Cancer therapy through TLR-induced local innate immunity activation and block of immune checkpoints or suppressive cells

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ABSTRACT

The immunostimulatory ability of oligonucleotides containing CpG motif (CpG-ODN), agonists of TLR9, can be harnessed to promote antitumor immunity by their application at the tumor site to stimulate local activation of innate immunity; however, this is not always true for lung tumor where the immunosuppressive microenvironment, crucial to avoid tissue damage by inflammatory response to the continuous exposition to inhaled particles, limits the power of immunotherapy. Thus, the tumor microenvironment is a critical factor for successful use of these immunotherapeutics, and strategies to shift a tumor-supporting milieu to a host-friendly one might lead to improved anti-tumor activity of CpG-ODN. Since bronchial and bronchoalveolar tumors are accessible via the endobronchial space, inhalation of aerosolized immunotherapeutic agents might represent a convenient and simple approach to shape a tumor microenvironment more favorable to induce an immune response against lung primary cancer and/or metastases.

Aim of this thesis is the evaluation of strategies to improve the antitumor activity of aerosolized TLR9 agonist in the lung. The thesis is divided in three tasks: 1) the evaluation of aerosol delivery CpG-ODN combined with Poly(I:C), a TLR3 agonist able to convert tumor-supporting macrophages to tumoricidal effectors, in the treatment of B16 melanoma lung metastases in C57BL/6 mice. 2) the investigation of the mechanism by which the two combined agonists could induce the activation of effector cells population. 3) the exploration of strategies to improve the therapeutic efficacy of TLR9/TLR3 agonists by including in the inhalant either an antibody directed to both Ly6G and Ly6C markers, to locally deplete myeloid-derived suppressive cells (MDSC), or IFNα, to directly activate innate immune cells in the lung.

Aerosolization of CpG-ODN with Poly(I:C) into the bronchoalveolar space reduced the presence of M2-associated arginase- and IL-10-secreting macrophages in tumor-bearing lungs and increased the anti-tumor activity of aerosolized CpG-ODN alone against B16 lung metastases without apparent signs of toxicity or injury of the bronchial-bronchiolar structures and alveolar walls. Moreover, CpG-ODN/Poly(I:C) aerosol combined with dacarbazine, a therapeutic agent used in patients with inoperable metastatic melanoma able to exert immunostimulatory effects, led to a significant increase in anti-tumor activity as compared to treatments with aerosolized CpG-ODN/Poly(I:C) or dacarbazine alone. This effect was related to an enhanced recruitment and cytotoxic activity of tumor-infiltrating NK cells in the lung. Our in vitro and in vivo experiments to elucidate the mechanism of NK cells activation by TLR9/TLR3 stimulation revealed that the two agonists were able to directly induce IFN-γ secretion by NK cells, but the stimulation of their cytotoxic activity required the presence of alveolar macrophages. Interestingly, our experiments demonstrated that, reciprocally, NK cells were able to influence macrophages polarization, since alveolar macrophages incubated with activated NK cells from the lungs of melanoma metastases-
bearing mice that were given aerosolized TLR9/TLR3 agonists, up-regulated M1- and down-regulated M2-related genes.

Addition of nebulized anti-MDSC antibody RB6-8C5 to aerosolized CpG-ODN/Poly(I:C) resulted in reduced mRNA levels of immunosuppressive molecules (IL10, Arg-1 and Nos2), increased activation of resident NK cells and improved treatment outcome, with a significant reduction in established B16 melanoma lung metastases, compared to treatment with CpG-ODN/Poly(I:C) alone. Likewise, addition of aerosolized IFN-α led to increased mRNA levels of pro-inflammatory cytokines (IL15 and IFN-γ) in the lung and recruitment of highly activated NK cells, with no evident signs of toxicity and with a significantly improved anti-tumor effect, as compared with aerosolized CpG-ODN/Poly(I:C). Combining both IFN-α and RB6-8C5 with CpG-ODN/Poly(I:C) did not produce additive effects.

In conclusion, the results of this thesis indicate that the pulmonary route is a feasible and non-invasive strategy to deliver immunodulatory molecules, including antibodies and cytokines, to reprogram the lung microenvironment shaping it more favorable to foster immune destruction of tumors.

**GRAPHICAL ABSTRACT**
INTRODUCTION

1. TOLL-LIKE RECEPTORS (TLRs)

The human body is provided with a system capable of defending against external assault to which we are daily exposed, that may generate serious damage to our body. The cells and molecules responsible of this protection compose the so-called immune system. The immune system has developed during evolution to defend the body from any form of chemical insult, traumatic or infective to their integrity.

In mammals the defense against foreign and tumor cells is based on early response, mediated by innate immunity, and on late responses, mediated by adaptative immunity.

The innate immune activation and discrimination between what is proper organism (self) and what is not part of it (non-self) is mediated by pattern-recognition receptors (PRRs), which recognize common molecular structures in groups of microbes similar between them. The PRRs include the Mannose receptors (MR), the family members of the Toll-like receptors (TLRs), the NOD-like receptors (NLRs) and the nucleotide-binding oligomerization domains proteins.

The PRRs generally recognize pathogen-associated molecular patterns (PAMPs), microbial components highly conserved, essential for the survival of micro-organisms, that include nucleic acids peptides and microbial cell wall components (eg. LPS peptidoglycan and lipoproteins). There are also other PRRs that are capable of recognizing also endogenous stress signals defined danger-associated molecular patterns (DAMPs).

The best known PRR family is the Toll-like receptors (TLRs). The TLR genes have been conserved in evolution; In fact, homologous receptors have been identified as those shown in mammals also in Caenorhabditis elegans and Drosophila melanogaster. The first member of this family called Toll was first identified in the Drosophila melanogaster, which represents a model for the study of the innate immune defense (Hoffmann J, 2003, Nature).

This receptor appears to be responsible for the embryonic development of the Drosophila dorsal-ventral and seems to play a fundamental role in the adult life of insect as an important factor in the antifungal protection. In fact, mutations in the Toll receptor lead to occurrence of fungal infections affecting the survival of insect (Hoffmann J, 2003, Nature).

The discovery of immune function of Toll in Drosophila led to the search for homologous receptors in mammals and the first receptor identified was the TLR4 (Medzhitov R, 1997, Nature).

In mammals there are 13 different TLR genes: 10 genes in human (TLR1-10) and 12 genes in mice (TLR 1-9 and 11-13). Some of them are homologous (Albiger B, 2007, J Intern Med; Akira S, 2004, Nat Rev Immunol). The TLRs are considered sensors for microbial infections and other "danger signals" and represent an important point of connection between the innate and the adaptive response.
1.1 MOLECULAR STRUCTURE AND CELLULAR DISTRIBUTION OF TLRs

The TLR receptors belong to the superfamily of interleukin-1 receptor (IL-1R) \((\text{Medzhitov R, 1997, Nature; Takeda K, 2003, Annu Rev Immunol})\) and are integral membrane type I glycoproteins. The intracellular domain, responsible for the signal transmission, is homologous to the receptor for interleukin-1 (IL-1R), and for this reason it takes the name of Toll/IL-1R domain or IRR \((\text{Akira S, 2006, Microbiol Immunol; Akira S, 2001, Nat Immunol})\). The extracellular domain differs from IL-1R to the presence of 19-25 of leucine-rich motifs repeated in tandem (LRR) each consisting of 24-29 amino acids forming a \(\beta\) sheet and \(\alpha\) helix connected to each other so as to create a loop. The extracellular portion of the IL-1R is instead characterized by a structure consisting of immunoglobulin domains (figure 1).

![Schematic representation of the superfamily of Toll-IL-1R.](image)

The LRR motif is responsible for the recognition of the specific PAMPs of bacteria, parasites, fungi and viruses \((\text{Albiger B, 2007, J Intern Med; Akira S, 2006, Microbiol Immunol})\).

The genes coding for TLRs are located on different chromosomes: those coding for the TLR1 and TLR6 map the human chromosome 4p14, TLR2 and TLR3 the chromosome 4q31.3-q35, TLR4 the chromosome 9q32-q33, TLR5 the chromosome 1q33.3-q42, TLR7 and TLR8 the chromosome Xp22 and TLR9 the chromosome 3p.21.3. Each of them is able to recognize a particular group of PAMPs.

The members of TLRs family are expressed predominantly and variably, depending on the specific receptor, on immune cells, such as macrophages, dendritic cells (DC), monocytes, B cells, mast cells, natural killer cells, neutrophils, eosinophils and T cells \((\text{Akira S, 2006, Microbiol Immunol})\).

