 50% of cancers, or drive protein synthesis; and ii) mutations that interfere with innate immune signaling pathways such as IRF3 and STAT1 mutations (IFN pathway) seen in a great variety of tumors. Some oncolytic viruses such as Parvovirus, Reovirus, Newcastle disease virus (NDV), Mumps virus or Moloney leukemia virus, have natural preference to replicate into cancer cells leading to the destruction of the cell and the spread of the infection to additional tumor cells (Russell, Peng, & Bell, 2012). They are not pathogenic for humans because of their sensitivity to innate antiviral signaling or dependence to oncogenic signaling pathways (e.g Ras signaling which is very frequent in different type of cancer, render cancer cells susceptible to Reovirus (Norman, Hirasawa, Yang, Shields, & Lee, 2004)). Natural oncolytic viruses have soon lost their interest because of the lack of means to control viral pathogenicity at that time. Shortly after, the idea was revived at the beginning of 1990 with the emerging development of genetically engineered viruses, such as adenoviruses and herpesviruses as cancer therapeutics (Lin & Nemunaitis, 2004)(M. S. Roberts, Lorence, Groene, & Bamat, 2006). In the modern era of oncolytic virotherapy, viruses such as measles virus, adenovirus, vesicular stomatitis virus (VSV), vaccinia virus (VV) and herpes simplex virus (HSV) can be engineered to confer them cancer specificity (Russell et al., 2012).

5.1.2) Mechanism of action OVs and host antiviral response: Although the molecular and cellular mechanisms of action are not fully understood, oncolytic viruses are thought to have antitumor activity via two mechanisms: 1) selective replication within cancer cells, resulting in a direct lysis of tumor cells, and 2) induction of systemic antitumor immunity, depending on cancer cell types, virus or the interaction of the virus with the host immune system (Kaufman, Kohlhapp, & Zloza, 2015). Oncolytic viruses can infect cancer cells; they induce cell death by jeopardizing the cellular machinery to produce the new viruses. Most oncolytic viruses can induce cancer cell death and directly eliminate tumor cells, but they also initiate systemic immune responses through different mechanisms such as inducing an immunogenic cell death, releasing danger associated molecular patterns (DAMPs) and TAA from virus-infected cells. They also release viral PAMPs (pathogen associated molecular patterns) contributing APCs maturation that conduct to activation of antigen-specific CD4+ and CD8+ T cell responses. Once activated, CD8+ T cells become cytotoxic effector cells that traffic to tumor sites, where they mediate anti-tumor immunity upon antigen recognition (Kaufman et al., 2015). The innate immune system represents the first line of defense against a viral infection and involves 2 main events: i) the virus detection within infected cells (by pattern recognition receptors (PRRs)) leading to type I IFN production by infected cells that through an autocrine and paracrine loop engages the type I IFN receptor and triggers a signaling pathway conducting to the inhibition of different stages of virus replication and to an anti-viral state in the host. ii) The infected cells are recognized by the innate immune system such as NK cells or macrophages (Seth, Sun, & Chen, 2006).

5.1.3) Tumor targeting mechanisms: Many oncolytic viruses have a natural tropism for cell surface proteins that are highly expressed by cancer cells such as nectins for HSV-1 entry (Yu et al., 2005) or the surface receptor CD46 for measles virus entry conferring a preferential site for measles virus infection (Anderson, Nakamura, Russell, & Peng, 2004) (Dörig, Marcil, Chopra, & Richardson, 1993). Others, like coxsackievirus, infect cells via intercellular adhesion molecule 1 (ICAM-1) and decay accelerating factor (DAF), which can be overexpressed in cancers such as multiple myeloma, melanoma, and breast cancer (Au, Lincz, Enno, & Shafren, 2007)(Guo et al., 2014). For Echovirus EV1, a member of the enterovirus family, for which infection occurs through the domain I of integrin $\alpha 2\beta 1$ overexpressed in ovarian cancer cells, mediates a therapeutic activity after a single intratumoral injection inducing rapid tumor regression (Shafren, Sylvester, Johansson, Campbell, & Barry, 2005). Oncolytic viruses can also be engineered to directly target unique cell surface receptors expressed by cancer cells such as adenovirus that has been engineered to target ovarian cancer cell by binding to the overexpressed coxsackievirus-adenovirus receptor (Car) and integrin at the surface of these cells (You et al., 2001). Measles virus has also been modified to express a single-chain antibody that recognizes carcinoembryonic antigen (CEA), which is expressed on

certain adenocarcinomas allowing targeting of CD46 negative cells (Hammond et al., 2001). Other viruses can infect cancer cells not only because of overexpression of cell surface receptor that favors virus entry, but as a consequence of aberrant signaling pathway in cancer cell. For example NDV targets cancer cells overexpressing BCL-XL, which make these cell resistant to apoptosis and as a consequence permits the incubation time needed for virus multiplicity (Mansour, Palese, & Zamarin, 2011). Many oncolytic viruses have shown specificity for cancer cells based on RAS transformation. Activated RAS prevents PKR-mediated suppression of translation in cancer cells, thereby allowing translation and promoting viral infection. It has been shown that RAS/Raf1/MEK/ERK signaling pathway, facilitates VV mediated oncolysis in cancer cells deficient for type I IFN (Noser et al., 2007). Another approach conferring tumor selectivity is to restrict virus replication to cancer cells where high expression of thymidine kinase (TK) is observed and not in normal cells, by deleting the TK gene in the virus genome to generate a viral dependence on cellular thymidine kinase expression (Thorne et al., 2007). This was done for VV. Alternatively; tumor-selective replicative viruses have been developed through the use of tumor-specific promoters to drive the expression of an essential virus gene only in the tumor. In addition to their direct cytotoxicity, oncolytic viruses have anti-tumor effect by inducing anti-cancer immune response after detection of viral genome and/or proteins by the immune cells or through specific activities of transgene-encoded proteins expressed from engineered viruses (Russell et al., 2012). In 1996 Bischoff and al, showed that the p53 deficient cancer cells (frequently observed in different type of cancer and refractory to chemotherapy and/or radiation) are sensitive to infection with E1B mutant adenovirus thanks to p53 inactivation. In fact, in wild type adenovirus, E1B gene encodes a protein that inactivates the cellular protein p53 in normal cells, and as a consequence can continue to replicate, but the adenovirus with E1B mutant gene, can only replicate in p53 deficient cells and kill them but not in cells with functional p53 (Bischoff et al., 1996). Interesting results were observed with very potent oncolytic coxsackievirus A21 (CVA21) that also causes fatal myositis due to off-target infection of normal muscle. Muscle toxicity was eliminated by muscle-specific microRNA, targets into the 3' UTR of CVA21 without compromising anticancer activity (Kelly, Hadac, Greiner, & Russell, 2008). This increased selectivity is normally achieved through the deletion of viral virulence genes that are redundant for viral replication in tumor cells. As a result viral replication is attenuated in normal tissues, but proceeds normally in cancer cells.

5.1.4) Advantages of OVs virotherapy: OVs have many features that make them advantageous for cancer immunotherapy: 1) there is a very low probability for the generation of resistance to virus (not seen so far), because OVs often target multiple oncogenic pathways and induce cytotoxicity in different ways 2) they are non-pathogenic, replicate and destroy cancer cells 3) virus dose in the tumor increases with time due to *in situ* virus amplification, which is opposite to classical drug

pharmacokinetics that decreases with time 4) OV's can be manipulated to include safety features such as drug and immune sensitivity allowing to control them (Chiocca & Rabkin, 2014).

5.1.5) OV's delivery in host: Another important aspect of OV therapy concerns its delivery into the tumor site. There are two way of delivery: intra-tumoral or systemic injection. The systemic administration is often inefficient because of the host defense, lack of extravasation into tumors and sequestration of the OV's in the liver. Many of the barriers that viruses encounter following systemic administration such as neutralizing antibodies or inactivation by complement, can be overcome by hiding oncolytic viruses inside carrier cells. Cellular carriers combine with oncolytic viruses and deliver them to the tumor beds. Interestingly, it has been demonstrated that stem and progenitor cells, immune cells, and finally cancer cells themselves possess such tumor-homing characteristics. Intra-tumoral delivery of the OV's can be a good strategy to minimize the sequestration of the virus in the spleen and liver (Russell et al., 2012)(Nakashima, Kaur, & Chiocca, 2010).

5.1.6) Clinical Trial: the oncolytic properties of many different viruses were studied in different preclinical model in cancer treatment. There are already two engineered OV's approved in clinic: E1B (associated with P53 inhibition)-deleted adenovirus, and talimogene laherparepvec virus (T-VEC). T-VEC is based on herpes simplex virus type 1 deleted for ICP 34.5 gene (Neurovirulence factor), ICP47 (block antigen presentation in HSV infected cell), over expressed US11 (viral RNA binding proteins) and inserted for GM-SCF (B. L. Liu et al., 2003) and is approved by the FDA for the treatment of melanoma (Fukuhara, Ino, & Todo, 2016).

5.1.7) Rotavirus: Rotavirus was not previously known as an oncolytic virus. In my thesis project we have identified its oncolytic properties and I would like to have a brief presentation on Rotavirus. Rotavirus (RV) is the member of the Reoviridae family which is icosahedral, non-enveloped, and composed of three concentric layers of proteins and a genome. It's genome contains 11 segments of double-stranded RNA, encodes 6 structural proteins (VP1-VP6) and 6 non-structural proteins (NSP1-6) (Patton, Silvestri, Tortorici, Vasquez-Del Carpio, & Taraporewala, 2006). Rotaviruses are the most common cause of severe, dehydrating diarrhea in children worldwide. Rotavirus infects the mature enterocytes in the mid and upper villous epithelium of the small intestine, which leads to cell death, villous atrophy, and diarrhea (Tafazoli, Zeng, Estes, Magnusson, & Svensson, 2001). It has been demonstrated that viral non-structural protein 4 (NSP4) acts as a viral enterotoxin to induce diarrhea and causes Ca²⁺ homeostasis disruption (Dong, Zeng, Ball, Estes, & Morris, 1997). Ca²⁺ homeostasis alterations in cell have been related to cytotoxicity and cell death, since the prevention or reduction of these changes results in a delay in cell death (Pérez, Chemello, Liprandi, Ruiz, & Michelangeli, 1998). Rotavirus types are distinguished by their surface proteins, VP7 and VP4, as G types and P types, respectively. There are at least 27 different G and 37 different P types

(Matthijnssens et al., 2011)(Lever & Desselberger, 2016). Rotavirus has two forms: triple layer virus particle (TLP) which is the infectious virion that binds to the target cells and internalized at the cytoplasm, and double layer virus particle (DLP) which is a non-infectious particle and forms through removal of the outer layer proteins (VP4 and VP7). Particles that lack the outer two layers are called the single-layered particles (SLP). DLPs are transcriptionally active forms of rotavirus and capable to produce virus within the target cells (Jayaram, Estes, & Prasad, 2004). The DLP is composed of the remaining four structural proteins encoded by the rotavirus genome of which VP6 is the major constituent of Rotavirus infection and replication in the cell. VP6 has two principal functions: cell entry and endogenous transcription, through its interactions with the outer layer proteins VP7 and VP4, and the inner layer protein VP2 (Jayaram et al., 2004). Rotavirus infection and replication in the host cell starts through the binding of outer layer proteins VP4 and VP7 to the different receptors on the cell. Several molecules including gangliosides GM1, GM3, integrins ($\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha 2\beta 2$ and $\alpha 5\beta 3$) and heat shock protein 70 have been involved as attachment receptors for rotaviruses (Hewish, Takada, & Coulson, 2000)(Graham et al., 2003). The dsRNA segments are transcribed within the structural elements of the DLP. DLPs bind into the endoplasmic reticulum, through the interaction between VP6 and C terminal of NSP4, which acts as an ER resident receptor to transfer viroplasm (distinctive cytoplasmic structures associated with their replication and assembly) to the endoplasmic reticulum (ER) lumen (Taylor, O'Brien, & Yeager, 1996). DLPs remain structurally intact during the process of transcription, and the nascent transcripts exit in to the cytoplasm through the type I channels of DLP structure (Lawton et al., 1997). The nascent (+) RNAs serve as template for viral proteins synthesis and genome replication. Non-structural protein 2 (NSP2) and NSP5 interact to form large viroplasms that sequester components required for genome replication and the assembly of subviral particles. Mature virions are released presumably following cell death and associated breakdown of host plasma membrane (Trask, McDonald, & Patton, 2012). Increased level of type I and II IFNs has been found in children and animals as a consequence of rotavirus recognition by host PRRs (Jiang et al., 2003). However, some studies have suggested that whereas rotaviruses are able to trigger IFN production, they also developed many antiviral pathways in a strain-specific manner and as a consequence can suppress the IFN effects (Sherry, 2009). Rotaviruses inhibit the IFN response through different mechanisms. It has been shown that NSP1 can mediate the degradation of IRF3, IRF5 and IRF7 proteins and as a consequence subvert induction of IFN (Barro & Patton, 2007). Rotavirus NSP1 can also inhibits NF- κ B activation through ubiquitin ligase complex protein beta-TrCP, resulting in stabilization of I κ B and repress NF- κ B (Graff, Ettayebi, & Hardy, 2009). Rotavirus also blocks type I and II IFN-induced gene expression by preventing the nuclear accumulation of activated STAT1 and STAT2 (Holloway, Truong, & Coulson, 2009). Moreover, repression of the IFN response is dependent on both the rotavirus strain and the cell type.

5.2) DC-based immunotherapy:

DCs are the most potent professional antigen presenting cells, that uptake the pathogens/antigens, process them, load ags on MHCII to present them to T helper cells or on MHCI to cytotoxic T cells (cross presentation) (Banchereau & Steinman, 1998). One of the strategies in active cancer immunotherapy is to mature the patients owns DC *in vitro* and to load them with tumor antigens by different methods and reinject them to the patients to allow activation of different T cells population. To prepare mature DCs, transfection with total protein isolated from the tumor cell (Salcedo et al., 2006), defined peptides of tumor-associated antigens (in prostate cancer) (Jähnisch et al., 2010), DNA/RNA/Virus (Steele et al., 2011) or fusion of tumor cells and DCs can be performed (Rosenblatt et al., 2011). Sipuleucel-T is FDA approved of autologous DC-based immunotherapy for the patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC) (Gulley et al., 2016).

5.3) Peptide- and DNA/RNA-based anticancer vaccines:

In this anticancer strategy, TAA peptides are injected in patients either intramuscularly, subcutaneously or intradermal to stimulate not only the DCs but also other APCs. They are very often combined with adjuvant to enhance DC maturation. Once these peptides are injected, the APC will capture and process them on MHC and consequently present them on different T cell populations to induce TAA specific immune responses. Tumor antigens, encoded by somatically mutated cancer genes, generate neoepitopes that are implicated in the induction of tumor-controlling T cell responses. An important step of determining the therapeutic potential of a vaccine is the detection of mutation reactive T-cell responses. DNA based vaccine correspond to TAA encoding in viral or bacterial vector or naked construction without any vector. MAGE-1 is the first gene that was reported to encode a human tumor antigen recognized by T cells (van der Bruggen et al., 1991). There is no DNA or peptide based anticancer vaccine approved by FDA, but their application in cancer is under evaluation in different study. In one interesting study, it has been observed that vaccination of women with HPV-16 positive, grade 3 vulvar intraepithelial neoplasia, with oncoprotein E6 and E7 leads to complete regression of neoplasia that was correlated to HPV-16 specific immunity (Kenter et al., 2009). RNA based vaccine is the messenger RNA (mRNA) synthesized *in vitro* encoding the antigen(s) of interest. Once administered and internalized by host cells, the mRNA is translated to the antigen in the cytoplasm and will be presented to antigen presenting cells to stimulate an immune response (McNamara, Nair, & Holl, 2015). These characteristics of RNA based vaccine make them interesting among cancer immunotherapy strategies. mRNA can be isolated from a limited tumor sample and amplified, using techniques such as polymerase chain reaction (PCR). RNA can also act as an adjuvant by providing costimulatory signals, for example, via toll-like receptors TLR3, TLR7,

B7 family: B7.1 (CD80), B7.2 (CD86), inducible costimulatory ligand (ICOS-L), programmed death-1 ligand (PD-L1), programmed death-2 ligand (PD-L2), B7-H3, and B7-H4 and four known members of the CD28 family: CD28, CTLA-4 (CD152), ICOS, PD-1. The first study of immune checkpoint blockade was done on cytotoxic T lymphocyte antigen 4 (CTLA-4) (Leach, Krummel, & Allison, 1996). CTLA-4 is a protein rapidly expressed at the surface of T cells upon activation and inhibits their function through the competition with CD28 receptor, sharing the same ligands B7.1 et B7.2, and also through the recruitment of phosphatases that dephosphorylate TCR/CD28 signaling pathway (Krummel & Allison, 1996). CTLA-4 role is so important in immunologic homeostasis as it has been shown that CTLA-4 deficient mice undergo massive lymphoproliferation and fatal multi organ destruction (Tivol et al., 1995). The essential role of CTLA-4 is the regulation of T cell activation through the interaction with its ligands on APC in the secondary lymphoid organs (Figure 8).

5.4.3) PD-1: The interaction between PD-1 receptor and its ligands, PD-L1 and PD-L2, delivers inhibitory signals that regulate the balance between T-cell activation, tolerance, and immune-mediated tissue damage. Similarly to CTLA-4, PD-1 is absent on naive and memory T cells and is expressed upon TCR engagement. In contrast to CTLA-4 that acts early in T cell activation in secondary lymphoid tissues, PD-1 inhibits effector T cell responses in tissue and tumors (Figure 8). PD-1 is highly expressed within tumor infiltrating lymphocytes which correlated with an exhausted phenotype and impaired function of these tumor infiltrated lymphocytes (TILs) (Ahmadzadeh et al., 2009). PD-1 knockout mice, will develop delayed autoimmune syndromes such as cardiomyopathy in Balb/c strain mice (Nishimura, 2001) and lupus-like glomerulonephritis and arthritis on a C57Bl/6 background mice (Okazaki, Iwai, & Honjo, 2002). PD-L1 is induced on activated hematopoietic cells and on epithelial cells by IFN γ , whereas PDL-2 is more expressed on activated DC and some macrophages (Ishida et al., 2002). One of the mechanism by which cancer cells escape from an antitumor immune response is PD-1 ligands upregulation by intrinsic or extrinsic mechanism as shown in many different type of cancer (Dong et al., 2002). Intrinsic mechanism such as genetic alteration like loss of PTEN function in glioma was correlated with increased expression of PDL-1 (Parsa et al., 2007) or/and the activation of signaling pathway like ALK signaling pathway induce the expression of PDL-1 by activating STAT3, which in turn acts as a transcriptional activator of the *PDL-1* gene (Marzec et al., 2008). Extrinsic mechanism like tumor inflammatory microenvironment also induce PDL-1 and PDL-2 expression through IFN γ which is expressed by activated CD8 cells, NK cells and CD4 Th1 cells in the inflammatory tumor microenvironment (Abiko et al., 2015).

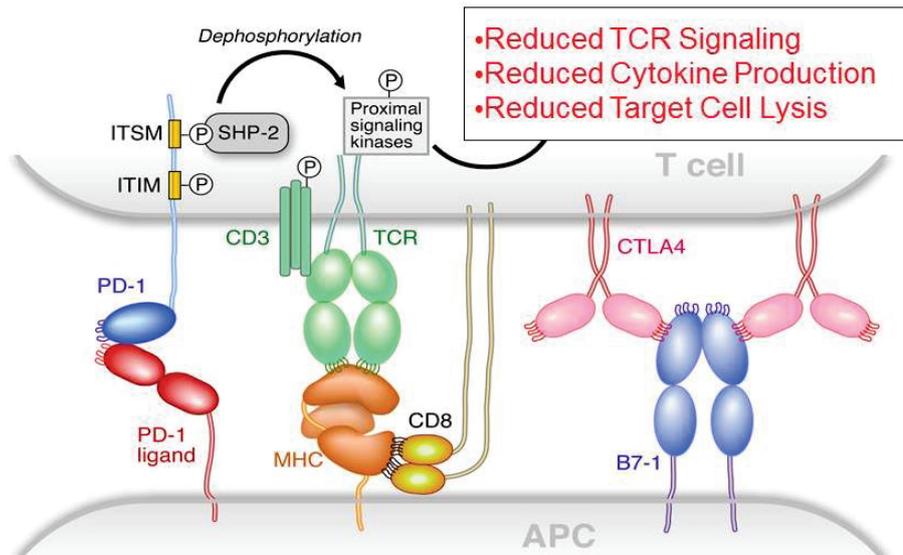


Figure 8: CTLA-4 and PD-1 engagement by their respective ligands, that are expressed on APCs and for PD-1 also expressed on cancer cells, trigger the signaling Pathway which Inhibits T Cell Responses by reducing TCR signaling and cytokine production. (G Freeman 2008; PNAS 105:10275)

5.4.4) *Other members of the CD28/B7 family:* ICOS/ICOSL are also members of CD28/B7 family and are positive costimulatory molecules. It has been shown before that they are preferentially expressed by tumor associated Tregs and induced their activation (Faget et al., 2012). Consequently the blockade of ICOS-ICOSL interaction would provide a potentially target for neutralizing Tregs function. Four other molecules belong to this family (B7H3, B7H4, B7H7 (HLA2) and VISTA), they are B7 homologues and also have inhibitory function on T cells although their receptors are not fully identified (Pardoll, 2012). BTLA receptor is a PD-1 and CTLA-4 homologue and also induce inhibitory signals by recruiting phosphatase after interacting with its Ligand HVEM (TNFR family) (Murphy & Murphy, 2010)(Gordon J Freeman, Casanovas, Umetsu, & DeKruyff, 2010).

5.4.5) *Other negative regulators of T cell activation:* Another inhibitory receptor expressed in majority on the surface of activated T cell, NK cell, B cell and pDC is lymphocyte activation gene 3 (LAG3). LAG3 binds to class II MHC with a greater affinity than CD4 (Huard, Prigent, Tournier, Bruniquel, & Triebel, 1995) and has been shown that play a role in T cell exhaustion (Blackburn et al., 2009). It was also reported that LAG-3 and PD-1 synergistically regulate T-cell function and their double (LAG-3/PD-1) expression is restricted to TILs. Their simultaneous blockade can be an efficient strategy to promote an antitumor immunity response (He et al., 2016). TIM3 is another inhibitory receptor which is induced during T cell activation. It interacts with Galectin (Gal-9), phosphatidylserine, HMB1 and CEAM-1 (Mahoney, Rennert, & Freeman, 2015)(Gordon J Freeman

et al., 2010). TIGIT is another inhibitory immune receptor expressed on lymphocytes from PVR/nectin family, and is associated with human cancers and T cell exhaustion phenotypes. TIGIT share the same ligand (CD155(PVR) and CD112(nectin-2)) with the costimulatory receptor CD226 (DNAM-1)(Manieri, Chiang, & Grogan, 2017).

5.4.6) TNFR family receptors: TNFR/TNFL family also has the activating and inhibitory receptors (Fas, Trail) on the T cells. Activating receptors such as CD137, CD27, GITR, OX40 are expressed on activated cells and their engagement with their ligand induced a co-activation of T cells and amplify their expansion and function (Mellman et al., 2011).

5.4.7) Targeting antibodies for these checkpoints:

5.4.7.1) anti CTLA-4: Multiple studies have assumed that the efficacy of α -CTLA-4 antibodies in murine tumor models relied on the inhibition of effector T-cells via an interaction with CD80 and CD86 (very high affinity compared to CD28 (Linsley et al., 1994)) and by preventing the engagement of the CD28 receptor and its stimulatory function on T-cells (Lenschow, Walunas, & Bluestone, 1996). Since the discovery that CD4⁺Foxp3⁺ Tregs express high levels of CTLA-4 on their surface, new mechanisms for α -CTLA-4 antibodies are proposed. There are four studies that have shown that the efficacy of CTLA-4 blockade relies on intra-tumoral Treg depletion mediated by ADCC or ADCP via Fc γ receptor expressing macrophages within the tumor microenvironment. Switching the isotype to ADCC or ADCP deficient α -CTLA-4 mAbs has been shown to lead to a defect in *in vivo* antitumor responses suggesting that blocking CTLA-4 pathway alone is not sufficient (Marabelle et al., 2013)(Simpson et al., 2013)(Bulliard et al., 2013)(Selby et al., 2013). Recent studies have shown that the blockade of CTLA-4 on both T-effector cells and Tregs is required for an efficient antitumor immune response (Peggs, Quezada, Chambers, Korman, & Allison, 2009)(Pentcheva-Hoang, Simpson, Montalvo-Ortiz, & Allison, 2014). The anti-CTLA-4 monoclonal antibody Ipilimumab has now become a standard therapy for patients with advanced metastatic melanoma based on two phase 3 clinical trials showing a long term overall survival benefit in treated patients (20% plateau at 5 years) compared to patients treated with chemotherapy (Hodi et al., 2010)(Robert et al., 2011)(Figure 9).

5.4.7.2) anti PD-1: mAbs targeting programmed cell death 1 (PD-1) and its ligand 1 (PD-L1) are currently in an advanced clinical development. Nivolumab and Pembrolizumab are anti PD-1 mAbs currently approved by the FDA and EMA. They have demonstrated in large randomized phase 3 trials that they provide a better overall survival when compared to conventional therapies for patients with relapsing/refractory cancers such as metastatic melanoma, non-small cell lung cancers (NSCLC), renal cell carcinomas, bladder cancers and head and neck cancers. Also they generate dramatic response rates in refractory/relapsing stage IV Hodgkin lymphoma. It has been shown that

patients with survival benefits to PD-1/PDL-1 blockade therapy have high level of somatic point mutations in their cancer cell genome or highly infiltrated tumors by T-lymphocytes (Rizvi et al., 2015a)(Tumeh et al., 2014)(Snyder et al., 2014b). To predict the responsiveness of patients to immune checkpoint blockade therapy, new biomarkers are needed. Several tumor criteria such as PDL-1 expression, density and location of intra-tumoral T cells, somatic point mutations and tertiary lymphoid structures are several of the key parameters that facilitate T cell recruitment within the tumor and allow for the coordination of the local adaptive antitumor immune response and which might be associated to the improved outcome of PD-1/PDL-1 blockade therapy (Dieu-Nosjean et al., 2008)(Topalian, Taube, Anders, & Pardoll, 2016) (Figure 9).

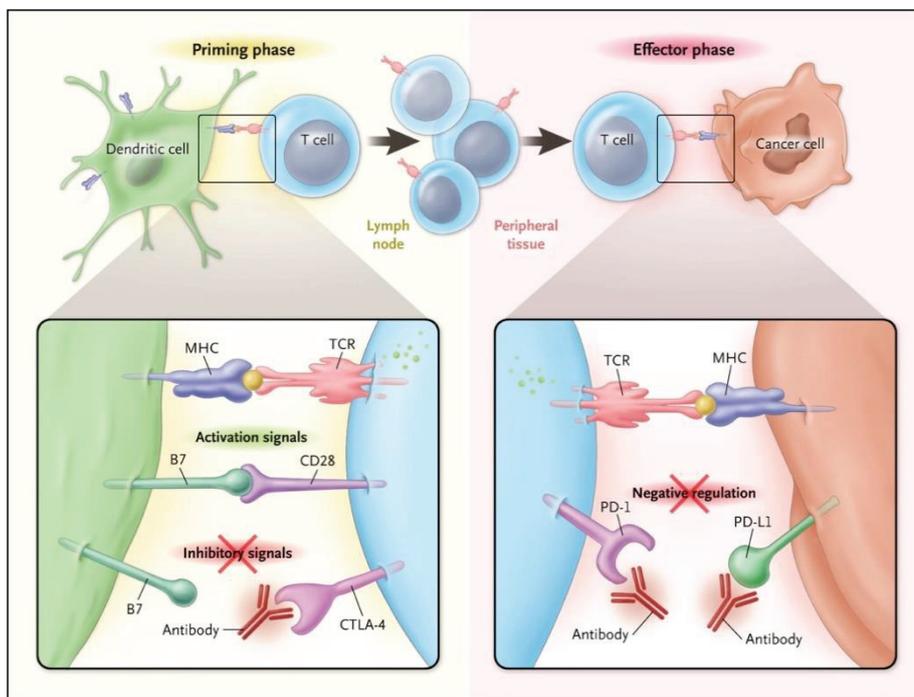


Figure 9: Difference between blocking CTLA-4/B7 and blocking PD-1/PDL1. CTLA-4 is upregulated shortly after activation and negatively regulates T-cell activation during the “priming” phase of T cell response in lymph node, whereas PD-1 binds to either of its ligands (PD-L1 or PD-L2), which are primarily expressed within inflamed tissues and the tumor microenvironment. Blockade of CTLA-4 or PD-1/PDL1 with antibodies results in the preferential activation of T cells with specificity for cancer cells. (Ribas A. *N Engl J Med.* 2012; 366:2517-2519)

5.4.8) Other antibodies:

5.4.8.1) *Antagonist antibodies*: some antagonist antibodies for other T cell inhibitory receptors such as BTLA, B7H3, B7H4, B7H5/VISTA, TIM3, LAG3 or TIGIT are also under development and are also the subject of different clinical trials (Mahoney et al., 2015)

5.4.8.2) *Agonist antibodies*: The other agonistic monoclonal antibody for co-stimulatory receptor of TNF superfamily expressed on T effector cells such as OX40 and CD137 (Melero et al., 1997) (Adler & Vella, 2013) as well as CD27 (D. J. Roberts et al., 2010) and GITR (Cohen, 2006) are under evaluation. It has been shown that in phase I clinical trial of OX40 in the patients with advanced cancer has shown the regression of at least one metastatic lesion (Curti et al., 2013). It exist two anti CD137 agonist mAbs: Urelumab and PF-05082566. Phase II study of Urelumab was designed in patient with metastatic melanoma. However, the study was terminated in May 2009 due to fatal hepatotoxicity whereas PF-05082566 was well tolerated, with evidence of disease stabilization in multiple patients (Yonezawa, Dutt, Chester, Kim, & Kohrt, 2015). In addition to immune checkpoint blockade monotherapies, combination approaches of anti-CTLA-4 or anti-PD-1 with the blockade of other immune-checkpoints or with activation of co-stimulatory molecules may also further amplify antitumor immune responses, as it has been shown that in melanoma patients the combination of PD-1 and CTLA-4 blockade was more effective than each agent alone (Valsecchi, 2015). In another study it has been observed that combination of anti-LAG-3/anti-PD-1 antibody treatment cured established tumors in the mice that were resistant to the monotherapy (Woo et al., 2012). Other study showed that in the CT26 colon cancer model, the combinatorial treatment of VISTA and PD-L1 mAbs led to tumor regression and long-term survival, whereas either mAb alone was less effective (J. Liu et al., 2015).

5.4.8.3) *Non T cell targeting checkpoint receptors*: Another class of antibodies that target other cells such as macrophages are also under development. These antibodies can either directly increase their antitumoral activity or neutralize their immunosuppressive activity. These antibodies target the molecules such as CD47/SIRP α , M-CSF/CSF-1R or CD40/CD40L on the surface of macrophages and APCs. SIRP α is an inhibitory receptor expressed on the macrophage and abolish it's phagocytose activity. It has been shown that blocking The interaction of SIRP α and its ligand CD47 which is highly expressed by tumor cells, leads to tumor destruction (Weiskopf et al., 2016). The antibodies neutralizing the interaction of CSF1-R with its ligands CSF-1 (M-CSF) have been shown that can induce the depletion of intra-tumoral immunosuppressive macrophages (Cassier et al., 2015). Anti CD40 agonist antibodies are also under development. Their objective is to induce the activation of APC such as DCs (Beatty et al., 2011). NK cell

targeting antibodies are the other class of antibodies. They target NK activatory receptors such as NKG2D and NKP46 or inhibitory receptors such as KIR and NKG2a and are under deep investigation (Mahoney et al., 2015).

5.4.8.4) Bi-specific antibodies: Bi-specific antibodies are antibodies that can bind to two different targets. One of the major strategies of these antibodies is the recruitment of immune effector T cells by targeting CD3 domain. They can target CD3 and another molecule which is abnormally expressed by the tumor cells. A bispecific CD3/CD19 antibody, has been approved in the US and Europe for the treatment of CD19+ acute lymphoblastic.

5.5) Inhibitors of immunosuppressive metabolism:

IDO is an intracellular immunosuppressive enzyme that converts Tryptophan into L-kynurenine. Decrease of local tryptophan by IDO enzyme activate the stress-response kinase GCN2 a component of the cellular stress response to amino acid withdrawal, which by its activation can inhibit T cell proliferation. It has been shown that GCN2-knockout T cells were resistance to IDO-induced anergy (Munn et al., 2005). Tryptophan depletion mediated by IDO can also play an important role in the regulation of naive CD4+ T cells toward differentiation into Treg cells (Fallarino et al., 2006). Also Kynurenine production through tryptophan degradation pathway by IDO, binds and activates the aryl hydrocarbon receptor (AhR) and promotes Treg differentiation. It can also induce tolerogenic dendritic cells that support FoxP3(+) Treg differentiation in a retinoic acid-dependent manner (Mezrich et al., 2010)(Quintana et al., 2010). In another study, increased IDO activity of tumor cells drives effector lymphocytes into either cell cycle arrest or apoptosis. Altogether these studies show, that IDO is potentially an attractive target for combinatorial immunotherapy because it affects inflammation, antigen cross-presentation, and the tolerogenic microenvironment. The IDO inhibitor is not already approved by FDA but phase I and II clinical trial has shown an antineoplastic effect in a subset of patients (Iversen et al., 2014)(Vacchelli et al., 2014). In the normal physiologic condition, mammalian cells have high concentrations of Adenosine triphosphate (ATP) whereas in pathologic conditions, such as inflammation or ischemia, intracellular ATP release to extracellular environment. These extracellular ATP and adenosine diphosphate (ADP), are both converted to adenosine monophosphate (AMP) through the ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and then converted to the Adenosine by ecto-5'-nucleotidase (CD73)(Eltzschig, Sitkovsky, & Robson, 2012). Adenosine inhibits inflammatory activity of different cell populations such as DCs and T lymphocytes and suppresses production of inflammatory cytokines to protects normal tissues from excessive damage due to hypoxia and inflammation (Ohta & Sitkovsky, 2001)(Panther, 2003). Adenosine binds to its receptors inhibiting the activation of T cell by inducing the accumulation of intracellular cyclic AMP which prevents TCR-induced CD25

upregulation and inhibiting effector T-lymphocyte proliferation and inflammatory cytokine secretion (Huang, Apasov, Koshiba, & Sitkovsky, 1997). In cancer, adenosine plays a significant role in protecting malignant tissue. It has been reported in some cancers such as human endometrial tumors the CD39 and CD73 expression upregulation that promote accumulation of extracellular adenosine that helps tumors evasion from antitumor immune response (Aliagas et al., 2014)(Clayton, Al-Taei, Webber, Mason, & Tabi, 2011). As a consequence targeting of extracellular adenosine or its receptors or CD39 and CD73 would be good strategies to induce antitumor immune response.

5.6) PRR agonists:

In this part I would like to discuss very widely the PRR agonists and their use in cancer immunotherapy by quoting our review that is currently under review in *Annals of oncology* (november2016) entitle” Pattern recognition receptor: new targets to enhance cancer immunotherapy”. You will find the review in the Annex.

5.7) Immunogenic cell death inducers:

Cellular death can be immunogenic or non-immunogenic based on different stimuli. Immunogenic cell death (ICD) has been defined by a) the composition change of dying cell surface that can promote immune reaction such as expression of Calreticulin at the surface of dead cell which can induce an “eat me” signal that facilitate phagocytose of dead body by the phagocytes and b) the release of cytoplasmic mediators such as HMGB1 functioning as alarmin and can activate the phagocytes after interaction with PRR expressed by APC. ICD was first proposed in 2005 by Guido Kroemer after observing that in vitro, murine colon cancer cells dying after the anthracycline doxorubicin treatment, were able to induce effective antitumor vaccination response that suppressed the growth of inoculated tumors or led to the regression of established neoplasia through the maturation of DC (Casares et al., 2005). In this regard ICD inducing molecules also can be classified within cancer immunotherapy strategies.

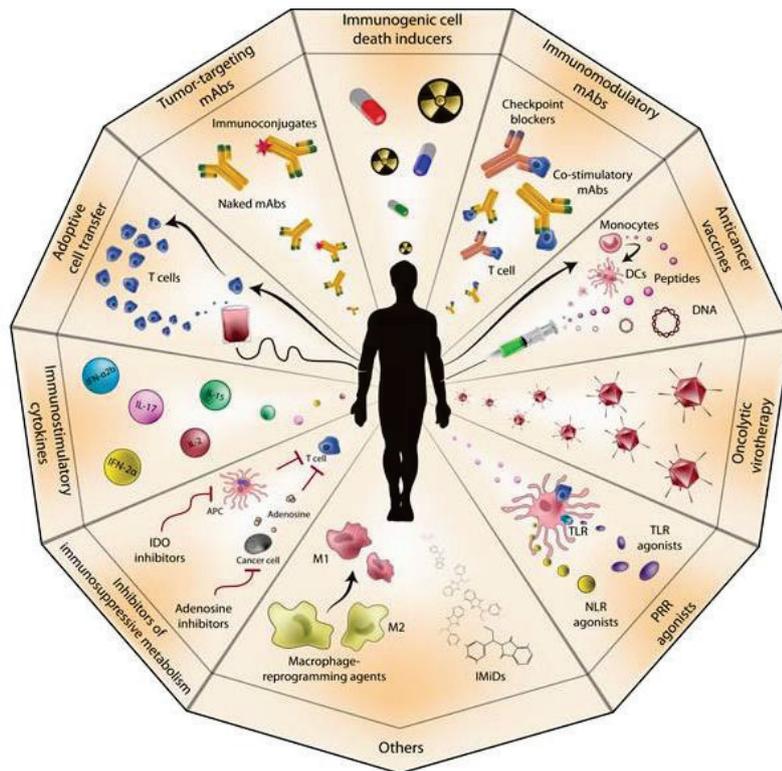


Figure 10: Anticancer immunotherapy strategie, Several anticancer immunotherapeutics have been developed during the last three decades, including tumor-targeting and immunomodulatory monoclonal antibodies (mAbs); dendritic cell (DC)-, peptide- and DNA-based anticancer vaccines; oncolytic viruses; pattern recognition receptor (PRR) agonists; immunostimulatory cytokines; immunogenic cell death inducers; inhibitors of immunosuppressive metabolism; and adoptive cell transfer. (Galluzi L and al. 2014 Dec 30;5(24):12472-508)

Hypothesis:

Immune checkpoint targeted therapies against PD-1, PD-L1 and CTLA-4 are currently revolutionizing cancer care. However, only a minority of patients generate objective tumor responses with these treatments. Therefore, new therapeutic interventions are needed to increase the immunogenicity of tumors in order to overcome the resistance to immune checkpoint blockade therapy. Pattern recognition receptors (PRR) such as toll-like receptor agonists have been shown to overcome resistance to immune checkpoint targeted therapy in pre-clinical models (Houot & Levy, 2009). Besides their intrinsic ability to stimulate PRR, the oncolytic properties of common viruses can be exploited also for the priming of anti-tumor immune responses. We made the hypothesis that commercially available anti-infectious vaccines, which are made of living pathogens or bacterial/viral extracts, could be used as PRR

agonists. These clinical grade source of PRR agonists could be of therapeutic interest for immunotherapy strategies in Pediatric cancers where little activity of immune checkpoint blockade monotherapy is expected based on their low level of somatic point mutations, low level of T-cell infiltrates and low level of clinical activity of the first clinical reports. One of our models of study is Neuroblastoma, one of the most aggressive tumor among pediatric cancers. Neuroblastoma patients have a 5 years overall survival of less than 40% despite intensive multi-modal therapies. Also, hematological malignancies such as lymphomas represent about a quarter of all pediatric cancers. Thus, novel therapeutic approaches are required to improve the survival of these children. In my PhD project we focused on building the rationale for new immunotherapy strategies of pediatric Neuroblastoma and Lymphoma through the combination of immune checkpoint blockade antibodies and clinical grade anti-infectious vaccines as a source of PRRs agonists (Figure 11).