Some studies have shown that these receptors may also be expressed on non-hematopoietic-derived cells, mainly epithelial cells \((\text{Parker LC, 2007, Clin Exp Immunol})\) localized at the level of potential entry sites of pathogens, such as the skin, the respiratory tract, intestinal and urogenital tracts. The TLRs 2, 3, 4, 5, 9 are expressed in epithelial bronchial and gastrointestinal tracts. The TLR3 appears to play a fundamental role on cefalorachidiana barrier and lastly different TLRs,
such as TLR4 and TLR9, are expressed in the gut with a different distribution in various intestinal compartments.

The specificity of different TLRs is partially influenced by their structure and by their cellular localization, which can be intracellular or at the cell surface. In general, most of the TLRs (TLRs as 1, 2, 4, 5, 6 and 10) are expressed on the cell surface, while others (such as TLRs 3, 7, 8, 9) are located in intracellular compartments, such as endosomes. The ligands of these last TLRs (mainly nucleic acids) therefore require an internalization inside the cell to bind to the receptors. Their expression also is not static but finely and rapidly modulated in response to various stimuli, such as contact with pathogenic microorganisms, various inflammatory cytokines and environmental stress (Akira S, 2006, Microbiol Immunol).

1.2 MAIN LIGANDS OF TLRs

The transduction of the signal through TLRs requires the dimerization of TLR proteins on the cell membrane, which sometimes involves homodimerization of same TLRs and other times heterodimerization of two different TLR proteins. The repertoire of the specificity of the TLR system is therefore extends to the ability of TLR to heterodimerize with each other (figure 2).

The TLR2 recognizes various molecules, such as bacterial lipopeptides, molecules localized at the level of the cellular membrane of yeast, molecules of parasites and viral proteins, the acid lipoteichoic acid (LTA) and peptidoglycan of Gram-positive bacteria and finally some glycolipids. The wide range of ligands recognized by TLR2 is due to the fact that this receptor may heterodimerize with two other TLRs, the TLR1 and TLR6. The heterodimer TLR1 / TLR2 recognizes triacetilate lipoproteins, while the heterodimer TLR2 / TLR6 recognizes diacetilate peptidoglycan and lipoproteins (Takeuchi O, 2002, J immunol; Lien E, 1999, J Biol Chem).

The TLR5 detects the conserved domain present in the flagellin monomers, the major structural protein of the flagella of both Gram-negative and Gram-positive (Hayashi, 2001, Nature). The TLR9 is a receptor capable of recognizing nucleic acids with not methylated CpG-rich motifs, characteristic of bacterial and viral organism prokaryotes (Bauer S, 2001, PNAS). Also the TLR3, TLR7 and TLR8 recognize nucleic acids, but unlike the TLR9, bind RNA sequences in double or single stranded. In particular, the TLR3 is designated to recognition of the double helix viral RNA, while the single-stranded RNA interacts with the TLR7 and TLR8 (Alexopoulou L, 2001, Nature; Heil F, 2004, Science).

The specificity of the TLRs are also affected by the binding capacity of various accessory molecules. For example, TLR4 is able to recognize also numerous ligands structurally different from each other, such as the lipopolysaccharide (LPS), viral proteins and some heat-shock proteins, thanks to the recruitment of other molecules, such as CD14, MD2 and CD11b/CD18. The different combinations of accessory molecules in the TLR complexes can therefore serve to increase the spectrum of microbial products able to induce innate responses. Finally, as regards the TLR 10, it is
not yet aware of the specific ligand, although recently it has been demonstrated its ability to form homodimers or heterodimers with TLR1 and TLR2.

Figure 2.
Examples of microbial products able to activate the various TLRs: lipopolysaccharide (LPS to TLR4), unmethylated oligonucleotides (CpG to TLR9) or double-stranded RNA molecules (poly (I: C) for TLR3). (from McInturff JM, 2005, J Invest Dermatol)

Were also identified endogenous ligands recognized by TLR, mainly represented by molecules produced under stress or cell damage. A potential sign of stress is the increase of the expression of heat shock proteins (Hsps), molecules involved in the assembly of the proteins and their translocation in the different cellular compartments. It is noted for example that the Hsp60 protein, both in bacteria and in mammals, once released from necrotic cells is able to activate the TLR4. Finally, also molecules generally released during an inflammatory response, such as various components of the extracellular matrix, can interact with family members of TLRs (Akira S, 2001, Nat Immunol).

1.3 THE PATHWAY OF TLRs SIGNALING
The dimerization of TLR induced by ligand allows the binding of cytoplasmic adaptors to the cytosolic tails through homotypic interactions of TIR domains present both on TLR receptors and on protein adapters. The activation of the cascade signal results in the induction of the transcription of genes involved in the activation of an inflammatory response and host antimicrobial response mechanisms. In particular, TLRs transmit the signal through four possible cytoplasmic adapters: MYD88 (myeloid differentiation factor 88), TIRAP (Tyr-associated protein), also called Mal (Myd88-like adapter), TRIF (Tir-domain-containing adapter inducing INFβ), also known as TICAM-1 (Tir-domain containing molecule1) and TRAM (adapters TRIF-related molecules), also called TICAM-2.

The various TLRs use different combinations of the adapters and this explains the different effects obtained following activation by the various ligands.
In particular, there are two possible signaling pathways: the way MyD88 independent and the way MyD88-dependent. The first pathway leads to the production of type I interferons, (IFN α or β). The second route uses for signaling the adapter MyD88 to activate the NF-kB nuclear factor, JUN kinase (JNK) and p38, with the consequent production of proinflammatory cytokines, such as TNFα, IL-12 and IL-1, and the induction of typical mechanisms of innate immunity (figure 3) (Iwasaki A, 2004, Nat Immunol).

All TLRs (except TLR3) and all members of the IL-1R family, recruit MyD88 at the level of the TIR portion of the receptor. The pathway independent from MyD88 involves TLR3, while TLR4 use both MyD88 dependent and independent pathway.

![Figure 3.](image)

*Figure 3.*
Pathways of TLRs signaling. The TLR, after the binding with their ligand, causing a signaling cascade leading the activation of proinflammatory responses. The route of transmission of the signal can be MyD88-dependent and MyD88-independent. The first way leads the production of proinflammatory cytokines, while the second to interferons of type I. (from Takeda K, 2003, Annu Rev Immunol)

The Activation of TLRs also induces maturation of dendritic cells (DCs), resulting in secretion of cytokines and chemokines and expression of costimulatory surface molecules such as CD40, CD80 and CD86. In particular, the activation of TLRs 3,4,7, and 9 involves the activation of T lymphocytes CD8+ CTL with high production of IFNγ and other cytokines important in the activity of the anticancer immune system (Peng G, 2005, Science).

The TLR can also regulate cell death. Stimulation of these PRR can cause the activation of the PI3K-AKT pathway that promotes cell survival and also appears to be associated with increased expression of anti-apoptotic proteins such as Bcl2-A1 (Bcl2-related protein A1) and other members the Bcl-2 family.

Depending on the type of infection, different immune cell subsets produce different cytokines and chemokines in order to limit the spread of infection. In general, it tends to affirm that the response induced by an extracellular pathogen is characterized by the production of pro-inflammatory
cytokines such as TNFα and IL-12, while in case of infection by an intracellular microbe, the response is characterized by the production of interferons type I (Krieg AM, 2006, Nat Rev Drug Discov). TLRs play a crucial role in the connection between the two types of immunity, therefore the use of TLR to promote the immune surveillance mediated by IFNγ and T lymphocytes, is a possible immunotherapeutics strategy against cancer.

2. CHARACTERISTICS OF TOLL-LIKE RECEPTOR 9 AND TLR3

The TLR9 and TLR3 are members of the TLR family on which many studies have focused in recent years. Their expression and effects resulting from their activation, make these PRRs a promising target for immunotherapy.

TLR9 is able to recognize specific sequences of nucleic acids, characterized by non-methylated CG dinucleotides (CpG motifs) very represented in bacterial and viral DNA. The genomes of bacteria and some viruses generally contain a CpG dinucleotide every 16 bases, while in vertebrates the frequency is greatly reduced and the CpG motifs are almost always methylated (Krieg A.M, 2007, Proc Am Thorac Soc). The activation of TLR3 is through the recognition of viral double-stranded RNA (Cheng YS, 2010, Cancer Biol Ther) which prime the homodimerization, but not induce conformational changes (Liu L, 2008, Science).

For therapeutic applications the TLR9 is generally activated through the use of synthetic oligonucleotides (ODN) that contain one or more CpG motifs (Krieg A.M, 1995, Nature), while the signaling of TLR3 is activated by Polyinosinic-polycytidylic acid (PolyI:C), a synthetic analog of double-stranded RNA (Cheng YS, 2010, Cancer Biol Ther).

2.1 STRUCTURE OF TLR9 AND TLR3

Currently it is still unknown the molecular structure of TLR9. There are in fact little information on structure of TLRs that have high clinical importance, such as TLR7, TLR8 and TLR9; on the contrary there are more detailed knowledge about the structure of other family members of TLRs such as TLR1/TLR2, TLR3 and TLR4, of which the binding mechanisms with the respective ligand are known. Crystallography data of ectodomain (ECD) of the TLR3 indicate that the enriched leucine region (LRRs) are responsible for the recognition of pathogen structures.

Recently, starting from the crystallographic structure of ECD of human TLR3, it was generated a homologue model of TLR9. With this model it is seen that, similarly to what happens for TLR3, also in the TLR9 there are two positively charged sites and there are other residues conserved positively loads on the N-terminal of EDC which would seem involved in the recognition of CpG motifs. These sites maintain a similarity of structure with the N-terminal binding site of TLR3 (Kubarenko A.V, 2010, Protein Sci). Of course all of these modeling studies should be validated with the corresponding crystallographic structure.
2.2 EXPRESSION AND LOCALIZATION OF TLR9 AND TLR3

TLR9 is predominantly expressed in immune system cells. In humans it is expressed exclusively in B cells (Bernasconi NL, 2003, Blood) and plasmacytoid dendritic cells (pDCs) (Kadowaki N, 2001, J Exp Med), a type of specialized dendritic cells responsible for most of the production of type I IFN in response to viral or intracellular pathogens (Iwasaki A, 2004, Nat Immunol). Some studies have also shown the functional expression of TLR9 on activated human neutrophils and on non-hematopoietic cells, such as epithelial cells of the respiratory and the gastrointestinal tract (Parker LC, 2007, Clin Exp Immunol; Hopkins PA, 2005, Clin Exp Immunol).

The expression profile of TLRs differs among different species and this does not allow to have accurately predictive animal models of the effects of the activation of TLR9. For example, in the mouse TLR9 is also expressed in monocytes, macrophages and myeloid dendritic cells, thus generating amplified effects than those observed in human following its activation.

In non-active immune cells, TLR9 is located in the endoplasmic reticulum (ER). The DNA recognition takes place in endolysosomes (Leifer CA, 2006, J Biol Chem). Some studies in the past had shown that TLR9, during the translocation from the ER to the early endosomes, do not cross the Golgi complex; on the contrary recent studies have shown that the TLR9 cross the Golgi complex before reaching the early endosomes and that export from the Golgi apparatus is a required step for optimal transduction of the activation signal (Chockalingam, 2009, Immunol Cell Biol).

In contrast, in epithelial cells the TLR9 is expressed on the cell surface, on the basolateral or on the apical membrane and the signal transduction seems to vary according to the different location. In fact, the basolateral stimulation of TLR9 leads to activation of the NFκB pathway, whereas stimulation of the apical TLR9 prevents the activation of NFκB, promoting the production of NFκB inhibitory protein, IκBα (inhibitory protein I kappa B-alpha).

TLR3 expression, such as TLR9, is limited to specific cell types. Both in human and mice, TLR3 is located on the ER or early endosome in macrophages, myeloid dendritic cell (DCs), NK cells and on the cell surface of non-immune cells like, fibroblast, alvelolar epithelial cells and intestinal epithelial cells (Cheng YS, 2010, Cancer Biol Ther).

2.3 TLR9 AND TLR3 ACTIVATION AND SIGNAL TRASDUCTION

Stimulation of TLR3 and TLR9 by dsRNA or CpG-DNA occurs only after the pathogens were endocytated or phagocytosed or after the virus has infected the cell. Once the microbial DNA is found in the endosomal or lysosomal compartment, inside the cell, it will be recognized by TLR9 and TLR3. Essential for the recognition of CpG-DNA by TLR9 is the acidification of the endosomes and lysosomes. Some drugs, such as china chlorine (CQ) and the bafilomycin A1 (ATP-dependent inhibitor of acidification of endosomes), in fact stop the activation of the signal cascade induced by TLR9, preventing acidification of the endosomal compartments.
It was demonstrated that the recruitment of MyD88 factor immediately after the activation of TLR9 determines the appearance of a truncated form of TLR9 into the endolysosome, indicating that the truncated receptor, rather than the full-length form, is functional (Ewald S, 2008, Nature).

The activation of TLR9 does not involve the mediator TIRAP / MAL. The intracellular signaling of TLR9 is mediated by MyD88, which are recruited to TIR domain and activate the pathway IRAK1-IRAK6-TAK1.

The cascade of the TLR9 signal involve mitogen-activated protein kinase, such as p38, JNK and ERK and the pathway of NF-kB-inducing kinase (NIK) -IKK-IκB and culminates with the activation of specific transcription factors, such as NF-kB, AP-1 (activating protein-1), C/EBP (CCAAT/enhancer binding protein) and protein CREB (cAMP-responsive element-binding protein), which directly induce gene expression of cytokines and chemokines. Some studies have shown that treatment with a drug called wortmannin (WM) leads to a reduction in the number and size of the endosomes containing CpG-DNA and TLR9. These studies suggest that the enzyme PI3K (phosphatidylinositol 3-kinase), which is the target of the WM, could be involved in vesicular trafficking of CpG-DNA (Ishii KJ, 2002, J Exp Med). In particular, the PI3K and its second messengers could play an important role at various stages of activation cell mediated by the binding CpG-DNA / TLR9 (figure 4).

![Figure 4.](image)

**Figure 4.** Summary scheme of the intracellular signaling mediated by TLR9/CpG DNA. PI3K of class III allow internalization of the ODNs in the endosomal vesicles containing TLR9. The same enzyme, together EEA1 antigen, mediates the maturation of endosomes containing CpG-DNA and TLR9 and trafficking inside the cells. The signal cascade begins with the recruitment of MyD88 at level of the TIR domain, which activates the complex IRAK-TRAF6-TAK1, which in turn determines the activation of JNK ½, p38 and IKK complex, causing the up-regulation of the transcription factors NF-kB and AP-1. (from Klinman DM, 2004, Nat rev. Immunol)

After binding to its ligand, the TLR3 recruits TRIF that binds the IKK-1 adapter which activates the transcription factors interferon regulatory factor 3 (IRF3), nuclear factor kappaB (NFkB) and Activator Protein 1 (AP1). This signaling induce the production of type I IFN and the production of inflammatory cytokines, causing the activation of NK cells and CD8+ T lymphocytes and
promoting the development and maturation of dendritic cells. The difference in the two pathways of TLR3 and TLR9 is that TLR9 activates mainly NFkB with the consequent production of pro-inflammatory cytokines at an early stage, while the agonists of TLR3 induce the activation of NFkB and IRF3 at late stage with the main effect to induce the production of IFN type I (Akira S, 2006, Microbiol Immunol).

3. SYNTHETIC AGONISTS OF TLR9 (CpG-ODN) AND TLR3 (Poly I:C) AND THEIR USE IN CANCER THERAPY

TLR9 recognizes nucleotide sequences enriched in not methylated CpG motifs characteristic of the bacterial and viral genome, where the frequency of unmethylated CG dinucleotides is greater than the frequency founded in the vertebrate genome.

Since their binding to TLR9 stimulate cascade of events that activates both innate and adaptive immune system, in the course of the years it appeared of interest to develop synthetic oligonucleotides CpG-rich capable of inducing optimal activation of TLR9, by modifications of nucleotide bases, of the skeleton or of secondary and tertiary structure. These sequences were produced with the aim to generate molecules able to stimulate an immune response versus the tumor cells in immunotherapeutic approaches.

Poly(I:C) is a synthetic analog of double-stranded RNA developed to improve antiviral response (Baron S, 1969, PNAS), able to activate both the innate and adaptive immune system by binding to TLR3. Different types of modified Poly(I:C) have been developed during the years to limit the toxicity. Several researchers have worked to modify the molecular structure of poly(I:C), generating a Poly(I:C) highly stable with increased specificity versus the TLR3, resulted very efficacious agent in immunotherapy against tumor (Carter WA, 1985, J Biol Response Mod, Comparative; Carter WA, 1985, J Biol Response, Preclinical; Hubbell, 1987, J Biol Response Mod; Bucur N, 1998, Neurol Med Chir; Akazawa, 2007, PNAS; Chin Al, 2010, Cancer Res; Harrison SD, 1990, J Biol Response Mod; Shime H, 2012, PNAS; Levy HB, 1975, J Infect Dis; Stephen EL, 1977, Science; Levy HB, 1978, J Infect Dis; Olsen GA, 1978, J Infect Dis; Machida H, 1982, Microbiol Immunol).