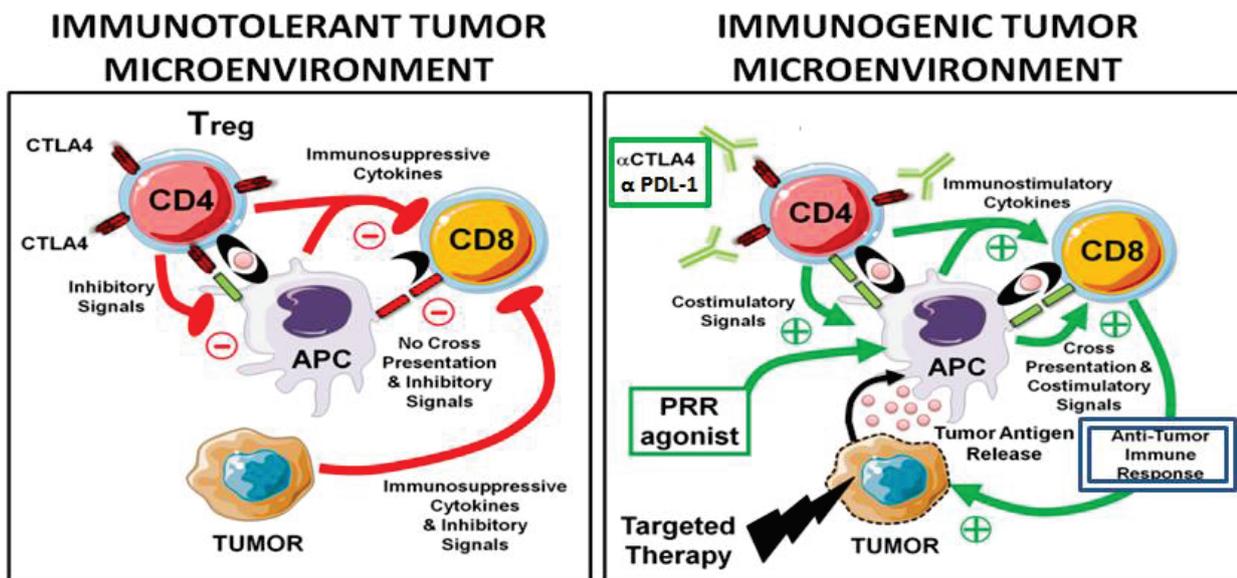


Figure 11. Hypothesis: Reprogramming of tolerogenic into immunogenic microenvironment by combining clinical grade of PRR agonists (anti-infectious vaccines) with immunecheckpoint targeted therapy. (image from Aurelien Marabelle)

A few words on Neuroblastoma: Neuroblastoma, an embryonal tumor of the sympathetic nervous system, is one of the most common extracranial solid cancer in children, with approximately 40% of patients presenting with metastatic disease at diagnosis. The tumor rise most frequently in adrenal glands (Neural crest cells) and in abdomen but it can also develop from nerve tissue in the neck, chest or pelvis. The "International Neuroblastoma Staging System" (INSS) stratifies neuroblastoma

according to the disease anatomical location at diagnosis from stage 1 localized tumors to stage 4 metastatic disease.

The etiology of Neuroblastoma is not fully understood, the majority of the cases are sporadic. Importantly, MYCN amplification is linked with advanced stage of the disease and poor prognosis. Among kids with metastatic Neuroblastoma, the amplification of MYCN (30% of tumors) is associated with high risk of disease relapse. The amplification of MYCN remains the best characterized genetic marker of aggressiveness in Neuroblastoma.

In addition to the MYCN status, the age of the children diagnosed with Neuroblastoma is another criteria of overall survival prognosis. Children diagnosed with metastatic NB at age ≥ 18 months often have tumors with recurrent segmental genomic alterations in CGH arrays and have high-risk disease with only 45% long-term survival. Children diagnosed with MYCN non amplified NB at age < 18 months frequently have tumors with whole chromosomal alterations and have greater than 90% overall survival. Within the cohort of patients with MYCN non amplified disease-NA, the age at diagnosis remains the most important prognostic factor (Schmidt et al., 2005).

The conventional therapy for Neuroblastoma depending on the staging of the cancer is: surgery, high dose chemotherapy followed by autologous hematopoietic stem cell transplantation and radiotherapy. In 2015, Dinutuximab (an anti-GD2 mAb) was approved by the US FDA and is currently used in combination with GM-CSF, IL-2 and isotretinoin (13-cis retinoic acid) for the treatment of children with high-risk neuroblastoma. As GD2 is rarely expressed on normal tissue and highly expressed on neuroblastoma cells, it became a very interesting target to treat children with high risk Neuroblastoma.

Immunological view of Neuroblastoma: Recent studies have shown that MYCN non-amplified Metastatic neuroblastomas have higher infiltration of TAMs (CD163) than loco regional tumors. Also, metastatic tumors diagnosed in children at age ≥ 18 months had higher expression of inflammation-related genes than those in patients diagnosed at age < 18 months. These data suggest that this inflammatory cells in the tumor microenvironment may contribute to the clinical metastatic neuroblastoma phenotype and reveal a novel rationale for immunotherapy of Neuroblastoma (Asgharzadeh et al., 2012a). In another study, the role of NK cells in the immune surveillance of High risk neuroblastoma was explored. It has been shown that NB (Neuroblastoma) cells expressing the NKp30 ligand B7-H6 (reported to be positively regulated by MYCN expression (Textor et al., 2016)) stimulated NK cells whereas serum concentration of soluble B7-H6 correlated with the down-regulation of NKp30, bone marrow metastases, and chemo resistance. Thus, interaction between NKp30 and B7-H6 may contribute to neuroblastoma immuno-surveillance and both NKp30

expression on circulating NK cells and the serum concentration of soluble B7-H6 may represent biomarkers for risk stratification (Semeraro et al., 2015). Danger signals provided by intra-tumoral PRR agonists could therefore be a relevant strategy to activate the phenotype of intra-tumoral TAMs and recruit tumor specific T-cells which could be further stimulated by a combination with immune checkpoint targeted antibodies.

Material, methods and Results:

Data generated during m PhD project are presented in the following paper which is under preparation and will be submitted soon. We were honored to receive for this work, the award from American society of immunotherapy of cancer (SITC) in November 2016.

Oncolytic properties of Rotavirus can overcome to Immune Checkpoint Blockade Therapy

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Keywords: cancer immunotherapy, oncolytic virus, immune checkpoint, rotavirus, in situ immunization, immunogenic cell death

Abstract:

Immune checkpoint targeted therapies against PD-1, PD-L1 and CTLA-4 are currently revolutionizing cancer care. However, only a minority of patients develop objective responses with these treatments. Therefore, new therapeutic interventions are needed to increase the immunogenicity of tumors in order to overcome resistance to immune checkpoint blockade therapy. Oncolytic properties of common viruses can be exploited for the priming of anti-tumor immunity and such oncolytic viruses (OVs) are currently in intense clinical development in combination with immune checkpoint targeted therapies. However the routine implementation of these therapies is limited by the ongoing regulations on GMOs. We have found that commercially available rotavirus vaccines do have oncolytic properties. This pediatric vaccine virus can directly

kill cancer cells with features of immunogenic cell death. Moreover, they have pro-inflammatory properties and can activate the NF- κ B pathway in a toll-like receptor and IRF3 independent manner. These in vitro biological properties translate in vivo into anti-tumor activity. Intra-tumoral rotavirus therapy has anti-tumor effects which are partly immune mediated. Interestingly, in immunocompetent murine pediatric tumor models, intra-tumoral rotavirus overcome resistance and synergize with immune checkpoint targeted therapy. Rotavirus vaccines are pediatric and adult clinical grade products. Therefore, in situ immunization strategies with intra-tumoral attenuated rotavirus could be implemented quickly in the clinic.

Introduction:

Immune checkpoint targeted therapies (ICT) against PD-1, PDL-1 and CTLA-4 are very promising strategies for the treatment of multiple cancers because they provide overall survival benefits over conventional therapies. However, only a minority of patients develop objective tumor responses with these new treatments. Several factors have been identified to correlate with the effectiveness of ICT, notably the high proportion of tumor infiltrating T-cells (Tumeh et al., 2014), and the high level of somatic point mutations that generate neoepitope (Rizvi et al., 2015b; Snyder et al., 2014a). Other elements have been shown to contribute to the resistance to ICT such as Myeloid cells (Gebhardt et al., 2015; Romano et al., 2015) and absence of MHC-I expression by tumor cells (Zaretsky et al., 2016). In this context, pediatric cancers should be poorly responsive to these novel immunotherapies as they display low levels of T-cell infiltrates, high levels of myeloid cell infiltrates, and usually have a low mutation load (Asgharzadeh et al., 2012b; Fujiwara et al., 2011; Lee, Stewart, & Carter, 2012; Molenaar et al., 2012; Pugh et al., 2013; Vakkila, Jaffe, Michelow, & Lotze, 2006). Intra-tumoral injections of oncolytic viruses (OVs), toll-like receptor agonists, or STING agonists are in situ immunotherapy strategies currently in active clinical development with the aim of stimulating pattern recognition receptors (PRRs) and prime anti-tumor immunity by turning non-infiltrated “cold” tumors into immune-infiltrated “hot” tumors. Intra-tumoral stimulation of PRRs has shown very promising results in cancer patients by inducing effective anti-tumor immune responses, in both injected and non-injected sites (so called “abscopal effect”) (Andtbacka et al., 2015; Brody et al., 2010; Kim et al., 2012). Also, intra-tumoral stimulation of PRRs can overcome ICT resistance in syngeneic tumor models (Fu et al., 2015; Houot & Levy, 2009; Marabelle et al., 2013; Zamarin et al., 2014) and patients with melanoma (Long et al., 2016; Puzanov et al., 2016).

However, the pediatric clinical development of PRR agonists will start many years after their adult development. Therefore, we sought to use anti-infectious vaccines as a potential source of

clinical grade pathogens which could be used as PRR agonists to prime the anti-tumor immunity of pediatric cancers and accelerate cancer immunotherapy development in pediatric oncology. Upon screening of most commercially available anti-infectious vaccines we have identified that rotavirus containing vaccines (Rotarix, Rotateq) have the ability to simultaneously activate NF- κ B in a TLR-independent manner and induce immunogenic cell death of multiple cancer cell lines in vitro. In vivo intra-tumoral injections of rotavirus vaccines results in type I interferon-response, and immune cell activation, which can culminate in complete regression of large, established tumors in some mice. Of utmost importance, oncolytic Rotavirus vaccines synergizes with anti-CTLA4 resulting in complete tumor regression associated to abscopal effect in to 100% of mice.

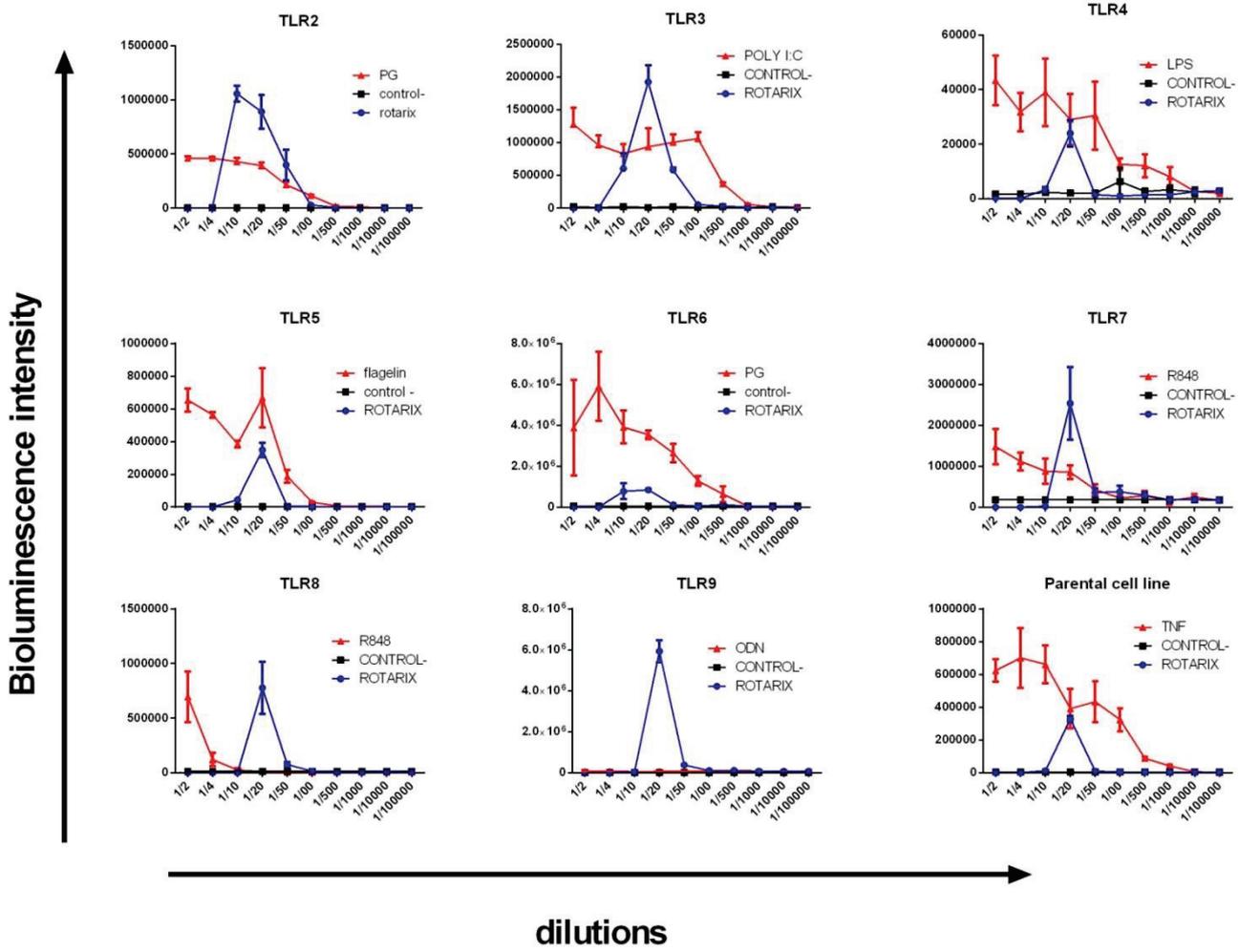
Results:

1) Rotavirus vaccine activates NF- κ B in TLR and IRF independent manner:

Because anti-infectious vaccines contain pathogens and pathogens extracts we wanted to test if they could be used as a clinical grade source of pattern recognition receptor agonists. To assess the intrinsic ability of anti-infectious vaccines to stimulate Toll-like receptors (TLRs), a major family of PRRs, we used transgenic cell lines transfected with the different TLRs and a luciferase reporter plasmid under the control of the NF- κ B binding site. A quantitative analysis of TLR ligand-induced luminescence after TLR stimulation by different vaccines was performed by measuring luminescence intensity. We incubated 10 different dilutions of 14 viral and bacterial anti-infectious vaccines with these transgenic cell lines overnight and measured the luminescence intensity after 12 to 14 hours. In these assays, we found that the Typhoid Fever vaccine (Tyavax®) and Mycobacterium tuberculosis vaccine (BCG Pasteur®) were potent activators of TLR4 and TLR2. More surprisingly, we found that the two rotavirus vaccines (Rotarix® and Rotateq®) were both activating the parental cell line that were devoid of TLR transgene, meaning that these vaccines were able to activate the NF- κ B pathway independently of any TLR. This effect was only and always true around 1/10-1/20 dilutions (Figure 1a and 1b). Because the rotavirus double stranded RNA (dsRNA) could activate IRF3 and NF- κ B via the RIG-I pathway, we performed the same assay on an ISRE transfected luciferase reporter cell line (Figure 1c). We could not find any activation of the IRF transcription factor, suggesting a direct activation of the NF- κ B pathway by the rotavirus vaccines independently of the TLRs, RIG-1 and MDA5 receptors.

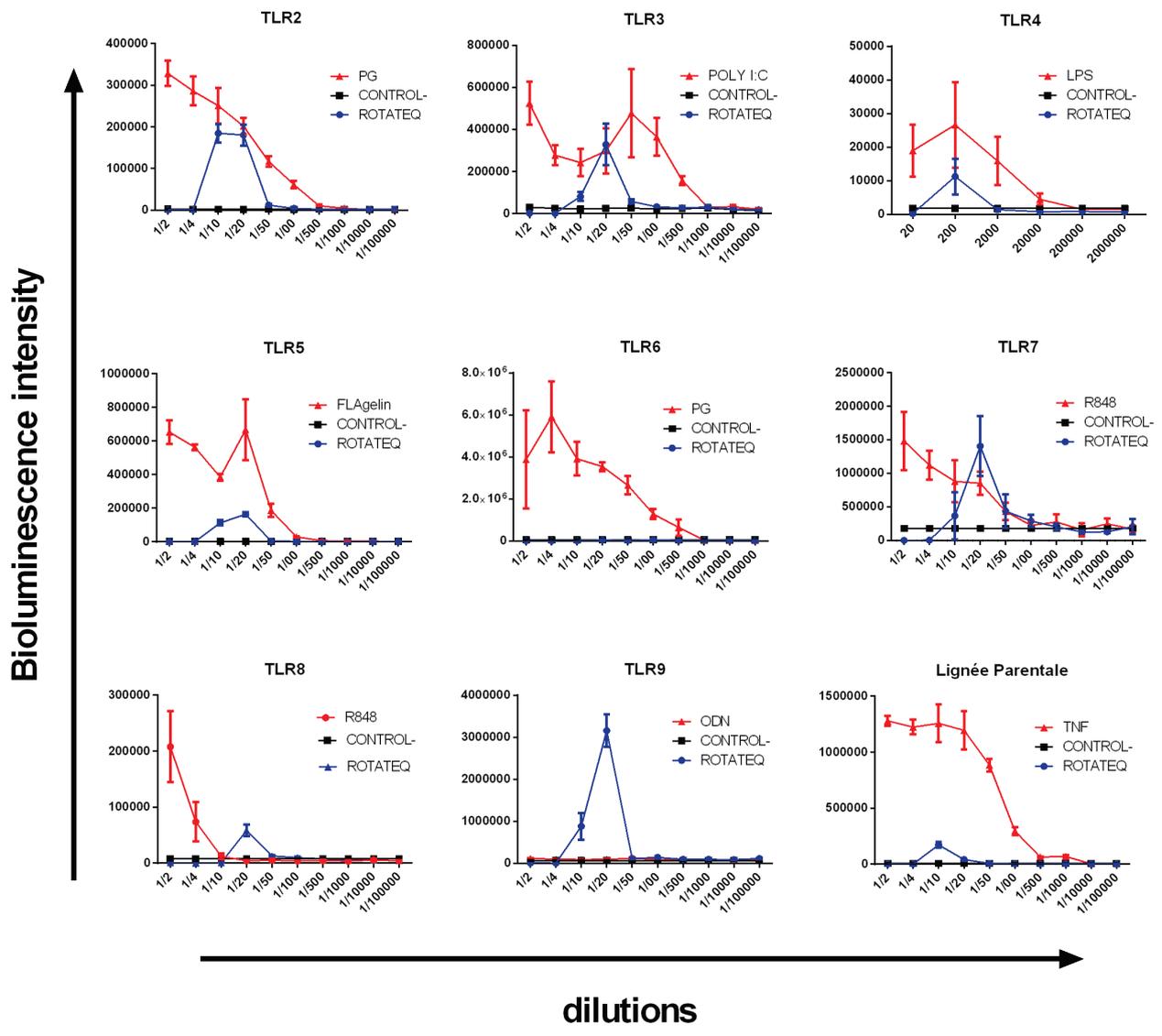
1a)

ROTARIX

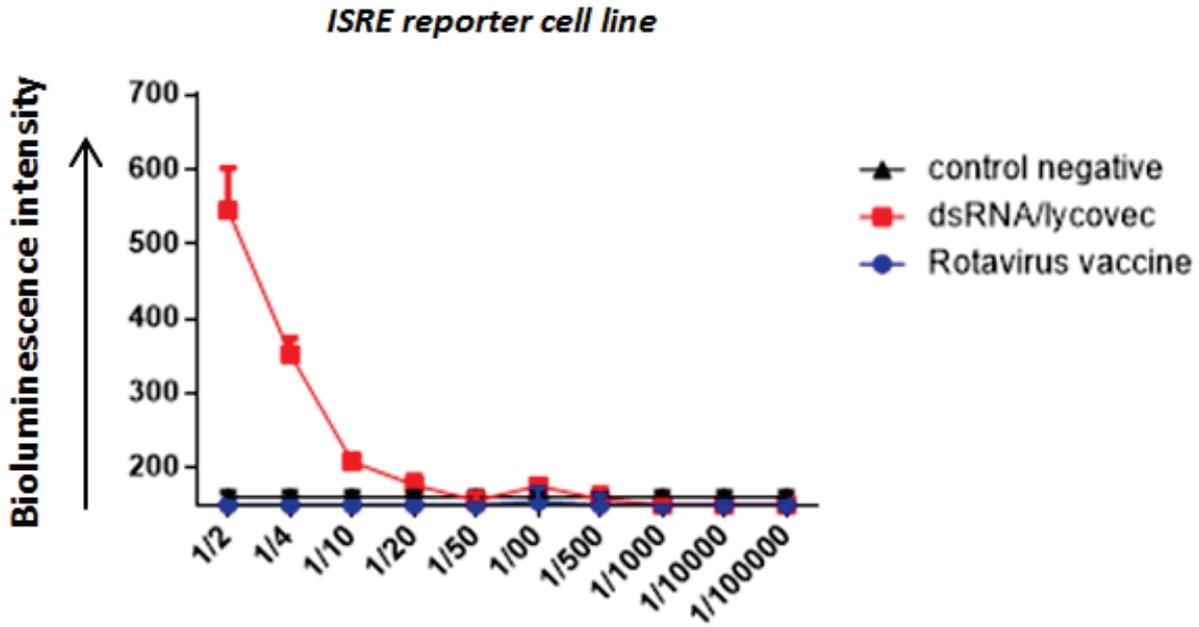


1b)

ROTATEQ



1c)



1d)

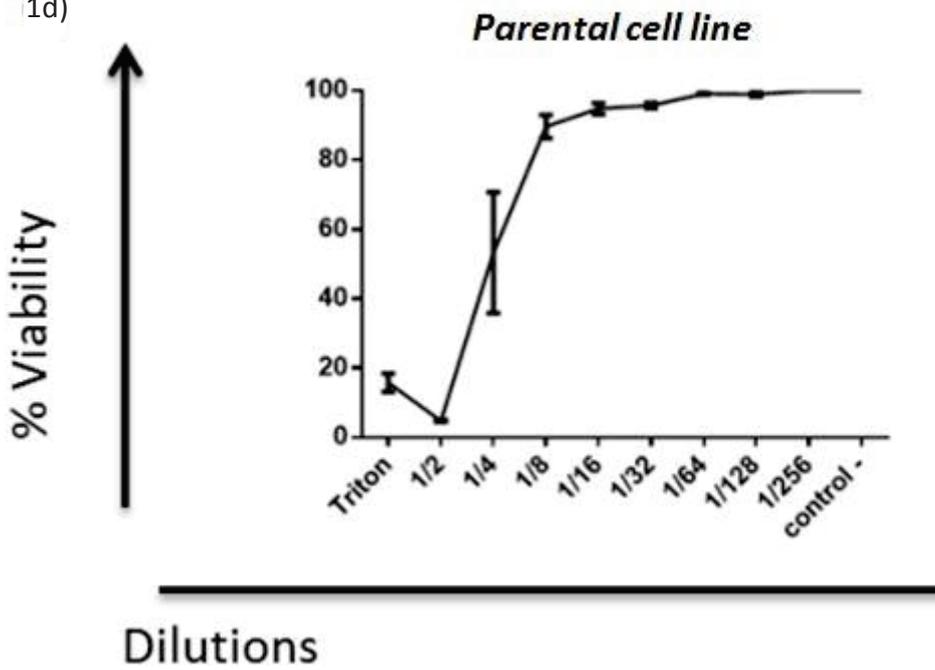


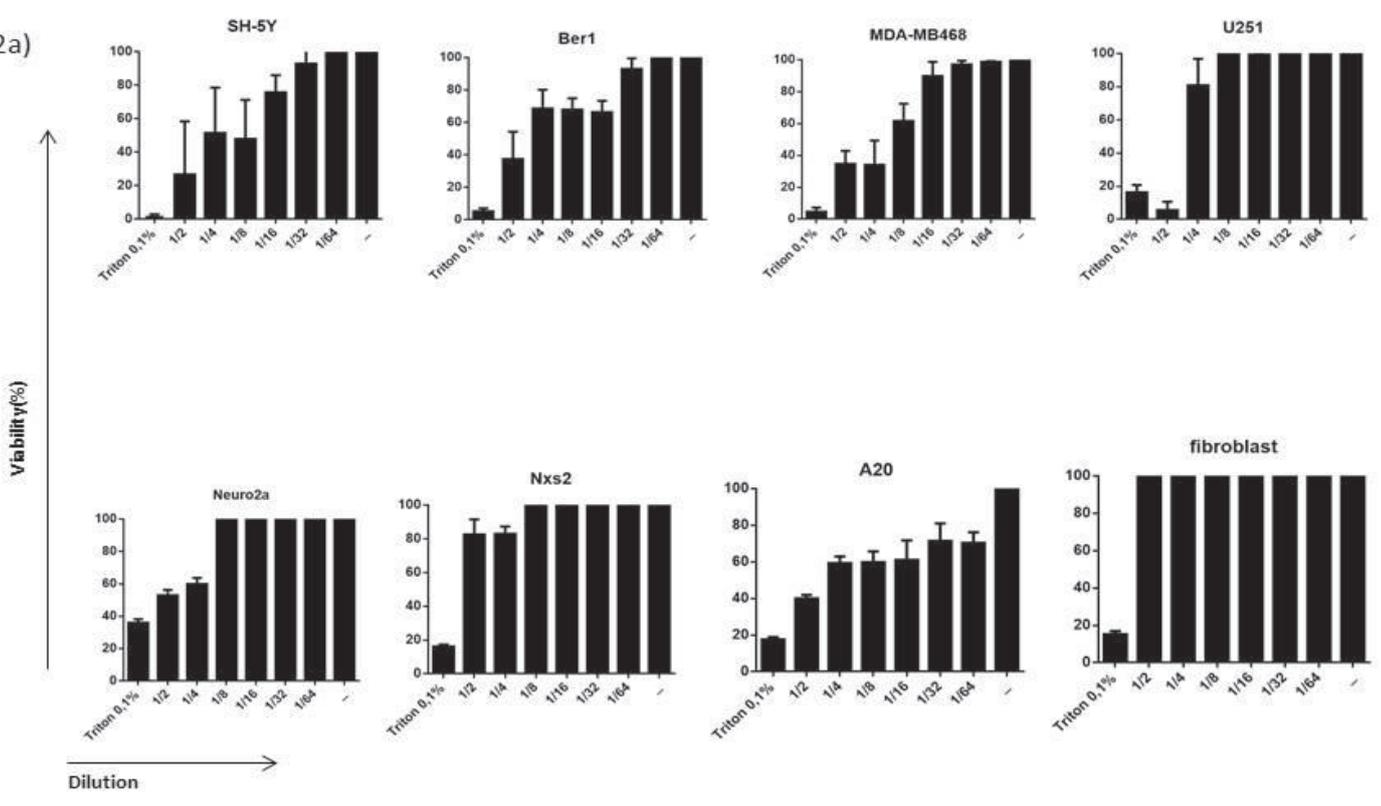
Figure 1: a) *NF- κ B* activation independently of TLR by Rotarix vaccine on transgenic cell lines that are transfected simultaneously by specific Toll like receptor and *NF- κ B* reporter. b) *NF- κ B* activation by Rotateq vaccine on transgenic

cell lines c) not IRF activation by Rotavirus containing vaccines (Rotateq and Rotarix) on transgenic cell line transfected by IRF3 reporter cell line .d) Dose-dependent cellular cytotoxicity induced by Rotavirus containing vaccines on parental cell line .Rotavirus containing vaccine is highly cytotoxic at high concentration on parental cell line. Triton was used as positive control. Cellular viability was studied by SRB test. Dilution of Vaccines was performed from 1/2 to 1/1000000, blue line is vaccine and the red line is natural ligand of each TLR and considered as a positive control.

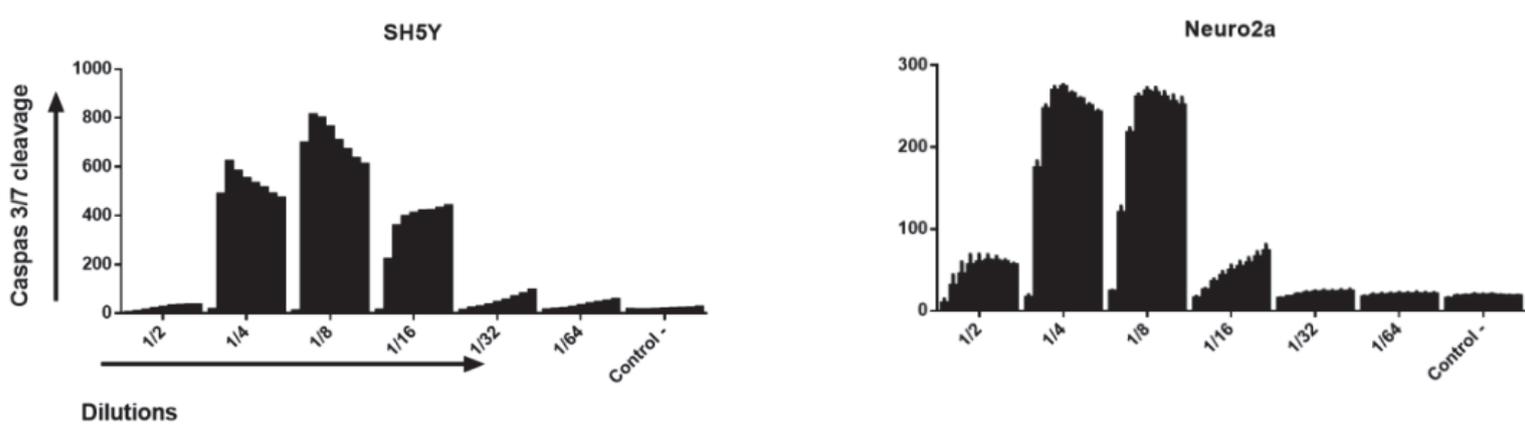
2) Rotavirus vaccines have oncolytic properties on cancer cells and induce immunogenic cell death:

In the previous bioluminescent assays, we observed that the NF-κB activation was dose dependent and diminishing upon dilutions of the Rotavirus vaccines. However, at high concentrations, this activation was lost. In order to explain this phenomenon, and because the cell viability was low in our assays at high rotavirus vaccine concentrations, we wondered if the vaccine had direct cytotoxicity at high concentration. Using the sulforhodamine B (SRB) cell cytotoxicity assay, we found that Rotavirus vaccines were killing cells at high concentrations while activating NF-κB at less cytotoxic dilutions (Figure 1c). Interestingly, using different types of human and murine cancer cell lines (Neuroblastoma, Triple negative breast cancer, lymphoma and glioblastoma) we observed a dose dependent cytotoxic effect of the Rotavirus vaccines on different tumor cell lines but not on fibroblast primary cell cultures (Figure 2a). Using a caspase assay, we found that Rotavirus vaccines induced a caspase 3/7 cleavage in dying cancer cells at diluted concentrations but not in high concentrations of Rotavirus vaccines (Figure 2b). We next studied if this cellular death induced by vaccine is immunogenic. We observed that vaccine 1/2 can induce a very high expression of Calreticulin almost as strong as Doxorubicin suggesting that Rotavirus vaccine can induce immunogenic cell death on different tumor cells type (Figure 2c).

2a)



2b)



2c)

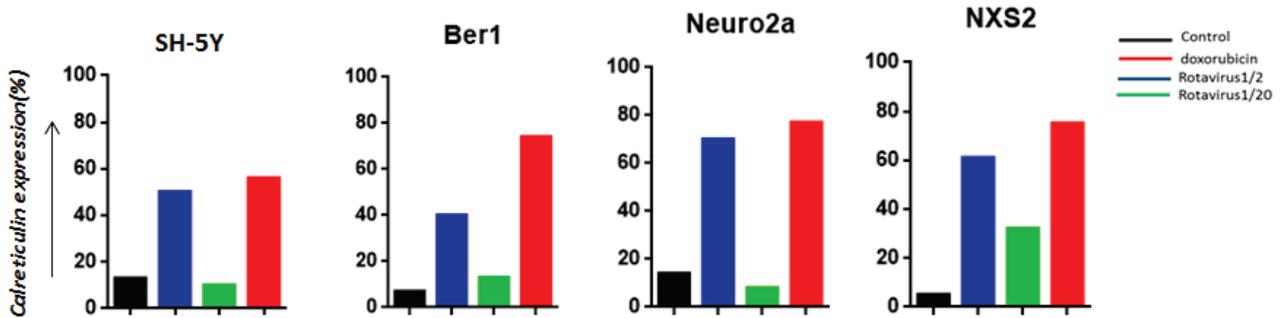
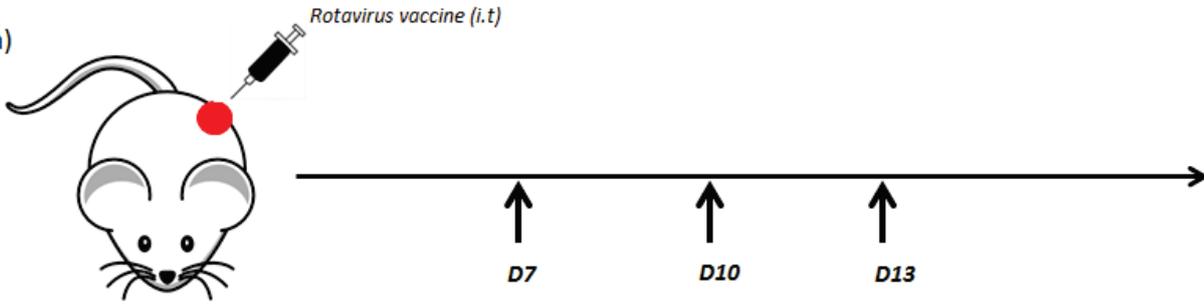


Figure 2: a) Cellular cytotoxicity induced by Rotavirus vaccine on different human cancer cell line (SH-5Y, Ber1, MDA-MB468 and U251) and murine cancer cell line (Neuro2a, Nxs2 and A20) but not on human normal fibroblast. b) Caspase3/7 cleavage on SH-5Y and Neuro2a tumor cell line was studied by incucyte microscopy after being exposed with different dilution of Rotavirus vaccine for 18 hours (each bar represents 2 hours). c) Calreticulin expression was studied on human (SH-5Y and Ber1) and murine (Neuro2a and NXS2) neuroblastoma cell line by flow cytometry in two different concentration (1/2 and 1/20) of Rotavirus vaccine. Doxorubicin is used as a positive control.

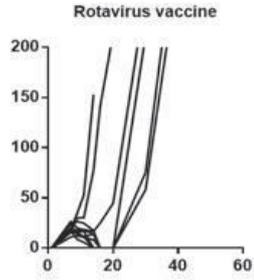
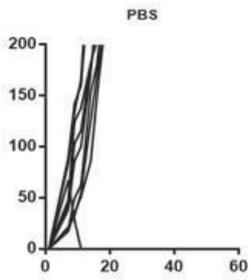
3) *In vitro* properties of cell killing translate into anti-tumor activity in vivo which is partly relying on the immune system:

In vitro oncolytic activity of Rotavirus vaccine leads us to study its anti-tumor effect in vivo in Neuroblastoma and Lymphoma models. 3 consecutive intra-tumoral injection of Rotavirus every 3 days conducted to complete tumor regression in around 20 to 30% of the mice and also slow down tumor growth in 70-80% of the mice (Figure 3a). To discriminate between direct anti-tumoral effect and immune mediated activity of the vaccine we performed experiments in immunodeficient mice (NSG mice). The rotavirus vaccine slows down tumor growth illustrating its direct oncolytic activity. However, its antitumor effect is diminished in NSG mice with no complete tumor rejection, demonstrating the contribution of the immune system in the antitumor effect of the vaccine (Figure 3b).