3.1 PRECLINICAL STUDIES ON MICE

Some of the most innovative studies concerning the therapeutic use of CpG-ODN and Poly(I:C) have been conducted in animal models (Iwasaki A, 2004, Nature; Carter WA, 1985, J Biol Response Mod, Comparative; Carter WA, 1985, J Biol Response, Preclinical; Hubbell, 1987, J Biol Response Mod; Bucur N, 1998, Neurol Med Chir; Akazawa, 2007, PNAS; Chin AL, 2010, Cancer Res; Harrison SD, 1990, J Biol Response Mod; Shime H, 2012, PNAS). Thanks to studies on TLR9 knockout mice, it was clearly shown that TLR9 is the receptor of CpG-ODN and proved that the CpG-ODN exerts its effects through the activation of this receptor.
The agonists of TLR9 and TLR3 were tested in different animal models of cancer and have shown moderate success in inducing the reduction of the tumor when administered alone or in combination with other cancer treatments, such as radiation or monoclonal antibodies (Krieg AM, 2004, Curr Oncol Rep; Carter WA, 1985, J Biol Response Mod, Comparative; Carter WA, 1985, J Biol Response, Preclinical; Hubbell, 1987, J Biol Response Mod; Bucur N, 1998, Neurol Med Chir; Levy HB, 1975, J Infect Dis; Levy HB, 1978 J Infect Dis; Stephen EL, 1977, Science; Olsen GA, 1978, J Infect Dis; Machida H, 1982, Microbiol Immunol).

### 3.1.1 Monotherapy

The antitumor efficacy of CpG-ODN and Poly(I:C) depends on the type of tumor. The mechanism of action of these agents depends on various factors, including the susceptibility of the tumor to immune effector cells (NK or T cells) and the expression of TLR9 and TLR3 on the tumor cells. Moreover, the treatment with CpG-ODN alone has been shown effective in some tumors only if administered peritumorally or intratumorally, it, but ineffective, if systemically administered (Heckelsmiller K, 2002, J Immunol; Kawarada Y, 2001, J Immunol).

In several studies Poly(I:C) showed antitumor activity, mostly since its ability to induce a change in the microenvironment from pro-tumoral to anticancer (Shime H, 2012, PNAS; Shime H, 2014, J Innate Immun), to activate the immune system (Talmadge JE, 1985, Cancer Res; Ebihara T, 2010, J Exp Med; Akazawa, 2007, PNAS) and to have direct effects on tumor (Cheng YS, 2010, Cancer Biol Ther).

### 3.1.2 Association with therapeutic agents

The therapeutic effects of CpG-ODN are higher when given in combination with other therapeutic agents (Krieg AM, 2004, Curr Oncol Rep). In animal models of cancer, treatment with CpG-ODN in combination with fluorouracil, with inhibitors of topoisomerase I (Balsari A, 2004, Eur J Cancer), with cyclophosphamide (Weigel BJ, 2003, Clin Cancer Res), or with paclitaxel resulted in an increased survival, compared to treatment with CpG-ODN alone.

The oligonucleotide was combined effectively even with cetuximab and cisplatin in ovarian cancer model (Sommariva M, 2011, Cancer Res; De Cecco L, 2013, PloS One) and with DCs based immunotherapy in colon carcinoma C26 animal model (Bourquin C, 2006, Int J Cancer).

### 3.2 CpG-ODN AND POLY(I:C) IN ANTITUMORAL CLINICAL TRIAL

The promising results showing antitumor efficacy of TLR9 agonist and of Poly(I:C) when used as a single agent in mice studies have prompted the development of several clinical trials.
3.2.1 CpG-ODN in Clinical Trial

3.2.1.1 Non-Hodgkin Lymphomas
Since Non-Hodgkin's lymphomas (NHL) usually well respond to treatment with immunomodulants (Liu Q, 2006, J Clin Oncol), the efficacy of monotherapy with CpG-ODN (Agatolimod) was tested in a clinical trial of phase I conducted in NHL patients, previously treated with standard therapy. Patients were divided into 7 cohorts (3 patients for each cohort) and received three i.v. infusions weekly of Agatolimod from one of six established doses (0.01, 0.04, 0.08, 0.16, 0.32, 0.64 mg/kg). By the second day after the treatment, these patients showed an increase in the absolute number of NK cells and an increased NK cell activity, compared to levels before treatment although After 42 days of treatment no clinical response was observed. This clinical study therefore showed that Agatolimod can be safely administered to patients previously treated for NHL, and that treatment with this drug produces immunomodulatory effects (Link BK, 2006, J Immunother).

A Phase I study was conducted in patients with relapsed B cells NHL to evaluate the tolerability, toxicity and antitumor activity of the combination of agatolimod with rituximab (a monoclonal antibody directed to the surface antigen CD20 widely expressed by B cells). It has been shown that the CpG-ODN increases the expression of the target of rituximab in various types of lymphomas. Agatolimod was administered by two routes (i.v. and s.c.) in combination with standard doses of rituximab. Patients were divided into 3 groups; all received rituximab i.v. at a dose of 375mg/m² for week, for 4 weeks, followed by weekly administration of agatolimod s.c. for the cohort 1 (0.01, 0.04, 0.08, 0.16 mg/kg), or Agatolimod i.v. for cohort 2 (0.04, 0.16, 0.32, 0.48 mg/kg) for 4 weeks. The third group received Agatolimod s.c. every week for 20 weeks, at 0.24 mg/kg dose. Among patients of Cohort 1, 21% achieved a complete response (CR) or partial response (PR). Similar results were observed in 11% of patients in the cohort 2. In cohort 3, however, the 50% of patients (6 of 12) achieved a PR or CR, while in 25% of cases a stable disease was maintained. In this cohort, it was observed an increase in the levels of cytokines and chemokines. From this study it was concluded that agatolimod can be administered to patients with NHL in combination with rituximab, either subcutaneously or intravenously, without increasing of toxicity related to infusions of rituximab alone (Leonard JP, 2007, Clin Cancer Res).

3.2.1.2 Renal Cell Carcinoma
In a multicenter phase I study the effects of the weekly s.c. administration of agatolimod (dose range 0.08-0.81 mg/kg) for 24 weeks was evaluated in patients with advanced renal cell carcinoma (Thompson JA, 2004, J Clin Oncol). 31 patients were recruited, 18 males and 13 females aged between 35 and 79 years. One patient had a durable partial response (8 months), 9 patients maintained stable disease, while 17 had disease progression despite treatment with Agatolimod. No side effects were observed and Agatolimod was well tolerated up to the weekly dose of 0.54 mg /
kg. There were biological responses, including increased levels of IP10 and OAS (oligoadenylate synthetase), consistent with the mechanism of action of the agonists of TLR9.

3.2.1.3 Melanoma

The main limitations of immunotherapy for melanoma and other cancers are the immune evasion mechanisms developed by the tumor, which make the host tolerant to the tumor antigens. It was noted that melanoma inhibits the maturation of APC, thus preventing the activation of T cells and the immunological response (Kirkwood JM, 2008, J Clin Oncol). The CpG-ODN was used in different protocols of treatment of melanoma, both as a single agent or in combination with other drugs.

A multicenter phase II clinical trial conducted to evaluate the effects of TLR9 chronic activation by weekly administrations s.c. of agatolimod in melanoma patients showed a moderate but consistent increase in CD86+ pDCs percentage in the blood and their activation, as demonstrated by the high levels of IFN type I. It was also observed a reduction in the number of CD56+/CD16+ NK cells in the blood of patients receiving agatolimod, probably as a result of recruitment at the tissue level. In two patients was confirmed PR, while 3 have maintained stable disease. This study therefore showed that the therapy based on TLR9 agonists in patients with melanoma may stimulate an innate immune response and led to the identification of biomarkers that may be associated with tumor regression induced by the activation of TLR9 (Pashenkov M, 2006, J Clin Oncol).

Phase II study was conducted in 184 patients divided randomly into 4 different cohorts: 1) treatment with 10 mg of agatolimod, 2) treatment with 40 mg of agatolimod, 3) the combination of 40 mg of agatolimod with DTIC (dacarbazine), 4) DTIC alone. Agatolimod was administered s.c. every week, while DTIC was administered every 21 days i.v. at a dose of 850 mg/m². Preliminary response in 92 patients was evaluated, showing a PR in 4 subjects who received the combination, while in the single DTIC group there were only 2 PR. This study concluded that the combination CCID and agatolimod could be more effective than treatment with DTIC alone in patients with metastatic melanoma (Wagner S, 2005, J Clin Oncol).

The CpG-ODN has also been used in immunotherapy protocol for melanoma as adjuvant. A pilot study was designed to investigate the immunogenicity of NY-ESO1 peptide in combination with 157-165V agatolimod and Montanide ISA 720 (another adjuvant) in 8 patients at stage III / IV with melanoma expressing NY-ESO1 (Fourcade J, 2008, J Immunother). The patients were divided into the following groups: 1) immunization with agatolimod and Montanide (3 patients); 2) immunization with Montanide and NY-ESO1 157-165V peptide (2 patients); 3) immunization with Montanide, agatolimod and NY-ESO1 157-165V peptide. The results of these studies show that vaccination with the peptide in combination with the two adjuvants promotes the expansion of CD8+ T cells specific for NY-ESO1 even in patients with advanced cancer.
Finally, a phase I study was conducted to evaluate the effects on cytokines serum levels, safety and clinical activity of agatolimod administered to lesions in patients with metastatic melanoma or with BCC (basal cell carcinoma) (Hofmann MA, 2008, J Immunother). Agatolimod was administered at doses up to 10 mg, every 14 days in 5 patients with BCC and in 5 melanoma patients with cutaneous or subcutaneous metastases. Both groups showed a local tumor regression and increased levels of IL-6 in all patients, increased IFNγ levels in 8 patients out of 10, increased IL-12 p40 levels in 7 out of 10 patients and an increase of TNF-α in 7 out of 10 patients.

A Phase Ib study has recently opened to evaluate the association of CMP-001, CpG-A oligodeoxynucleotide (ODN) formulated within a virus-like particle, with pembrolizumab (anti-PD-1 antibody) to facilitate activation of the innate immune response within a tumor that will enhance response to anti-PD-1/L1 therapy. The trial enroll patients with advanced melanoma and present with either progressive disease, or stable disease after at least 12 weeks of pembrolizumab therapy (Clinical trial information: NCT02680184).

**3.2.2 POLY(I:C) in clinical trial**

The efficacy of Ampligen (PolyI:C) is being tested in combination with different types of agents, such as celecoxib, IFNα and Hiltonol in various clinical trials performed in different types of cancers, such as breast cancer, colon rectal cancer and cancer of reproductive tract (Galluzzi L, 2012 Oncoimmunology).


**3.2.2.1 Hematological Cancer**

A phase I-II trials with 19 patients with different tumors and 6 patients with acute leukemia were performed. Patients, Poly(I:C) administered at a dose of 0.5-27 mg/m², showed a significant increase of interferon production that resulted directly proportional to the dose administered. Adverse reactions were fever, nausea, hypotension in relation to the dose used of TLR3 agonist (Levine As, 1978, Cancer Treat Rep). A similar effect was observed also in another phase II clinical trial on patients with acute lymphoblastic leukemia and not acute lymphoblastic, where an increase of interferon in 50% of patients was detected (Lampkin B, 1985, Cancer Res).
Seven patients with refractory multiple myeloma in a phase II trial were treated with Poly(I:C). In one patient a partial response (PR) was observed and in 3 patients an objective response was observed. Even in these patients was detected an increase of interferon (Giantonio BJ, 2001, Invest New drugs).

3.2.2.2 Melanoma
In a phase I-II study, 16 patients with metastatic melanoma were treated with Poly(I:C). In this study no anti-tumor responses were observed, but there was an increase in the concentration of interferon in the serum of patients eight hours after treatment with TLR3 agonist (Hawkins MJ, 1985 J Biol Response Mod).

3.2.2.3 Association with immunomodulators and chemotherapy
25 patients with different types of tumor were enrolled in a phase IB study, where the association of TLR3 agonists with interleukin 2 was evaluated. A slight toxicity and the absence of objective tumor response was observed, but all patients treated with this combination showed an increase in NK cells activity (Ewel CH, 1992, Cancer Res).
Morse et al. has conduct a study to evaluate the combination of poly(I:C) with CDX-1307, a vaccine composed of human chorionic gonadotropin beta chain fused with MR-specific monoclonal antibody in epithelial cancer patients. The combination of TLR3 agonists with the vaccine induced a high immune response and clinical benefit represented by an increase in the duration of stable disease (Morse AM, 2011, Clin Cancer Res).
Two other Phase II studies evaluated the association of TLR3 agonists with chemotherapy. In the first, survival increase was observed in patients with glioblastoma treated with Poly(I:C) in combination with temozolomide (Rosenfeld MR, 2010, Neuro Oncol) while in the second a significant increase in disease-free survival and a reduction in the incidence of metastases in patients with breast cancer treated with radiotherapy in combination with TLR3 agonist (Laplanche A, 2000, Breast Cancer Res Treat).
Overall, these studies have shown that the use of TLR3 agonist in patients is safe and is able to induce an activation of the immune system.

4. IMMUNE MICROENVIRONMENT IN THE LUNG AND APPROACHES FOR ITS MODULATION
4.1 IMMUNE MICROENVIRONMENT IN THE LUNG
The airways every day filter considerable amount of air and this determines a continuous contact with solid particles, allergens and microbes. The continuous exposure to external environment induces the formation of a pulmonary immunosuppressive microenvironment, governed by several

4.4.1 Dendritic cells resident in the lung:
Lung resident DCs derive from Hematopoietic Stem Cell in the bone marrow that undergo maturation becoming Pre-DC; these cells reach the lung through blood and differentiate into conventional or plasmacytoid DCs. The cytokine Flt3L and GM-CSF appear to be essential elements for all stages of dendritic cells differentiation (Liu K, 2009, Science; Greter M, 2012, Immunity).

DCs are mainly divided in conventional DCs (CDCs), plasmacytoid (pDCs) and monocyte-derived DCs (moDCs). These cells can be identified by immunofluorescence as cells expressing the CD11c and MHCII marker. Moreover, the analysis of markers CD103 or CD11b and PDCA-1 or B220 allows to discriminate between conventional and plasmacytoid subset, respectively (Kopf M, 2015, Nat Immunol).


4.1.2 Alveolar macrophages (AMs):
Is another population closely involved in maintaining lung homeostasis, which colonize the alveoli in the first day of life (Guilliams M, 2013, J Exp Med). AMs differ from interstitial and bronchial macrophages for their function, location and live-turnover.

In health condition, AMs is about 95% of the cell population contained in the alveoli together with a small number of T lymphocytes (Ely KH, 2006, J Immunol; Snelgrove RJ, 2011, Trends Immunol). They have reduced phagocytic capacity, compared to interstitial macrophages (Holt PG, 1978, Am Rev Respir Dis) and low antigen presentation capacity (Lipscomb MF, 1986, J Immunol; Lyons CR, 1986, J Immunol; Toews GB, 1984, J Immunol). They also produce TGF-β that inhibits

Alveolar macrophages in health conditions have the duty to respond to infections by their activation, to eliminate apoptotic cells and to inhibit the activation of the immune system in the presence of harmless antigens. (Martinez FO, 2008, Front Biosci; Edwards JP, 2006, J Leukoc Biol; Mosser DM, 2008, Nat Rev Immunol).


4.2 IMMUNE-SUPPRESSIVE CELLS IN TUMOR MICROENVIRONMENT

The growth and tumor development may be promoted through a cross-talk between tumor cells and immune system cells, that exacerbates an immunosuppressive microenvironment. In this process are involved mainly myeloid-derived suppressor cells (MDSC), tumor associated macrophages (TAM) and dendritic cells, found in different types of cancer (Gabrilovich DI, 2012, Nat Rev Immunol; Qian BZ, 2010, Cell). These cells have been identified as responsible of the failure of numerous studies to evaluate different immunotherapeutic approaches in cancer and high density of these cells in tumors are often associated with poor prognosis and resistance to therapy (DeNardo DG, 2009, Cancer Cell; Steidl C, 2010, N Engl J Med; Mazzieri R, 2011, Cancer Cell). Moreover,
different studies demonstrated that TAMs and MDSCs depletion can re-establish the efficacy of immunotherapy (Srivastava MK, 2012, PloS One; Germano, 2013, Cancer Cell).