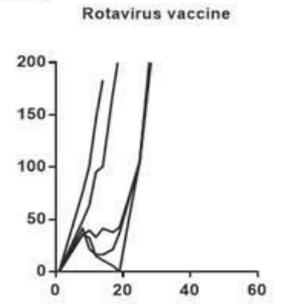
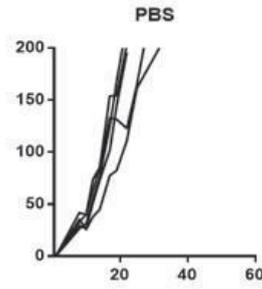
3a)



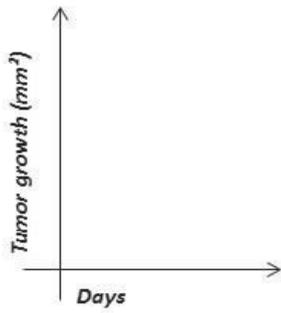
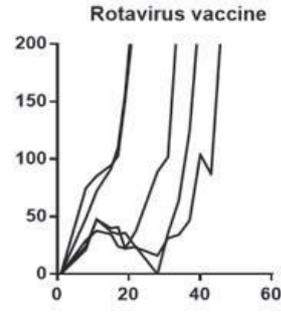
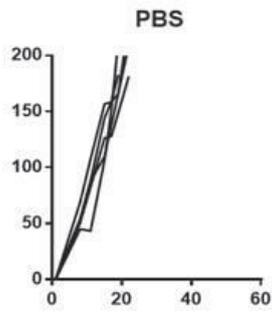
Neuroblastoma (Neuro2a)



Neuroblastoma (NXS2)



Lymphoma (A20)



3b)

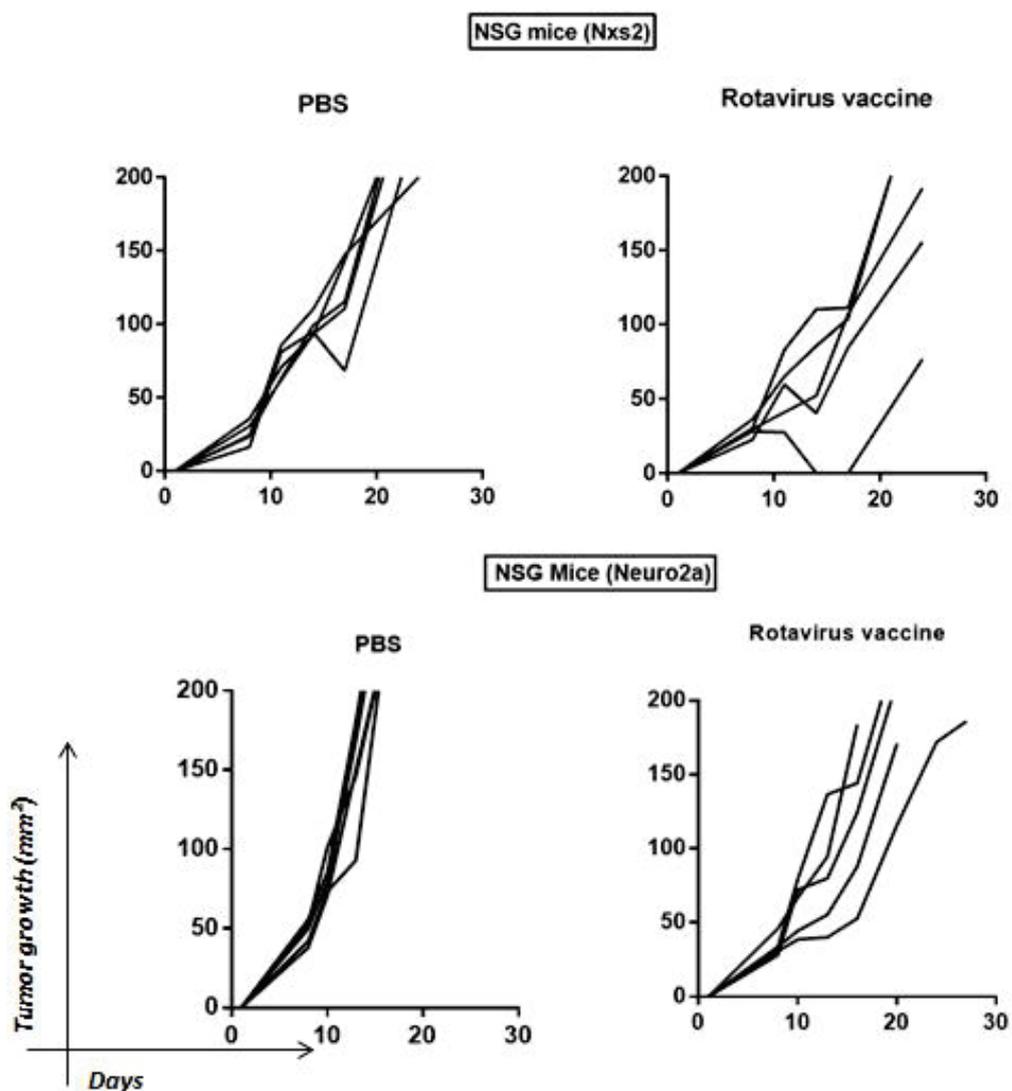
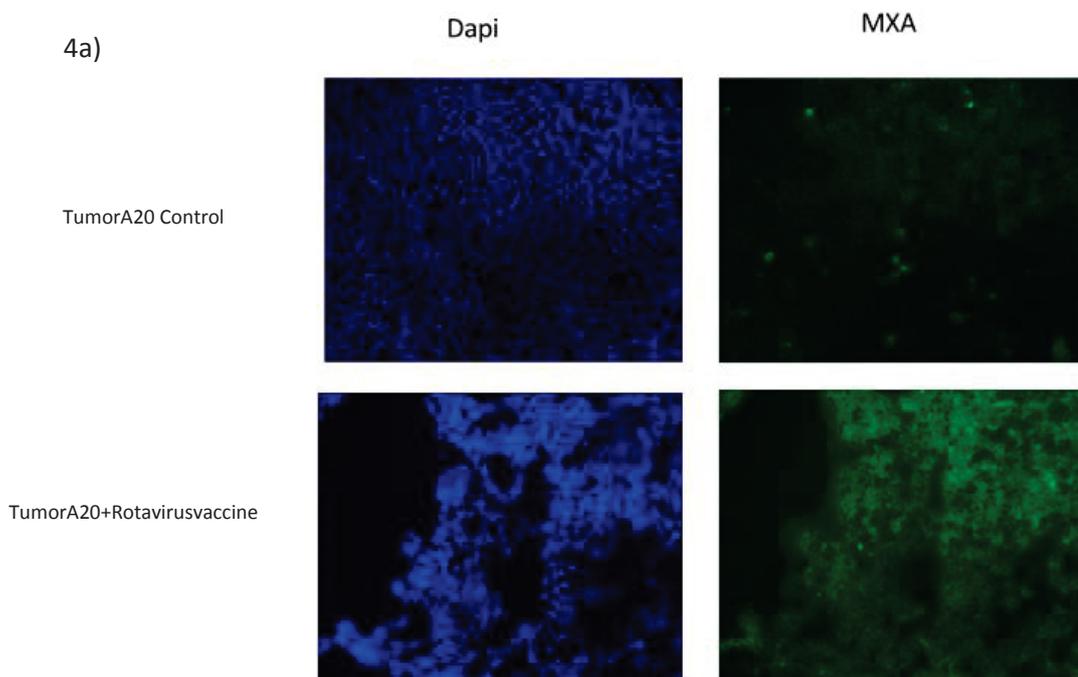


Figure 3: a) Antitumor activity of Rotavirus vaccine in immunocompetent mice was observed in Neuroblastoma (Neuro2a and NXS2) and Lymphoma (A20) model. 50 μ l intra-tumoral injection of the vaccine was done 3 times each 3 days b) Antitumor activity of Rotavirus vaccine in immunodeficient (NSG) mice in Neuroblastoma model by the same protocol of treatment.

4) Immunostimulatory properties of intra-tumoral Rotavirus vaccine injection:

To study the inflammation signature after intra-tumoral injection of Rotavirus vaccine we studied by immunofluorescent technic the interferon-inducible MxA protein expression that is highly

regulated by type I and type III interferons(Haller & Kochs, 2011). 24 hours after intra-tumoral injection of Rotavirus vaccine, a high expression level of MxA protein was observed in treated mice compared to the control mice (Figure 4a). The tumor-infiltrating cells were also analyzed 24 hours after the first vaccine injection. We observed a remarkable increase of myeloid infiltrating cells expressing up-regulated level of CD86, suggesting the activation of these myeloid infiltrating cells. Although the total CD8 T cell number remains stable, activation markers (CD137, OX40) were strongly up-regulated at their surface. Also we observed the increase of T cell activation markers on Cytotoxic T cells. Interestingly CTLA-4 was also strongly up-regulated at the cell surface of both CD8 and Foxp3+ Regulatory T cells (Figure 4b).



4b)

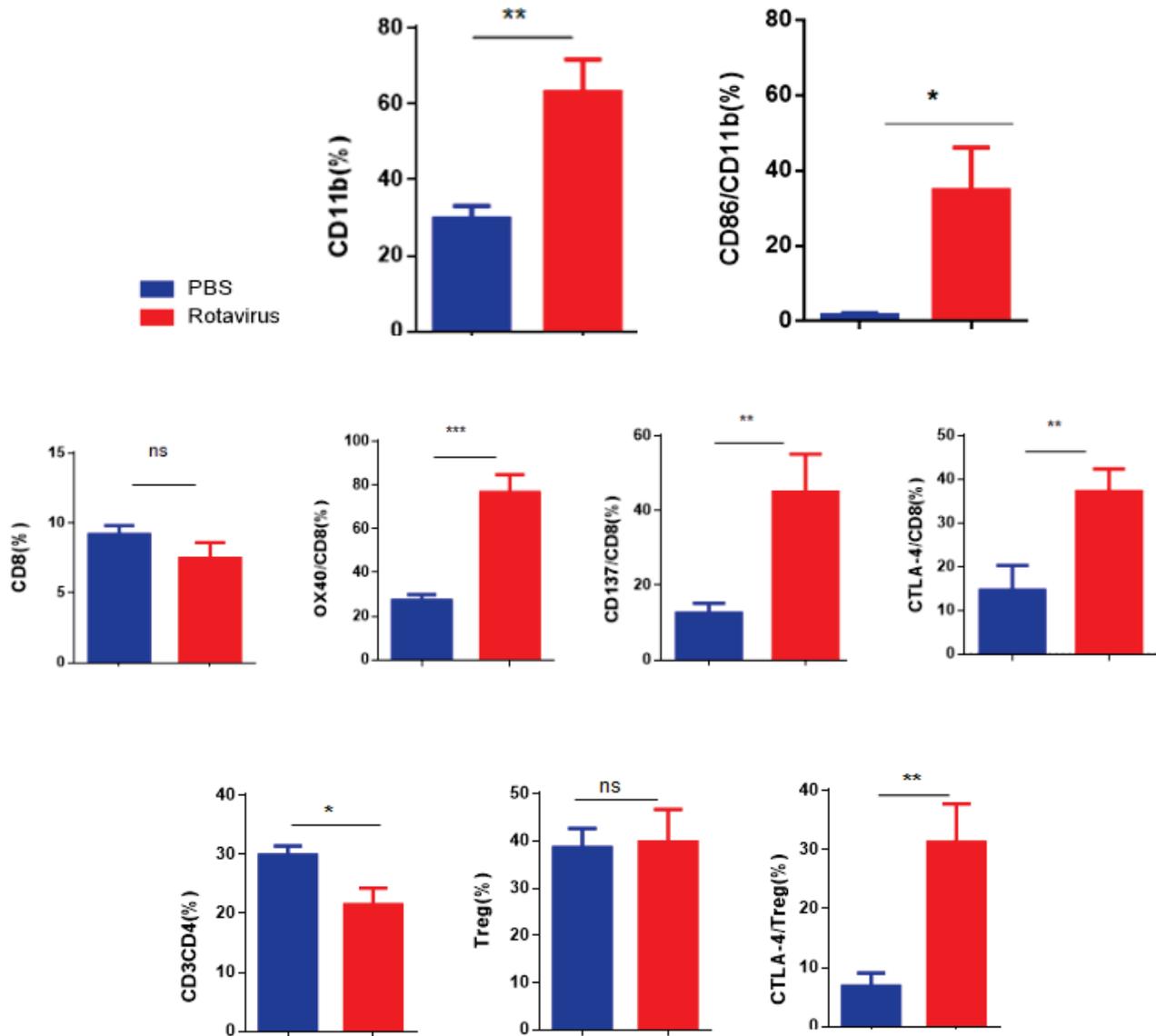
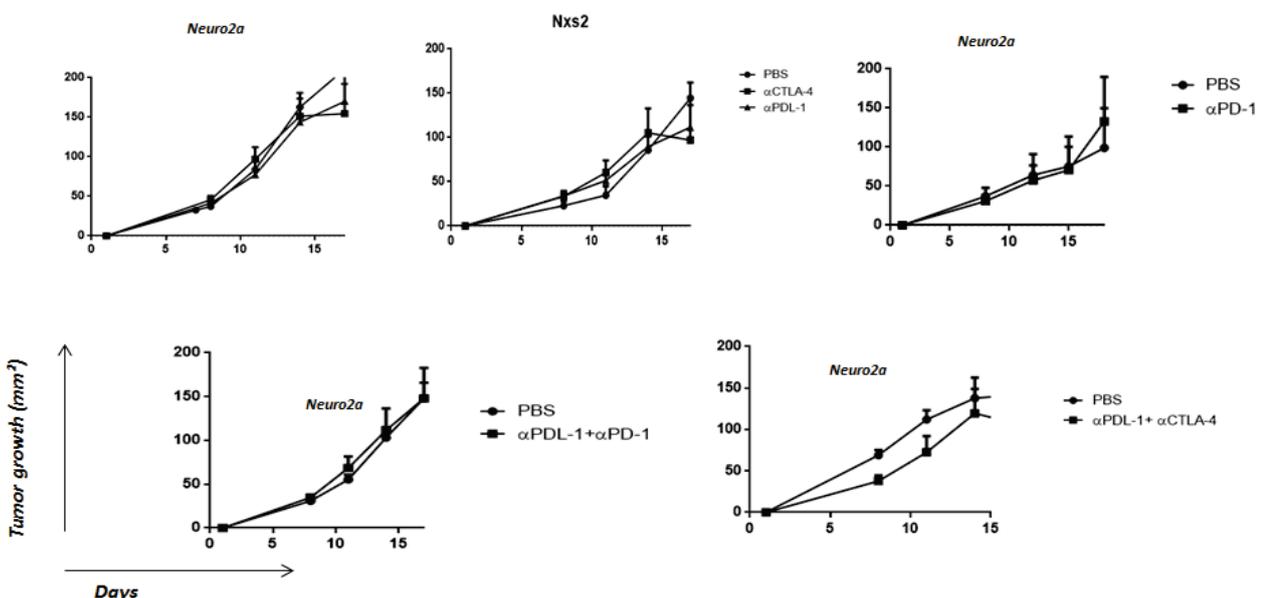


Figure 4: a) *MxA* expression was studied by IF, 24h after the first injection of the Rotavirus vaccine, in treated and control mice with Lymphoma tumor. B) Immune activation phenotype was studied by flow cytometry on tumor infiltrated immune cells 24h after the first intra-tumoral injection of Rotavirus vaccine in Neuroblastoma model (Neuro2a).

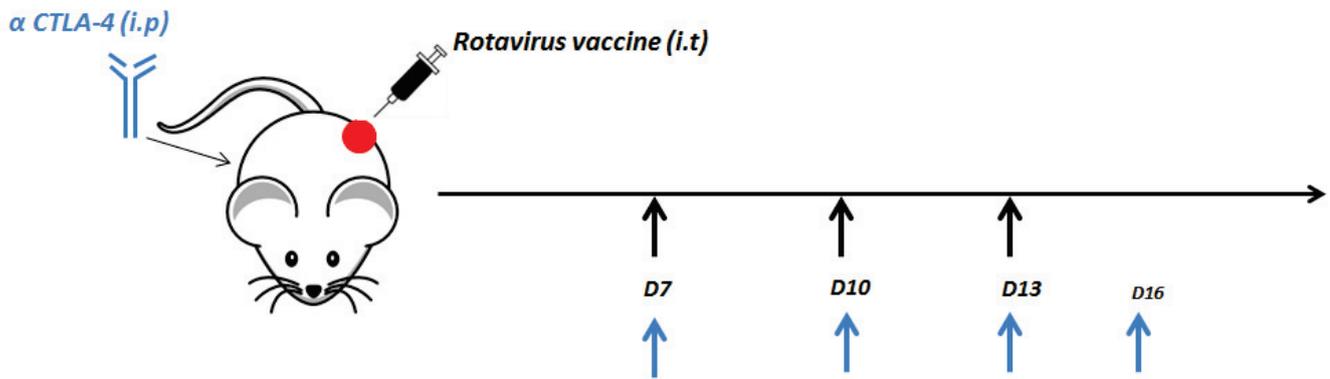
5) Synergy between Rotavirus Vaccine and anti-CTLA-4 combination in different tumor types:

Following the observed CTLA4 and CD86/PDL1 up-regulation on T cells and myeloid cells, respectively, we designed combination experiments between Rotavirus and ICB. First, mice were treated with anti-CTLA-4 anti-PDL-1 or PD-1 alone or in combination in systemic injection (intra peritoneal). We observed no significant effect in monotherapy or in association in the 2 syngenic tumor models, Lymphoma and Neuroblastoma (Figure 5a). Very interestingly we observed a synergic effect of the combination of Rotavirus+anti-CTLA-4 antibody with up to 60% of complete tumor regression in Neuroblastoma (Figure 5b) and 70% in lymphoma (Figure 5c) In contrast, anti PDL-1 antibody didn't synergize with Rotavirus vaccine (Figure 5d). The cured mice were protected upon rechallenge with the same tumor, demonstrating induction of active immune memory response in cured mice (Figure 5e). After confirming the oncolytic properties of the live rotavirus vaccine strain in vitro (not shown), we studied its anti-tumor activity in vivo. The initial virus stock is highly concentrated compared to the vaccine (mixt of 5 strains between around 100 to 1000 times more concentrated compare to the vaccine) we thus compare intra-tumoral injection of pure virus, 1/10 and 1/100 diluted virus alone. We observed an anti-tumor effect of rotavirus at all virus dilutions with around 50% of complete regression. In combination with anti CTLA-4 a potent synergy was observed with, 100% of complete regression at all virus dilution tested (Figure 5f).

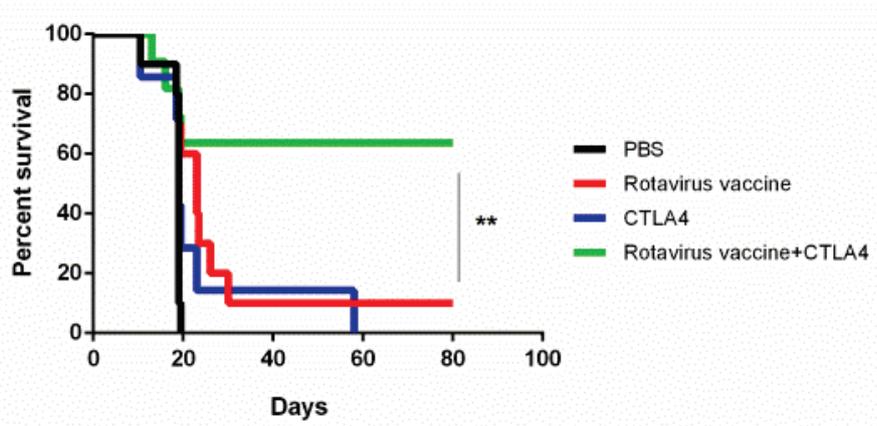
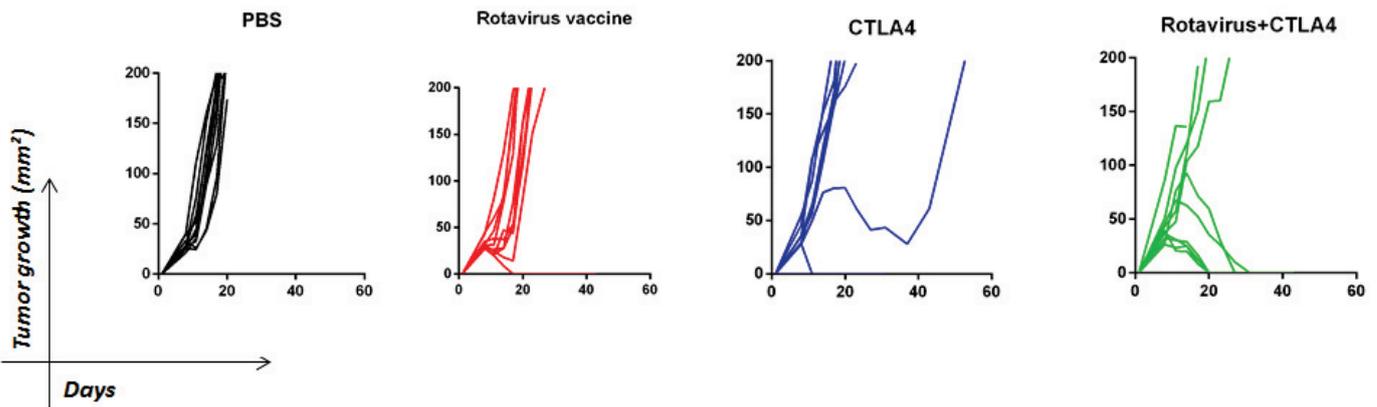
5a)



5b)

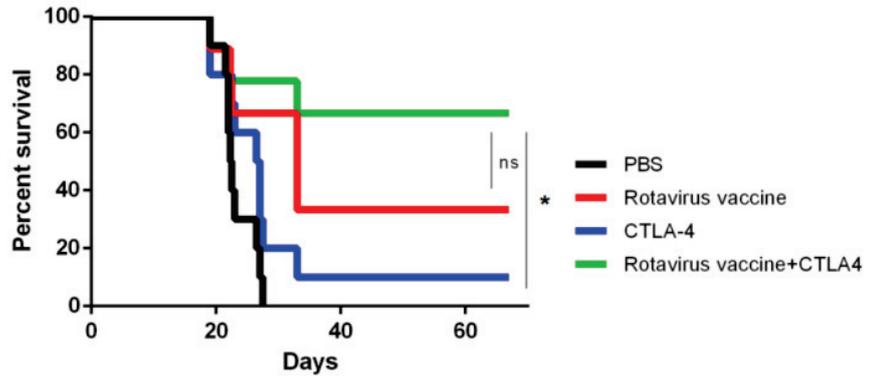
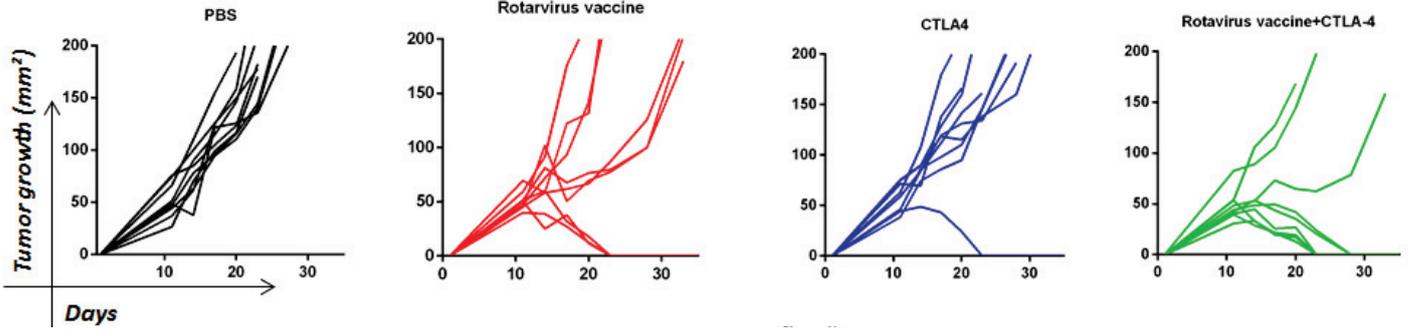


Neuroblastoma(Neuro2a)



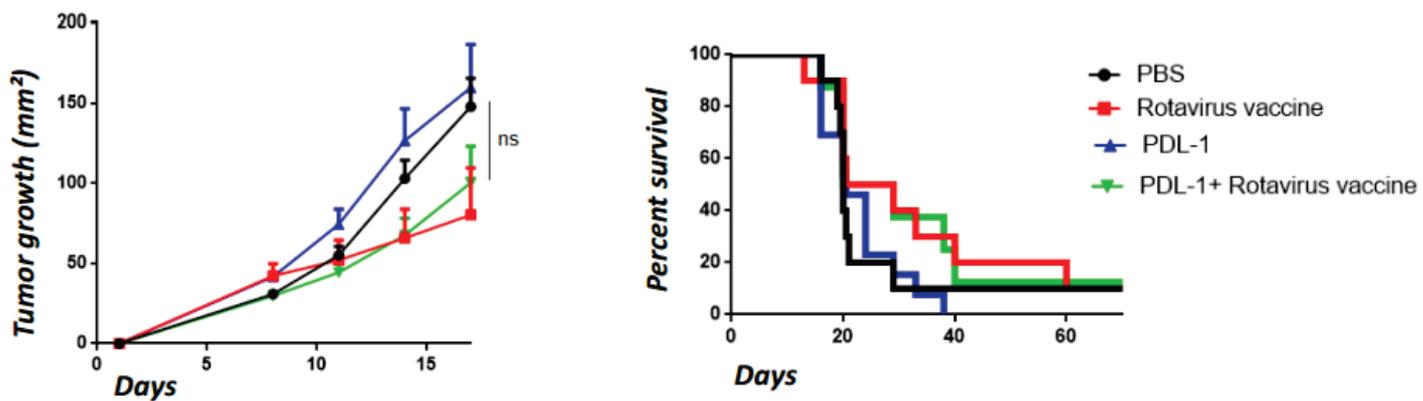
5c)

Lymphoma (A20)



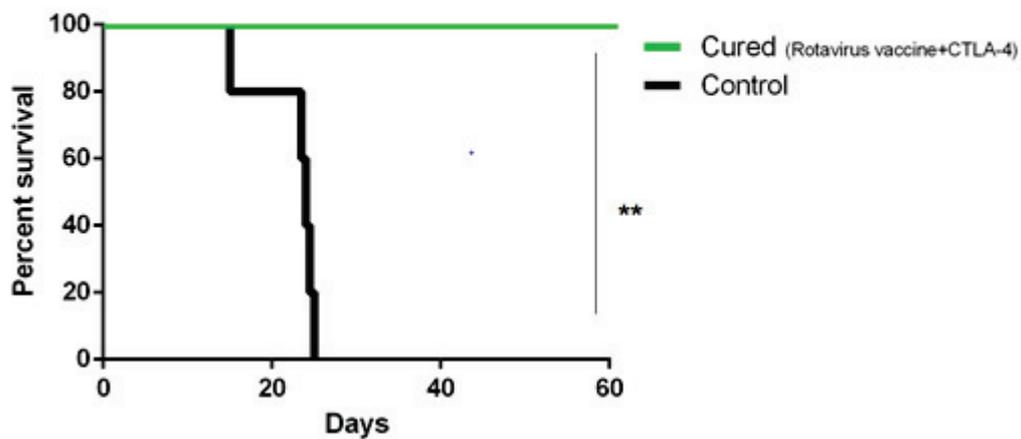
5d)

Neuroblastoma (Neuro2a)

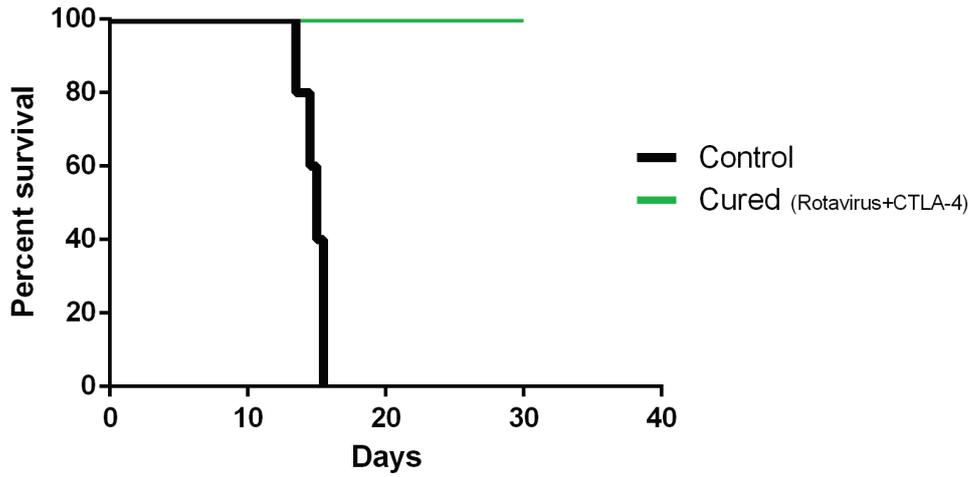


5e)

Neuroblastoma (Neuro2a)



Lymphoma (A20)



Lymphoma (A20)

5f)

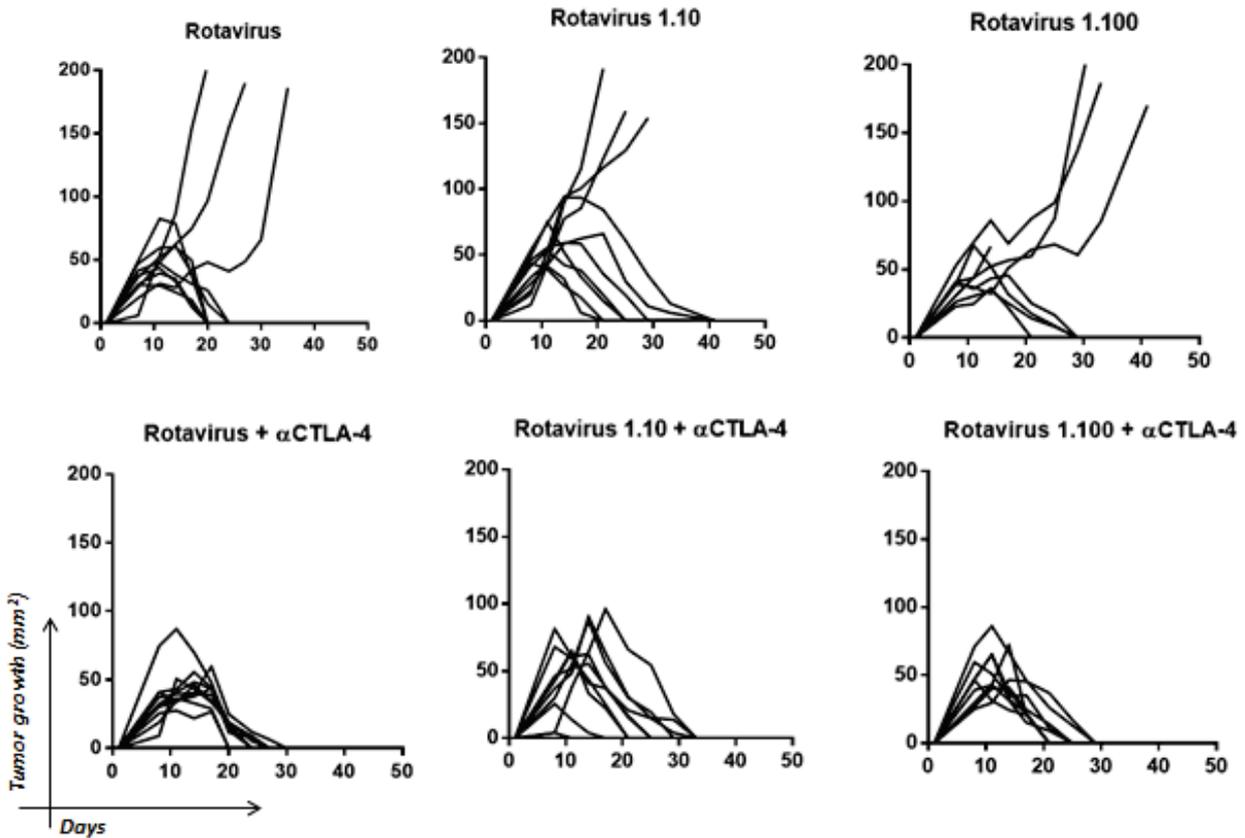
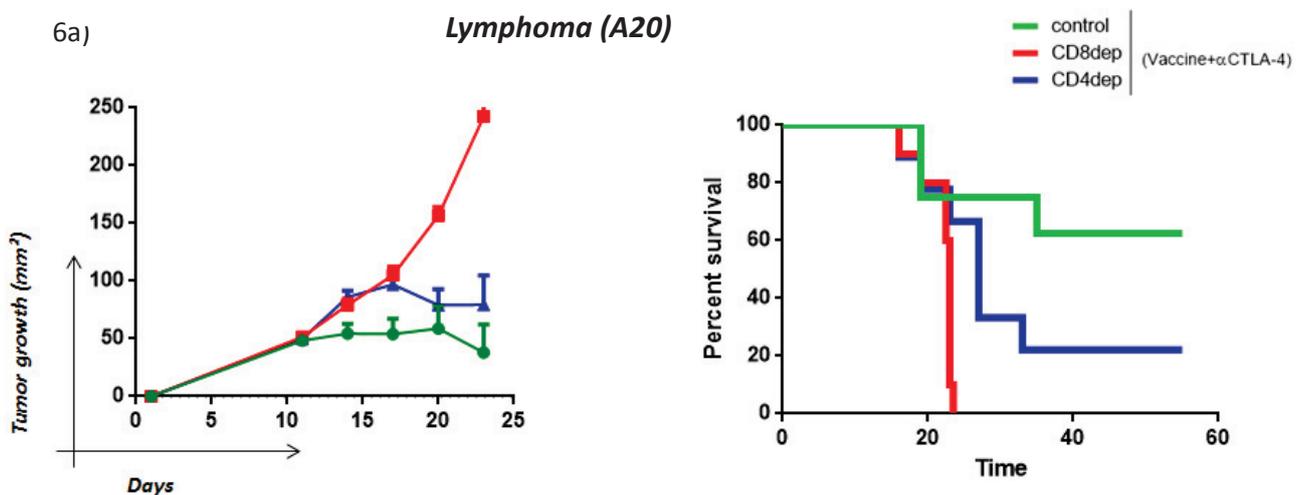


Figure 5: a) Immune checkpoint blockade monotherapy with anti-CTLA-4, PD-1, PDL-1 and their combination in NB model. 200µg of the antibodies were injected systematically 4 times each 3 days. B) Intra-tumoral injection of Rotavirus vaccine combined with systemic injection of anti CTLA-4 in NB model c) Rotavirus and anti CTLA-4

combination in Lymphoma. d) Rotavirus combined with anti PDL-1 in NB. e) Protection of the cured mice in 2 models of NB and Lymphoma from tumor rechallenge assay. The cured mice from their primary tumor were reinjected by tumor cells but they were all protected from tumor growth .f) In vivo titration of Rotavirus vaccine strain. Antitumor effect of Rotavirus vaccine strain was studied in 3 different concentration (pure, 1/10 and 1/100 diluted Rotavirus vaccine strain).

6) Tumor specific immunity response was induced after the combination of Rotavirus vaccine with ICB antibodies:

To demonstrate the contribution of the immune populations in this therapeutic synergy we depleted CD4 and CD8 T cells in Balb/c mice transplanted by murine lymphoma. We observed that in CD8 depleted mice the anti-tumor effect of the combination therapy was drastically abolished. In contrast, the depletion of the CD4 T cells has virtually no impact on the therapeutic response. This demonstrates the important role of CD8 population in the response to the treatment (Figure 6a). Then to determine the specificity of the anti-tumor response, Balb/c mice cured from A20 lymphoma, were reinjected with both 4T1 breast cancer carcinoma and A20 lymphoma. 4T1 breast carcinoma developed in these cured mice whereas A20 lymphoma tumor didn't (Figure 6b). In another experience, splenocytes from A20 lymphoma cured mice and control mice, were exposed to A20 cell line in vitro for 4 days. After 4 days CD8 cells were stained for intracellular IFN- γ , surface CD137 and OX40 activation markers. We observed that cured mice produce higher levels of intracellular IFN γ and expressed up-regulated surface activator marker such as CD137 and Ox40 compared to control mice. All of these data suggest that Rotavirus + anti-CTLA4 combination therapy induced anti-tumor specific immune response.



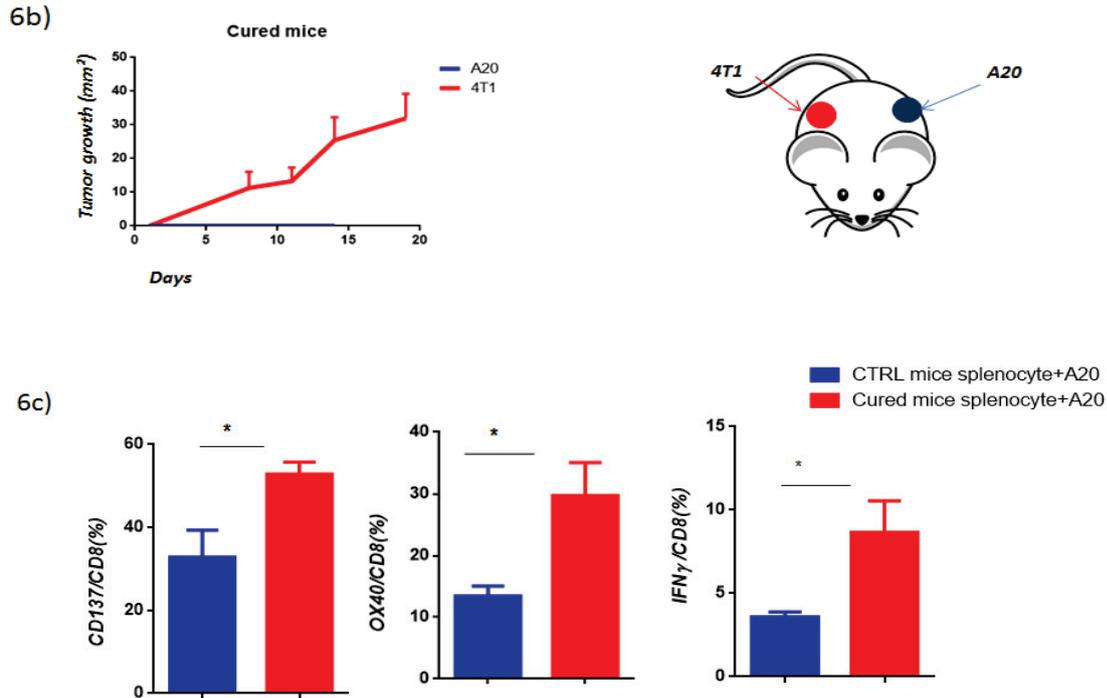
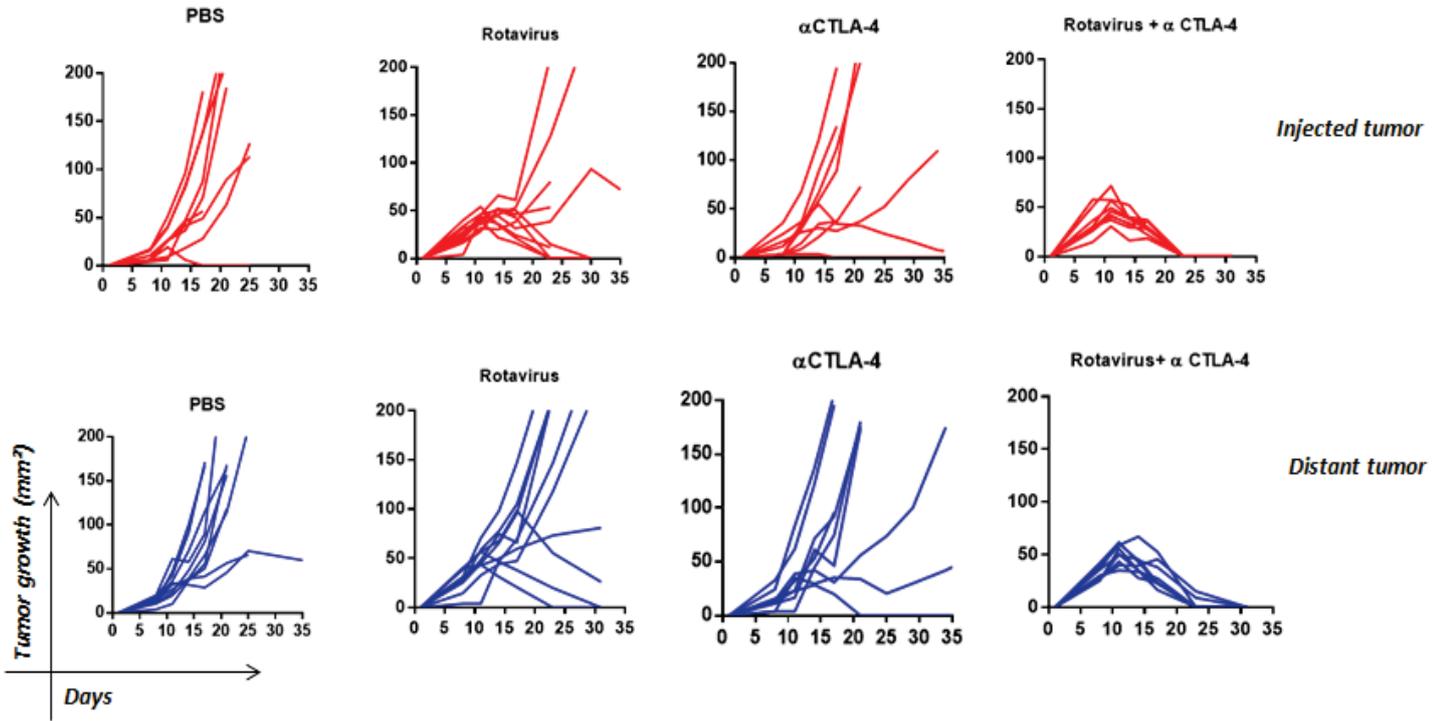


Figure 6: a) CD4 and CD8 depletion in Balb/c mice treated with Rotavirus and anti CTLA-4. b) Lymphoma (A20) cell line and breast cancer (4T1) cell line are injected into the Balb/c mice cured from lymphoma. c) Lymphoma cured mice splenocyte and control mice splenocyte exposed to A20 cell line in vitro for 4 days and CD8 cell's activation was studied by flow cytometry and by staining of activation markers (OX40, CD137 and IFN γ).