TAMs and MDSCs accumulation in a tumor microenvironment is mainly caused by chemotactic products derived from cancer cells. It has been reported that TAMs and MDSCs support tumor growth by different mechanisms: The block of anti-tumor response of lymphocyte T cells and natural killer (NK), the promotion of angiogenesis, the secretion of growth and proangiogenic factors and matrix-degrading enzymes (Gabrilovich DI, 2009, Nat Rev Immunol; De Palma M, 2013, Cancer Cell).

4.2.1 Myeloid-derived suppressor cells:

are a heterogeneous population of myeloid progenitor cells that infiltrate all tumors (Ostrand-Rosenberg S, 2012, Semin Cancer Biol). In mice MDSCs are characterized of CD11b+ Gr1+ expression (Gabrilovich DI, 2012, Nat Rev Immunol). CD11b+Gr1+ myeloid progenitor cells are usually present in healthy mice, but they not have immunosuppressive activity and differentiate into mature myeloid cells. Conversely in tumor bearing mice, the differentiation is blocked and MDSC accumulates in different body districts (Almand B, 2001, J Immunol; Diaz-Montero CM, 2009, Cancer Immunol Immunother; Ostrand-Rosenberg S, 2009, J Immunol).

These cells are divided into two subtypes: granulocytic and monocytic by the expression of surface markers. Granulocytic-MDSCs (G-MDSCs) (Ly6GhiLy6Clow) are much more than Monocytic-MDSCs (M-MDSCs) (Ly6GlowLy6Chi), but their suppressive activity is lower compared to than activity of M-MDSCs (Youn J, 2008, J Immunol; Youn J, 2013, Nat Immunol; Movahedi K, 2008, Blood; Peranzoni E, 2010, Curr Opin Immunol). Distributed in the body, MDSCs are recruited into tumors site through chemokines, such as CCL2 or Bv8 (Huang B, 2007, Cancer Lett; Shojaei F, 2007, Nature; Sawanobori Y, 2008, Blood; Sinha P, 2008, J Immunol)

They promote tumor growth both through mechanisms involving suppression of other immune cells, and through non-immunologic mechanisms. For example, MDSC inhibit the activation of T lymphocytes through the production of ROS that down regulate the activity of the TCR (Ezernitchi AV, 2006, J Immunol; Nagaraj S, 2010, J Immunol) and the depletion from the microenvironment of arginine and L-cysteine required for their proliferation (Rodriguez PC, 2004, Cancer Res; Srivastava MK, 2010, Cancer Res). Moreover, they promote also the development of regulatory T-cells through the production of IL-10, TGF-beta and CD40-CD40L (Huang B, 2006, Cancer Res; Pan PY, 2010, Cancer Res; Serafini P, 2008 Cancer Res).

MDSC have been reported to also block the activity of the innate immune system, by inhibiting the cytotoxic activity and the IFN-gamma production of NK cells by cell-cell contacts. Membrane-bound TGF-β1 on MDSCs induces anergy of NK cells (Li H, 2009, J Immunol; Liu C, 2007, Blood; Suzuki E, 2005, Clin Cancer Res; Elkabets M, 2010, Eur J Immunol). These cells also
inhibit NK cells activation through blocking the expression of NKG2D (Nausch N, 2008, Blood) (figure 5).

Figure 5.
Roles of MDSCs in tumor growth and progression. Summary of MDSCs immune-suppression mechanisms; these cells can suppress T cells activation and proliferation, lead induction of regulatory T cells and TAMs and suppress DC activity. (from Shime H, 2015, Inflammation and Immunity in Cancer)

4.2.2 Tumor-associated macrophages (TAMs):
infiltrate various type of cancer both in humans and mice models (Solinas G, 2009, J Leukoc Biol) ad can induce tumor growth both through immunologic and non-immunological mechanisms. Studies with macrophages deficient models (Lin EY, 2001, J Exp Med) or macrophages depletion by bisphosfonate treatment (Sfondrini L, 2013, Int J Cancer) demonstrate that TAMs play an important role in growth and cancer progression.

Tumor and stromal cells secrete many chemokine and chemoattractants that determine the recruitment of peripheral monocytes at tumor site. These cells evolve in TAM after microenvironment stimulation (Solinas G, 2009, J Leukoc Biol).


TAMs are characterized by low expression levels of pro-inflammatory cytokine (IL12, IL6, IL1beta) and high levels of IL10, CD206, MMR and Arginase-1 that down regulate the activity of cytotoxic T lymphocytes, NK cells and DC. In addition, IL10 induces the activation of regulatory T cells. TAM also produce VEGF and PDGF that stimulate angiogenesis and matrix-degrading enzymes causing tissue remodeling in the tumor environment (Shime H, 2015, Inflammation and Immunity in Cancer; Mantovani A, 2008, Nature; Lin EY, 2007, Cancer Res) (figure 6).

Figure 6.
Roles of TAMs in tumor growth and progression. Circulating monocytes recruited by chemoattractant at tumor site and differ in TAMs with M2 phenotype. TAMs induces immune-suppression and tumor development producing cytokines, proangiogenic and growth factors and matrix-degrading enzymes. (from Shime H, 2015, Inflammation and Immunity in Cancer)
4.2.3 Dendritic cells:


Therefore, the mutual interaction among these different immunological populations that accumulate in the tumor microenvironment causes an intensification of the immunosuppression, favoring tumor development. The depletion of these cells from the tumor microenvironment by the use of antibodies or the conversion through the TLRs ligands can induce a differentiation and maturation of tumor-associated myeloid cells, “re-educating” these cells and inducing a shift towards an antitumor phenotype.

For example, a shift of TAMs versus a M1 phenotype through activation of immune signal of TLRs have been observed by several authors (Shime H, 2012, PNAS; Akazawa, 2007, PNAS; Azuma M, 2012, Oncoimmunology; Seya T, 2012, Oncoimmunology). Moreover, Eriksson et al., reported that the use of Bacteriophages determines production of M1-like cytokines and expression of proteins related to antigen presentation in TAMs (Eriksson F, 2009, J Immunol). Shime at al. demonstrated that Poly(I:C) administration on tumor-bearing mice result in a M1 polarization of TAMs with a delay of tumor growth (Shime H, 2012, PNAS) (figure 7).
Figure 7.

M1 phenotype shift of TAMs by TLR pathway. TLR agonists, such as CpG-ODN and Poly(I:C), are able to induce re-education of M2 TAMs to a M1 Macrophages. M1 tumor associated macrophages produce type I IFNs, IL-12 and other Th1 cytokines that induce the activation of CTLs and NK cells. Moreover, M1 TAMs produce TNF-alpha that is responsible of killing of tumor cells. (from Shime H, 2015, Inflammation and Immunity in Cancer)

TRL ligands can also modify immunosuppressive activity of MDSCs. Tumor injection of CpG-ODN stimulates MDSCs to differentiate into M1 macrophage and to lose their suppressive activity on T cells (Shirota Y, 2012, J Immunol). Moreover, Toll Like Receptors signal have been demonstrated to induce Type-I IFNs production that in turn can modulate the function of MDSCs and CpG-ODN and Poly(I:C) have been reported to induce Type-I IFNs secretion by pDCs, which result in MDSCs maturation (Zoglmeier C, 2011, Clin Cancer Res; McCartney S, 2009, J Exp Med) (figure 8).
Figure 8.

Conversion of MDSCs activity by TLRs pathway. TLR3 and 9 agonists and type I IFNs induces maturation and activation of MDSCs, which result in a direct cytotoxic activity on tumor and IFN-alpha production that activate NK cells cytotoxic activity. (from Shime H, 2015, Inflammation and Immunity in Cancer)

4.3 PRECLINICAL STUDIES WITH TRL AGONISTS FOR THE TREATMENT OF LUNG CANCER

4.3.1 Preclinical studies with TRL9 agonists

Preclinical studies in murine models have shown limited efficacy of CpG-ODN in promoting antitumor activity in the lung. In fact, the CpG-ODN result capable to control the growth of lung metastases only when administered before injection of tumor cells or in a state of minimal disease. A study evaluated the efficacy of CpG-ODN in controlling the development of metastasis induced by i.v. injection of N202.1A cells, derived from a spontaneous mammary carcinoma. The results showed that the growth of the metastasis was inhibited only when the systemic treatment with CpG-ODN, was carried out immediately before or immediately after the inoculation of tumor cells, but not when the CpG-ODN was administered 2 days after tumor injection. Therefore, the study has indicated that repeated systemic CpG-ODN injection in this tumor model is able to prevent the tumor grow in the lung, but is poorly effective when administered few days after tumor injection (Sfondrini L, 2002, FASEB J).