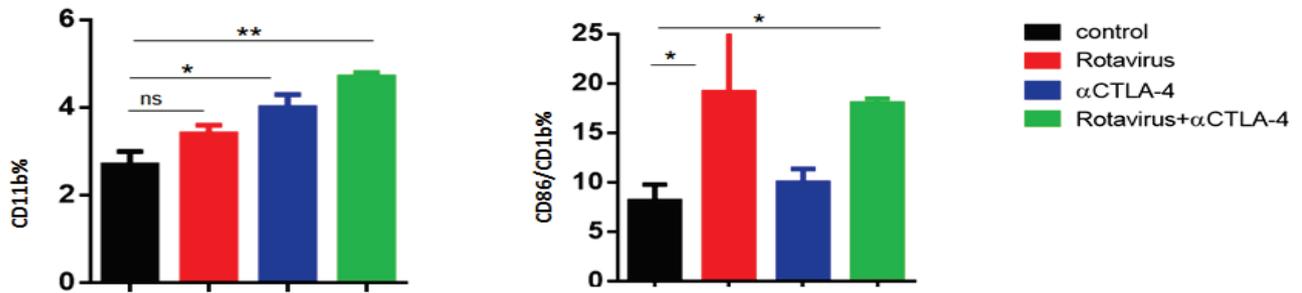
7) Abscopal effect of combination therapy:

To assess if this treatment by Rotavirus and anti CTLA-4 antibody can also induce antitumor effect on distant tumor, we injected Lymphoma (A20) tumor in two flanks of BALB/C mice at the same time. Rotavirus was injected in one tumor site and CTLA-4 was injected systemically. Despite very aggressive feature of the two lymphoma tumors, to the treatment rescued 100% of the mice with complete regression of both tumor (Figure 7a). 24 hours after the second injection of Rotavirus and anti CTLA-4 (D4 of the treatment), we observed an increase in the total number of the myeloid cell only in the injected tumor with Rotavirus (1/100) alone and combined to anti CTLA-4 group, however their activation (CD86 upregulation) was detected in both injected and distant tumors in Rotavirus groups with and without anti CTLA-4. Despite the same number of CD4 and CD8 T cells a high CD8 T cell activation was observed in both injected and distant tumor. These results suggest a systemic antitumor immunity response leading to complete regression of primary tumor and metastatic lesion.

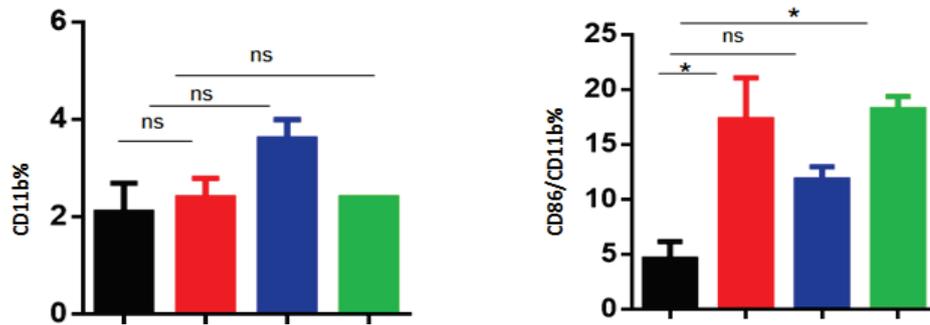
7a)



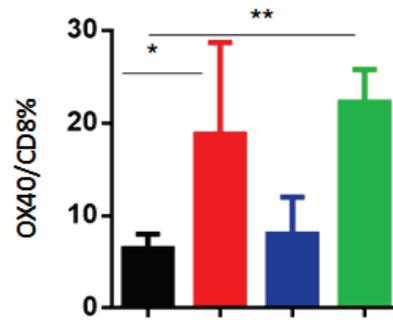
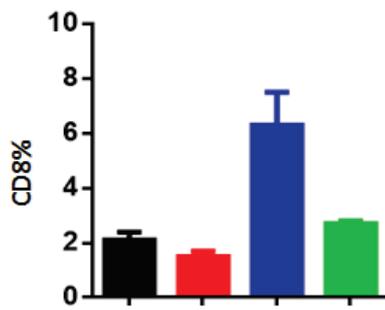
7b) *Injected tumor*



Distant tumor



Injected tumor



Distant tumor

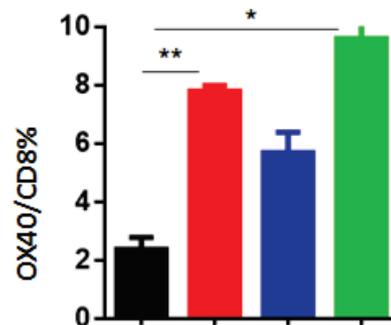
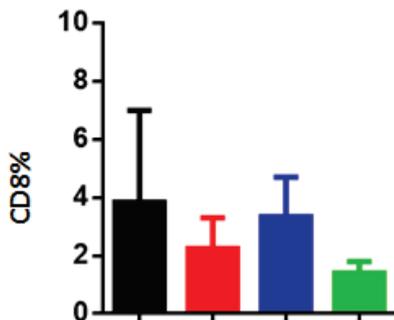


Figure 7: a) Abscopal effect in Balb/c mice transplanted by 2 tumor at the same time. The intra-tumoral injection of the vaccine was done in only one tumor. b) Immune infiltrated cells analyzed by flow cytometry, 24h after the second injection, in injected and distant tumors. In black PBS treated mice, in red Rotavirus treated mice, in blue CTLA-4 treated mice and in green Rotavirus combined with CTLA-4 treated mice.

Discussion:

The immune checkpoint blockade antibody therapy is a very successful therapeutic strategy in different types of cancers both in preclinical and clinical trials. However, there is still an important fraction of the patients that are resistant to these therapies. This study was designed to evaluate strategies to overcome this resistance. We previously showed that intra-tumoral injection of PRR agonists can induce an anti-tumor immune stimulation that can synergize with ICT (Marabelle et al., 2013). Commercially available anti-infectious vaccines represent a potential alternative source of PRRs agonists. While evaluating the TLR stimulating capacity of different viral and bacterial based vaccine, we identified that Rotavirus vaccine has the capacity to stimulate NF- κ B independently of TLRs and IRFs. The NF- κ B activation mechanism by Rotavirus remains unclear. It can be direct by one of the viral structural or non-structural protein as it has been shown before in different study that viral protein interact with host protein implicating In IFN response (Barro & Patton, 2007). This activation might be indirect via the cytotoxicity induced by the vaccine on the tumor cell resulting in the release of DAMP or the production of cytokines and

the activation of different pathway that leads to NF- κ B activation. The cytotoxic effect of Rotavirus on tumor cells occurs simultaneously with caspase 3/7 cleavage that is observed at the same virus concentration that the NF- κ B activation, strengthening the link between the two effect. The cell death induced by Rotavirus is not yet well understood but it has the feature of immunogenic cell death through the induction of expression of Calreticulin at the surface of apoptotic (AnnexinV+) cells. It has been shown in Kroemer lab that Calreticulin expression can be considered as a specific marker of immunogenic cell death (Obeid et al., 2007). However this immunogenic cell death induced by Rotavirus needs to be confirmed by measuring the release of ATP and HMGB1. Rotavirus has shown specificity for tumor cells as it is inducing cell lysis of all tumor lines evaluated but cannot kill normal fibroblasts. The definitive proof that Rotavirus is an oncolytic virus addicted to an oncogenic pathway and not just targeting proliferating cells, it should be studied in more detail in different cell lines from normal, immortalized and transformed cell lines and in healthy proliferating cells such as PBMC. The sensitivity of the tumor cells to Rotavirus cytolysis might be associated to a defect in type I IFN production, as previously reported for measles virus cytolysis of human pleural mesothelioma cells (Achard et al., 2014). In this context, 24h after intra-tumoral injection of Rotavirus we observed the expression of MxA protein by immunofluorescence but the source of type I IFN leading to MxA protein expression may originate from cancer cells or tumor infiltrating immune cells. We also observed the activation of tumor infiltrating immune cell which demonstrate the immune stimulating properties of the virus. As expected the therapeutic activity of the combination of Rotavirus and CTLA-4 was mainly CD8 T cell dependent and specific anti-tumor immunity was demonstrated. In mice bearing 2 tumors we demonstrate a clear abscopal effect of the combination of Rotavirus with CTLA-4 in 100% of the mice. In addition to this complete regression, we also observed immune cell activation in both injected and non-injected tumors. Our work is in concordance with the work of Allison's lab reporting that intra-tumor injection of New castle disease virus can overcome to a systemic resistance to anti CTLA-4 therapy (Zamarin et al., 2014). Through the induction of the tumor immunogenic cell death and the production of type I IFN the Rotavirus injection is leading to activation of the myeloid antigen presenting cells likely resulting to CD8 T cell priming. CTLA4 and it ligands (CD80, CD86) are clearly upregulated upon virus injection on both T cells and myeloid cells respectively, explaining the potent synergy between Rotavirus and anti-CTLA4. In addition anti-CTLA4 may also block Treg suppressive function that shows upregulated CTLA4 expression upon virus infection. Another subject that needs to be discussed is the route of virus delivery to the host. Intra-tumoral injection of the virus will prevent the side effect of systemic injection. This way of administration is also simple for easily accessible cancer such as melanoma or in different types of cancer with skin metastasis. Furthermore, intra-tumoral injection will prevent virus neutralization in vaccinated patients. In fact, data obtained with NDV

virus (Auteur, SITC 2016, not published yet) have suggested that immunized mice with the NDV respond better to the treatment because of the switch from antiviral response to anti-tumor response. We have shown that our combination strategy is very efficient in 2 tumor model with totally different immune infiltrated cells. In Neuroblastoma which is a pediatric cancer, highly infiltrated myeloid cell and very low level of somatic mutation and consequently low level of T cell called “cold tumor” as well as in Lymphoma which is infiltrated with very low level of myeloid cells and moderate T-cells frequency. With the intra-tumoral injection of Rotavirus we succeed to reheat our tumors and induce an antitumor inflammation conducting to systemic eradication of cancer, and protection against an eventual recurrence. Rotavirus vaccinal strain is a very simple virus compared other oncolytic engineered viruses, and can be easily enter to the phase I clinical trial.

Material and methods:

Reagents:

Commercially available Rotavirus vaccines Rotateq (Merck), Rotarix (Sanofi) have been both tested in the experiments and will be referred as Rotavirus vaccine throughout the manuscript. Because multiple of infections cannot be established for these commercial vaccines, dilutions in culture media of pure vaccines have been used for *in vitro* experiments. Pure vaccines have been injected intra-tumorally for *in vivo* experiments.

Cell culture:

SY5Y cells (a neuroblastoma cell line) were provided by the American Type Culture Collection (ATCC) but other Neuroblastoma tumors were obtained from patients diagnosed at the Leon Berard Comprehensive Cancer Center between 1993 and 2004. For some patients, tumor cell lines were derived in the lab. These cell lines were cultured in RPMI 1740 medium (Gibco-life technologies) supplemented with 10% of heat inactivated fetal bovine serum (FBS), 1% amino acid not essential, 1% Glutamine, 1% antibiotic (penicillin and streptomycin) (Life technologies). Transgenic hTLR-LUC cell lines were seeded in DMEM (Gibco-life technologies) supplemented with 2% fetal bovine serum, 1% Glutamine, 1% Sodium Pyruvate and 1% amino acid not essential (Gibco-life technologies). Each cell line was selected by its respective antibiotic (TLR2, 4 by Zeocin, Hygromycin, Blastidicin and TLR3, 5, 6, 7, 8, 9 by Zeocin, Blastidicin and LUC by Zeocin) (Invivogen). All cell lines were incubated at 37°; 5% CO₂. A20 cell lines were obtained

from were a kind gift from Prof Ronald Levy lab, Stanford University, California, USA. Skin fibroblast cell lines from Caucasian individuals were obtained from the cell culture collections of the Centre de Biotechnologie Cellulaire, CBC Biotec, CRB-Hospices Civils de Lyon, France

Bioluminescence assay:

Human embryonic kidney cells 293 (HEK 293) were stably transfected with plasmid allowing the constitutive expression of TLRs of human origin (hTLR) (InvivoGen San Diego, CA, USA). The resulting cell lines 293/h TLR2-CD14, 293/hTLR3, 293/hTLR4-MD2-CD14, 293/ hTLR5, 293/hTLR2/6, 293/hTLR7, 293/hTLR8 and 293/hTLR9 were purchased from Transgene Inc (www.transgene.fr). Cells were cultured in DMEM supplemented with 10% inactivated Fetal bovine Serum (FBS), 40 µg Gentamycin (Sigma), 2mM Glutamine, 1 mM Sodium Pyruvate and 1x non-essential amino acids (life technologies), in the presence of 10 µg/ml Blasticidin. Hygromycin B (Roche) was added to a concentration of 100 µg/ml per cell lines 293/hTLR2-CD14 and 293/hTLR 4-MD2-CD14.

To generate reporter model of TLR activation, HEK-293 stably expressing hTLRs or control HEK293 cells were then stably transfected with the NF- κ B inducible reporter plasmid pNiFty-luc (Invivogen) using the Calcium phosphate method. pNiFty-luc encodes Zeocin-resistance gene as well as the firefly luciferase gene under the control of an engineered ELAM1 promoter comprising five NF- κ B sites and the proximal ELAM promoter. Stable transfectants were selected in the presence of 100 µg/ml Zeocin (Invivogen). For stimulation assay cells were seeded in DMEM supplemented with 2% FBS , 40 µg/ml Gentemycin, 2 Mm L-Glutamin, 1Mm Sodium Pyruvate and 1x MEMAA on flat-bottom 96 well plates. The next day, medium was replaced by fresh medium and respective ligands. 18-20 hours later, cells were lysed in 30 µl of 5x lysis buffer (Promega). Firefly luciferase activity was quantified using 20 µl lysate by integrated measurement of flash luminescence over 1 sec (Bethold Tristar LB941, Grenier) in 96 wells after injection of 20 µl 1x "In-House" luciferase revelation buffer (20 mM Tris Ph7.8, 1.07 mM MgCl₂, 2.7 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 470 µM luciferine, 530 µM ATP and 270 µM CoEnzyme A) by TECAN machine.

Vaccine preparation:

Commercially available anti-infectious vaccines were tested for their PRR agonist abilities. The selection of the vaccines used in our study was based on their structural properties in order to cover the spectrum of life pathogens. The vaccines were diluted in culture media (classical

DMEM) in 10 serial dilutions Vaccines assays was performed in triplicate on each TLR transgenic cell line.

Cytotoxicity test (SRB):

SRB assay (Sigma-Aldrich TOX6-1KT) was used to evaluate the cytotoxicity activity of different dilution of Rotavirus vaccine on human tumoral cells .Triton 0.1% was used as the positive control. Briefly, cancer cells (100,000) were plated in a 96-well plate and then treated with serial dilution of Rotavirus vaccine in medium for 12 hours. The treated cells were fixed with 50 µl of 10% cold trichloroacetic acid solution for 1 h at 4°C, washed with distilled water five times, and then dried at room temperature. The dried cells were stained with 50 µl of 0.4% SRB for 10 min and rinsed with 1% acetic acid solution five times. After being dried, dye was dissolved in 10 mM Tris buffer and measured at 565 nm on a TECAN machine.

IncuCyte microscopy:

The caspase cleavage after being exposed with the vaccine was studied by using IncuCyte Caspase-3/7 Apoptosis Assay Reagent (Cat No 4440 Essen Bioscience) and incuCyte microscopy.

Calreticulin Expression:

Calreticulin expression were measured using mouse monoclonal anti-calreticulin antibody (Abcam ab22683) stained by goat anti mouse conjugated antibody (Abcam Dylight 488). Different type of cellular death was identified by AnnexinV /PI staining and calreticulin expression was measured on different population by Flow cytometry.

Mice:

6 to 8 week A/J and Balb/C mice were provided from Harlan, the NSG mice from Charles Rivers. All of the experiments were conducted in the Leon Berard Center animal facility.

Tumor transplantation:

All the tumor cell lines (Neuro2a, Nxs2, A20) were transplanted 5×10^6 and 4T1 in 10×10^5 in 100 μ l of PBS in the Right flank (unilateral tumor) and in left flank. On day 7 after tumor transplantation the treatment were started.

Tumor treatment:

Sub-cutaneous tumor sizes were monitored with a digital caliper (Mitutoyo) every 2 to 3 days and expressed either as surface (length \times width) Mice were killed when s.c. tumor size reached 2 cm².

Tumor immunotherapy:

Treatment started when s.c. tumors reached 0.7–1 cm in largest diameter, which usually occurred around day 7 after tumor inoculation. Intra-tumoral injection of rotavirus vaccine combined with i.p injection of anti CTLA-4 was 4 at the concentration of 200 μ g in 100 μ l of PBS 4 times every 3 days.

CD4 and CD8 T cell depletions:

Anti-CD4 (rat IgG2b) and anti-CD8 (rat IgG2b) depleting mAbs were obtained from in Bioxcell laboratory, 200 μ g of these antibodies were injected intraperitoneally 2 and 1 day before treatment and 5 and 8 days after beginning of the therapy.

Immune cell processing and transplant:

Tumors, lymph nodes, and spleens were turned into single cell suspensions by processing them through a 70- μ m cell strainer (BD Biosciences).

Flow cytometry:

1×10^6 Cells were surface stained in PBS with 1 μ g antibodies, Mouse Fc receptors were blocked with 1 μ g Fc γ RIII/II-specific antibody (clone 2.4G2, rat IgG2b κ ; BD Bioscience) CD45 (clone 30-f11), CD8 (clone 53-6.7), CD4 (GK1.5), foxp3 (clone FJK-16S), CTLA-4 (clone UC10-4B9), Ox40 (clone ox-86), CD137 (clone-17B5) IFN γ (clone XMG1.2) antibodies also Foxp3 staining kit (00-5523-00), Intracellular Fixation and permeabilization buffer (88-8824-00) and Brefeldin A (00-4506-51) provided from eBioscience, CD3 (clone 17A2) and CD11B (clone

M1/70) was provided from Biolegend. All of the staining was detected and analyzed by flow cytometry on a FACS Canto II (BD Bioscience).

Immunofluorescence:

Immunofluorescence staining was done using mouse anti Mxa (2G12)(NPB1-47859) provided by Novus and goat anti mouse antibody conjugated to FITC (00030748) provided from Dako.

Splenocyte stimulation with cancer cells:

Splenocytes of cured and control mice were exposed to A20 tumor cell line for 4 days *in vitro*. The ratio of splenocyte to tumor cell line was 1000:1 in RPMI medium. After 4 days the surface markers and intracellular IFN γ were stained by the antibodies (the reference were mentioned before in the flow cytometry part) and were analyzed by flow cytometry.

General discussion and perspectives:

Pediatric cancers belong to the so called “non-inflamed tumor” family with low somatic mutations, low T cell infiltration and high protumoral myeloid cells contexture. Such tumor types might need to be “warmed up” and become infiltrated by T-cells in order to respond to immune checkpoint blockade (ICT) therapy. The original idea of my thesis project was to develop innovative immunotherapy strategies to overcome the probable resistance of pediatric cancers to immune checkpoint blockade therapy by using local injections of PRRs agonist in order to stimulate the infiltrated myeloid cells. This idea was based on the rationale of previous studies showing that intra-tumoral injections of PRRs agonist can induce an anti-tumor immune stimulation that can synergize with ICT (Marabelle et al., 2013) (Houot & Levy, 2009). However waiting for the clinical development of GMP (good manufacturing practice) PRR agonists, especially in pediatric oncology, could take ages. As they contain bacterial or viral components, anti-infectious vaccines represent a potential alternative source of clinical grade PRRs agonists. The use of such commercially available “off the shelf” products allows to envision a rapid translation to the clinic of this strategy. While evaluating the capacity of almost 22 viral and bacterial based vaccines to stimulate TLRs, we identified that Rotavirus vaccines have the capacity to stimulate NF- κ B independently of TLRs and IRFs. Serendipously, we discovered that Rotavirus vaccines have also direct cytotoxic effects on tumor cells. The cell death induced by

Rotavirus is not yet fully understood and remains one of our principle questions to be elucidated. We have shown that it occurs with caspase 3/7 cleavage. Also, the cell death induced by Rotavirus has features of immunogenic cell death (ICD) such as the induction of expression of Calreticulin at the surface of apoptotic (AnnexinV+) cells (Obeid et al., 2007). We need to further analyze if this ICD implies a particular type of death (apoptosis, necrosis, necroptosis, ferroptosis, pyroptosis, autophagy...) as well as the impact of the virus virulence factor and tropism for ER stress of target cells. This very exciting discovery, lead us to further characterize the oncolytic properties of the Rotavirus and it's *in vivo* application. Rotavirus has shown specificity for tumor cells as it is inducing cell lysis of all tumor lines evaluated but cannot kill normal fibroblasts. The direct oncolytic potential of the Rotavirus was confirmed *in vivo* in immunodeficient mice suggesting a direct lysis of cancer cells by the virus. The definitive proof that Rotavirus is an oncolytic virus addicted to an oncogenic pathway and not just targeting proliferating cells, should be studied in more detail in different cell lines from normal, immortalized and transformed cell lines and in healthy proliferating cells such as PBMC. The sensitivity of the tumor cells to Rotavirus cytolysis might be associated to a defect in type I IFN production, as previously reported for measles virus cytolysis of human pleural mesothelioma cells (Achard et al., 2014). In immunocompetent mice, we demonstrated a synergic effect of Rotavirus when used in combination with anti-CTLA4 mAb. This might be explained either by up regulation of CTLA4 on intra-tumoral Treg population after treatment (even if we have not yet proven intra-tumoral Treg depletion) or by priming of T-cells thanks to CTLA4 blockade on CD8 cells and local reactivation of myeloid cells through the release of TAA, DAMPs generated by Rotavirus mediated lysis and the PRR stimulation through agonists properties of Rotavirus itself leading to type I IFN release (MXA induction). Surprisingly, whereas up-regulation of PDL1 was also observed, no synergic effect could be obtained with rotavirus vaccine in neuroblastoma. First, these experiments need to be repeated with higher concentration of viral strain instead of vaccines, and second, as it was previously demonstrated with other ICD and cancer type models, anti-CTLA4 therapies gave generally better results in mice models compare to humans. Therefore, combination of rotavirus with PD1-PDL1 blockade is an important issue that will deserve further exploration in mouse preclinical models investigating different tumor types (pediatric vs adult cancer, cold vs inflamed hot tumor, etc..). In Lymphoma mice model with moderate T-cell frequency, PDL1 or PD1 therapy combine with rotavirus could be as efficient as CTLA4 therapy. This will be address soon.

Abscopal effect observed with our combination therapy demonstrates a systemic anti-tumor immune reactivation and memory conducting to eradication of disseminated cancer cells and protection against eventual recurrences. Compared to other oncolytic engineered viruses,

Rotavirus vaccine strain is a very simple virus that can easily enter to phase I clinical trials. In conclusion, we identified a new oncolytic virus that can be quickly transfer to clinic for new immunotherapy approaches in different types of cancers by re-awaking immune system.

My work reveals a number of issues that will need to be addressed in the future. First the oncolytic properties will need to be consolidated as discussed above. Second, the type of cell death and its immunogenic nature will also deserve further investigations (HMGB1, ATP release, *in vivo* vaccination...). Third, the role of type I IFN in both the oncolytic properties and in the immunogenicity of the Rotaviruses will also be of high relevance to our team. Fourth, the characterization of the type of antigen presenting cells involved in the Rotavirus induced anti-tumor immunity will represent another line of investigation for the team. Fifth, the *in vivo* mechanism of action and the role on Treg depletion versus CTLA4 neutralization on CD8 T cells will also be important to address. Sixth, the exploration of anti-PD1/L1 blockade with Rotavirus injection will need to be extend to other tumor models known to respond to anti-PD1 (MC38 for example). Seventh, the impact of Rotavirus vaccination of the mice before tumor implantation and treatment will need to be evaluated. Last, the possibility to move this strategy toward clinical application in patient will be evaluated by the CLB immunotherapy team.

Annexes:

In this part I would like to attach all the collaborations that we have done during my PhD and the published papers (after the references you will find the papers).

1) Paper 1 (mutagenesis 2015): paradigm shift in oncology: targeting the immune system rather than cancer cells. *Tala SHEKARIAN, Valsesia-Wittmann S, Caux C, Marabelle A.*

2) Paper 2 (Sci Transl Med. 2015 Apr): Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients. *Semeraro M, Rusakiewicz S, Minard-Colin V, Delahaye NF, Enot D, Vély F, Marabelle A, Papoular B, Piperoglou C, Ponzoni M, Perri P, Tchirkov A, Matta J, Tala SHEKARIAN, Valsesia-Wittmann S, Kroemer G, Valteau-Couanet D, Zitvogel L.*

3) Paper 3 (European journal of cancer 2016 jan): Stability of Ipilimumab in its original vial after opening allows its use for at least 4 weeks and facilitates pooling of residues. *Pascale Bardo-Brouard, Victoire VIEILLARD, Tala SHEKARIAN, Aurélien MARABELLE, Alain ASTIER, Muriel PAUL,*

4) Paper 4 (accepted in annals of oncology 2017 mars): Pattern Recognition Receptors: New Immune Checkpoint Targets for Cancer Immunotherapy accepted in Annals of Oncology. *Tala SHEKARIAN, Sandrine Valsesia-Wittmann, Christophe Caux and Aurélien Marabelle.*

5) Paper 5 (thesis paper under preparation): Oncolytic properties of Rotavirus can overcome to Immune Checkpoint Blockade Therapy. *Tala SHEKARIAN, Anne catherine Jallas, Nadege goutagny, Stephane Depil, Sandrine Valssesia Wittmann* , Christophe Caux*, Aurelien Marabelle**

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Review

Paradigm shift in oncology: targeting the immune system rather than cancer cells

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Abstract

The clinical benefits obtained with rituximab in the treatment of CD20⁺ B-cell malignancies and of imatinib in the treatment of Phi⁺ leukaemias have opened a new era in oncology, transforming the concepts of tumour-targeted therapies and personalised medicine into reality. Since then, many tumour-targeted monoclonal antibodies and tyrosine kinase inhibitors have been approved for the treatment of cancers. Compared to conventional chemotherapies, these new drugs have more specificity against cancer cells and less systemic toxicities. However, like conventional chemotherapies, they often provide limited therapeutic benefits with short-lasting tumour responses as the vast majority of cancers become resistant to these drugs over time. Therefore, tumour-targeted therapies are an incremental innovation as compared to historical chemotherapies. Recently, a paradigm shift has been brought to the clinic with drugs targeting immune cells rather than cancer cells with the aim of stimulating the anti-tumour immune response of patients against their own cancer. Immunomodulatory drugs such as anti-CTLA4 and anti-PD-1 have generated long-lasting tumour responses when used as single agent in patients with refractory/relapsing cancers such as metastatic melanomas, renal cell carcinoma or non-small-cell lung carcinoma. These new immune-targeted therapies are therefore a disruptive innovation in cancer treatment: they demonstrate that long-lasting clinical benefits could be obtained by targeting molecules involved in the immune tolerance of cancer cells rather than by targeting oncogenic drivers or antigens expressed by cancer cells.

Introduction

Despite more than a century of dedicated scientific and clinical research, curing cancer remains one of the biggest medical challenges to date. So far, cancer treatments have mainly relied on the combination of surgery, radiotherapy and cytotoxic chemotherapies. None of these strategies is recent. The first report of a radical mastectomy for breast cancer was performed by Bernard Peyrilhe in 1773 and published by the Academy of Science of Lyon, France (1). Its practice became subsequently widely spread, thanks to the work of William Halsted in the USA at the end of the 19th century (2). The first successful use of radiotherapy for cancer treatment was also performed in Lyon, France by Victor Despeignes in 1896 (3). The

interest of chemotherapies, such as alkylating agents, was discovered by Paul Ehrlich in the 1930s (4), but their real development in oncology was performed by Alfred Gilman and Louis Goodman, thanks to their work on nitrogen mustard during World War II (5). During the subsequent 70 years or so, a great international collaborative effort has allowed to define the best treatment combinations through randomised clinical trials involving thousands of cancer patients. Thanks to optimised doses and schedules, the overall survival (OS) has dramatically improved for some cancers such as Wilms tumours, paediatric lymphoblastic leukaemias, lymphomas or Hodgkin disease. However, combinations of conventional therapies did not provide much improvement in the survival of other cancers such as high-grade glioblastoma, lung or pancreatic cancers. Moreover,

many of these treatment regimens have shown high toxicity profiles and late side effects (6). Therefore, most of the cancer research efforts of the last 20 years have been focusing on finding drugs with better specificity against tumour cells and less toxicity against the host.

The limits of tumour-targeted therapies

The discovery of imatinib, a tyrosine kinase inhibitor, and its dramatic effect against chronic myeloid leukaemia (CML) bearing the Philadelphia chromosome (bcr-abl gene translocation) has opened a new era in the field of oncology (7). Thanks to the progress made in biochemistry, it is now possible to imagine small molecules which can bind specifically to proteins over-expressed or over-activated in cancer cells and inhibit their proto-oncogenic activity (8). These targeted proteins can be membrane receptors (e.g. erlotinib on the EGFR tyrosine kinase), intracytoplasmic kinases (e.g. everolimus on mammalian target of rapamycin) or intra-nuclear chromatin-associated proteins (e.g. the bromodomain and extra terminal inhibitor GSK525762). At best, they inhibit the single over-activated pathway responsible for the proliferation of the tumour cells and, by doing so, make the disease disappear without too much toxicity for the patient. This situation represents the ideal type of cancer, with a single dominant driver mutation, sensitive to a monotherapy blocking specifically the designated pathway (Table I). Thanks to a small mutational load in these cancers, the patients will rarely develop resistance to the drug. Imatinib represents this ideal clinical situation for patients with CML. More recently, ibrutinib might also provide such clinical benefits for patients having B-cell lymphomas relying on the hyper-activation of the Bruton tyrosine kinase (BTK) pathway [although with shorter OR and progression-free survival (PFS)] (9). However, most cancers present with multiple driver mutations and have a large mutational load. These patients would therefore require multitargeted therapies to control the proliferation of their disease and would be highly susceptible of relapse due to early acquired resistance (Table I).

The validity of the strategy of tumour-targeted therapies also relies on the misinterpreted concept of the clonal origin of cancer (10). Indeed, if cancer cells have a clonal origin, it does not mean that they remain monoclonal over time. We know now that each cancer cell can have an independent genomic evolution over time (11, 12), and that their genome (and therefore the genome of their daughter cells) will acquire new mutations that would not necessarily exist in other tumour cell clones (13). The consequence of this phenomenon is that cancer can be in fact an extremely heterogeneous disease with different mutations found in cancer cells at different sites within the primary tumour and at the metastatic sites (Figure 1) (14). The practical clinical consequences of these results are that:

- (i) the identification of a target for therapy based on a single tumour biopsy made at the time of diagnosis might miss other driver

mutations present at other sites within the primary tumour or metastases, or which appeared subsequently after the biopsy.

- (ii) a targeted therapy directed against one driver mutation will control the burden of the disease only temporarily but will finally allow sub-clones not expressing (or not exclusively depending on) the targeted molecule to finally expand and spread throughout the body.

The relevancy of these conceptual limits has been demonstrated recently in several clinical trials of patients bearing cancers with complex genomics. For instance, metastatic melanoma patients bearing a V600 mutation in the BRAF gene of their tumour cells and treated with a BRAF inhibitor can present dramatic tumour responses (15). However, within a few months, all the responding patients subsequently relapse with a metastatic melanoma sub-clone not sensitive to the BRAF inhibitor, leading to benefits in PFS but few benefits in OS (Figure 2). Similar patterns of transient sensitivity to tumour-targeted therapies followed by a subsequent relapse due to acquired treatment resistance have been described in many tumour types—for instance, in patients with metastatic medulloblastoma treated with a hedgehog inhibitor (16) or patients with metastatic lung cancer bearing ROS1 translocations and treated with crizotinib (17).

Interestingly, tumour-targeted monoclonal antibodies (mAbs) can present the same limitations as tumour-targeted small molecules. Thanks to their antigen specificity, these antibodies can destroy selectively antigen-positive tumour cells (e.g. rituximab against CD20⁺ B-cell lymphoma cells). However, like for small molecules, tumour-targeted mAbs exert a pressure of selection on cancer cells and can eventually favour relapses of antigen negative tumour cells (18, 19).

Therefore, tumour-targeted therapies appear to be an incremental improvement of conventional chemotherapies, with better selectivity and toxicity profile, but like chemotherapies, offering long-term disease control only for a minority of patients and a high level of drug resistance at relapse (Figure 3).

Cancer immunotherapies: what are we talking about?

When people talk about using immunotherapy as a therapeutic strategy in oncology they usually mean ‘to use the immune system to fight cancer’. At the end of the 19th century, some physicians in Europe, such as Robert Koch, Louis Pasteur or Emil von Behring, had recorded observations of erysipelas infection coinciding with cancer regression. Notably, Prof. Busch published, in 1868 in Germany, his successful treatment of neck sarcoma after intra-tumoral inoculation of an extract of erysipela (a cutaneous bacterial infection) taken from another patient (20). Dr William Coley, a surgeon at what would become later the Memorial Sloan Kettering hospital in New York City, turned such observation into a medical practice. He confirmed that intra-tumoral injections of bacteria collected from erysipela could cure some solid tumours (21). Even if the mechanistic was not unravelled at that time, it was clear to them that they were injecting pathogens to their patients, and that the reaction of their body to the pathogens would help to eradicate their tumour. However, at a time where there were no antibiotics, and where radiotherapy showed high rates of reproducible efficacy against tumours, the number of adepts of the Coley therapy vanished over time. After World War II, major scientific discoveries concerning the immune system were done while studying tumour models in mice such as the concept of tumour immunosurveillance, the discovery of the major histocompatibility complex, dendritic cells (DCs), cross-presentation of antigens, etc. (22).

Table I. Complexity in cancer genomes and its consequences on tumour-targeted therapies sensitivity

Cancers with simple genomic	Cancers with complex genomic
Single dominant mutation	Multiple mutational drivers
Small mutational load	Large mutational load
Monotherapy is effective	Multitargeted therapy required
Resistance is rare, late and involves same pathway	Resistance is common, early and involves different pathways

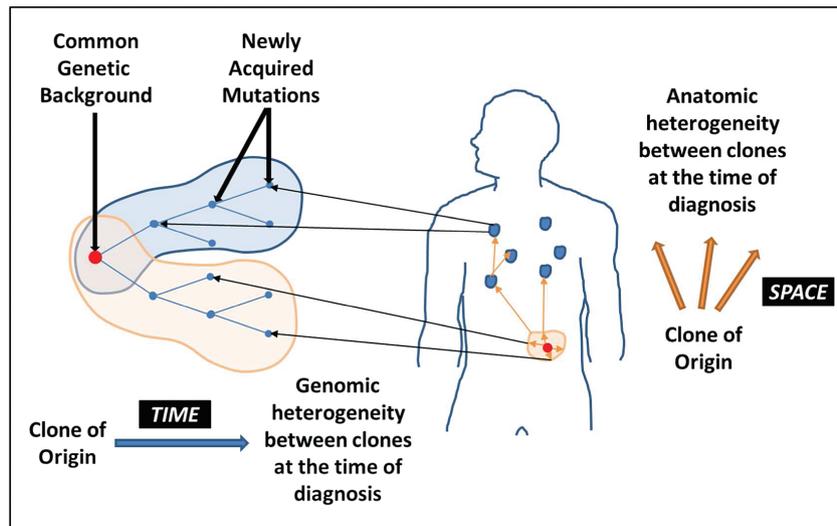


Figure 1. Genomic and anatomic heterogeneity of cancers. The clonal origin of cancer does not mean that the disease remains monoclonal. Indeed, cancer cells can evolve independently from each other and acquire mutations that are not necessarily shared by other clones at other sites in the body.

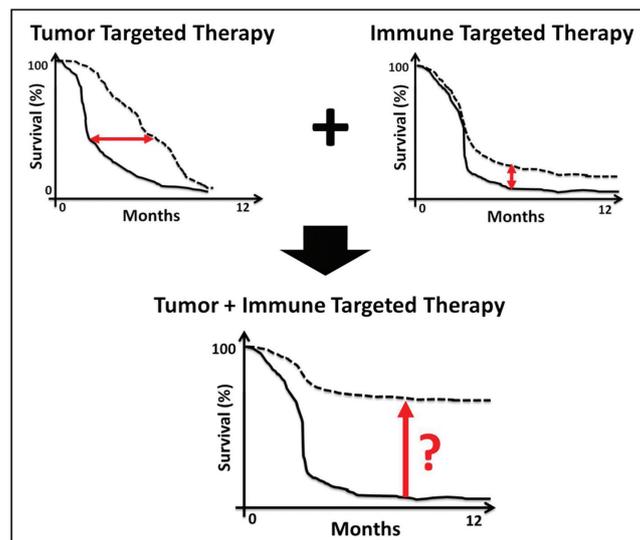


Figure 2. Clinical benefits of tumour-targeted and immune-targeted therapies and expected benefits of combinations strategies. The clinical consequence of single-agent tumour-targeted therapies is an improvement of the median PFS but no benefits in terms of long-term OS. On the other hand, single-agent immune-targeted therapies provide long-term tumour responses and improved OS, but only in some patients. Strategies combining tumour-targeted and immune-targeted therapies should improve the proportion of patients developing long-lasting tumour responses. ___ line: standard of care survival profile. --- line: targeted therapy survival profile.

These discoveries led to the development of various strategies of cancer immunotherapy, relying on the different types of effector cells of the immune system. Some of these strategies were considered as passive immunotherapy because they provided directly to the patient the immune effectors needed to fight his cancer (Figure 4). Some were not specific of the cancer antigens such as adoptively transferred lymphokine-activated killer cells, cytokine-induced killer cells, Haplo-identical natural killer (NK) cells, allogeneic hematopoietic stem cell transplantation or donor lymphocyte infusions. Others, such as tumour-targeted mAbs, *ex vivo* expanded tumour-infiltrating lymphocytes (TILs) and chimeric antigen receptor T cells, were used to passively but specifically target an antigen expressed by the cancer cells. Inversely, active immunotherapy strategies were built to enhance the anti-tumour immune response of the host against his

own cancer. One of these nonspecific active immunotherapy strategies that have been widely used in the 1990s is the infusion of immunostimulatory cytokines such as interleukin-2 or interferons (IFNs). Many tumour antigen-based vaccines have been (and still are) tested as tumour-specific active immunotherapies in order to generate an adaptive immune response against cancers. For a comprehensive review on the history of cancer immunotherapy strategies, see Zhao *et al.* (22).

Reasons of failure of the historical cancer immunotherapies trials

The major breakthrough in cancer immunotherapy happened in 2001 in an academic area very far from oncology. That year, three

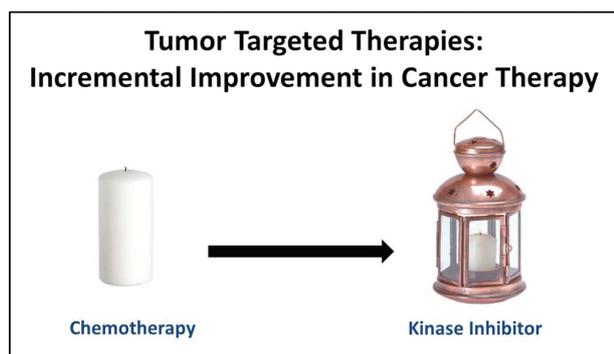


Figure 3. Tumour-targeted therapies are by design more specific and less toxic than chemotherapies. However, they do not offer the conceptual disruptive thinking required to get substantial innovation and therefore suffer the same limitations as chemotherapies: limited efficacy over time, treatment resistance at relapse.