Kim et al. (Kim HA, 2009, Cancer Lett) evaluated the ability of CpG-ODN in preventing the growth of lung metastases derived from intravenous inoculation of B16F10 murine melanoma cells. In this study, CpG-ODN was administered i.p. at day -7 or -3 before B16F10 injection, or at day of injection. Fourteen days after inoculation animals were sacrificed and the number of lung
colonies was counted. In this study, a reduction of 62%, 82% and 54% in the number of lung metastases was observed in the groups treated at day -7, -3 and 0 before the injection, compared to the group control.

To evaluate the therapeutic effect of CpG-ODN on the growth of already implanted B16F10 melanoma cells, the experiment was repeated administering the oligonucleotide after tumor injection. Although CpG-ODN treatment induced a significant inhibition in lung tumor colonization, the inhibition was much lower compared to the CpG-ODN administration before tumor injection.

The therapeutic activity of CpG-ODN in countering tumor growth in the lungs was evaluated in another study (Sorrentino R, 2010, J Immunol). The aim of this study was to clarify the effect of CpG-ODN in the murine Lewis lung carcinoma model and to evaluate the immune populations activated by CpG-ODN. The mice were inoculated i.v. with LLC1 cells and 10 days after injection, CpG-ODN was administered i.p. The animals were sacrificed 3 or 7 days after administration. The results of this study have shown that a single administration of CpG-ODN induced an increased tumor growth evaluated 3 and 7 days after treatment.

The study shows that CpG-ODN increased an immunosuppressive environment in the lung associated with an increased influx of plasmacytoid dendritic cells (pDCs), that resulted in turn responsible of an increased recruitment of T regulatory cells. Depletion of pDCs by a specific Ab (m927) in mice treated with CpG was able to revert this immunosuppressive environment, resulting in an increased percentage of activated myeloid dendritic cells, CD8+ T lymphocytes and an increased production of Th1 and Th17-like cytokines. The induction of this type of immune response in the animals treated with CpG-ODN in combination to pDC depletion was associated with a reduction of the tumor size.

Therefore, this study shows that treatment with CpG-ODN in this model of lung cancer induced an increase in tumor growth linked to the increased of the immunosuppressive environment.

Yan et al. (Yan J, 2012, Acta Pharmacol Sin) evaluated an immunotherapeutic approach against tumor metastases, based on combination of activatory anti-TLR2 antibodies with CpG-ODN. C57BL/6 mice were inoculated i.v. with melanoma B16-F10 cells and mice were treated with CpG-ODN (0.5 mg/kg) or with the anti-TLR2 antibody (200 mg/kg) alone, or in combination.

The results of this study revealed that only treatment with the CpG-ODN in combination anti-TLR2 antibody resulted able to suppress significantly the growth of metastases compared to single treatments. Indeed, combinatory treatment induced a higher infiltration of NK cells, CD8+ T lymphocytes, a reduced recruitment of M2 macrophages and of regulatory T cells and a reduced expression of immunosuppressive factors (TGF-β1, cyclooxygenase-2 and indoleamine 2,3-dioxygenase). The anti-metastatic effect of the combination therapy was confirmed also in a metastatic spontaneous mouse model of Lewis lung carcinoma. Therefore, the study demonstrated
that combination of TLR9 agonist with an anti-TLR2 antibody is able to eliminate immunosuppressive factors from the tumor, that block an effective immune response.

4.3.2 Preclinical studies with TRL3 agonists


Shime H et al. (Shime H, 2012, PNAS) showed as Poly(I:C) injection in a model of LLC carcinoma was able to trigger a shift of TAM to macrophage with a M1 phenotype. LLC bearing mice treated with Poly(I:C) shown increased TNF-α production by TAMs and expression of M1-related genes, which in turn induced hemorrhagic tumor necrosis and block of tumor growth. This effect was not observed in TNF-α−/− and Ticam-1−/− mice. This study demonstrated that the conversion of a microenvironment that supports the tumor to an anti-tumor microenvironment through the administration of the TLR3 agonist is able to efficiently block tumor growth.

The same group of researchers demonstrated that Poly(I:C) treatment induces maturation and activation of MDSCs, making them competent for priming of NK cells, that activated produce IFNγ. The maturation of the MDSC and NK cells activation results in a reduction in tumor growth. This effect is partially abrogated in Ticam-1−/− and MAVS−/− mice and totally in the double KO mice (Shime H, 2014, J Innate Immun).

Another study evaluated the effect of TLR3 agonists administration on B16 induced metastatic lung cancer. Mice were i.v. injected with B16 melanoma cells and after one week treated with Poly(I:C) i.p. After 9, 5 or 10 days from melanoma cells injection, mice were sacrificed and metastases were counted.

Poly(I:C) treatment resulted in a growth reduction of lung metastases due to activation of dendritic cells and IFNγ and IL17A production, which in turn induced the recruitment into the lung of several cells types with cytotoxic activity as NKT and CD8+ lymphocytes. This study shows that the activation of the immune system and the simultaneous reduction of an immunosuppressive microenvironment are able to effectively counteract the growth of lung metastases (Forte G, 2012, J Immunol).

4.4 LOCAL DELIVERY OF TLR AGONIST

Several studies (De Cesare M, 2010, J Immunother; De Cesare M, 2008, Clin Cancer Res; Sommariva M, 2011, Cancer Res; Lou Y, 2011, J Immunother) suggested that the route of
administration is a critical factor in influencing the activity the CpG-ODN. A greater anti-tumor effect was observed when the CpG-ODN was inoculated directly and repeatedly in tumor growth site, where ensures effective and continuous activation and recruitment of both innate and adaptive immune response. Indeed, the anti-tumor activity of CpG-ODN showed limited efficacy in the therapy of lung cancer. Various studies in preclinical models show that the CpG-ODN is capable of controlling the growth of lung metastases only when administered before tumor injection, or immediately after in a state of minimal disease (Sfondrini L, 2002, FASEB J; Kim HA, 2009, Cancer Lett; Sorrentino R, 2010, J Immunol). Moreover, was not observed any benefit from the addition of CpG-ODN administration to chemotherapy in phase III clinical trials in patients with non-small cell Lung Cancer (NSCLC) (Jaehrdsorfer B, 2008, Update Cancer Ther; Kelly RJ, 2010, Clin Lung Cancer). Thus, a local, instead of systemic, TLR9 agonists administration, could be a novel approach to improve the therapeutic efficacy of CpG-ODN in the treatment of lung cancer or preventing lung metastases derived from different primary tumors.

4.4.1 Experimental approaches for local delivery of therapeutic agents in the lung


Intranasal administration, however, is not applicable in a clinical setting, where inhalers (device used for inhalations) or nebulizers (device which reduces a liquid into fine droplets and spreads in the air), which are the most convenient and simple approach to repeated application and to ensure a uniform distribution and penetration in the pulmonary alveoli, are generally used.

Several studies in mice have evaluated the use of aerosol administration of various therapeutic agents in the lung. In particular, this approach has been used to administer: cytokines as interleukin-12 (Jia SF, 2003, Clin Cancer Res), interleukin-2 (Khanna C, 1997, Cancer) and stimulating granulocyte/macrophage factor (Anderson PM, 1999, Clin Cancer Res); chemotherapeutic agents such as gemcitabine (Koshkina NV, 2005, Int J Cancer) and docetaxel in combination with celecoxib (Haynes A, 2005, Pharm Res); antisense oligonucleotides alone or in combination with anticancer drugs (Templin MS, 2000, Antisense Nucleic Acid Drug Dev;

The conclusion derived from these studies is that the local administration of these molecules by aerosols allows their location at the level of the bronchial epithelium, alveolar epithelium, endothelium. Moreover, compared to systemic administration, this route of administration results in a better retention of the different agents in the lungs, minimizes their penetration into the systemic circulation, and thus limits adverse side effects.
AIM

TLRs recognize several conserved pathogen-associated molecular patterns, allowing to detect microbial pathogens. Toll-like receptors agonists, in light of their immunostimulatory activity, are included in the National Cancer Institute list of immunotherapeutic agents with the highest potential to cure cancer. Our and other previous studies in experimental and clinical cancers highlighted the importance and the superior antitumor activity of the locoregional delivery of TLR agonists compared to the systemic administration, revealing the relevant role in this activity of innate immune cells, that must be activated locally unlike cells of the adaptive immune response, which can reach the antigen wherever they are activated.