	PASSIVE	ACTIVE
NON SPECIFIC	LAK, CIK NK, allo	Cytokines
SPECIFIC	mAbs TILs CAR CTLs	Vaccines

Figure 4. Historical cancer immunotherapy strategies. Allo, allogeneic hematopoietic stem cell transplantation; CAR CTLs, chimeric antigen receptor cytotoxic T lymphocytes; CIK, cytokine-induced killer cells; LAK, lymphokine-activated killers; mAbs, tumour-targeted monoclonal antibodies; vaccines, tumour-associated antigen-based vaccines.

teams published back to back in *Nature Genetics* their discovery of a new gene called FOXP3 (forkhead box P3) which is specifically expressed in a subset of CD4⁺ T-cell lymphocytes called regulatory T cells (Tregs). The FOXP3 mutation is responsible of a neonatal pan-autoimmune disease called IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) (23–25). Rapidly, people understood that the key role played by Tregs in the physiology of immune self-tolerance was also involved in the immune cancer tolerance (26). It is now clear that within the tumour microenvironment, tumour cells, together with cells from the myeloid lineage such as tumour-associated macrophages, DCs and myeloid-derived suppressor cells, sustain the development of Tregs and protect cancer cells from anti-tumour immune effector cells (27). Interestingly, Tregs express high levels of the interleukin-2 receptor α subunit (CD25), and IL-2 therapy results in the stimulation and expansion of Tregs (28). This phenomenon of cancer tolerance enhancement *via* Treg stimulation mainly explains the failures of the IL-2 immunotherapy strategies developed in the 1990s in cancers such as melanoma and renal cell carcinoma (29–31). Another unexpected immunosuppressive effect on macrophages and DCs might explain why type I IFN cytokine immunotherapy also had little success in the clinic (32). Therefore, it is not surprising to find that these

immunosuppressive (or tolerogenic) tumour-infiltrating cells have a bad prognostic value in patients with cancer (33, 34). It also explains why performing a lymphodepletion prior the adoptive transfer of tumour-specific immune cells (CTLs or TILs) greatly enhances their therapeutic efficacy (35).

mAbs: from incremental to disruptive paradigm in cancer therapy

mAbs have been the historical milestone of both concepts of targeted therapies and personalised medicine in oncology. Indeed, in 1982, Prof. Ronald Levy at Stanford University, CA showed that a monoclonal antibody could be designed to target specifically the idiotype of each B-cell lymphoma (36). This strategy was tested in patients and resulted in dramatic tumour responses. It opened a large series of blockbuster anti-cancer mAbs over the last 20 years. The understanding of the biology of mAbs efficacy allowed subsequently designing them for specific functions. For instance, mAbs could be designed to have an intrinsic direct pro-apoptotic effect on tumour cells or a cytotoxic effect mediated by immune cells such as NK cells (antibody-dependent cell-mediated cytotoxicity) and macrophages (antibody-dependent cell-mediated phagocytosis). The typical example is anti-CD20 antibodies which have a mode of action that encompasses these different mechanisms. MABs can also be designed to be antagonistic against a growth hormone receptor such as trastuzumab (anti-HER2). These mAbs turned out to be also versatile platforms for the local delivery of cytotoxic molecules such as chemotherapies and radioactive particles (37). In terms of therapeutic paradigm, these different types of mAbs were an incremental innovation in cancer therapy, providing drugs that are more specific of tumour cells, but less toxic against the healthy ones.

Over the last 15 years, it has been increasingly clear in pre-clinical tumour models that very significant anti-tumour efficacy could be obtained when mAbs are designed to target co-stimulatory or co-inhibitory molecules expressed on the surface of immune cells (38, 39). As a matter of facts, these either agonistic or antagonistic mAbs resulted in breaking the tumour tolerance and/or boost the anti-tumour immune effectors. These so called ‘immunomodulatory antibodies’ capable of ‘immune checkpoint blockade’ were then translated to the clinic. In 2010, one of them called ipilimumab, directed against the immunosuppressive CTLA4 (cytotoxic T lymphocyte antigen 4) molecule expressed by CD4 T cells (notably Tregs), showed in a randomised Phase III monotherapy clinical trial to improve the survival of patients with relapsed/refractory metastatic melanoma (40). Ipilimumab therapy resulted in long-term tumour responses with benefits in OS in about one patient out of five (20%). The proof of concept that immunomodulatory mAbs could be of interest in oncology was subsequently confirmed with another checkpoint blockade antibody directed against PD-1 (programmed death receptor-1), a molecule also expressed by T cells. A monotherapy Phase I trial showed long-term tumour responses upon nivolumab (anti-PD-1 mAb) therapy in one third of patients with metastatic melanoma and non-small-cell lung carcinoma (41). Interestingly, concomitant combinations of anti-CTLA4 with anti-PD-1 seem very synergistic with more than two third of melanoma patients developing durable tumour responses (42).

Immunomodulatory antibodies are disruptive by design. Indeed, these molecules are not designed to target the tumour cells but to target the immune system in order to break the cancer tolerance and boost the anti-tumour immune response (Figure 5). As opposed to tumour-targeted therapies, this paradigm shift offers tumour

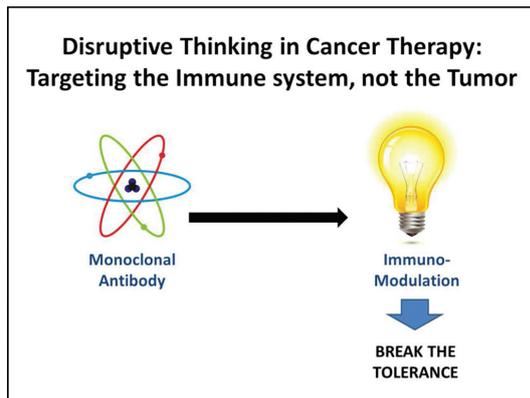


Figure 5. Immune-targeted therapies are aimed to break the cancer tolerance and boost the anti-tumour immune response. They are a disruptive therapeutic innovation because as opposed to the other cancer drugs that have been used so far in the clinic, they are not designed to directly damage cancer cells.

responses, but it also provides long-term immune protection against relapses, thanks to the generation of a polyclonal adaptive (memory) anti-tumour immune response.

However, the major limitation of use of these immunomodulatory mAbs is the auto-immune toxicity triggered upon therapy. Indeed up to 60% of patients treated with ipilimumab present with auto-immune symptoms, one third being of grade 3–4, and all requiring high doses of steroids therapy in order to dampen the side effects. The proportion of patients developing such auto-immune toxicity upon the anti-CTLA4 + anti-PD-1 combination was even higher.

Tolerogenic versus immunogenic tumour cell death

Recent progress has been made in our understanding of the biology of dying cells. Several types of cell death processes have now been defined based on discriminant cytological and molecular criteria: apoptosis (either intrinsic or extrinsic, either caspase dependent or independent), regulated necrosis, autophagy and mitotic catastrophe (43).

In the field of oncology, some research teams have focused on the type of tumour cell death happening upon chemo- or radiotherapy. Interesting results have shown recently that the therapeutic efficacy of these conventional therapies do not only depend on their direct toxic effect on the tumour cells. Back-to-back experiments in immunocompetent and immunocompromised murine tumour models have shown that part (sometimes all) of the activity of chemotherapies *in vivo* rely on the ability of the immune system to mount an anti-tumour immune response (44).

These results have led to the concept of therapy-induced immunogenic cell death (ICD). Indeed, upon chemotherapy, tumour cells can undergo ICD *via* the exposure of calreticulin and heat-shock proteins on their plasma membrane. They can also release in the tumour microenvironment molecules such as ATP and HMGB1 which also have immunogenic properties. These damage-associated molecular pattern molecules (DAMPs) interact subsequently with specific receptors [e.g. CD91 for calreticulin, Toll-like receptor 4 (TLR4) for HMGB1] expressed on DCs and activate their ability to engulf dying cells and efficiently present tumour antigens to the anti-tumour immune effectors (45). Moreover, either conventional chemotherapies or recent tumour-targeted therapies also have immunostimulating effects through a direct action on anti-tumour

immune effectors such as antigen-presenting cells (APCs), CD8 and CD4 effector T cells, or a direct dampening of Tregs function (44).

The demonstration of the ICD phenomenon and of the immune effects of conventional therapies is in striking contrast to the historical view that the clinical efficacy of anti-cancer drugs relies only on tumour cells *via* a direct cytotoxic effect. Taking into account, these results should improve the efficacy of combination therapies. Indeed, the choice of drug regimen should focus on their ability to generate an ICD and improve the cross-talk between the dying cells and the immune system.

In accordance with this consideration, some teams have recently shown that the anti-tumour effects of tumour-targeted therapies such as imatinib, dasatinib or radiotherapy synergise with immunomodulatory mAbs such as anti-CTLA4, anti-OX40 or anti-PD-L1 to generate long-term tumour responses in mice (46–49). Therefore, combination of ICD-inducing tumour-targeted therapies with immune-targeted therapies seems a promising strategy in oncology (50).

From Coley's toxins to the 2011 Nobel prize: rationale for the use of pattern recognition receptor agonists as adjuvants for cancer immunotherapy

The 2011 Nobel Prize of medicine rewarded J. Hoffman, B. Beutler and R. Steinman for their work on the role of pattern recognition receptors (PRRs) to activate the innate and adaptive immune systems. PRR agonists are made of pathogen-associated molecular pattern molecules (PAMPs) and DAMPs (also known as danger-associated molecular pattern molecules). Within the PAMPs, TLR agonists have been widely used in clinical trials with the idea of reproducing William Coley's work (51). We understand now that their therapeutic effect is probably mediated by the activation of APCs switching from a tolerogenic to an immunogenic phenotype upon TLR stimulation (52).

Enhancing anti-tumour efficacy via the combinations of immunomodulatory drugs

The abscopal effect is a very rare physiological phenomenon where an irradiation of a tumour site has a bystander effect on other distant, non-irradiated, tumour sites. Recently, two teams have reported that a local irradiation resulted in tumour responses on distant sites (abscopal effect) upon combination with a systemic anti-CTLA4 therapy in patients with metastatic melanoma (53, 54).

Interestingly, the team of Prof. Ronald Levy at Stanford University showed recently in a Phase I trial of patients with metastatic lymphoma that local irradiation of a single lymphoma site could trigger a systemic anti-tumour immune response when combined to intra-tumoral injections of CpG (a TLR-9 agonist) into the same irradiated site (55). This result illustrates in humans the above-mentioned synergy between a conventional anti-tumour therapy (irradiation) with an immunomodulatory drug (CpG). Notably, in this synergistic strategy, both therapies were delivered locally at the same tumour site, suggesting that the ignition of an anti-tumour immune response at a single site might be sufficient to trigger a systemic tumour response. However, this systemic anti-tumour response seemed to be limited by the expansion of Tregs (55). The same team showed in a pre-clinical murine model of lymphoma that, like in humans, intra-tumoral CpG monotherapy triggers an anti-tumour immune response eradicating mainly the injected tumour site but had little effect

on the growth of a distant, non-injected, tumour site (56, 57). Surprisingly, the combination of intra-tumoral CpG with immunomodulatory mAbs (such as anti-CTLA4, anti-OX40, anti-FR4 or anti-GITR) allowed the generation of a systemic anti-tumour immune response able to eradicate the distant (non-injected) tumour site (57). Interestingly, the anti-tumour effect of these immunomodulatory mAbs seems to be mediated by the intra-tumoral depletion of tumour-specific Tregs (58–60). Notably, better systemic anti-tumour efficacy can be obtained when injecting low doses of mAbs directly into one tumour site, while preventing their systemic toxicity (61, 62).

In accordance with these pre-clinical results, intra-tumoral delivery of immunomodulatory mAbs directly into tumour sites is currently sought as an alternative way of using these new drugs in order to maintain their therapeutic benefits but prevent their systemic, off-target, toxicity (63).

Conclusion

Tumour-targeted therapies are still the main focus of drug development in oncology these days. However, immune-targeted therapies aimed at boosting the anti-tumour immune response in cancer patients are now a game changer in the field and many immunomodulatory mAbs are currently in development in the clinic. There is a strong scientific rationale to believe that combinations of ICD-inducing tumour-targeting drugs should synergise with immune-targeted mAbs. However, the doses, schedules and ways of administrations of these drugs remain to be specified for each cancer type in order to obtain gains in survival with less toxicity. The whole oncology practice should therefore be completely transformed over the next decades.

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CANCER

Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients

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The immunosurveillance mechanisms governing high-risk neuroblastoma (HR-NB), a major pediatric malignancy, have been elusive. We identify a potential role for natural killer (NK) cells, in particular the interaction between the NK receptor NKp30 and its ligand, B7-H6, in the metastatic progression and survival of HR-NB after myeloablative multimodal chemotherapy and stem cell transplantation. NB cells expressing the NKp30 ligand B7-H6 stimulated NK cells in an NKp30-dependent manner. Serum concentration of soluble B7-H6 correlated with the down-regulation of NKp30, bone marrow metastases, and chemoresistance, and soluble B7-H6 contained in the serum of HR-NB patients inhibited NK cell functions *in vitro*. The expression of distinct NKp30 isoforms affecting the polarization of NK cell functions correlated with 10-year event-free survival in three independent cohorts of HR-NB in remission from metastases after induction chemotherapy ($n = 196$, $P < 0.001$), adding prognostic value to known risk factors such as N-Myc amplification and age >18 months. We conclude that the interaction between NKp30 and B7-H6 may contribute to the fate of NB patients and that both the expression of NKp30 isoforms on circulating NK cells and the concentration of soluble B7-H6 in the serum may be clinically useful as biomarkers for risk stratification.

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid pediatric malignancy, and about 45% of patients present with metastatic disease at diagnosis (1, 2). Metastatic NB patients aged more than 1 year exhibit a 30% event-free survival despite the use of conventional induction chemotherapy, surgical resection of the primary tumor, and consolidation with a myeloablative multimodal chemotherapy followed by autologous stem cell transplantation (ASCT), local irradiation, and maintenance

with 13-cis-retinoic acid (3–5). On the basis of historical (6, 7) and renewed interest supported by the experimental evidence that NB is controlled by the immune system (8, 9), investigators are currently including anti-disialoganglioside ch14.18 monoclonal antibody (mAb), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2) in the maintenance therapy (10) of high-risk NB (HR-NB). Adoptive T cell therapy with chimeric receptors recognizing GD2 antigen (11), CD3×GD2 bispecific antibodies (12), and vaccines are in early clinical development (13).

Several lines of evidence support the role of natural killer (NK) cells in keeping NB in check. First, Moretta's group identified activating receptors (notably NKp46 and NKp30) involved in the recognition of NB (14, 15). Second, in animal models, NK cell depletion reduced antitumor activity of the immunocytokine consisting of human IL-2 linked to the hu14.18 humanized anti-GD2 mAb (hu14.18-IL-2 IC) (16). Third, in children with relapsed/refractory NB receiving the hu14.18-IL-2 IC immunocytokine in the Children's Oncology Group phase 2 trial, the autologous KIR/KIR ligand mismatch was associated with clinical response (17). Hence, it is conceivable that NK cell-based immune biomarkers might predict clinical outcome in HR-NB.

NKp30/NCR3 is the most NK-specific receptor (18–22). The human NCR3 gene encodes six differentially spliced transcripts that differ in their immunoglobulin (Ig) domains (V versus C type), and each of the two extracellular domains can be linked to one of three distinct intracellular domains, depending on which particular exon 4 they use (23). Several reasons prompted us to functionally characterize the different NKp30 isoforms. First, NKp30 appears to play a role in the prognosis of infectious diseases (18–22, 24, 25) and cancers (26). Second, NKp30

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has been involved in both dendritic cell (DC) killing and DC maturation (27, 28). Third, the identification of NKp30 ligands rekindled the interest in this NCR and established that basal or stressed leukemic or solid tumor cells may express specific NKp30 ligands (29–31). Alternative splicing of exon 4 of the *NCR3* gene can affect the intracellular domain of NKp30, compromising its effector functions and severely affecting NKp30-dependent functions in gastrointestinal stromal tumor (GIST) patients. GIST cells express NKp30 ligands and are sensitive to NK cell attack, which can be stimulated by tyrosine kinase inhibitors such as imatinib that are commonly used for GIST treatment. GIST patients differ in the transcription of NKp30 isoforms, which in turn affect NK cell function. Whereas the NKp30A and NKp30B isoforms of NKp30 mediate cytotoxicity and T helper 1 (T_H1) cytokine secretion, respectively, the NKp30C isoform only promotes IL-10 release. The predominant expression of NKp30C in GIST patients is associated with reduced patient survival, decreased NKp30-dependent tumor necrosis factor- α (TNF α) release and CD107a degranulation, and an IL-10-mediated suppression of the NK/DC crosstalk resulting in defective interferon- γ (IFN γ) and IL-12 secretion (32).

Here, we show that NKp30 isoforms also correlate with the event-free survival of patients with HR-NB responding to induction chemotherapy in three independent cohorts (a test cohort of 70 and two validation cohorts of 55 and 71 children). This could be related to several functional observations. First, distinct NK cell phenotypic abnormalities selectively characterize HR-NB. Decreased CD16⁺ NK cell frequencies were associated with chemoresistance. Moreover, NKp30 expression was markedly down-regulated in CD16⁺ NK cells from metastatic bone marrow (BM), where neuroblasts and monocytes express the NKp30 ligand B7-H6. Second, soluble B7-H6 (sB7-H6) serum concentrations inversely correlated with NKp30 expression and IFN γ secretion by NK cells, and directly correlated with NB dissemination and resistance to chemotherapy. Third, NK cells from patients with metastatic NB exhibited deficient transcription of the A and B isoforms of NKp30 [compared to patients with localized NB and healthy volunteers (HVs)]. HR-NB patients presenting with circulating NK with a favorable ratio of activating NKp30B over regulatory NKp30C exhibited a longer time to progression after remission induced by myeloablative chemotherapy and hematopoietic stem cell transplantation. Finally, depletion of the NKp30C isoform transcripts by means of specific small interfering RNAs (siRNAs) restored NKp30 effector functions, opening up potential therapeutic avenues in HR-NB.

RESULTS

NK cell abnormalities associated with NB dissemination and chemoresistance

We prospectively analyzed various immune subsets in fresh peripheral blood (PB) and BM of HR-NB children treated in the Pediatric Department of the Gustave Roussy Cancer Campus (GRCC) from 2010 to 2013. We focused on NK lymphocytes and, particularly, on the immature secreting CD56^{bright} NK subset and the cytotoxic CD56^{dim} subset. A cohort of 36 patients with metastatic NB was compared to 23 patients with localized NB at the time of diagnosis (cohort 1, Table 1). The percentages of neutrophils (fig. S1A), monocytes (fig. S1B), CD4⁺ T lymphocytes (fig. S1C), CD8⁺ T lymphocytes (fig. S1D), $\gamma\delta$ T cells (fig. S1E), and CD3⁺CD56⁺ T cells (fig. S1F) did not significantly differ in localized versus metastatic NB-bearing children, but the percentage of

CD3⁺CD56⁺ NK cells was significantly increased in the PB of patients with metastatic NB ($P = 0.01$) and the CD3⁺CD56^{bright} NK subset was significantly increased in the BM of patients with metastatic NB compared to PB ($P = 0.03$) (fig. S2, A and B). As previously described for other malignancies (33, 34), regulatory CD4⁺Foxp3⁺ T cells were also more abundant in metastatic stages (fig. S2C). The percentages of CD3⁺CD56⁺ and CD3⁺CD56^{bright} NK cells were also associated with a minor or no response to induction chemotherapy (35) (fig. S2, D and E). Conversely, the percentages of CD16⁺ NK cells correlated with clinical responses to induction treatment (fig. S2F).

Next, we phenotyped peripheral NK cells from HR-NB. One major phenotypic difference between localized and metastatic NB was the down-regulation of NKp30 expression in BM NK cells in metastatic NB compared with BM of localized NB (Fig. 1A, left panel). Moreover, in PB, the frequency of circulating CD3⁺CD56^{dim} NK subset diminished in metastatic as compared to localized NB (Fig. 1A, right panel). In contrast, PB or BM NK cells from metastatic NB and localized NB did not differ significantly with regard to the expression of other activating NK cell receptors (such as NKp46, NKG2D, NKp44, DNAM-1, and NKp80) (Fig. 1, B to F), whereas CD16⁺ NK cells increased in BM of patients bearing metastatic NB compared to PB from metastatic NB (Fig. 1G). Additionally, KIR receptors were not differentially expressed

Table 1. Characteristics of NB patients. Cohort 1: prospective flow cytometry analysis of NB patients sampled at diagnosis. Test cohort used for the retrospective correlation between NKp30 isoform profiling and PFS. Validation cohort 1 used for the retrospective correlation between NKp30 isoform profiling and PFS. Validation cohort 2 used for the retrospective correlation between NKp30 isoform profiling and PFS. INRGSS, International Neuroblastoma Risk Group Staging System; CR, complete response; PR, partial response.

	Cohort 1 (n = 59)	Test cohort (n = 70)	Validation cohort 1 (n = 55)	Validation cohort 2 (n = 71)
Age at diagnosis (n)				
<18 months	8	14	8	9
≥18 months	51	56	47	62
Mean age at diagnosis in months (range)				
	48 (4.8–192)	36 (2–181)	35.8 (2–219)	39.6 (4–383)
INRGSS stage (n)				
L1	12	0	0	0
L2	11*	0	0	5 [†]
MS	2	2	2	2
M	34	68	53	64
MYCN amplified (n)				
	6	16	14	19
Response after induction chemotherapy (for metastatic patients) (n)				
CR or PR	19	70	55	71
Time of sampling				
Diagnosis	59	17	18	0
Before ASCT	0	53	37	71

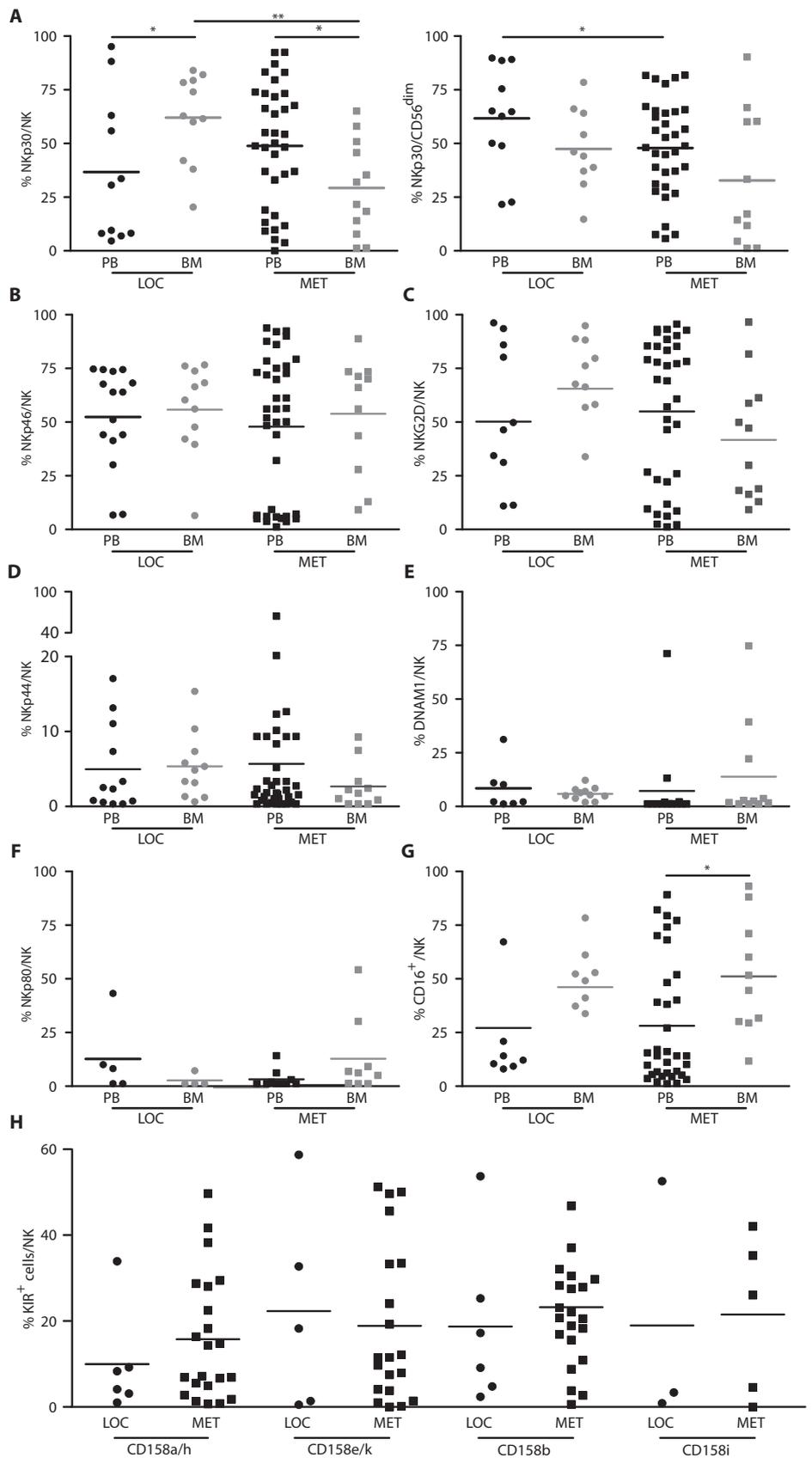
*Included three with amplified N-Myc. [†]Five of five with amplified N-Myc.

Fig. 1. Selective down-regulation of NKp30 in BM of metastatic NB. (A to H) Phenotype of PB and BM NK cells in children with localized (LOC) or metastatic (MET) NB, evaluated for the expression of NKp30 on all (A, left panel) versus CD56^{dim} NK cells (A, right panel) cells and for other receptors such as NKp46 (B), NKG2D (C), NKp44 (D), DNAM-1 (E), NKp80 (F), CD16 (G), and four different KIR inhibitory receptors (H), gating on CD3⁺CD56⁺ NK cells using flow cytometry on the same specimens described in fig. S1. **P* < 0.05, ***P* < 0.01 by Mann-Whitney analysis. Exact *P* values are provided in table S3.

in localized and metastatic NB (Fig.1H). Together, metastatic NB was thus characterized by a high frequency of NK cells with a selective NKp30 down-regulation in the blood for CD56^{dim} NK cells and in BM for the whole NK cell population.

NKp30 ligands expressed by metastatic BM

Because NKp30 appeared to be down-regulated in the BM from patients with metastatic NB, we hypothesized that NKp30 ligands were overexpressed in the BM. B7-H6 has been recently identified as one of the ligands for NKp30 (28, 31). Although *B7-H6* transcripts were not detected in most normal adult tissues, they were measurable in 15 of the 17 BM specimens from metastatic NB that we analyzed, yet undetectable in all 4 BM samples from patients with localized NB (Fig. 2A). B7-H6 was originally described in leukemia and some primary cancer lines (31, 36), but its surface expression could be observed in two of four cases in the GD2⁺ neuroblast-enriched fraction of the BM of HR-NB-bearing patients at the time of diagnosis (Fig. 2B). Moreover, all the NB cell lines tested demonstrated constitutive B7-H6 expression (Fig. 2, C and D), contrasting with other tumor cell lines of neuroectodermal origin such as glioblastomas that were less likely to express high amounts of B7-H6 (Fig. 2D). As recently reported, B7-H6 can also be expressed by inflammatory monocytes, and sB7-H6 can be found in the serum of patients suffering from a Gram-negative septic shock (37). Indeed, in two of six metastatic NB cases, BM monocytes expressed B7-H6 (Fig. 2D). We therefore monitored serum concentrations of sB7-H6 in HR-NB patients. High concentrations of sB7-H6 were found in the serum of some patients with metastatic disease and were associated with an abnormally low NKp30 expression on CD3⁺CD56^{dim} NK cells (Fig. 2E). sB7-H6 serum concentration was associated with NB



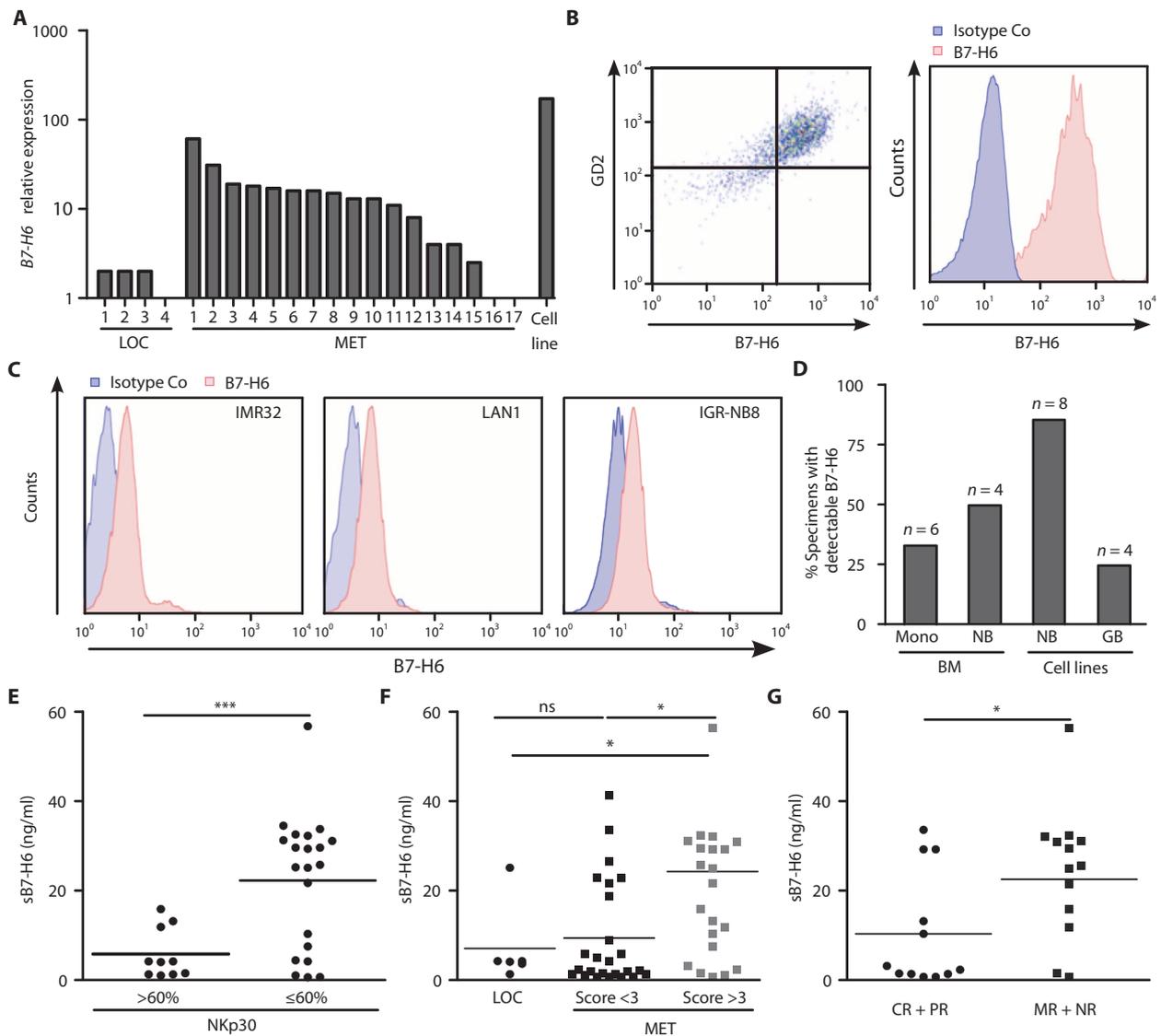


Fig. 2. B7-H6 expression by neuroblasts and serum accumulation of sB7-H6. B7-H6 expression on NB was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry. (A) mRNA levels were analyzed by qRT-PCR in 17 metastatic and 4 localized NB. NB-8 is a cell line exhibiting a positive membrane expression of B7-H6. (B) BM cells were analyzed by flow cytometry, gating on CD45⁺GD2⁺ NB cells (left panel) for the expression of B7-H6 molecules. An overlay of a representative surface B7-H6 expression is depicted (right panel). The isotype control (Co) is shown as solid lilac. (C) Constitutive expression of B7-H6 was demonstrated by flow cytometry on three NB cell lines. The isotype control antibody is shown with a solid lilac. (D) Proportions of B7-H6-expressing specimens among several sources. Absolute numbers of analyzed specimens are

indicated above BM monocytes, BM neuroblasts, and NB and glioblastoma cell lines. (E) Serum concentrations of sB7-H6 from 54 HR-NB patients (47 with metastatic and 7 with localized NB at diagnosis) were correlated with NKp30 expression on peripheral CD56^{dim} NK cells from metastatic patients (cutoff value, 60%; established on median NKp30 expression in the localized NB cohorts) (Fig. 1A, right panel). (F) The dissemination of NB as assessed by bone scan (MIBG-SIOPEN criteria) was correlated with sB7-H6 concentration. Score 3 means detection of three meta-iodo-benzyl-guanidine (MIBG) distinct lesions. (G) The clinical response to induction chemotherapy in patients with metastases at the time of diagnosis was correlated with sB7-H6 concentration. Each dot represents one patient. * $P < 0.05$, *** $P < 0.001$ by Mann-Whitney analysis. ns, not significant. Exact P values are provided in table S3.

dissemination (38) (Fig. 2F) and tumor progression despite therapy (Fig. 2G). We set up an in vitro assay admixing peripheral blood mononuclear cells (PBMCs) and a B7-H6⁺ K562 cell line to monitor NKp30-dependent NK cell activation by flow cytometry (Fig. 3A). Using this in vitro assay, we determined whether high sB7-H6 concentrations interfere with NK cell activation. Incubation of NK⁺ K562 cocultures with patients' sera containing high (>3 ng/ml, considered "positive" on the graph) concen-

trations of sB7-H6 reduced IFN γ release by NK cells (Fig. 3, B and C). Accordingly, a negative correlation between sB7-H6 serum levels and IFN γ -producing NK cells was found in a group of 18 individuals (Fig. 3D).

Together, these data indicate that the NKp30 ligand B7-H6 is over-expressed in the invaded BM from patients with HR-NB. Moreover, high sB7-H6 serum concentrations are associated with NKp30 down-regulation, NK cell dysfunction, metastatic spread, and chemoresistance.

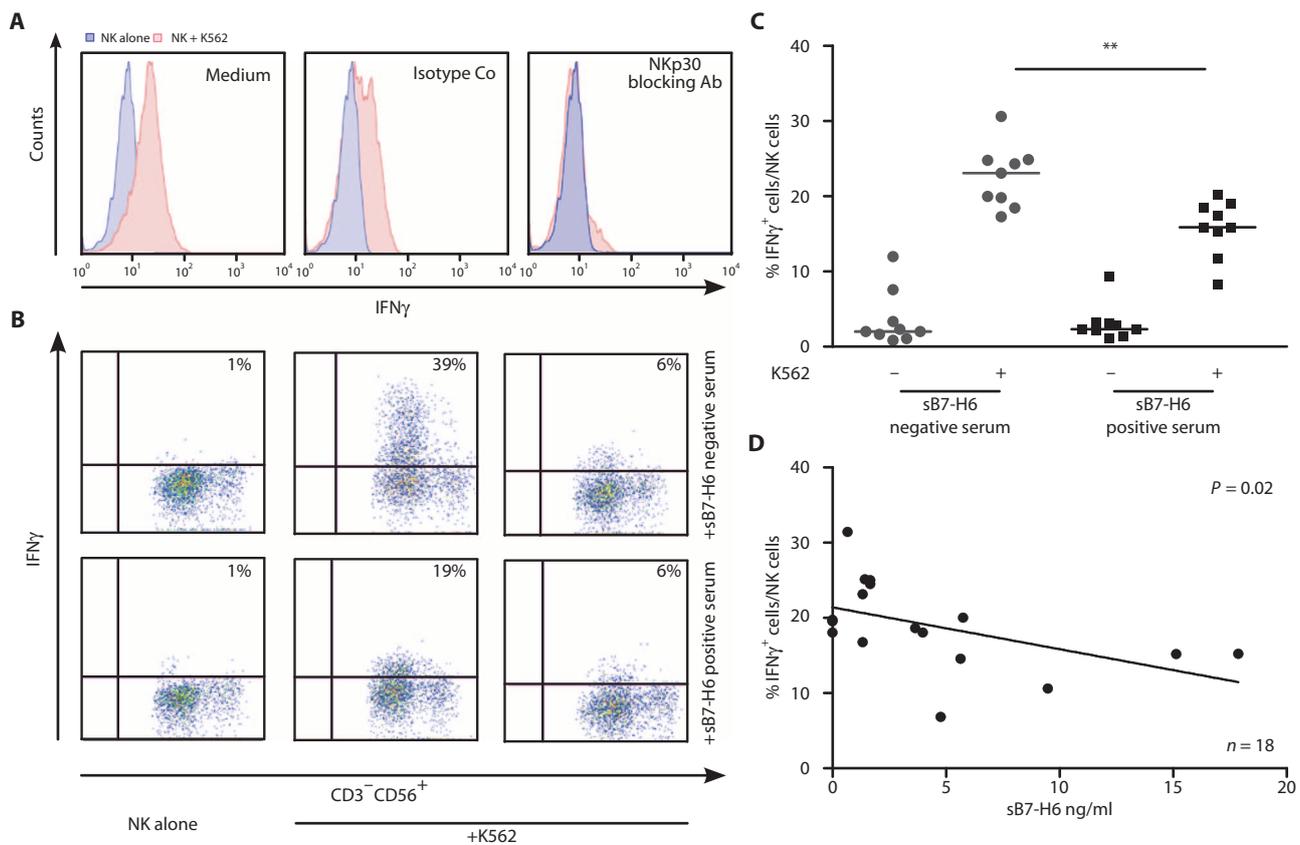


Fig. 3. Inhibitory effect of sB7-H6 on NK cell IFN γ secretion. (A and B) Coculture of purified NK cells with B7-H6–expressing K562 (erythroleukemia cell line) with (right dot plots) or without neutralizing anti-NKp30 (or isotype control, middle dot plots) antibody (Ab) and flow cytometry analyses of intracellular staining for IFN γ (A and B), gating on CD3[−]CD56⁺ NK cells in the presence of patients' serum containing detectable concentrations of sB7-H6 (B, lower dot

plots) or not (B, upper dot plots). Representative overlays (A) and dot plots (B) are depicted. (C) Results obtained with 18 sera incubated in such cocultures. (D) Correlation between sB7-H6 serum concentration and proportion of IFN γ –producing NK cells during the NK/K562 crosstalk. The threshold of sB7-H6 detection in our enzyme-linked immunosorbent assay (ELISA) was 0.6 ng/ml. * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney analysis. Exact P values are provided in table S3.

Correlation between NKp30 isoforms and progression-free survival in chemosensitive HR-NB

Because total NKp30 surface expression (irrespective of isoforms) was decreased in BM from NB patients, yet failed to allow a clinically meaningful stratification of the cohort (fig. S3), we determined the transcriptional profile of the three NKp30 isoforms by means of qRT-PCRs using specific primers for NKp30A, NKp30B, or NKp30C on PBMCs. The analysis of *NKp30* mRNA expression for each of the three isoforms (presented as relative expression) revealed a lower expression of A and B isoforms in metastatic patients compared to HVs and patients with localized NB (fig. S4A). However, the quantity of each NKp30 isoform did not correlate with NKp30 protein surface expression (fig. S4B). Moreover, the mRNA expression of each NKp30 isoform did not affect progression-free survival (PFS) if patients were stratified in two groups, based on the criterion of NKp30 isoform expression below or above the median (fig. S4C).

Therefore, considering that each individual NK cell harbors a different amount of each NKp30 isoform (32), we next considered the relative expression of the different isoforms (A, B, or C) compared with each other. We calculated the relative expression of the three isoforms (A, B, or C) using the “delta” formula $\Delta\text{NKp30}_x/\text{NKp30}_y = \text{NKp30}_y - \text{NKp30}_x$. We found that ΔBC and ΔAC were both significantly lower ($P = 0.0008$ and $P = 0.002$, respectively) in metastatic

compared with localized disease (Fig. 4A), whereas ΔAB was in the same range, supporting the observation that isoforms A and B were relatively underexpressed in HR-NB (fig. S4A). As already reported for normal volunteers and GIST and HIV patients (32, 39), the NKp30 isoform profile was stable over time in NB patients, as confirmed by longitudinal analyses (fig. S5A). Moreover, the NKp30 isoform profile was similar in different anatomical locations of the same individual, suggesting that information on circulating NK cells can be extrapolated to tumor sites (fig. S5B). ΔBC (but not ΔAC) levels were positively correlated with normal (high) expression of NKp30 (Fig. 4B), and there was a trend, which was not statistically significant, toward a negative correlation between ΔBC and sB7-H6 serum levels (fig. S6). The $\Delta\text{BC}^{\text{low}}$ phenotype was accompanied by a loss of NKp30-dependent T_H1 cytokine release (TNF α) (Fig. 4C). The analysis of PFS according to the median of each delta isoform was subsequently performed in a first (test) cohort of 120 HR-NB. Although the ΔBC analysis could not predict PFS in the whole cohort of 120 HR-NB, subgroup analysis only including 70 HR-NB (test cohort, Table 1) with minimal residual disease (MRD) after induction treatment showed a correlation between a high ΔBC and longer PFS (Fig. 5A). A first validation cohort of 55 HR-NB children with MRD (validation cohort 1, Table 1) confirmed the impact of ΔBC on PFS (Fig. 5B). A second validation cohort of

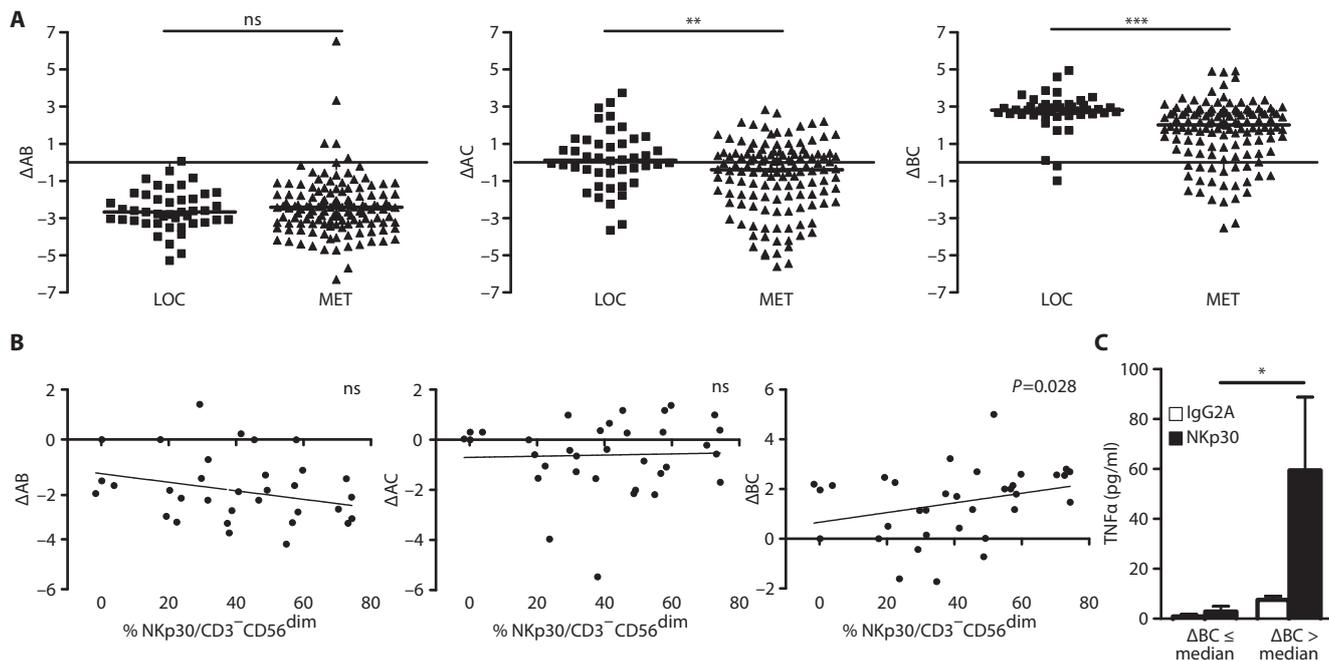


Fig. 4. Down-regulation of NKp30 expression and function in the ΔBC^{low} phenotype. (A) Total RNA was isolated from PBMCs of children bearing a localized or metastatic NB, and all three NKp30 isoforms were quantified by qRT-PCR relative to $\beta 2$ -microglobulin housekeeping gene. The delta of the three NKp30 isoforms was calculated using the following formula: $\Delta NKp30_{(\gamma)} = NKp30_{\gamma} - NKp30_{\alpha}$. Each dot represents the value for one child. (B) Correlations

between the ΔBC values and total NKp30 expression on CD56^{dim} NK. (C) Patients' NK cells exhibiting a ΔBC^{high} ($n = 3$) or ΔBC^{low} phenotype ($n = 3$) (relative to ΔBC median 1.88) were activated using anti-NKp30 (or isotype control) antibody and monitored by ELISA for TNF α release. The mean value \pm SD of TNF α secretion is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t test. Exact P values are provided in table S3.

71 HR-NB children with MRD (validation cohort 2, Table 1) further confirmed the effect of ΔBC on PFS (Fig. 5C). Considering the whole population composed of 196 children with MRD in these three cohorts, we conclude that ΔBC^{high} is a valuable variable to consider for analyzing PFS (Fig. 6A) in a Cox regression model. Multivariate analyses on this whole cohort of HR-NB patients with MRD demonstrated that high ΔBC contributes additional prognostic value even after stratification of NB patients according to their age at diagnosis or the presence of *N-MYC* amplification (Table 2). Thus, the ΔBC^{low} phenotype was associated with increased relapse rates among children with *MYC* nonamplified NB (Fig. 6B) and >18 months at diagnosis (Fig. 6C). Supervised microarray analysis focusing on T_H1 and NK cell versus T_H2/T_H17 inflammatory genes in 33 HR-NB BM samples harvested at the stage of MRD highlighted the link between the ΔBC^{high} phenotype and NK-related gene products such as *TNF*, *CCL4*, *NKp46/NCR1*, and *KLRK1* (*NKG2D*) (Table 3). Together, the existence of stable phenotypes in the preferential exon 4 usage in *NKp30* transcripts appears clinically relevant in HR-NB in that an imbalance between the predominant expression of the immunosuppressive NKp30C isoform over the immunostimulatory NKp30B isoform is associated with shorter PFS.

Restoring NKp30 functions in cells from individuals with a ΔBC^{low} phenotype

The NKp30/B7-H6 receptor/ligand interaction can result either from contact between monocyte cells and NK cells in lymphoid organs (32, 40, 41) or from interaction between NK cells and cancer targets expressing NKp30 ligands in tumors. In cocultures between dendritic and NK cells (32), NKp30A and NKp30B isoforms favored the secre-

tion of TNF α , IFN γ , and IL-12p70, whereas NKp30C isoforms promoted IL-10 production. Similarly, cocultures with B7-H6-expressing targets (such as the NB8 neuroblastoma cell line) resulted in NKp30C-driven IL-10 release (with low TNF α production) (fig. S7A) and NKp30A- or NKp30B-driven TNF α production (with low IL-10 release) (32). We speculate that, within the NB-infiltrated BM, neuroblasts and NK cells (and perhaps monocytes) engage in a regulatory cytokine network that is influenced by physical interactions between B7-H6 and functionally distinct NKp30 isoforms.

Therefore, we analyzed the regulation of *B7-H6* transcription in NB cell lines by distinct cytokines. Whereas IFN γ markedly reduced the expression of mRNA encoding *B7-H6* (and *BAT3* but not *B7-H1*; fig. S7, B to D), IL-10 increased the transcription of *B7-H6* (fig. S7, B to D). The exposure of tumor cells to IFN γ was accompanied by a reduced IL-2 release by NKp30-expressing effectors (fig. S7E). In contrast, TNF α did not play any major role in modulating *B7-H6* mRNA levels in any of these cell lines (fig. S7). Hence, it is conceivable that NKp30C induces IL-10 release that further enhances *B7-H6* transcription and NKp30 signaling, in a vicious feedback loop, culminating in NKp30 down-regulation and tumor escape. Conversely, by inducing IFN γ , NKp30A and NKp30B might limit the constant exposure of NKp30 to its ligand and prevent NKp30 exhaustion (42).

Considering the established prognostic significance of M2-like macrophages in HR-NB (43) and the fact that *B7-H6* is expressed in monocytic cells (Fig. 2D), we analyzed the differential expression of *B7-H6* on M1- and M2-like polarized macrophages. M1 and M2 expressed similarly low levels of *B7-H6*, but M1 cells expressed rather high levels of *B7-H1* (fig. S8). Both M1 and M2 could equally trigger NK

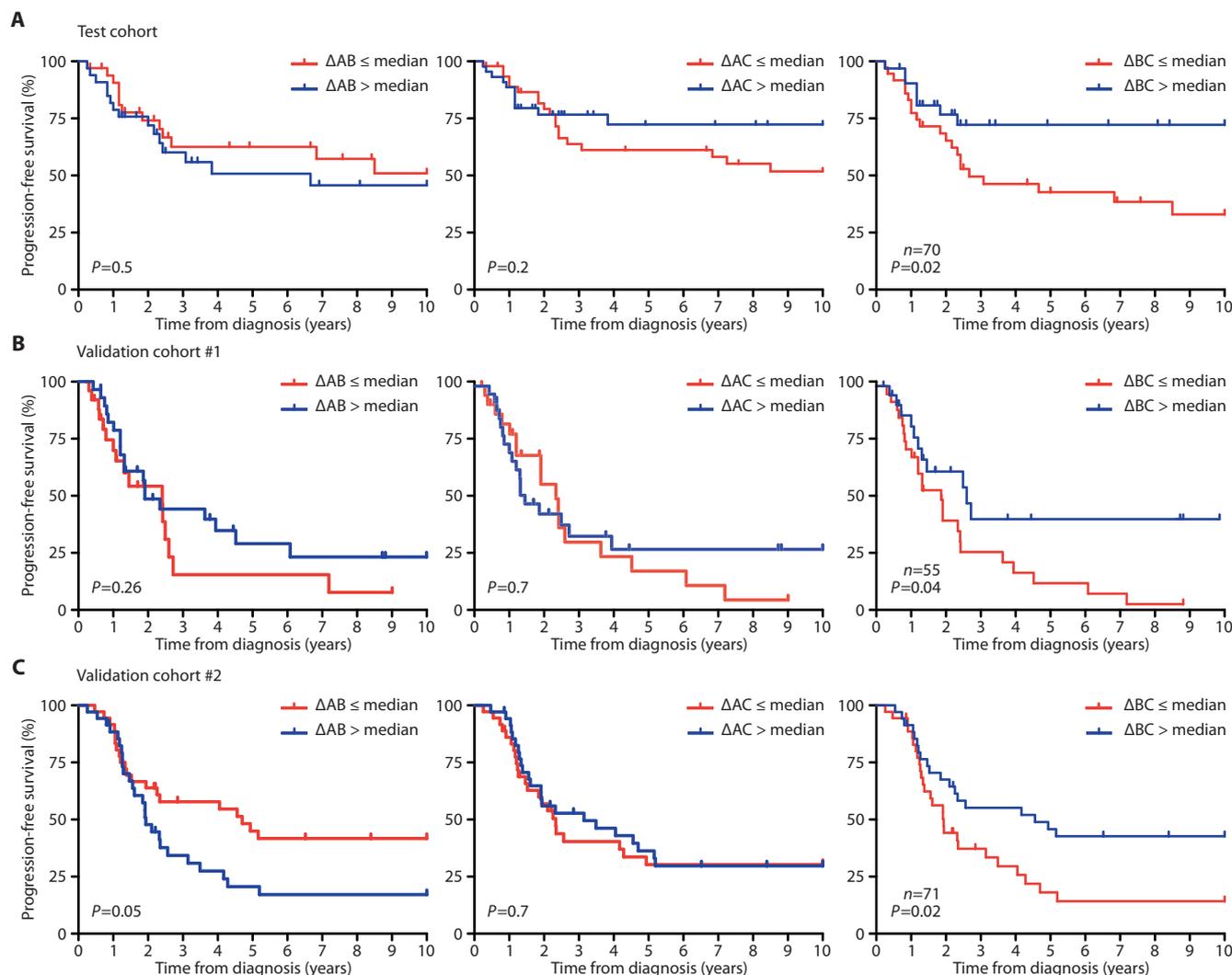


Fig. 5. Prognostic value of the NKp30 isoforms in HR-NB in multivariate analyses. HR-NB patients were clustered into two groups with respect to the median value of ΔBC , ΔAC , or ΔAB . (A to C) Kaplan-Meier curves of PFS are shown for the test cohort of 70 HR-NB children (A), for validation cohort 1 of $n = 55$ HR-NB children (B), and for validation cohort 2 of $n = 71$

HR-NB (C), all selected with MRD after intensified induction regimen. NKp30 profiling was determined on samples drawn at diagnosis or during or after the induction chemotherapy, before myeloablative therapy and ASCT. Notably, NK profile is stable over time (fig. S5). All P values are based on the log-rank test.

cell TNF α production (fig. S9A). Release of TNF α (but not CCL20) was NKp30/B7-H6-dependent only in the M2/NK cell dialogue (fig. S9, A to C). In contrast to M2, M1 also promoted IFN γ production in an NKp30-independent manner (fig. S9B). Recombinant TNF α could induce apoptosis in several NB cell lines (fig. S10). Ex vivo coculture assays revealed that monocytes amplified the NKp30-dependent stimulation of NK cells by NB cell lines. IL-10 and TNF α played differential roles, such that the former inhibited, and the latter facilitated, NK cell triggering (fig. S11). In conclusion, we propose a model in which M2-like macrophages affect the prognosis of HR-NB by influencing the secretion of the proapoptotic cytokine TNF α by NK cells from patients with an NKp30 ΔBC^{high} phenotype (fig. S12).

Next, we attempted to restore the NKp30-dependent IFN γ and TNF α secretion in patients exhibiting a ΔBC^{low} phenotype, who indeed present with low T H 1 cytokine release (Fig. 4C). Transfection of NK cells with

Lipofectamine combined with siRNA specific for NKp30C isoform reduced NKp30C RNA as measured by qRT-PCR (fig. S13A) and restored T H 1 cytokine release from NK cells derived from NB patients or healthy individuals presenting with a ΔBC^{low} phenotype (fig. S13, B and C). No significant modulation of NKp30 functions was obtained using a scrambled siRNA (fig. S13).

DISCUSSION

Very few biological prognostic factors identify patients with HR-NB that are amenable to cure or long-term survival (35). Dense infiltration of primary tumors by inflammatory macrophages or elevated expression of inflammation-related genes predicts short-term PFS in children aged >18 months without *N-MYC* amplification (44). Here, we show that the

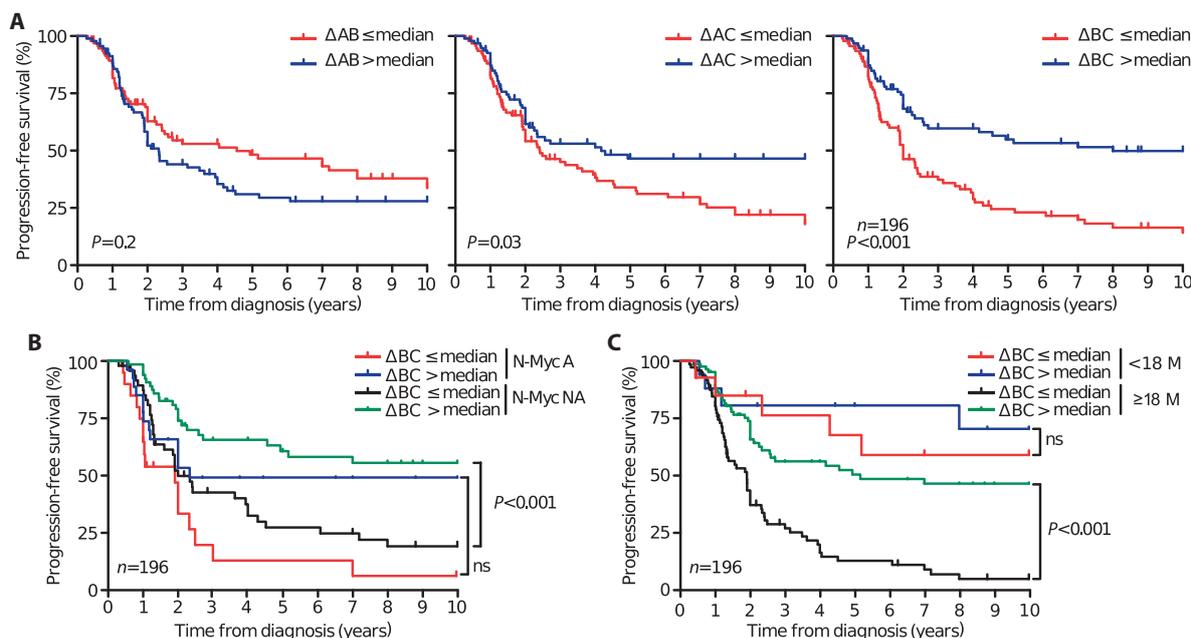


Fig. 6. Added value of NKp30 isoforms to clinicobiological parameters determining the prognosis of HR-NB. (A to C) Cox regression analyses and Kaplan-Meier curves of PFS in all three cohorts (test cohort + validation cohort 1 + validation cohort 2) of 196 HR-NB patients stratified according to the median (low or high) of ΔAB (median -2.37), ΔAC (median -0.51), and ΔBC (median 1.88) (A), according to the presence or absence of *N-MYC* amplifica-

tion (NA, not amplified; A, amplified) (B), or according to age <18 or ≥ 18 months (M) at diagnosis (C) (Table 3 presents the uni- and multivariate analyses). Notably, the NKp30 isoform phenotype remains highly predictive in the subset of NB children ≥ 18 months old and bearing *N-MYC* nonamplified tumors. *N-MYC* NA, nonamplified *N-MYC* oncogene; *N-MYC* A, amplified *N-MYC* oncogene. All *P* values are based on the log-rank test.

Table 2. Univariate and multivariate analyses of survival according to NKp30 profile and clinico-biological parameters. CI, confidence interval.

		ΔAB		ΔAC		ΔBC		
		<i>n</i> event/ <i>n</i> *	HR (95% CI), <i>P</i>	<i>n</i> event/ <i>n</i> *	HR (95% CI), <i>P</i>	<i>n</i> event/ <i>n</i> *	HR (95% CI), <i>P</i>	
Univariate analysis	$\Delta NKp30 \leq Median$	49/98		65/98		69/98		
	$\Delta NKp30 > Median$	62/98	1.32 (0.90–1.93), <i>P</i> = 0.1496	46/98	0.74 (0.50–1.09), <i>P</i> = 0.1235	42/98	0.52 (0.35–0.78), <i>P</i> = 0.0015	
Multivariate analysis [†]	$\Delta NKp30 \leq Median$	44/88		54/81		55/78		
	$\Delta NKp30 > Median$	48/80	1.46 (0.96–2.22), <i>P</i> = 0.0806	38/87	0.79 (0.51–1.22), <i>P</i> = 0.2788	37/90	0.50 (0.32–0.80), <i>P</i> = 0.0035	
	Age	<18 months	6/25		6/25		6/25	
		≥ 18 months	86/143	3.85 (1.65–8.99), <i>P</i> = 0.0018	86/143	3.18 (1.36–7.42), <i>P</i> = 0.0074	86/143	3.38 (1.47–7.79), <i>P</i> = 0.0042
	N-Myc	Nonamplified	61/119		61/119		61/119	
		Amplified	31/49	1.63 (1.04–2.56), <i>P</i> = 0.0332	31/49	1.57 (1.00–2.46), <i>P</i> = 0.0478	31/49	1.47 (0.94–2.31), <i>P</i> = 0.0926
Missing	19/28		19/28		19/28			

*HR-NB patients (pooled test cohort, validation cohort 1, and validation cohort 2, as depicted in Table 1) with MRD after induction chemotherapy according to the MIBG-SIOPEN criteria and the immunocytologic quantification of NB cells infiltrating the BM. [†]Event-free survival adjusted for *N-Myc*, age group (cutoff, 18 months), and $\Delta NKp30$ profile (data available for these three parameters for *n* = 168). Events are considered as local or metastatic relapses and disease-related deaths.

relative expression of stimulatory and inhibitory NKp30 isoforms may influence the 10-year PFS in three independent cohorts of HR-NB treated with induction chemotherapy.

Several arguments support the probable clinical relevance of this classifier. First, among all hematopoietic cells, NK cells represented

the only cell subset of those tested that was considerably altered in metastatic (as compared with localized) NB. Second, NKp30, which is an NK cell-specific receptor, was the only activating receptor of those tested to be severely down-regulated in the BM from metastatic NB patients. Third, NKp30 ligand B7-H6 was highly expressed in BM

Table 3. T_H1- and NK-related genes according to ΔBC^{high} expression. Microarray analyses have been performed on 33 BM samples of HR-NB in MRD after induction treatment. Log₂ fold changes (with 95% CIs) correspond to the

differences in gene expression between BM from patients exhibiting a ΔBC^{high} phenotype versus ΔBC^{low} phenotype. Supervised analysis of the expression of T_H1- and NK-related genes according to the ΔBC^{high} cohort is presented.

Symbol	Gene name	Gene location	95% CI	P	Fold change
CCL4	Chemokine (C-C motif) ligand 4	17q12	0.12 to 1.35	0.02*	0.73
CCL5	Chemokine (C-C motif) ligand 5	17q11.2-q12	-0.13 to 1.4	0.11	0.63
TBX21	T-box 21	17q21.32	-0.19 to 1.32	0.15	0.56
TNF	Tumor necrosis factor	6p21.3	0.02 to 1.05	0.04*	0.53
GZMA	Granzyme A	5q11-q12	-0.28 to 1.32	0.21	0.51
GZMB	Granzyme B	14q11.2	-0.25 to 1.11	0.22	0.43
KLRK1	NKG2D	12p13.2-p12.3	0.03 to 0.79	0.04*	0.41
FCGR3A	CD16	1q23	-0.23 to 1	0.22	0.38
EOMES	Eomesodermin	3p24.1	-0.25 to 0.88	0.28	0.31
NCR1	NKp46	19q13.42	0 to 0.47	0.05	0.24
CD274	PDL1	9p24	-0.4 to 0.86	0.47	0.24
CD226	DNAM-1	18q22.3	-0.07 to 0.53	0.14	0.23
PDCD1	PD-1	2q37.3	-0.41 to 0.61	0.68	0.11

*P < 0.05.

neuroblasts, and its serum soluble form correlated with tumor load and disease dissemination. Fourth, both the frequency of circulating NK cells and sB7-H6 serum concentrations were associated with chemoresistance. In conclusion, on one hand, NKp30 may play a major role in the natural immunosurveillance against NB in the equilibrium state of the host-tumor interaction; on the other hand, at late stages of tumor escape, the NKp30/B7-H6 dialogue is disabled, reflecting and/or participating in immunosubversion.

Together, these results obtained in NB patients are reminiscent of another tumor type of mesenchymal origin, GIST, which mostly affects adults (32, 40). GISTs also express B7-H6 and present with detectable sB7-H6 at diagnosis (45). Moreover, the ratio of NKp30 isoforms influences GIST prognosis (32). Pharmacological inhibition of the GIST-associated oncogenic tyrosine kinase by imatinib reduced infiltration by T regulatory cells (T_{regs}) and augmented that by effector CD8⁺ Tc1 and NK cells (45, 46). The number of NK cells within tumors positively correlated with overall survival in GIST (45). Whether primary tumors from HR-NB patients are infiltrated by NK cells remains elusive. However, BM cells from HR-NB patients do host numerous immune cells (including NK cells, among others, such as NKT and $\gamma\delta$ cells) that can interact with B7-H6⁺ neuroblasts, suggesting that they can influence malignant progression.

Although NK cells may play an important role in the natural immunosurveillance of NB because of the NB cells' low major histocompatibility complex class I expression and their high density of activating NCR ligands such as PVR (15), therapies aimed at directly boosting NK cell functions have so far been unsuccessful. Thus, phase 1/2 trials using recombinant IL-2 alone (47) or lenalidomide, which promotes IL-2 secretion by T cells and indirect NK cell triggering (48), did not yield objective responses in refractory or relapsing NB (49). Several reasons may account for the failure of these clinical trials. NB cells can suppress NK cell functions by multiple mechanisms [sMICA (50), B7-H3 (15), HLA-G (51), IL-6, TGF- β , and IL-10 (44, 52)]. NB with dismal prognosis expresses N-MYC, which, like other oncogene products

such as TP53, MYC, and BCR-ABL, promotes immune escape (43, 53, 54). As demonstrated in this article, NKp30 transcriptional status could compromise the NKp30-dependent effector functions of NK cells. Hence, future therapies of HR-NB might include specific methods to overcome local immunosuppression by myeloid-derived suppressor cells, as well as BM engineering to restore proper transcription of NKp30A or NKp30B and/or to suppress NKp30C expression, or epigenetic manipulations aimed at restoring the preferential expression of stimulatory NKp30 isoforms (55).

There are several limitations to the present study. At the cognitive level, the lack of an animal model for the NKp30 investigations (the mouse ortholog of *NCR3* is a pseudogene) makes it impossible to establish true cause-effect (rather than correlative) associations. Establishing a humanized mouse model of BM reconstitution with patient-derived autologous neuroblasts and NK progenitors from various NKp30 isoform profiles would be helpful to screen drugs capable of modulating this phenotype. At the clinical level, it may be critically annotated that our study only involves a total of 196 patients (although in three independent cohorts) from one single country (with a mostly Caucasian population), meaning that further validation studies must be performed on ethnically different populations. Moreover, the three cohorts investigated here only include HR-NB patients in complete remission that are treated by intensive chemotherapy. Thus, further studies of NKp30 isoform expression must include low-risk patients with NB that do not receive chemotherapy. Finally, at this stage, even if we may conclude that the presence of a preferential NKp30C isoform expression constitutes a negative prognostic marker for a specific population of patients with HR-NB, this does not affect the practical management of the disease, awaiting additional innovation in NK-centered immunotherapy.

In light of the results presented, it is tempting to envisage a vicious feedback loop, in which IL-10 produced by NKp30C-expressing NK cells enhances B7-H6 transcription by NB cells, hence allowing the tumor cells to down-regulate NKp30 expression and to condition an immunosuppressive environment. Conversely, ligation of NKp30B on

NK cells may trigger the release of TNF α , which is cytotoxic for NB cells and exerts antiangiogenic effects in conjunction with IFN γ (56). In addition, M2-like polarized macrophages could represent a major regulator of NK cell functions, as indicated by the findings that M2 macrophages express B7-H6 and that they trigger the release of TNF α by NK cells in an NKp30/B7-H6–dependent fashion. Hence, in those patients exhibiting a Δ BC^{low} phenotype (and thus defective production of the NKp30-dependent TNF α), M2 macrophages might contribute to the immunosuppressive microenvironment, whereas they could have the opposite effects in Δ BC^{high} patients (who produce a high amount of TNF α after NKp30 engagement). Therefore, blocking IL-10 or promoting IFN γ /TNF α secretion by CD4⁺ T cells (for instance, by means of immune checkpoint blockers such as ipilimumab) may constitute alternative strategies to correct the cytokine imbalance of Δ BC^{low} patients. It is also conceivable that antibodies neutralizing sB7-H6 serum molecules without impairing the membrane-bound form of B7-H6 could be therapeutically active, more specifically in Δ BC^{low} phenotypes. Yet, another strategy might consist in the generation of bispecific antibodies targeting GD2 and NKp30, mostly in Δ BC^{high} patients, where NK lymphocytes are able to release T_H1 cytokines in an NKp30-dependent manner. The up-regulation of B7-H1/PD-L1 on NB cell lines after IFN γ stimulation should also prompt the evaluation of PD-1/PD-L1 blockade in Δ BC^{high} patients. In conclusion, this study strongly supports the development of immunotherapies for the treatment of HR-NB that might be guided in their design or in their application by NK-specific biomarkers.

MATERIALS AND METHODS

Study design

The primary objective of this study was to evaluate the modulation of immunological parameters in PB and in BM samples from patients with localized versus metastatic NB at diagnosis. On the basis of these initial results, the overall goal of this study was to elucidate the role of the NKp30–B7-H6 axis in the long-term protective anti-NB response in HR-NB patients. Monitoring of B7-H6 expression and its soluble form (sB7-H6) was carried out on cell line specimens and patients' samples. Analysis of the NKp30 isoforms was performed by RT-PCR on PB samples isolated from patients with HR-NB. All studies were carried out with protocols that were reviewed and approved by institutional ethics committees and with informed consent from all participants. In particular, the NKp30 study on NB specimens was approved by the local ethics committee (2007-A00923-50).

Patients and specimens

The immunomonitoring study was carried out on a total of 48 fresh PB samples (12 from localized NB and 36 from metastatic NB) and 23 fresh BM samples (11 from localized NB and 12 from metastatic NB) collected at the time of diagnosis. The test cohort and the two validation cohorts included, respectively, 70, 55, and 71 frozen samples of PBMCs from HR-NB patients, with most samples being collected after induction chemotherapy, before myeloablative therapy and ASCT. Patients' characteristics are depicted in Table 1. The Pediatric Department of GRCC (Villejuif, France) provided the cohort 1, test cohort, and validation cohort 1 samples, and the Centre Léon Bérard (Lyon, France) provided the validation cohort 2 samples. HVs' PBMC samples were obtained from the Gustave Roussy Institute biobank (Centre Ressources Biologiques).

Immunomonitoring

The antibodies used for nine-color flow cytometry analysis are listed in table S1. Cells were incubated with conjugated antibodies (except for the GD2 antibody, which was revealed by a secondary antibody with indirect staining) for 20 min in the dark, washed, and fixed with 1% paraformaldehyde. Stained cells were acquired within 24 hours on a CyAn flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Cell lines and cultures

The human NB cell lines SH-SY5Y, NJB, N91, NB8, LAN-1, SK-N-SH, SK-N-BE, and IMR-32 were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Life Technologies) or in R10 medium, consisting of RPMI medium (Invitrogen, Life Technologies) with 10% heat-inactivated fetal bovine serum (Invitrogen, Life Technologies), 1% sodium pyruvate, 5 mM L-glutamine, and 1% penicillin-streptomycin, in 5% CO₂ at 37°C.

Monitoring of B7-H6 and sB7-H6

Fresh BM samples from four patients with BM-infiltrating NB at the time of diagnosis, as well as NB cell lines, were stained with conjugated antibodies, as detailed in table S1 for neuroblasts (CD45⁺, CD235ab⁺, CD9/CD81⁺, CD56⁺, and GD2⁺) and monocytes (CD45⁺ and CD14⁺). Anti-B7-H6 mAbs (17B1.3) conjugated to Alexa Fluor 647 were a gift from E. Vivier (Centre d'Immunologie de Marseille-Luminy, Marseille). Staining was analyzed on a CyAn flow cytometer (Beckman Coulter) using the FlowJo software (Tree Star Inc.). sB7-H6 was detected in the sera of patients by the following ELISA method. First, 96-well Nunc-Immuno plates (Thermo Scientific) were coated with 17B1.3 mAbs at 5 μ g/ml in 0.1 M NaHCO₃ solution overnight at 4°C. Blocking solution [phosphate-buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA)] was then added overnight at 4°C. After discarding this solution, serial dilutions of sB7-H6 were incubated in each well for 3 hours at room temperature. Then, biotin-conjugated B7-H6 was added to each well (1 μ g/ml in PBS supplemented with 1% BSA) for 1 hour at room temperature. Anti-biotin horseradish peroxidase (Sigma-Aldrich) was then added for 1 hour at room temperature. Finally, BD OptEIA TMB substrate (BD Biosciences) was used to reveal the staining and left to incubate for 15 to 30 min at room temperature. The reaction was stopped with 1 M HCl, and the optical density at 450 nm was read with Apollo LB 911 from Berthold. The threshold of sB7-H6 detection in our ELISA was 0.6 ng/ml. In a cohort of healthy individuals, sB7-H6 serum concentrations were below 3 ng/ml (37).

NK cell functional assays

Enriched NK cells (STEMCELL Technologies) from an HV were incubated overnight with sera that did or did not contain sB7-H6 (sB7-H6 level considered positive if higher than 3 ng/ml) before being cocultured with B7-H6⁺ K562 cells and brefeldin A (eBioscience) for 5 hours. In some experiments, anti-human NKp30 F(ab')₂ or anti-human NKp46 F(ab')₂ antibody used as control (10 μ g/ml, gift from C. Bottino, Genoa, Italy) was incubated with NK cells for 1 hour at 37°C before the coculture. The cocultured NK cells were harvested and stained with CD3 VioBlue (Miltenyi), CD56 PC7 (Beckman Coulter), and the dead cell removal reagent Vivid Yellow (Molecular Probes, Invitrogen). Cells were fixed, permeabilized with Fix/Perm reagents (BD Biosciences), and stained with IFN γ -phycoerythrin (BD) and TNF α -Alexa Fluor 647 (BioLegend).

siRNA transfection of NK cells using Lipofectamine RNAiMAX

NK cells were enriched from PBMCs of HV or patients using a STEMCELL Technologies kit. NK cells were transfected with a final concentration of 5 pmol NKp30C-specific siRNA (siRNA ID: s453382, sequence: UGUGUCCCAUGUGACAGUGG) or scrambled siRNA (Ambion Life Technologies) in Lipofectamine RNAiMAX reagent in Opti-MEM medium according to Life Technologies recommendation. After 48 hours, NK cells were harvested, the NKp30 transcript levels were assessed by qRT-PCR, and 5×10^4 NK cells were seeded in 96-Maxisorb plates (Nunc) coated with mouse IgG2a anti-NKp30 (2.5 µg/ml) (clone 210847, R&D Systems) or isotype control for 18 hours at 37°C. TNFα and IFNγ cytokines were monitored by commercial ELISA (BD Biosciences) in supernatants.

qRT-PCR and determination of NKp30 profiling

One hundred sixty-one of our 196 patients (gathered from the test cohort, validation cohort 1, and validation cohort 2) were tested during or after induction chemotherapy, before myeloablative therapy and ASCT. The other samples were drawn at diagnosis. Notably, the phenotyping of NKp30 isoforms was stable over time in HR-NB children in different locations (BM versus blood), as was previously observed in patients with GISTs (32) (fig. S5).

Total cellular RNA was isolated from PBMCs or cell lines with the RNeasy kit (Qiagen). First-strand complementary DNA (cDNA) was synthesized from 5 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen, 18080-044) and random primers (Promega, C1181) according to Invitrogen's instructions. For the qRT-PCR, 5 µl of first-strand cDNA was mixed with 12.5 µl of 2× TaqMan Gene Expression Master Mix (Applied Biosystems) and 0.75 µl of NKp30 primers (10 µM) and probe (5 µM) or 0.5 µl of β2M primers (10 µM) and probe (5 µM) in a final volume of 25 µl. Temperature cycling and real-time fluorescence measurement were done using a StepOnePlus System (Applied Biosystems). The PCR conditions were as follows: initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C (for *NKp30A*, *NKp30B*, *NKp30C*, and *β2M* transcripts) for 1 min. The PCR primers and TaqMan probes are listed in table S2. B7-H1 PCR primer and TaqMan probe were provided by Life Technologies. Analysis of relative gene expression was performed with the $2^{-\Delta C_t}$ method normalized to the level of expression of the housekeeping gene *β2-microglobulin*. The formula used is $2^{-\Delta C_t} = 2^{[C_t(\text{reference gene}) - C_t(\text{sample})]} \times 10^6$.

The expression of the distinct NKp30 isoforms compared with each other in each patient (delta) was calculated using the following formula: $\Delta NKp30_x NKp30_y = NKp30_y - NKp30_x$. The median of each delta was used for the survival analysis.

Coculture assays and cytokine release

IGR-NB8, LAN-1, or SKN-BE cells were cocultured with various cytokines (rTNFα, rIFNγ, and rIL-10) at various doses (32) or with medium for determination of annexin V/propidium iodide by flow cytometry at 24 hours. IGR-NB8 cells were cocultured with NKp30B-transduced Jurkat cells (1 IGR-NB8: 1 Jurkat NKp30B) for 24 hours in the presence of monocytes at a 1:1:1 ratio with or without various neutralizing IgM or IgG antibodies (specific for NKp30, B7-H6, IL-10, or TNFα). IL-2, IL-10, and TNFα in the supernatants were measured by ELISA (R&D Systems ELISA Kits).

Regulation of B7-H6 transcription with inflammatory or T_H1/Tr1 cytokines

NB cell lines were incubated with IFNγ, TNFα, or IL-10 for 6 hours. Cells were collected and stored in RNAlater solution (Qiagen). RNA

extraction and RT-PCR were performed as detailed before, and qRT-PCR was performed to determine B7-H6 expression (StepOne software version 2.0). Data were analyzed using the $2^{-\Delta C_t} \times 10^6$ method normalized to the level of expression of the housekeeping gene *β2-microglobulin* according to the manufacturer's recommendations.

Monocyte and macrophage isolation

Monocytes were isolated from PBMCs or BM using the Human Monocyte Isolation Kit from Miltenyi Biotec. Cells were cultured for 7 days with M-CSF (100 ng/ml) and then stimulated for 24 hours with rIL-4 (20 ng/ml) to obtain M2-polarized macrophages or with IFNγ (20 ng/ml) and lipopolysaccharide (100 ng/ml) to obtain M1-polarized macrophages. Cells were then cocultured with autologous isolated NK cells at a 1:1 NK/macrophage ratio.

Microarray analysis

Global gene expression analyses were performed on 33 cDNA samples from BM of HR-NB patients in complete remission of metastases after induction treatment, using the Human Gene Expression 8 × 60 K Agilent Human Microarray Kit. The microarray analysis was performed using the Agilent Human Gene Expression Microarray technology, and the Agilent data were extracted using the software provided by the manufacturer. Gene relative expression is expressed in arbitrary units of fluorescence intensity. The data presented in Table 3 are log₂ fold changes between differences of gene expression in BM from patients exhibiting a ΔBC^{high} phenotype versus ΔBC^{low} phenotype (57).

Statistical analyses

Fisher's exact test, χ^2 test, one-way analysis of variance (ANOVA), and the nonparametric Mann-Whitney test were used for comparison of different groups. These statistical analyses were performed with the GraphPad Prism software version 5. The survival curves were plotted according to the Kaplan-Meier method and compared using the log-rank test (Mantel-Cox).

SUPPLEMENTARY MATERIALS

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- Fig. S1. Enumeration of various leukocyte subsets in PB and BM of localized or metastatic NB.
 Fig. S2. Clinical relevance of the selective increase in CD3⁺CD56^{bright} NK cells in HR-NB.
 Fig. S3. Survival of HR-NB according to NKp30 NK cell surface expression.
 Fig. S4. Expression of NKp30 isoforms and prognosis in HR-NB.
 Fig. S5. Stable expression of the NKp30 isoforms in HR-NB.
 Fig. S6. sB7-H6 serum concentrations not correlated with the relative expression of NKp30 isoforms.
 Fig. S7. Regulation of B7-H6 expression.
 Fig. S8. Membrane expression of B7-H6 in M1- and M2-like polarized macrophages.
 Fig. S9. Crosstalk between M1- or M2-like polarized macrophages and autologous NK cells.
 Fig. S10. Direct proapoptotic effects of rTNFα and rIFNγ on various NB cell lines.
 Fig. S11. Opposite roles of IL-10 and TNFα in the dialogue between monocytes, NB, and effector cells.
 Fig. S12. Schematic representation of the interactions between NK, NB, and macrophages in the context of NKp30 isoform profiling.
 Fig. S13. Restoration of NKp30 function by down-regulation of NKp30C with siRNA.
 Table S1. Details of antibodies used for the immunophenotyping.
 Table S2. Oligonucleotides used in this study.
 Table S3. Exact *P* values.

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Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients

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Editor's Summary

Unleashing the natural killer within

Natural killer cells, a part of the innate immune system, can kill cancer cells. Neuroblastoma is a common pediatric cancer that is difficult to treat, especially in older children with metastatic disease. The immune system helps to control the spread of neuroblastoma, and immune-based treatments for this cancer are under active investigation. Now, Semeraro *et al.* analyzed the role of natural killer cells in neuroblastoma and sought to understand why they are not always equally effective against the tumor. The authors found that the key lies in the predominant isoform of a receptor that natural killer cells use to interact with neuroblastoma cells and that the balance between isoforms of this receptor on a patient's cells can help predict survival.

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Original Research

Stability of ipilimumab in its original vial after opening allows its use for at least 4 weeks and facilitates pooling of residues



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KEYWORDS

Ipilimumab;
Monoclonal antibody;
Stability;
Immunotherapy

Abstract Purpose: Well-documented stability data of monoclonal antibodies are generally missing. That is why we studied the physicochemical and biological stability of undiluted ipilimumab (IPI) in glass vial (5 mg/ml) over 28 d after opening, stored at 4°C and 25°C.

Method: A stressed study (60°C) was performed to validate our analytical methods as 'stability indicating'. The different methods used were turbidimetry, dynamic light scattering (DLS), second-derivative ultraviolet and chromatographic methods as size-exclusion chromatography (SEC) and cation-exchange (CEX). Biological characterisation was performed by an *in vitro* functional binding inhibition bioassay.

Results: We demonstrated that ipilimumab in opened vials stored at 4°C and 25°C remained stable for at least 28 d. No physical, chemical or structural instability was found. No aggregation was observed by turbidimetry, SEC and DLS. Hydrodynamic diameters remained unchanged, as chromatographic profiles in CEX and thermal aggregation curves. Functionally, the ability of IPI to antagonise CTLA-4/B7.2 binding remained stable over 1 month at 4°C.

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Conclusion: These results indicate that unused residues of IPI in their original vials can be safely kept up to 28 d, following good manufacturing procedures, allowing re-use for another patient or in case of cold-chain rupture.

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1. Introduction

A paradigm shift is currently occurring in oncology where monoclonal antibodies (mAb) are targeted against immune cells rather than cancer cells [1,2]. The revolution started with ipilimumab (IPI, Yervoy[®], BMS), an IgG1 κ mAb designed to antagonise the inhibitory immune checkpoint molecule cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). A maximum of four intra-venous infusions of this immunomodulatory mAb can reinvigorate an efficient antitumour cytotoxic T-cell immunity and cure 20% of metastatic melanoma patients [3–5].

Like other biotherapies, the main problem of mAb is the difficulty to assess their stability. These sensitive products can undergo more complex degradation pathways than classic drugs [6]. Their activity depends on their primary but also secondary or tertiary structures, which could be altered under in-use stresses [7]. MAB are prone to undergo a variety of physical and chemical instability reactions [8,9], like aggregation, unfolding or absorption onto surfaces. Chemical instability is commonly caused by deamidation of Asparagine residues. These instabilities may lead to a loss of efficacy or appearance of toxic effects such as immune reactions. That is why complementary methods should assess their structural properties and their rate of aggregation or chemical degradation during storage [10].

Because of its proteic nature, IPI may go through instability during practical use. Thus, the manufacturer recommends use of IPI solution in vial within 24 hours after opening. But our team has previously demonstrated that some widely used mAb remained stable in solution for several months, like Rituximab [11]. Thus, it can be hypothesised that stability of undiluted IPI opened under aseptic conditions would be more than 24 hours.

It is of paramount importance for hospital pharmacists to have well-documented data about the real stability of an opened drug formulation, because of the centralised preparation in pharmacy departments. Moreover, these data may be helpful for specific medical applications. Different authors showed in pre-clinical and clinical studies that the intratumoural delivery of very low doses of an anti-CTLA-4 antibody resulted in an antitumour effect, while avoiding the systemic auto-immune toxicity [12–16]. In case of human application, a vial opening to withdraw only few milligrammes would result in an enormous waste, considering the very high cost of IPI [17]. Extended stability of unused vial

residues of IPI beyond 24 hours, both in pharmacies and in oncology departments, would allow hospitals to realise significant cost savings.

The objective of this study was to assess the physicochemical and functional stability of IPI in commercial vial (5 mg/ml) over 28 d after opening in aseptic environment and kept under optimal storage temperature (4°C), and higher (25°C) to test possible temperature excursions. Several complementary analytical methods were used to determine changes in physicochemical properties of IPI. Then, concerning biological activity, binding of IPI to CTLA-4 was determined using a cell-based competitive binding assay. According to international conference of consensus (ICH) Q5C [18], forced degradation studies may help to evaluate analytical methods as indicators of stability and to elucidate the degradation profile of the drug. That is why we also conducted some experiments in highly stressing conditions i.e. 60°C as a methods' validation tool.

2. Material and methods

Stability of IPI was studied in original vials after opening, from the commercially product Yervoy[®] (Bristol-Myers Squibb, United States of America [USA]), presented as a ready-to-use solution of 5 mg/ml in glass vials. One vial was stored at each storage temperature tested: 4°C [2–8°C] and 25°C [20–25°C], with protection against light. A reference sample was obtained after the immediate opening of vials (D0) and analyses were next performed at days D14 and D28. For functional activity, assays were performed only on vial stored at 4°C upon opening (D0) and after 30 d of storage (D30). Each measurement was done in triplicate except for thermal denaturation curves (in duplicate). A parallel study was done to demonstrate that our methods were stability indicating by heating IPI (5 mg/ml) at 60°C in an ICH climatic incubator with 75% of relative humidity (Climacell[®]; Bioblock Fisher Scientific, France) and analyses were performed at hours H0, H8, H24, H48, H72 and H96.

For chromatographic analysis, the samples were diluted at 1 mg/ml with a dilution buffer [20 mM K₂HPO₄ pH 6.0] and centrifuged at 5000 rpm for 5 min.

2.1. Physico-chemical stability

Analytical methods used to assess physico-chemical stability were those used and validated by our team for similar mAb as rituximab or cetuximab [11,19].

Briefly, an ultraviolet-visible (UV-VIS) spectrophotometer (Cary 50 Probe, Varian, USA) was used to measure the optical density (OD) at 350 nm (turbidity) of samples not diluted. The second derivative UV spectrum, in the near-UV region (250–320 nm), was used to assess the tertiary structure. Dynamic light scattering (DLS) measurements were done with a Malvern Zetasizer Nano ZS (Worcestershire, United Kingdom) on undiluted samples, giving the size and hydrodynamic diameter distributions of the different populations. DLS was also used to trace denaturation curves and determine the protein melting temperature (T_m), by following protein size during heating from 55°C–80°C. Chromatography was performed with an Ultimate 3000 system coupled with a Chromeleon[®] workstation and an UV detector (Dionex Corporation, Sunnyvale, CA, USA). The different ionic variants of IPI were separated by cation exchange chromatography (CEX) with a ProPac WCX-10 analytical column (4 mm × 250 mm; Dionex, Sunnyvale, CA) at ambient temperature and monitored at 280 nm. 100 µl of samples were eluted with a linear gradient (1 ml/min) of buffer B [20 mM MES + 200 mM sodium chloride, pH 6.5] from 0%–50% over 50 min, followed by an equilibrium phase of 10 min to obtain 100% of buffer A [20 mM MES, pH 6.5]. Size-exclusion chromatography (SEC) was performed using a Yarra 3µm-SEC3000 column (300 × 7.8 mm, Phenomenex, Le Pecq, France) preceded by a GFC3000 guard column (4 mm × 3.0 mm), and the elution was monitored at 280 nm at room temperature. 40 µl of samples were eluted with a pH 7 buffer [0.2 M K_2HPO_4 and 0.25 M KCl], at a flow rate of 0.6 ml/min.

2.2. Functional bioassay

To demonstrate the ability of IPI to block the interaction of CTLA-4 to his ligand B7.2, a competition assay was performed by flow cytometry. The CTLA-4 producing hybridoma cell line (CTLA4 Ig-24, ATCC[®] CRL-10762TM) was cultured in complete Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with 10% of foetal bovine serum, 1% glutamine, 1% antibiotic (penicillin and streptomycin, Life Technologies) supplemented with 0.2 mM proline (Sigma-Aldrich) and 1 µM methotrexate (Mylan) at 37°C in 5% CO₂. These CTLA-4-Ig producing cells were detached

with 0.05% Trypsin (Life Technologies) then fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). The cells were treated with freshly opened (D0) or 30-day-old IPI (D30), or rituximab as a negative control, for 30 min. The cells were stained with recombinant human B7.2 (rhB7-2-Ig, R&D system) conjugated with Alexa Fluor 647 Protein Labeling Kit (Life Technologies) or an isotype control. Then the cells were washed two times and analysed by flow cytometry (BD, Canto II). The results were analysed with FlowJo software.

2.3. Statistical analysis

The results are presented as mean ± standard deviation from three separate samples. Non-parametric Wilcoxon tests were used, if required, to compare D14 or D28 results to the reference sample (D0) (KaleidaGraph 4.0, synergy Software). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Physical stability

Turbidity was not modified after 28 d at 4°C and 25°C (Table 1). In contrast, after only 8 hours at 60°C, an important increase in OD was found (0.2477 ± 0.0047). This increase was linear ($r^2 = 0.99$) up to 48 hours of heating, following by a decrease reaching a plateau after 72 hours.

The 2nd order derivative UV spectrum showed no shift for the maxima corresponding to the aromatic amino acids (Phe: 253, 259 and 266 nm ; Tyr: 276 nm ; Trp: > 280 nm) during the 28 d storage at 4°C and 25°C. However, there was a significant structural change after 4 d at 60°C, resulting in a major red shift (1 nm) at 259 nm and other weak shifts as showed in Fig. 1.

Hydrodynamic diameter of IPI remained around 12 nm after 28 d at both temperatures with a low polydispersity index (< 0.1) (Table 2). However, after 8 hours of heating, the main peak diameter increased, associated with the appearance of two additional peaks (Fig. 2) and polydispersity (Table 2).

The T_m values were not significantly different between D0 and D28 (73.44 ± 0.16 versus 73.47 ± 0.04 °C

Table 1
Optical density of IPI (5 mg/ml) at 350 nm after different storage conditions.

	D0	D14	D28			
4°C	0.0644 ± 0.0003	0.0657 ± 0.0011	0.0672 ± 0.0002			
25°C	0.0649 ± 0.0005	0.0666 ± 0.0006	0.0681 ± 0.0005			
	H0	H8	H24	H48	H72	H96
60°C	0.0854 ± 0.0009	$0.2477 \pm 0.0047^*$	$0.2154 \pm 0.0032^*$	$0.3019 \pm 0.0054^*$	$0.1705 \pm 0.0089^*$	$0.1393 \pm 0.0039^*$

No significant difference was observed between D0 and D28 for both storage temperatures while turbidity was significantly increased after heating at 60°C. (*: $p < 0.05$ versus D0 at 4°C and 25°C).

IPI, ipilimumab.

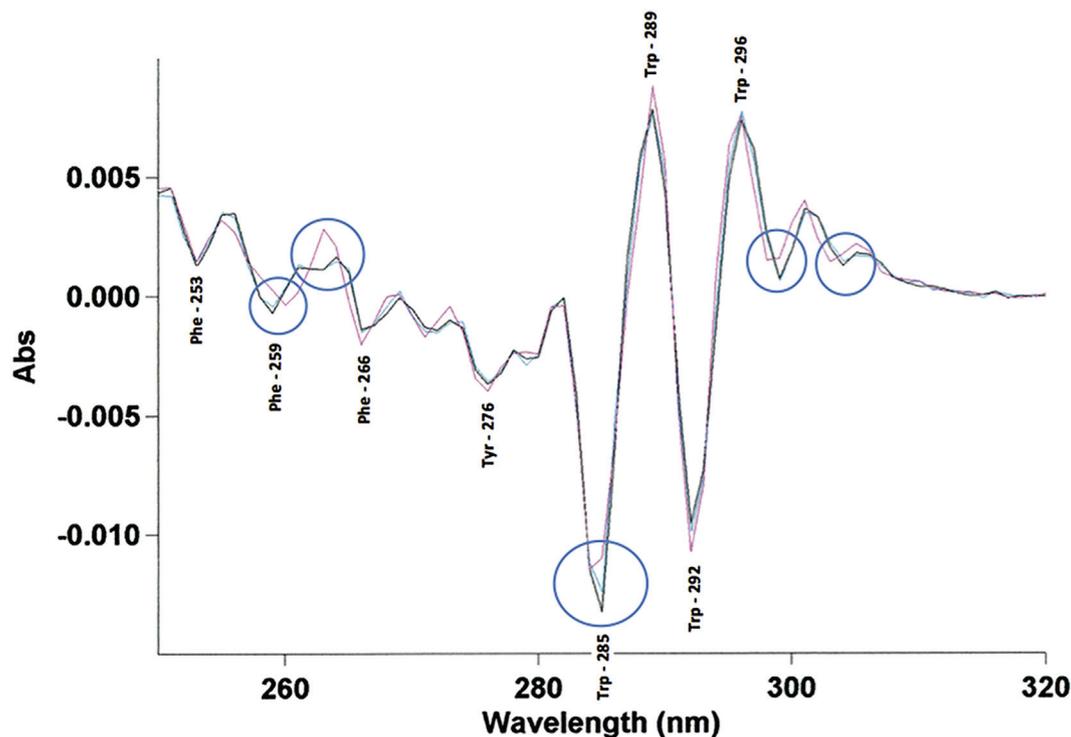


Fig. 1. Second derivative UV spectra of IPI samples. Comparison of spectra obtained immediately after opening (blue line) or after storage at 25°C during 1 month (black line) and 4 d at 60°C (red line). The concentration of samples was 0.4 mg/ml. No difference was observed in the maxima and minima corresponding to the aromatic amino acids (Tyr, Phe and Trp) between control and sample stored 1 month at 25°C, indicating no modification of the tertiary structure. Whereas, for heated sample, shifts (indicated by circles) were observed in several minima (259, 285, 299 and 304 nm) and maxima (285 and 263 nm). IPI, ipilimumab. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 4°C; and 73.37 ± 0.12 versus $73.33 \pm 0.04^\circ\text{C}$ at 25°C). Nevertheless, at 60°C, a weak but significant decrease was observed ($73.16 \pm 0.03^\circ\text{C}$ after 48 hours of heating).

3.2. Chemical stability

At the initial state (D0), the commercial solution showed a major peak in SEC, corresponding to the native protein, and two front shoulders revealing the pre-existence of dimers and high molecular weight species (HMW) (Fig. 3 and Table 3). This profile remained unchanged and total AUC and the relative percentage of peaks did not change up to 28 d at 4°C and 25°C (Table 3). After heating for a week, a linear decrease in the total AUC was found ($r^2 = 0.95$).

CEX showed a chromatographic profile of IPI (D0) compounded of five peaks (Fig. 4), with a major isoform. In both storage conditions, AUC and proportion of each peak remained unchanged at 28 d (Table 4). On the contrary, a linear decrease in total AUC was observed at 60°C reaching 41% after 4 d ($r^2 = 0.98$). Furthermore, the principal peak's AUC decreased, as the last basic peak, and the first acidic peak was increased of 25% (Fig. 4).

3.3. Functional activity

The ability of D30 IPI to antagonise the CTLA-4-B7.2 interaction has been compared to the functionality of IPI tested the day of the vial opening (D0). With

Table 2

Mean hydrodynamic diameter (nm) of IPI (5 mg/ml) in different storage conditions, determined by dynamic light scattering.

	Storage (d)	Main peak	2 nd peak	3 rd peak	PdI
4°C	D0	12.04 ± 0.07	-	-	0.075 ± 0.030
	D28	11.77 ± 0.09	-	-	0.046 ± 0.010
25°C	D0	11.94 ± 0.26	-	-	0.053 ± 0.020
	D28	11.89 ± 0.09	-	-	0.067 ± 0.015
60°C	H96	$13.39 \pm 0.41^{**}$	56.62 ± 7.94	1539.25 ± 249.84	$0.422 \pm 0.024^{**}$

Each value represents the mean \pm SD of 3×2 separate determinations. No significant difference was observed after 28 d of storage, while mean diameter and polydispersity index (PdI) were significantly increased after 96 hours of heating. (**: $p < 0.001$ versus D0 at 4°C and 25°C).

IPI, ipilimumab; SD, standard deviation.

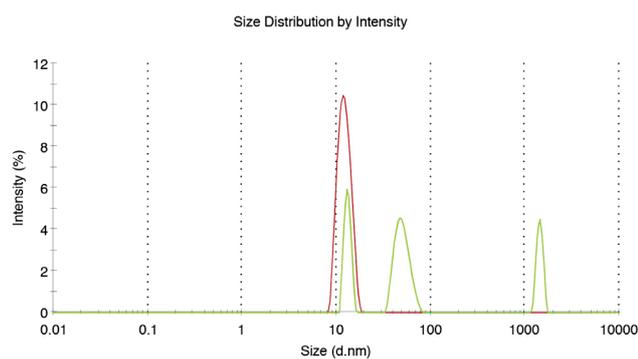


Fig. 2. Size distributions of the hydrodynamic diameters (nm) of IPI (5 mg/ml) determined by dynamic light scattering. Results are presented by intensity with the size in log scale. Control (D0; red line) showed a monodisperse population of around 12 nm (monomer and dimer), whereas after 4 d at 60°C (green line), two new populations of higher diameters were observed, indicating a trend to aggregation. IPI, ipilimumab. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negative control rituximab, there was no competition at all, while an important fluorescence decrease was observed with IPI at D0 as well as at D30 (Fig. 5). This functional binding inhibition assay could demonstrate that D30 IPI was as good as D0 IPI to prevent the B7.2 binding to the CTLA-4 positive cells.

4. Discussion and conclusion

The study of IPI stability using several orthogonal methods demonstrated that IPI in opened vials (5 mg/ml) stored at 4°C and 25°C remained stable for at least 28 d. No physical instability was found, translated by the absence of aggregation observed by turbidimetry, SEC and DLS. SEC did not show either fragmentation. Ionic profiles in CEX and thermal aggregation curves remained unchanged. Also, the functional ability of IPI to antagonise CTLA-4/B7.2 binding remained stable over 30 d, in the opened vial stored at 4°C. These results indicate that the time frame for the practical use of unused residues of IPI can be safely extended to 1 month, at 4°C and following good manufacturing procedures, to ensure maximal security in terms of sterile conditions and quality controls. The good stability at room temperature also supports the possibility of not throwing away IPI's vials in case of unexpected short-time cold-chain rupture.

To our knowledge, this work is the first studying the physicochemical and functional properties of IPI and its stability beyond the 24 hours manufacturer's stability limit after opening. However, mAb can undergo long-term stability either in original vial or in diluted solutions, as showed by several authors [20,21]. Also, mAb are generally stable enough at room temperature, as reported by Brader [22] with 8 mAbs, stable in glass

vials over 6 months at 25°C. And a long-term incubation at relatively high temperature (i.e. 40°C or 37°C) was necessary to observe significant physicochemical instability [23,24]. Therefore, our results converge with these data to conclude that mAb in solution are relatively stable over time. Moreover, these results are not surprising considering the high T_m values of mAb (range of 70–80°C) compared with other proteins [25]. In order to elucidate degradation mechanisms, we exposed IPI at 60°C during several days. Hawe et al. recommended to use a temperature at least 10–20°C below the T_m , during thermal stress testing [26], where unfolded molecules are already present, leading to other degradation reactions. Above the T_m , severe aggregation may occur, hiding intermediate degradation products.

Physical instability, the first response to stress, results mainly in aggregation, which may potentially enhance immunogenicity [27]. Then, stability studies should ensure that aggregate contains stay minimal, with different methods, to cover a broad size range [7]. Furthermore, sub-visible particles are difficult to assess because they are too large for analysis by SEC (> 0.1 μm) but too small to be visible to the naked eye (< 100 μm) [28]. But DLS analysis is very sensitive in detection of these submicron aggregates (0.6–6 μm) [29]. Concerning IPI, aggregation was observed only after 8 hours at 60°C stressed conditions. Medium-sized aggregates were formed, showed by turbidity, as small-sized aggregates, with the appearance of two sub-populations in DLS at 60°C (Table 2). SEC also revealed the increase in soluble oligomers. Finally, chromatographic analyses showed the formation of insoluble aggregates, demonstrated by the decrease of total AUC after heating, because large aggregates were removed by centrifugation. On the contrary, IPI solutions stored for 28 d at 4°C or 25°C neither showed this kind of aggregation signs, whatever the size range studied. Denaturation did not occur considering the absence of new peak of fragmentation in SEC.

MAb are heterogeneous due to post-translational modifications and degradation events that may occur during manufacturing process or shelf-life of the product [30]. Such modifications may alter structure, stability or biological functions. No alteration appeared with the methods employed for IPI, whereas under stressed conditions (60°C), proportion of IPI isoforms was modified in CEX. The increase of acidic species observed is coherent with a thermal-induced deamidation.

After showing the integrity of the physico-chemical properties of IPI, we wanted to confirm its functional activity. Therefore, we characterised the ability of IPI to inhibit binding between CTLA-4 and its costimulatory ligand B7.2 [31], leading to its immune stimulatory activities [2]. IPI displayed the same binding profile of CTLA-4-positive cells after 1 month storage at 4°C

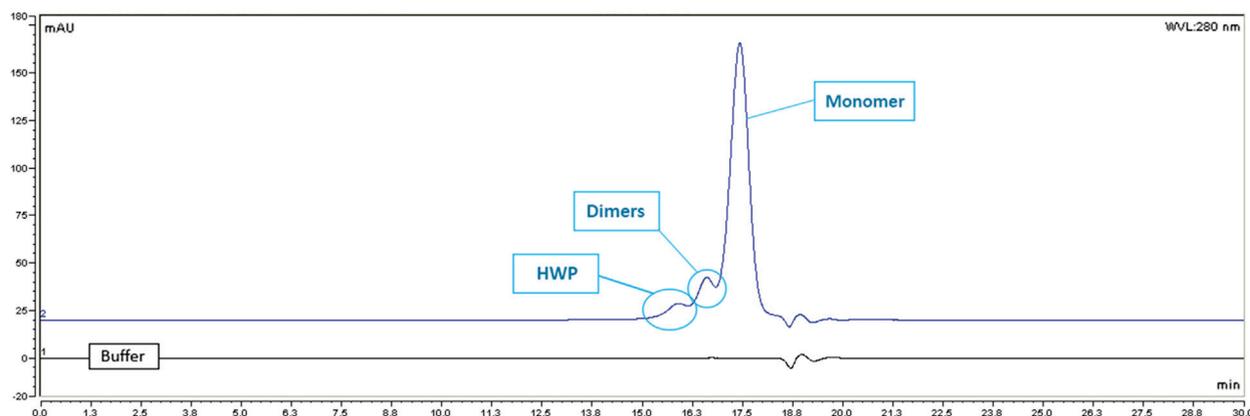


Fig. 3. Elution profile of IPI (1 mg/ml) by SEC. Centrifuged sample of IPI at initial state (D0; blue line), eluted using a Yarra 3u-SEC3000 column with detection at 280 nm. Inferior curve (black = buffer) represents the elution of 40 μ l of dilution buffer [20 mM K_2HPO_4 pH 6.0]. IPI, ipilimumab; SEC, size-exclusion chromatography; HMW, high molecular weight species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Mean total area under the curve (AUC : mAU \times min) and relative percentages of monomer, dimer and high molecular weight species (HMW) of IPI, estimated by SEC.

	4°C		25°C		60°C
	D0	D28	D0	D28	H96
Total AUC	93.91 \pm 0.45	93.37 \pm 1.23	90.78 \pm 1.13	94.20 \pm 1.45	74.37 \pm 0.55*
HWP %	4.45 \pm 0.07	5.12 \pm 0.11	4.06 \pm 0.35	5.22 \pm 0.05	7.25 \pm 0.38*
Dimer %	10.61 \pm 0.04	11.25 \pm 0.06	10.51 \pm 0.15	11.63 \pm 0.17	19.12 \pm 0.07*
Monomer %	84.94 \pm 0.06	83.63 \pm 0.17	85.43 \pm 0.21	83.15 \pm 0.15	73.63 \pm 0.98*

The percentages were calculated by dividing the respective peak areas by the sum of the three peaks and multiplying by 100. No significant difference was observed between D0 and D28 for both storage temperatures (4°C and 25°C), whatever the peak, in contrast with heated sample (mean \pm SD, $n = 3$). (* : $p < 0.05$ versus D0 at 4°C and 25°C).

IPI, ipilimumab; SEC, size-exclusion chromatography; SD, standard deviation; AUC, area under the curve; HMW, high molecular weight; AU = arbitrary unit.

(Fig. 5). This was expected given that structure and micro-heterogeneity profile of IPI was maintained. Bioassays studying functional activity of mAb give complementary data, but should not be used alone

without confirmation of physico-chemical stability and absence of potentially immunogenic aggregates [7].

The very high cost of IPI prevented us to carry out our stability study on three different batches, as

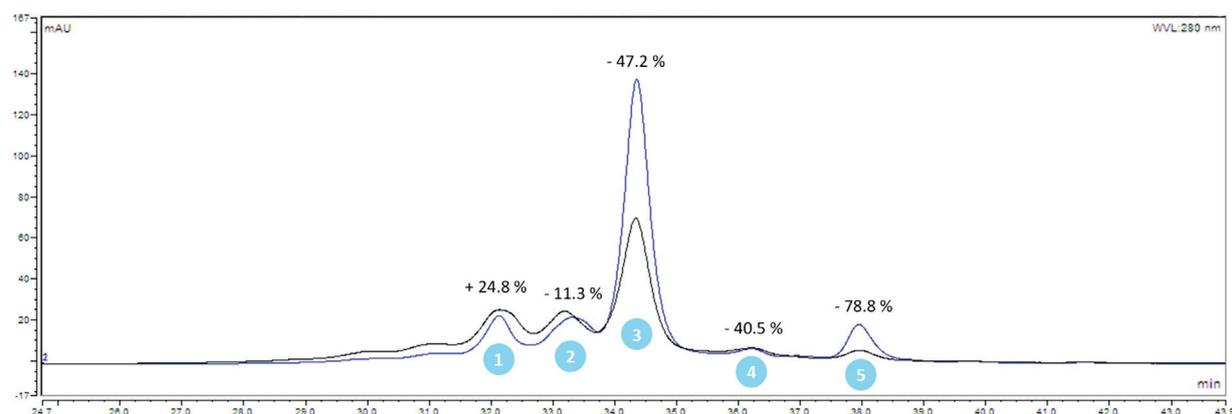


Fig. 4. Cation-exchange chromatographic profile of IPI (1 mg/ml). CEX separation was done using a WCX-10 column at pH 6.0 eluted with a linear salt gradient from 0–50% of 200 mM NaCl and detection at 280 nm. H0 profile (blue line) was compared to 4 d profile after heating at 60°C (black line). AUC of the different peaks was modified (+24.8%, –11.3%, –47.2%, –40.5% and –78.8% for peaks 1, 2, 3, 4 and 5 respectively). IPI, ipilimumab; CEX, cation-exchange; AUC, area under the curve. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4

Mean area under the curve (AUC: mAU × min) of total peak and relative proportion of each peak of IPI, in cation-exchange chromatography.

	4°C		25°C		60°C
	D0	D28	D0	D28	H96
Mean total AUC	99.97 ± 2.48	99.99 ± 1.79	100.10 ± 1.21	101.01 ± 6.72	67.94 ± 0.41*
Peak 1 %	8.86 ± 0.09	9.00 ± 0.06	8.96 ± 0.27	9.39 ± 0.09	18.36 ± 0.36*
Peak 2 %	13.4 ± 0.20	12.76 ± 0.14	13.21 ± 0.13	12.97 ± 0.03	18.82 ± 0.32*
Peak 3 %	66.13 ± 0.16	66.87 ± 0.39	65.88 ± 0.04	67 ± 0.27	56.50 ± 0.38*
Peak 4 %	3.44 ± 0.18	3.22 ± 0.17	3.7 ± 0.02	3.1 ± 0.15	3.34 ± 0.16
Peak 5 %	8.17 ± 0.13	8.15 ± 0.04	8.25 ± 0.07	7.54 ± 0.07	2.99 ± 0.07*

Separation was done using a WCX-10 column at pH 6.0 eluted with a linear salt gradient from 0–50% of 200 mM NaCl and detection at 280 nm. The percentages were calculated by dividing the respective peak areas by the sum of all the peaks and multiplying by 100. No significant difference was observed between D0 and D28 for both storage temperatures whatever the peak, in contrast with heated sample (mean ± SD, $n = 3$). (*: $p < 0.05$ versus D0 at 4°C and 25°C).

IPI, ipilimumab; SD, standard deviation; AUC, area under the curve.

recommended by ICH. But for practical studies, the European consensus admits to use only one batch for licenced drug [7]. For biological products, intrinsic variability between production batches exists, with variations of physicochemical properties. Experiments using three batches would allow us to calculate a batch-to-batch variability for the different parameters, which could be used as the basis for acceptance limits as proposed by Visser et al. [32]. However, since our study was comparative and showed no modification for all the

tested parameters, it can be concluded that IPI remains stable 1 month at 4°C and 25°C under our experimental conditions.

Together, our results support the pooling of IPI residues in pharmacy practice, beyond 24 hours and until 4 weeks after vial opening. Because of its positive economic impact for hospitals and clinical research funding, we believe these data will contribute to the elaboration of academic sponsored trials with IPI, notably for new cancer indications. Moreover, as the current use of anti-

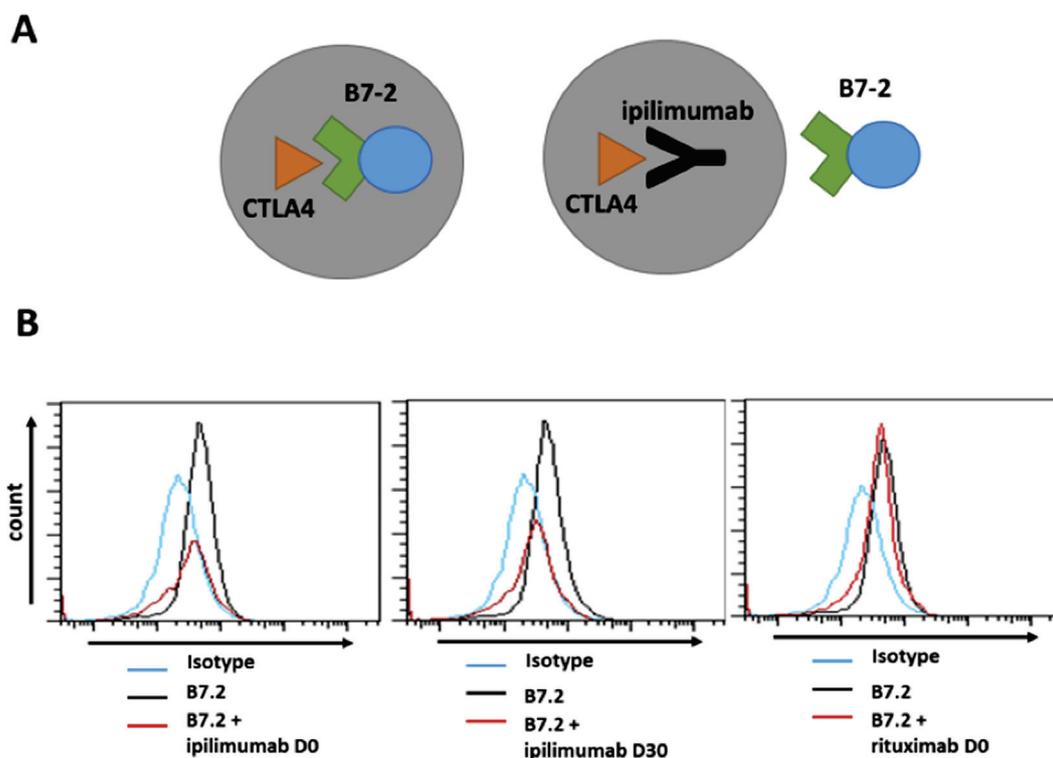


Fig. 5. Functional activity of IPI determined using a cell-based competitive binding assay. A: Inhibition of CTLA-4/B7.2 interaction by the anti-CTLA-4 antibody IPI on a CTLA-4-Ig cell line. B: The mean fluorescence intensity was measured by flow cytometry. IPI upon opening (D0, left box) showed a major inhibition of B7.2 binding to CTLA-4 cells, compared to the negative control (rituximab, right box). After 1 month of storage of IPI vial at 4°C (D30, middle box), the binding inhibition profile remained similar to the reference sample (D0). IPI, ipilimumab; CTLA-4, cytotoxic T-lymphocyte-associated protein 4.

CTLA-4 antibodies has been recently questioned, our results could foster the development of new regimen and new routes of administration of IPI [33].

Conflict of interest statement

Authors declare no conflict of interest concerning this article.

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Pattern Recognition Receptors: New Immune Targets to Enhance Cancer Immunotherapy

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Keywords:	cancer, immunotherapy, immune checkpoints, pattern recognition receptors
Abstract:	Durable tumor responses and significant levels of disease control rates have been described in more than 20 advanced/metastatic cancer types with B7-family immune checkpoint-targeted anti-CTLA-4, anti-PD-1, and anti-PD-L1 monoclonal antibodies. These results and the recent approvals of ipilimumab, pembrolizumab, nivolumab and atezolizumab are currently revolutionizing the way we envision the future of cancer care. However these clinical benefits are not observed in all cancer types and in every patient. Therefore, our clinical challenge is to identify therapeutic strategies which could overcome the primary and secondary resistances to these novel cancer immunotherapies. Pattern recognition receptors (PRRs) are other critical checkpoints for the activation of immune cells, notably myeloid cells (macrophages, dendritic cells,...). They were initially described as sensors for "danger signals" released by pathogens (e.g viral DNA, bacterial proteins,...). We know now that PRRs can also be recruited and activated upon recognition of endogenous stress signals such as molecules released upon self-cell death (e.g ATP, HMGB1,...). Natural endo/exogenous or synthetic PRRs agonists have notably the ability to activate phagocytosis and antigen presentation by myeloid cells residing in

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	<p>the tumor micro-environment. In pre-clinical models, these PRRs agonists have also been shown to overcome the resistance to T-cell targeted immune checkpoints anti-CTLA-4 and anti-PD-1/PD-L1. This manuscript reviews the current knowledge on this major family of immune receptors and the molecules targeting them which are currently in clinical development.</p>

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REVIEW

Pattern Recognition Receptors: New Immune Targets to Enhance Cancer Immunotherapy

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Key words : cancer, immunotherapy, immune checkpoints, pattern recognition receptors

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ABSTRACT

Durable tumor responses and significant levels of disease control rates have been described in more than 20 advanced/metastatic cancer types with B7-family immune checkpoint-targeted anti-CTLA-4, anti-PD-1, and anti-PD-L1 monoclonal antibodies. These results and the recent approvals of ipilimumab, pembrolizumab, nivolumab and atezolizumab are currently revolutionizing the way we envision the future of cancer care. However these clinical benefits are not observed in all cancer types and in every patient. Therefore, our clinical challenge is to identify therapeutic strategies which could overcome the primary and secondary resistances to these novel cancer immunotherapies. Pattern recognition receptors (PRRs) are other critical checkpoints for the activation of immune cells, notably myeloid cells (macrophages, dendritic cells,...). They were initially described as sensors for “danger signals” released by pathogens (e.g viral DNA, bacterial proteins,...). We know now that PRRs can also be recruited and activated upon recognition of endogenous stress signals such as molecules released upon self-cell death (e.g ATP, HMGB1,...). Natural endo/exogenous or synthetic PRRs agonists have notably the ability to activate phagocytosis and antigen presentation by myeloid cells residing in the tumor micro-environment. In pre-clinical models, these PRRs agonists have also been shown to overcome the resistance to T-cell targeted immune checkpoints anti-CTLA-4 and anti-PD-1/PD-L1. This manuscript reviews the current knowledge on this major family of immune receptors and the molecules targeting them which are currently in clinical development.

Words: 225/300

INTRODUCTION

At the end of the 19th century, an American surgeon named Dr. William Coley showed that inoperable cancers could be treated by repeated injections of live or inactivated *Streptococcus pyogenes* and *Serratia marcescens* bacteria [1]. More than 120 years later, Coley is considered to be one of the pioneers of cancer immunotherapy. Indeed, based on our current understanding of tumor immunology, his controversial strategy of injecting pathogen extracts into tumors to stimulate the anti-tumor immunity has become of great interest.

It is now known that within the cancer microenvironment reside immunosuppressive lymphoid and myeloid cells such as regulatory T cells (T_{reg}), tumor associated macrophages (TAMs), tolerogenic plasmacytoid or myeloid dendritic cells (pDCs and mDCs), and myeloid derived suppressor cells (MDSCs) [1,2]. Together with the tumor cells and immunosuppressive cytokines (e.g. IL-10, TGF- β), these cells hamper the generation of an efficient anti-tumor immune response and therefore favor tumor growth and metastasis. Novel cancer immunotherapy strategies are now focusing on the depletion, blockade or reprogramming of these tolerogenic immune effectors. The aim of this review is to gather the scientific rationale for the use of PRR agonists in cancer therapy, and discuss routes of administration which might potentiate their clinical benefits in combination with existing immunotherapy strategies.

THE FAMILY OF PATTERN RECOGNITION RECEPTORS

In 2011, the Nobel Prize of medicine rewarded J. Hoffman, and B. Beutler for their discovery and description of Pattern Recognition Receptors (PRRs) and their role in activating cells from the innate and adaptive immune systems. PRRs are germline encoded receptors which play a central role in the immune response against pathogens but also in immune healing upon self-injuries. PRRs consist of five families including Toll-Like Receptors (TLRs), RIG-I-like Receptors (RLRs), Nucleotide-binding Oligomerization Domain (NOD)-like receptors (NLRs), C-type Lectin Receptors (CLRs) and DNA sensors. They detect Pathogen-Associated Molecular Patterns (PAMPs) such as LPS (Lipopolysaccharide), flagellin, lipoproteins, but also structural proteins, RNA and DNA from bacteria, virus, fungi and parasites [3]. They can also recognize

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4 endogenous Damage Associated Molecular Patterns (DAMPs) that are released upon cellular
5 stress, apoptosis or necrosis, such as chromatin associated protein high-mobility group box1
6 (HMGB1), heat shock proteins (HSPs) and proteins from the extracellular matrix that are
7 generated following tissue injury like hyaluronan [4]. DAMPs/PAMPs recognition by PRRs
8 leads to transient pro-inflammatory gene expression, and immune cell activation. However,
9 inappropriate activation of PRRs has been linked to chronic inflammation and autoimmune
10 diseases notably TLR9 and DNA sensors in systemic lupus erythematosus [5] or psoriasis [6].

17 **TARGETING TOLL-LIKE RECEPTORS FOR CANCER IMMUNOTHERAPY**

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19 Toll-Like Receptors (TLRs) are trans-membrane proteins which function as homo or hetero-
20 dimers. They can be either expressed at the cell surface of (TLR 1, 2, 4, 5, and 6) or within the
21 endosomes (TLR 3, 7, 8, 9 and 10). They are conserved among species except for TLR 11, 12
22 and 13 which are not found in humans. Following recognition of their cognate PAMPs or
23 DAMPs, TLRs become active and trigger signaling pathways via their intracellular TIR domain
24 and the recruitment of specific adaptor proteins such as MYD88, TRIF, TIRAP or TRAM (Fig 1).
25 MYD88 is an adaptor protein for all TLRs except TLR3 that signals via TRIF [7]. The
26 downstream signaling leads separately to the translocation of NF- κ B and IRF3/7 transcription
27 factors into the nucleus, and the regulation of the expression of genes involved in several
28 cellular processes such as survival, proliferation, and inflammatory cytokine and type-I IFN
29 secretion [8].

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41 Until recently, it was believed that TLR expression was restricted to immune cells (Fig 2) or in
42 cells involved in the first line of defense (such as epithelial intestinal cells or endothelial cells).
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44 Indeed the activation of TLRs on Antigen Presenting Cells (APCs) enhances antigen
45 presentation, leads to the expression of inflammatory cytokines and up regulates the
46 expression of adhesion and co-stimulatory molecules at the cell surface [9]. Also, TLRs play
47 multiple roles in the activation and differentiation of B cells in combination with other
48 signaling pathway triggered by the B-cell receptor (BCR) stimulation. Notably, TLR signaling
49 has been shown to regulate the antibody responses including germinal center formation and
50 autoantibody production in an autoimmune disease model [10]. TLRs can also function as co-
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4 stimulatory receptors for T cells to enhance their proliferation, survival and cytokine
5 production. Notably, it has been shown that TLR2 engagement on CD8(+) memory T cells can
6 directly contribute to the maintenance of their diversity in the organism and induce their
7 proliferation and IFN gamma secretion both in vivo and in vitro [11]. TLRs can also modify
8 directly or indirectly the immunosuppressive functions of CD4+CD25+ Treg cells, as it has
9 been shown that TLR2 ligands (Pam3Cys) can significantly increase Treg cell number but
10 resulting in a transient loss of the suppressive Treg phenotype *in vitro* [12]. It has been also
11 observed that engagement of TLR4 by LPS (TLR4 agonist) on regulatory T-cells
12 (CD4+CD25+FOXP3+ T-cells or Tregs) can enhance their proliferation, their survival and more
13 importantly their immunosuppressive capacities [13].

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23 More recently, it has been proposed that TLR could also be expressed by tumor cells [14].
24 Interestingly, DNA-damage and cell stress inducing agents such as doxorubicin chemotherapy,
25 can induce a p53-dependent upregulation of TLR expression [15]. The stimulation of such
26 tumor associated TLR by synthetic agonists can either promote or inhibit tumor progression.
27 For instance, *in vitro* exposure of TLR-expressing tumor cell lines derived from neuroblastoma
28 [16], lymphoma [17], prostate adenocarcinoma [18] and breast carcinoma [19] to their
29 cognate TLR agonists inhibited cell growth and induced apoptosis. In other instances, TLR
30 agonists paradoxically enhanced tumor progression as it has been shown that regulatory T
31 cells can become activated after being exposed to LPS [13]. Other effects have been linked to
32 the sustained endogenous stimulation of TLRs and the subsequent continuous secretion of
33 pro-inflammatory cytokines which promote chronic inflammation, angiogenesis and
34 metastasis [20]. In breast cancer cell lines, stimulation with LPS strongly up-regulated TLR4
35 expression but also matrix metalloproteases (MMP-2 and -9) and VEGF at mRNA and protein
36 levels, suggesting a direct role of TLRs in tumor aggressiveness [21]. These results were
37 confirmed in vivo in a nude mouse breast carcinoma model, where stimulation of TLR4 by LPS
38 promoted tumorigenesis and metastasis in the liver [21]. TLR expression measured by tissue
39 microarray and immunohistochemistry on 73 biopsies, has been also found in Stage I-II oral
40 squamous cell carcinoma [22]. In this type of cancer, a high TLR-2, 4 and 9 expression was
41 correlated with higher tumor invasiveness, whereas high TLR5 expression correlated with a
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4 lower tumor grade. In another study, Chatterjee & Cremer have observed that the stimulation
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6 of TLR7 in immunodeficient and immunocompetent mice transplanted with TLR7 positive
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8 NSCLC tumor cell lines has a protumoral effect and induces resistance to chemotherapy [23].
9
10 Also, patients with high TLR7 expression correlate to poor prognosis outcome [23]. In acute
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12 myeloid leukemia (AML), it has been shown that TLR8 activation promotes AML
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14 differentiation and cell growth inhibition [24]. It was also demonstrated that the TLR7/8
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16 agonist R848 impairs the growth of human AML cells in immunodeficient mice [24]. Therefore
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18 a single TLR stimulation can lead to either anti- or pro-tumoral signaling depending on the
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20 targeted TLR and the type of cancer. However these contradictory results on the role of TLRs
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22 could also be the consequence of the experimental settings. For instance, TLR gene
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24 expression data do not inform on the effects of therapeutic stimulation of TLRs. Also, in vitro
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26 TLR stimulation on cancer cell line or xenograft models might not lead to the same results in
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28 vivo on tumors with an immunocompetent microenvironment.

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30 Multiple TLR agonists are currently in clinical development (Table I). Intra-tumoral injections
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32 of a TLR-9 agonist, the CpG rich oligonucleotide PF-3512676, has already shown significant
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34 activity in both injected and non-injected lesions of B- and T-cell lymphomas when used
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36 concomitantly with low dose (2x2Gy) local irradiation. Out of 15 patients with low-grade B-
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38 cell lymphoma, 27% developed an objective response (1 CR + 3 PR) and 2 other patients
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40 showed stable but continually regressing disease (disease control rate of 40%) [25]. Similar
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42 results have been also reported in patients with T-cell lymphoma [26].

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44 As shown in Table 1, several compounds, mainly represented by imiquimod and imiquimod
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46 derivatives, target TLR 7 and 8. TLR7 in pDC is functionally associated with the production of
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48 IFN- α - and IFN-regulated cytokines, similar to the role of TLR9. TLR8 functions in monocytes
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50 and myeloid DC and is involved in the production of proinflammatory cytokines such as TNF- α
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52 [27]. Intra-tumoral administration of the TLR7/8 agonist 3M-052/MEDI9197 in a mouse
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54 melanoma model generated antitumor response and has been shown to potentiate the
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56 effects of anti-CTLA4 and anti-PDL1 therapies [28]. Several studies have shown that synthetic
57
58 dsRNA induce apoptosis in tumor cells in a TLR3-dependent manner [19,29]. So activation of
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60 TLR3 by specific ligands may induce apoptosis of tumor cells and activate the immune system

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4 at the same time. In a randomized clinical trial including 194 breast cancer patients, adjuvant
5 treatment with poly(A:U) dsRNA was associated with a significant decrease in the risk of
6 metastatic relapse in TLR-3 -positive but not in TLR3-negative breast cancers [30].
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9 **TARGETING RIG-I-LIKE RECEPTORS FOR CANCER IMMUNOTHERAPY**

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11 RIG-I-Like Receptors (RLRS) are cytosolic PRRs that have the ability to detect viral and
12 endogenous double stranded RNAs. Three members of the RLRs family have been identified
13 so far: Retinoic acid-Inducible gene I (RIG-I), Melanoma Differentiation-Associated gene 5
14 (MDA-5) and Laboratory of Genetics and Physiology 2 (LGP2). They trigger a signaling pathway
15 through their Caspase Activation and Recruitment Domains (CARD). Their downstream
16 signaling activates the key transcription factors IRF3/7 and NF- κ B leading to the secretion of
17 type-I interferons (IFN) and pro-inflammatory cytokines, respectively (Fig 1). RLRs are
18 ubiquitously expressed in tissues but have been described to play a key role in innate immune
19 activation in epithelial cells, myeloid cells and central nervous system cells. Their expression
20 remains at low levels but can increase after viral infection and exposure to type-I IFN in a
21 large variety of other cell types, including cancer cells [31]. Recent studies have highlighted an
22 anti-tumor activity of RLR activation [32]. RIG-I stimulation can inhibit leukemia cell
23 proliferation via STAT-1 expression and transcription of several genes implicated in type-I and
24 II IFN induction [33]. Interestingly, it has been shown recently that blocking TGF- β by siRNA
25 can simultaneously stimulate RIG-I and induce high levels of type-I IFN and CXCL10 in the
26 serum and tumor tissue which subsequently activates systemic immune cells and tumor cell
27 apoptosis in vivo in murine pancreatic tumors [34]. In melanoma cells the activation of RIG-I
28 and MDA-5 can induce a type-I IFN-independent apoptosis [35].
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46 The interaction of MDA-5 and RIG-I with their ligands triggers a signaling pathway through the
47 binding to the mitochondrial membrane adaptor protein MAVS (mitochondrial antiviral
48 signaling protein) which eventually results in the nuclear translocation of the transcription
49 factors IRF3 and NF- κ B and type-I IFN production [36]. Meanwhile, their interaction triggers
50 another signaling pathway independent of the tumor suppressor p53 resulting in
51 mitochondrial apoptosis through caspase-9 and Apaf-1 as well as activation of the
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4 proapoptotic BCL-2 family member NOXA [35]. Also, recent data has shown that the
5 stimulation of RIG-I in human ovarian cancer cells by short double stranded RNA with
6 uncapped 5'-triphosphate (direct RIG-I agonist) [37] or stimulation of MDA-5 with poly I:C [38]
7 leads to HLA-class-I upregulation, proinflammatory cytokines secretion, tumor cell apoptosis
8 and phagocytosis by monocytes and dendritic cells. Moreover the cytoplasmic transfection of
9 breast cancer cells with poly I:C has been shown to trigger a caspase dependent apoptosis and
10 a cell growth arrest via MDA-5 stimulation [39]. A recent study reported that Polyinosine-
11 polycytidylic acid (poly-IC), a synthetic dsRNA which is known to act as a TLR3 and RLR
12 agonist, can stimulate apoptosis in pancreatic cancer cells while sparing normal pancreatic
13 epithelial cells. The mechanism of action of this effect involved the inhibition of survival, XIAP
14 expression and the activation of an immune response by inducing MDA-5 and RIG-1.
15 Interestingly, the in vivo administration of poly-IC can inhibit tumor growth in subcutaneous
16 and orthotopic pancreatic tumor models [40]. Altogether this data illustrates the anti-tumor
17 potential of RLRs activation through the induction of apoptosis in tumor cells and the
18 stimulation of anti-tumor immunity. Some RIG-I agonists should reach soon the clinic in early
19 phase trials.
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33 34 **TARGETING NOD-LIKE RECEPTORS FOR CANCER IMMUNOTHERAPY**

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36 Nod-Like Receptors (NLRs) are another family of pro-inflammatory cytosolic PRRs. They are
37 divided into four subfamilies, each with a suffix to denote their unique type of N-terminal
38 domain: NLRA-A in reference to their acidic trans-activating domain, NLRB-B in reference to
39 their Baculovirus Inhibitor of apoptosis protein Repeat (BIR) domain, NLRC-C in reference to
40 their Caspase Activation and Recruitment Domain (CARD), and NLRP-P in reference to
41 their Pyrin Domain (PYD) [41]. Some NLRs including NOD1, NOD2 Apoptosis Inhibitory Protein
42 (NAIP) and NLRC4 can detect conserved bacterial molecular signatures such as γ -D-glutamyl-
43 meso-diaminopilemic acid (iE-DAP) or muramyl dipeptide (MDP) that are produced during
44 synthesis or degradation of bacteria cell wall peptidoglycan within the host cytosol. Other
45 members of the NLR family can detect danger signals such as DAMPs. NLR stimulation leads to
46 NF- κ B activation and pro-inflammatory cytokines secretion, leading to inflammation and
47 enhanced immune response. However, it has been shown recently that NLRP12 can suppress
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4 colon inflammation and tumorigenesis through negative regulation of non-canonical NF- κ B
5 signaling [42].
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8 Most NLRs play an important role in a multiprotein complex called the inflammasome, that
9 activates caspase-1 and leads to the secretion of proinflammatory cytokines IL-1 β and IL-18
10 via proteolytic cleavage of pro-peptides [43]. However, NLRP10 seems to be an exception as it
11 is the only NLR lacking the common ligand-binding leucine-rich-repeat domain. Moreover, it
12 does not function through an inflammasome to regulate caspase-1 activity nor does it
13 regulate other inflammasomes. Interestingly, NLRP10 has been recently described to play an
14 important role in the migration of DCs and the initiation of adaptive immune responses [44].
15 Other reports have shown that ATP released from apoptotic cells can be sensed by DCs
16 through NLRP3 inflammasome activation via the generation of Reactive Oxygen Species (ROS).
17 This NLRP3 activation can lead to the secretion of IL-1 β by DCs, and can induce an anti-tumor
18 immunity by effective recruitment of CD8 T-cells and can promote tumor clearance by
19 phagocytes [45]. Mifamurtide (liposomal muramyl tripeptide), a NOD-2 agonist, is currently
20 approved in the E.U. for the treatment of non-metastatic osteosarcoma in conjunction with
21 chemotherapy, and this molecule showed immunomodulatory properties via the activation of
22 monocytes and macrophages [46].
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36 **TARGETING CYTOSOLIC DNA SENSORS FOR CANCER IMMUNOTHERAPY**

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38 Stetson and Medzhitov reported in 2006 the induction of type-I IFN by cytosolic DNA in cells
39 lacking TLR9, the only previously known sensor of foreign DNA [47]. Since then, it has been
40 shown that cytoplasmic aberrant DNA can activate 3 different TLR9-independent pathways.
41 First, DNA can be recognized by cytoplasmic sensors such as DAI (DNA-dependent Activator of
42 IFN-regulatory factors) and lead to the production of type-I IFNs via IRF3 nuclear translocation
43 [48]. Second, foreign DNA can be retro-transcribed by RNA polymerase-III and become a
44 ligand for RNA sensor like RIG-I [49]. Third, several groups have identified AIM2 (Absent In
45 Melanoma 2) as a cytosolic DNA sensor whose activation promotes the assembly of an
46 inflammasome leading to the cleavage of caspase-1, the expression of IL-1 β and IL-18. This
47 pathway leads to the induction of a unique caspase-1-dependent cell death called pyroptosis
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4 [50]. Recent data has revealed that AIM2 stimulation in colorectal cancer cells can lead to the
5 upregulation of interferon stimulated genes such as the MHC-II molecule HLA-DR [51]. This
6 novel function seemed independent of caspase-1 and resulted in cell cycle arrest but
7 conferred an invasive phenotype to colorectal cancer cells [52]. AIM2 contradictory role was
8 also recently linked to the development of benign prostate hyperplasia and prostate cancer
9 by sustaining chronic inflammation [53]. In another study it was shown that electroporation
10 of a DAI-encoding plasmid *in vivo* in mice can augment the transcription of genes encoding
11 proinflammatory cytokines, type-I IFN and costimulatory molecules [54]. Moreover, DAI used
12 as an adjuvant with a DNA vaccine (encoding for a tumor antigen) led to an anti-tumor
13 cytotoxic T-cell (CTL) response and induced-memory CTLs [54].
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23 Recently a 5 spanning membrane protein playing a crucial role in recognition of cytosolic DNA
24 has been identified and named STING (Stimulator of IFN genes) also known as TMEM173,
25 MITA, ERIS and MPYS. Cytosolic DNA binds to cGAS (cGAMP synthase) and catalyzes cGAMP
26 (cyclic GMP-AMP) synthesis from ATP and GTP. cGMP then binds and activates STING [55] and
27 induces a conformational change which leads to activation of transcriptional factors IRF3 and
28 NF- κ B through the kinase TBK1 and IKK [56]. STING has been shown to play an important role
29 in the innate immune response against cancer. It was recently demonstrated that in the
30 tumor microenvironment, tumor cells DNA detected by APCs is correlated with activation of
31 STING pathway that leads to IFN- β production and spontaneous T cell response against
32 tumors [57]. The rationale of targeting STING in the tumor microenvironment is to activate
33 cross-priming of tumor-specific antigens to CD8+ T cells and induce the production of
34 chemokines to enhance the trafficking of effector T cells [58]. Some studies have shown that
35 the administration of different doses of STING ligand, cyclic di-GMP, can reduce the number
36 of metastasis and tumor size in a metastatic breast cancer mouse model. Interestingly, low
37 dose of cyclic di-GMP enhances the production of IL-12 by MDSC and improve T-cell
38 responses but high dose of cyclic di-GMP leads to activation of caspase-3 in tumor cells that
39 can directly kills them [59]. STING agonists are currently in clinical development. For instance
40 the molecule ADU-S100 (Aduro Biotech/Novartis), has been reported as stimulating the
41 different variants of human STING receptors. ADU-S100 is currently evaluated in a phase I
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4 clinical trial using intratumoral (IT) administration. Encouraging preclinical results were
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6 obtained with the lead molecules, which induced, after IT administration, tumor regressions
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8 in different syngeneic mouse models, with immune protection after re-challenge with the
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10 same tumor [58]. No significant local or systemic toxicity was reported in those preclinical
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12 mouse models.

13 **TARGETING C-TYPE LECTINS RECEPTORS FOR CANCER IMMUNOTHERAPY**

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16 C-Type Lectins Receptors (CLRS) are large trans-membrane receptors characterized by a
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18 carbohydrate recognition domain (CRD) which binds in a calcium dependent manner to
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20 glycans present on the surface of microorganisms [60]. They are principally implicated in the
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22 recognition (and clearance) of fungi and facilitate the antigen internalization and processing
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24 by DCs. Based on their molecular structure, CLRs can be separated in two groups of
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26 transmembrane CLRs and secreted CLRs. Among transmembrane CLRs, a first subgroup
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28 contains several CRDs or CRD-like domains and includes receptors such as DEC-205 (CD205) or
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30 the macrophage mannose receptor (MMR). These CLRs can play a role in antigen uptake by
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32 APC, resulting in the presentation of peptides by MHC class I and II molecules [61]. A second
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34 subgroup presents a single CRD domain and includes molecules such as Dectin-1, Dectin-2,
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36 macrophage-inducible C-type lectin (Mincle), DC-specific ICAM3-grabbing non integrin (DC-
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38 SIGN), and DC NK lectin group receptor-1 (DNGR-1 or Clec9A). These receptors play notably a
39
40 critical role in fungal recognition and the subsequent activation of innate immune responses
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42 [62]. A second group of CLRs is composed by soluble CLRs like for instance the Mannose-
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44 Binding Lectin (MBL), an oligomeric protein that binds to carbohydrate patterns on pathogen
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46 surfaces [63]. CLR engagement by their ligands triggers specific signaling pathways (Fig 1).
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48 These pathways lead to the expression of different cytokines directly or by modulating the
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50 TLR signaling pathways, and result in T cell polarization [60]. It was recently shown that oral
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52 administration or intraperitoneal injection of MD-fraction (a highly purified soluble β -Glucan
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54 extract from the oriental mushroom *Grifola frondosa*) in tumor bearing mice lead to the
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56 induction of systemic anti-tumor immunity [64]. MD-fraction was taken up by antigen
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58 presenting cells and induced their maturation via Dectin-1 pathway, leading to an infiltration
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60 of activated T cells into the tumors and a decrease of immunosuppressive cells such as Tregs

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4 and MDSCs. More recently, new data showed an essential role of CLRs in the induction of
5 inflammation after necrotic cell death. Indeed, Mincle and Clec9A have been shown to
6 recognize DAMPs released upon necrosis but not apoptosis [65,66]. Genetic variants of C-type
7 lectin genes were associated with colorectal cancer susceptibility [67]. Imprime PGG, a beta
8 1,3/1,6-glucan PAMP derived from yeast is currently in clinical development [68]. At ASCO
9 2015, the company reported the results of a 2:1 randomized Phase II trial testing Imprime
10 PGG w/o carboplatin + paclitaxel + bevacizumab in 92 patients with non-squamous NSCLC.
11 The combination arm with Imprime PGG showed rapid and durable objective responses in
12 60% of the patients vs 43% in the control arm [69].
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21 **PRR AGONISTS TO OVERCOME IMMUNE CHECKPOINT BLOCKADE RESISTANCE**

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23 The current revival of cancer vaccines is supported by the hypothesis that the pre-existing
24 anti-tumor immunity might be insufficient in anti-PD-1/PD-L1 refractory patients and that
25 tumor-antigen exposure might trigger a better priming of anti-tumor T-cells. Thanks to their
26 immuno-stimulatory properties, notably in terms of activation of antigen presenting cells, PRR
27 agonists are now being extensively used as cancer vaccines adjuvants (Table 1). Alternatively,
28 the administration of PRR agonists could be done directly into a tumor lesion with the idea
29 that all the relevant tumor antigens are there and that local PRR stimulation could prime or
30 boost the anti-tumor T-cell responses. Interestingly, the rationale for such strategy has been
31 shown in both murine models and humans. First, it has been shown that intra-tumoral PRR
32 agonist injections can trigger a more potent anti-tumor immune response than upon distant
33 or systemic administrations [17,70]. Indeed, intra-tumoral injections of PRR agonists can
34 induce a local inflammation with chemokines which can recruit infiltrative myeloid cells in the
35 tumor microenvironment. Moreover, PRR agonists can activate these myeloid cells and
36 enhance their ability to efficiently present tumor antigens to T-cells and activate the anti-
37 tumor immune response. PRR agonists can also have an intrinsic direct anti-tumor activity on
38 the injected tumors [17,71,72] and generate regression of non-injected, distant tumor sites
39 [58]. The proof of concept of the benefits of local PRR agonist delivery has been recently done
40 in humans with lymphoma [25,26]. Injections of CpG, a TLR9 agonist, into a single tumor site
41 together with a local low dose (2x2Gy) irradiation were able to trigger a systemic anti-tumor
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4 immune response and generate objective tumor responses at distant, non injected, sites.
5 Concerning the route of administration, an IT injection of PRRs agonists not only achieve a
6 maximal local therapeutic effect with compounds often characterized by poor
7 pharmacokinetic properties, but also allows to limit the side effects of a systemic immune
8 activation. Interestingly, this so called “*in situ* immunization” strategy just follows the initial
9 practice of Dr William Coley more than a century ago.
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15 PRR agonists can also potentiate the systemic activity of conventional therapies when used in
16 combination with chemotherapy [17], tumor-targeted antibodies [73], or tumor-targeted
17 tyrosine kinase inhibitors [74]. Most of all, PRR agonists can overcome the resistance to
18 immune checkpoint targeted antibodies therapy. This has been recently demonstrated in
19 immunocompetent mouse models which are not sensitive to anti-CTLA4, anti-OX40, or anti-
20 PD-1/PD-L1 therapies and where the combination of immune checkpoint targeted antibodies
21 with a STING-based cancer vaccine or intra-tumoral injections of a TLR9 agonist (CpG) could
22 trigger a systemic anti-tumor immune response able to eradicate both the injected tumor site
23 and the distant, untreated, tumor sites [72,75].
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32 **PATHOGENS AS NATURAL SOURCES OF PRR AGONISTS**

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34 Beyond synthetic molecules, entire pathogens with multiple potential PRR agonistic function
35 via their constitutive proteins and nucleic acids can be used for cancer therapy. Beyond its
36 approved use for local bladder cancer, cutaneous and intra-tumoral BCG therapy has been
37 extensively used to treat cancers in the past with significant clinical activity, including
38 responses in non-injected lesions (see [76] for review). More recently, in a randomized phase
39 2 study, another mycobacteria (*Mycobacterium obuense*) has shown activity in metastatic
40 pancreatic cancer when injected sub-cutaneously in combination with gemcitabine. Besides
41 bacteria, viruses can also be used to stimulate PRRs and infect/kill cancer cells in an
42 immunogenic manner. The whole field of oncolytic viruses is currently in active development.
43 Indeed, Talimogene laherparepvec (aka T-Vec), a GM-CSF expressing, herpes virus (HSV-1)
44 derived, oncolytic virus, has been recently approved for the treatment of metastatic
45 melanoma following the positive results of a randomized phase III trial [77]. Intratumoral T-
46 VEC has been shown to induce objective responses in 64% of injected lesions, 34% of non-
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4 injected non-visceral lesions and 15% of non-injected visceral lesions [78]. Pexa-Vec, a GM-
5 CSF expressing vaccinia virus derived oncolytic virus is currently in phase 3 in
6 hepatocarcinoma following promising activity in both injected and non-injected tumor lesions
7 in phase I & II trials [79,80]. Interestingly, early data of intra-tumoral oncolytic virus therapy
8 seem to show that it can synergize with anti-CTLA-4 and anti-PD-1 therapy with objective
9 response rates above 50% and including responses in non-injected lesions [81,82].
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15 **CONCLUSION**

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17 Due to their immune stimulatory properties, PRR agonists can contribute to the induction of
18 systemic and long lasting adaptive immunity against tumors. Moreover, PRR agonists can also
19 directly induce an immunogenic death of tumor cells. Cancer vaccines or intra-tumoral
20 administrations with PRR agonists can induce potent anti-tumor immune responses.
21 However, therapies with PRR agonists might not be sufficient to overcome tumor antigen
22 tolerance within the tumor microenvironment and its draining lymph nodes. Combination of
23 therapies based on PRR agonists with immune checkpoint targeted antibodies such as anti-
24 CTLA-4 or anti-PD-1 could address this issue and overcome the resistance to immune
25 checkpoint blockade monotherapy.
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FIGURE LEGENDS

Figure 1: Signalling pathways upon Pattern Recognition Receptor (PRR) stimulation. The stimulation of PRRs by PAMPs (Pathogen Associated Molecular Patterns) or DAMPs (Damage Associated Molecular Patterns) results in Type I IFN expression in target cells and release proinflammatory cytokines.

Figure 2: Expression of Toll-like Receptors (TLRs) in immune cells. Myeloid cells such as Monocytes and myeloid or plasmacytoid dendritic cells (mDCs and pDCs respectively) express a wide range of TLRs and are therefore major sensors of PAMPs (Pathogen Associated Molecular Patterns) or DAMPs (Damage Associated Molecular Patterns).

Figure 3: Combinations of immunomodulatory therapies to overcome tumor tolerance and induce effective anti-tumor immunity. Pattern recognition receptor (PRR) stimulation can induce tumor cell death, recruitment of Antigen Presenting Cells (APCs) in the tumor stroma, turn APCs (notably macrophages) into an activated phenotype for better antigen uptake and presentation to T-cells. Also, PRR stimulation can directly activate adaptive immune cells (B- and T-cells). Together, the immune stimulatory properties of PRR agonists might upregulate the co-stimulatory signals that are necessary to obtain the full therapeutic potential of anti-CTLA-4 and/or anti-PD-1/PD-L1 antibodies.

Table 1: Ongoing Clinical Trials using PRR agonists as cancer vaccine adjuvants or for intratumoral delivery.

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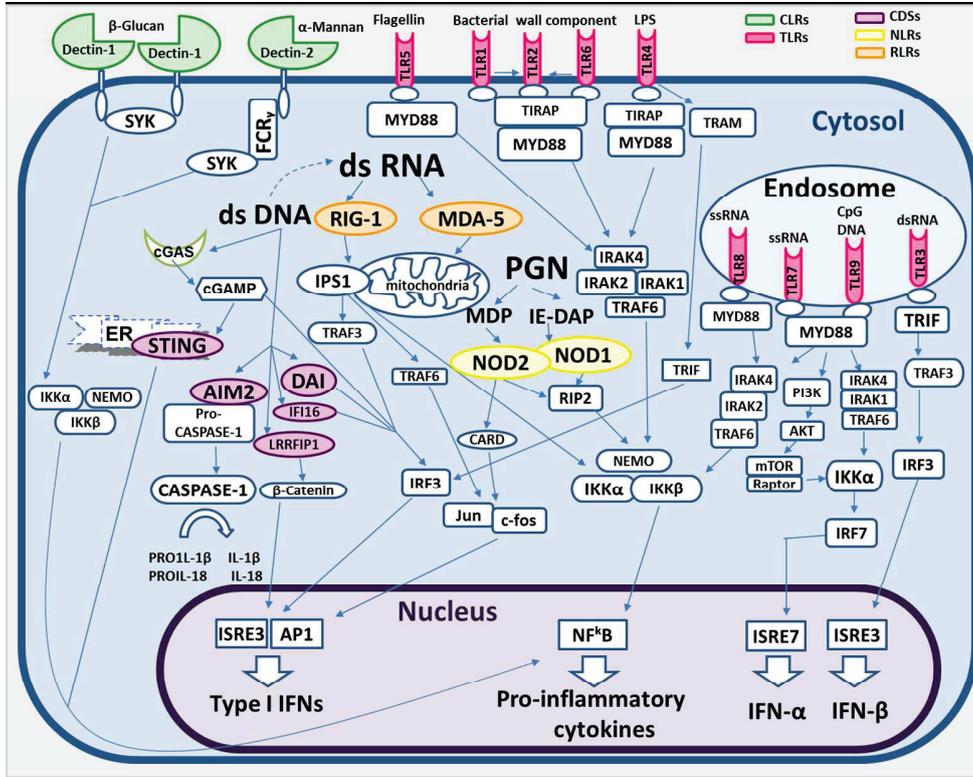


Figure 1: Signalling pathways upon Pattern Recognition Receptor (PRR) stimulation. The stimulation of PRRs by PAMPs (Pathogen Associated Molecular Patterns) or DAMPs (Damage Associated Molecular Patterns) results in Type I IFN expression in target cells and release proinflammatory cytokines.

Figure 1
271x217mm (150 x 150 DPI)

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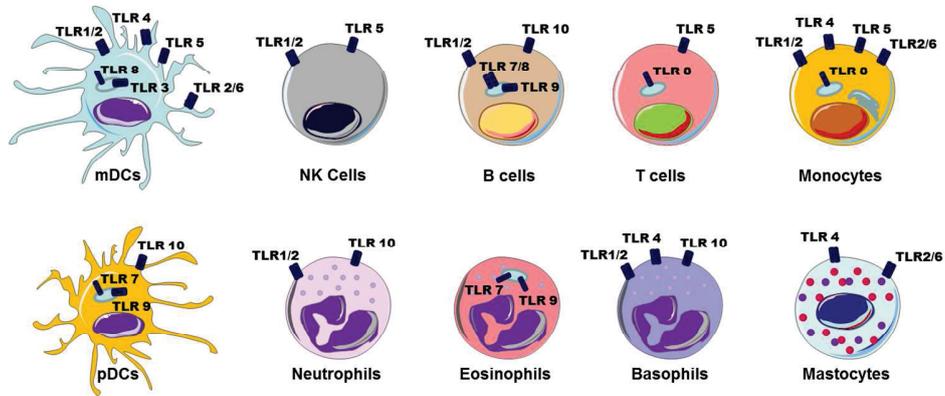


Figure 2: Expression of Toll-like Receptors (TLRs) in immune cells. Myeloid cells such as Monocytes and myeloid or plasmacytoid dendritic cells (mDCs and pDCs respectively) express a wide range of TLRs and are therefore major sensors of PAMPs (Pathogen Associated Molecular Patterns) or DAMPs (Damage Associated Molecular Patterns).

Figure 2

254x114mm (150 x 150 DPI)

Peer Review

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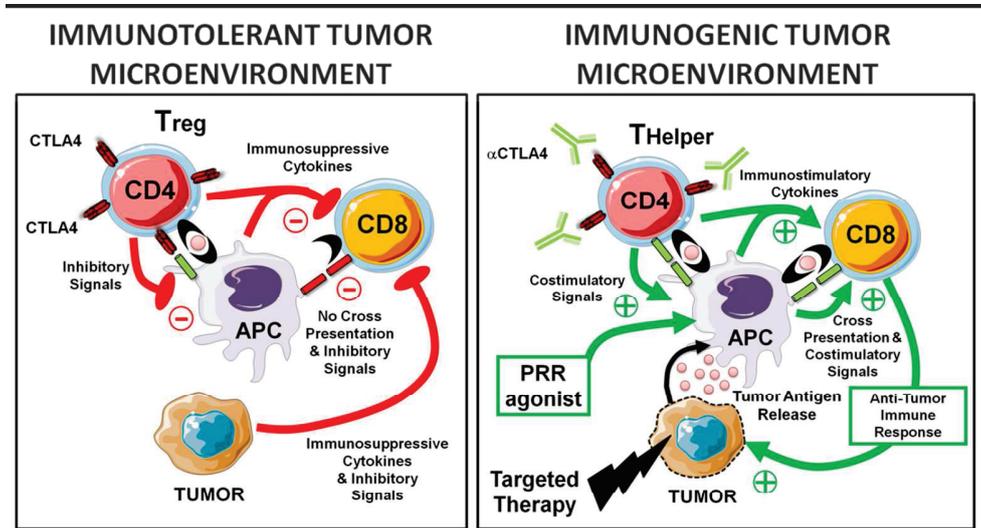


Figure 3: Combinations of immunomodulatory therapies to overcome tumor tolerance and induce effective anti-tumor immunity. Pattern recognition receptor (PRR) stimulation can induce tumor cell death, recruitment of Antigen Presenting Cells (APCs) in the tumor stroma, turn APCs (notably macrophages) into an activated phenotype for better antigen uptake and presentation to T-cells. Also, PRR stimulation can directly activate adaptive immune cells (B- and T-cells). Together, the immune stimulatory properties of PRR agonists might upregulate the co-stimulatory signals that are necessary to obtain the full therapeutic potential of anti-CTLA-4 and/or anti-PD-1/PD-L1 antibodies.

Figure 3
161x97mm (220 x 220 DPI)

Review

PRR targeted	PRR agonist	Tumors	Phase	Title	Clinical Trial #	
1	STING	MW815 (ADU-S100)	Solid Tumors & Lymphomas	1	Study of the Safety and Efficacy of MW815 (ADU-S100) in Patients With Advanced/Metastatic Solid Tumors or Lymphomas	NCT020675439
2	TLR3+TLR7/8	Poly(I:CLC)+Resiquimod	melanoma	1/2	Phase I/II Trial of a Long Peptide Vaccine (LPV7) Plus TLR Agonists (MEL60) (Vaccine adjuvant)	NCT02126579
3	TLR3	Poly-I:CLC	Low grade B-cell lymphoma	1/2	In Situ Vaccine for Low-Grade Lymphoma: Combination of Intratumoral Flt3L and Poly-I:CLC With Low-Dose Radiotherapy	NCT01976585
4	TLR3	Rintatolimod	HER2-Positive Breast Cancer	1/2	Vaccine With Rintatolimod and/or Sargramostim in Patients With Stage II-IV HER2+ Breast Cancer	NCT01355393
5	Poly-I:CLC	Colorectal adenoma	2	Study of the MUC1 Peptide-Poly-I:CLC Vaccine in Individuals With Advanced Colorectal Adenoma(ADJ)	NCT00773097	
6	Pediatric Low Grade Gliomas	2	A Trial of Poly-I:CLC in the Management of Recurrent Pediatric Low Grade Gliomas	NCT01188096		
7	Pediatric Gliomas	0	A Pilot Study of Glioma Associated Antigen Vaccines With Poly-I:CLC in Pediatric Gliomas(ADJ)	NCT01130077		
8	Triple-negative Breast Cancer	0	MUC1 Vaccine for Triple-negative Breast Cancer(ADJ)	NCT00986609		
9	TLR3 + TLR4	Poly-I:CLC + LPS	Stage III or IV melanoma	1	A Multi-peptide Vaccine Plus Toll-Like Receptor Agonists in Melanoma Patients (MEL58)(ADJ)	NCT01585350
10	TLR4	GSK1572932A	NSCL cancer	3	GSK1572932A Antigen-Specific Cancer Immunotherapy as Adjuvant Therapy in Patients With Non-Small Cell Lung Cancer	NCT00480025
12	TLR5	CBLB502	Inoperable solid tumors	1	TLR5 Agonist CBLB502 in Patients With Inoperable Locally Advanced or Metastatic Solid Tumors	NCT01527136
13	Stage III-IV or recurrent HNSCC	1	Entolimod in Treating Patients With Stage III-IV HNSCC Receiving Cisplatin and Radiation Therapy	NCT01728480		
14	TLR7/8 or TLR3	Imiquimod or PolyI:CLC	Malignant Glioma	2	Dendritic Cell Vaccine for Patients With Brain Tumors(ADJ)	NCT01204684
16	TLR7/8	Imiquimod	Metastatic melanoma	1/2	Peptide Vaccination Associated With Tumoral Immunomodulation in Patients With Advanced Metastatic Melanoma (ADJ)	NCT01191034
18	Breast Cancer	2	Topical Imiquimod and Abraxane in Treating Patients With Advanced Breast Cancer	NCT00821964		
19	Lentigo Maligna	2/3	Imiquimod to Detect Residual Lesions and Prevent Recurrence of Lentigo Maligna	NCT01088737		
20	Non-melanomatous Skin Cancer	3	Topical Imiquimod Compared With Surgery in Treating Patients With Basal Cell Skin Cancer	NCT00066872		
21	Breast Cancer	1/2	Toll-like Receptor (TLR) 7 Agonist and Radiotherapy for Breast Cancer With Skin Metastases	NCT01421017		
22	Vulvar Neoplasia and/or anogenital Condyloma	2	Immunevasion of Human Papillomavirus (HPV) in Vulvar Intraepithelial Neoplasia 2/3 and Anogenital Warts and Efficiency and Mechanisms of Imiquimod Treatment	NCT00941811		
24	Resiquimod	Metastatic melanoma	2	Tumor and Vaccine Site With a Toll Like Receptor (TLR) Agonist	NCT00960752	
25	MEDI9197	Solid Tumors	1	A Study of MEDI9197 Administered in Subjects With a Solid Tumor Cancer	NCT02556463	
26	VTX-2337	Ovarian Cancer	1	VTX-2337 and Liposomal Doxorubicin Hydrochloride in Treating Patients With Recurrent or Persistent Ovarian Epithelial, Fallopian Tube, or Peritoneal Cavity Cancer	NCT01294293	
28	HNSCC	1	TLR8 Agonist VTX-2337 and Cetuximab in Treating Patients With Locally Advanced, Recurrent, or Metastatic HNSCC	NCT01334177		
29	Ovarian, fallopian tube or primary peritoneal cancer	2	VTX-2337 and Pegylated Liposomal Doxorubicin (PLD) in Patients With Recurrent or Persistent Epithelial Ovarian, Fallopian Tube or Primary Peritoneal Cancer	NCT01666444		
31	TLR9 + TLR4	CpG	Melanoma	0	Safety and Immunological Response of Stage IIB-IV Resected Melanoma After Treatment With MAGE-A3 ASCI	NCT01425749
32	+ MPL (AS15)	2	High-dose Interleukin-2 (HDIL-2), Combined With reCMAGE-A3 + AS15 ASCI	NCT01266603		
34	TLR9 + anti-PD-1	CMP-001 + pembrolizumab	Melanoma	1	A Multicenter, Open-Label, Phase 4B Clinical Study of CMP-001 in Combination With Pembrolizumab in Subjects With Advanced Solid Malignancies	NCT02680184
35	TLR9	MGN1703 + ipilimumab	Advanced Solid Malignancies	1	Phase I Study of Ipilimumab and MGN1703 in Patients With Advanced Solid Malignancies	NCT02668770
36	anti-CTLA-4	1	Phase I Study of Ipilimumab and MGN1703 in Patients With Advanced Solid Malignancies	NCT02668770		
37	TLR9	SD-101	Low-Grade B-cell Lymphoma	1/2	TLR9 Agonist SD-101, Ipilimumab, and Radiation Therapy in Treating Patients With Low-Grade Recurrent B-cell Lymphoma	NCT02254772
38	MGN1703	Metastatic colorectal cancer	3	Evaluation of MGN1703 Maintenance Treatment in Patients With mCRC With Tumor Reduction During Induction Treatment	NCT02077868	
39	1018 ISS	Colorectal Neoplasms	1	1018 ISS With Irinotecan and Cetuximab in Patients With Previously Treated Metastatic Colorectal Cancer	NCT00403052	
40	Agatolimod (CpG 7909)	Esophageal Cancer	1/2	Phase I/II of URLC10-177 and TTK-567 Peptide Vaccine With CpG7909 in Patients With Esophageal Cancer	NCT00669292	
41	Mantle Cell Lymphoma	1/2	Phase I/II of a CpG-Activated Whole Cell Vaccine Followed by Autologous Immunotransplant for MCL	NCT00490529		
42	Breast Cancer	2	Agatolimod and Trastuzumab in Treating Patients With Locally Advanced or Metastatic Breast Cancer	NCT00824733		
44	CLL	1	CpG in Patients With Chronic Lymphocytic Leukemia Who Have Been Previously Treated	NCT00233506		
45	Lymphoma	1/2	CpG 7909 in Treating Patients With Cutaneous T-Cell Lymphoma	NCT00091208		

For Peer Review

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