Since bronchial and bronchoalveolar tumors are accessible via the endobronchial space, lung cancers are candidate tumors for taking advantage of local delivery. Several therapeutic agents, such as cytokines, oligonucleotides and chemotherapeutic agents have been explored for inhalation delivery in malignancies. These studies demonstrated the feasibility of aerosol delivery, its potential anti-tumor effects and the reduced side-effects compared to systemic treatment. Thus, inhalation of aerosolized TLR agonists could represent a convenient and simple approach to treat/prevent primary lung cancer and lung metastases and might favour frequent local replenishment of innate immune effectors.

Our previous studies showed that aerosolized CpG-ODN, TLR9 agonist, was able to reach the bronchoalveolar space, locally activated an immune response without any signs of toxicity and was more efficacious than systemic administration against lung metastases of N202.1A mammary carcinoma cells. In contrast, aerosol delivery of CpG-ODN was not very effective against metastases of B16 melanoma cells, which selectively recruit CD68+ M2 macrophages and induce an immunosuppressive environment that hinder the recruitment of NK cells. Studies in mice showed that Poly(I:C), TLR3 agonist, through the TICAM-1/TRIF adaptor was able to convert lung tumor-associated macrophages (TAM) from tumor supporters (M2) to those with tumoricidal properties (M1). Thus, aerosol-delivered TLR3 agonists could increase the antitumor activity of CpG-ODN even in the presence of an immunosuppressive tumor microenvironment.

Besides TAM, other immune populations can contribute to the tumor-induced immunosuppressive microenvironment. MDSCs represent one of the most relevant of these populations that progressively accumulate within the tumor mass and suppress effector cell function both directly and by amplify the macrophage immunosuppressive activity through a continuous cross-talk.

Therefore, the microenvironment is a critical factor for successful use of immunotherapies with immunomodulants, and strategies to shift a tumor-supporting milieu to a host-friendly one might lead to improved antitumor activity of TLR agonists. Thus, the aim of this project of thesis are to:
1) Evaluate the combined aerosol administration of Poly(I:C)/CpG-ODN to simultaneously blocking TAM-induced immunosuppression and maintain a continuous activation of innate immune cells in the treatment of experimental B16 melanoma lung metastases.

2) Investigate the mechanism by which the two combined agonists could induce the activation of effector cells population.

3) Develop strategies to locally shape a lung microenvironment that further could favor effector cells activation against B16 melanoma lung metastases, by combined inhalation-based immunotherapies.
MATERIAL AND METHODS

1. Cell lines, culture and reagents

B16 mouse melanoma cells and YAC-1 mouse lymphoma cells (ATCC) were routinely maintained at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) and RPMI-1640 (EuroClone), respectively, supplemented with 10% fetal bovine serum (FBS, Gibco) and 2 mM glutamine (LONZA). Purified phosphorothioated TLR-9 agonist ODN1826 (5’-TCCATGACGTTCCTGACGTT-3’), containing CpG motifs (CpG-ODN), was synthesized by TriLink Biotechnologies. Low-molecular-weight polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analog of the double-stranded RNA (dsRNA) agonist of TLR-3, was from InvivoGen. ^51^Cr (1 mCi) was purchased from Perkin-Elmer (Waltham). Dacarbazine was provided by Medac GmbH. Intron A (interferon-alpha-2b recombinant) was provided by Schering-Plough. InVivo MAb anti-mouse Ly6G (Gr-1), RB6-8C5, was from Bio X Cell. Bronchoalveolar lavage (BAL) was performed in euthanized mice by 5 intratracheal instillations of 1 ml PBS, containing 0.5% BSA and 2 mM EDTA, followed by gentle aspiration. The recovered BAL fluid was then centrifuged. The resulting cells were plated for 2 hr at 37°C to allow macrophages to adhere to the culture plates, and non-adherent cells were removed by washing with PBS. To evaluate NK cell degranulation and the composition of lung immune cells, lungs from mice that were injected with tumor cells and treated with CpG-ODN, poly(I:C), IFN-α, RB6-8C5 and Dacarbazine were digested in DMEM that contained collagenase (300 U/ml) and hyaluronidase (100 U/ml) (Stemcell Technologies) for 1 hr at 37°C. Cell suspensions were filtered through 70-µm cell strainers (Falcon), and after red blood cell lysis by incubation in 1x RBC Lysis Buffer Solution (eBiosciences), they were analyzed by flow cytometry or plated for 2 hr 37°C to separate adherent cells, containing primarily macrophages, from non-adherent cells, containing effector cells, such as NK cells. Splenocyte preparations were obtained by gently flushing the spleen in serum-free RPMI 1640 to release the cells. After red blood cell lysis by incubation in 1x RBC Lysis Buffer Solution (eBiosciences), naive NK cells were purified using the Mouse NK Cell Isolation Kit II and MACS separation columns (Miltenyi), according to the manufacturer’s instructions.

2. Mice and experimental protocols

All experiments were carried out using 6- to 12-week-old female C57BL/6 mice (Charles River) maintained in laminar-flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan according to the Declaration of Helsinki regarding the use and care of animals. Mice were injected intravenously with 5×10^7 B16 melanoma cells and treated starting 4 or 7 days later with aerosol at
72- to 96-hr intervals or with dacarbazine administered intraperitoneally at 70 mg/Kg, 5 days/week for 3 or 2 weeks, respectively. Mice were weighed twice weekly.

CpG-ODN1826, Poly(I:C) and Intron A were aerosolized using a whole-body exposure system (EMMS) as described (Sfondrini L, 2013, Int J Cancer). Briefly, Poly(I:C) (15 mg), CpG-ODN (1.5 mg) or Intron A (4,000,000 IU) were dissolved in 5 ml of saline and placed in the nebulizer to treat up to 10 mice placed in the aerosol box and exposed to aerosol for 15 min. The monoclonal antibody RB6-8C5 was administered endotracheally (Guillemainault L, 2014, J Control Release; Herve V, 2014, MAbs). Briefly, mice were anesthetized and given 25 μg/mouse of RB6-8C5 MAb using a Microsprayer Aerosolizer Model IA-1C connected to a FMJ-250 high-pressure syringe (Penn-Century) introduced just before the first trachea bifurcation (Bivas-Benita M, 2005, Eur J Pharm Biopharm). In all experiments, mice were weighed twice weekly, euthanized at the end of experiments and macroscopic lung metastases counted. All in vivo experiments were repeated at least twice.

3. Histological, immunofluorescence and immunohistochemical examination of lungs

Lung samples obtained from healthy mice or from mice i.v. injected with B16 cells and treated with aerosolized Intron A, CpG-ODN, Poly(I:C), or CpG-ODN/Poly(I:C) as described above or untreated were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 mm and stained with hematoxylin and eosin for histological evaluation by light microscopy (Scan Scope Aperio, Nikon).

Immunofluorescence and immunohistochemical analyses were performed to analyze macrophage infiltration on lung sections collected from mice after i.v. injection of B16 cells (4 mice/group) untreated or treated with aerosolized CpG-ODN alone or combined with Poly(I:C). For immunohistochemistry, tissue samples were fixed in 10% buffered formalin and paraffin-embedded, sectioned (4-μm thick), deparaffinized and rehydrated. Antigen unmasking was performed using pH 9 Tris/EDTA buffer (Novocastra) in a PT Link Dako unit at 98°C for 30 min. Sections were then brought to room temperature and washed in PBS. After neutralization of endogenous peroxidase with 3% H2O2 and Fc blocking by a specific protein (Novocastra), samples were incubated with polyclonal rabbit anti-mouse arginase antibody (1:200, Genetex International Corp.) or polyclonal rabbit anti-mouse CD163 antibody (1:100, Abcam) for 1 hr at room temperature. Staining was revealed by a polymer detection kit (Novocastra) and AEC (3-amino-9-ethylcarbazole, Dako) substrate-chromogen. Slides were counterstained with Harris hematoxylin (Diapath). For immunofluorescence analysis, polyclonal rabbit anti-mouse CD68 (1:100, Abcam) and rat anti-mouse IL-10 (1:200, Novus Biologicals) were used. Antigen unmasking was performed using pH 6 citrate buffer (Novocastra) in a PT Link Dako unit at 98°C for 30 min. After Fc blocking, primary antibody binding was revealed by Alexa 488-conjugated
goat anti-rat (Invitrogen Molecular Probes) and Alexa 568-conjugated goat anti-rabbit (Invitrogen Molecular Probes) secondary antibodies. Rat isotype control (eBioscience Rat IgG1 isotype control) and an unrelated rabbit antibody (Invitrogen Rabbit isotype control), used as controls to verify the specific reactivity of arginase and IL-10 staining, did not reveal any staining. Slides were counterstained with DAPI nucleic acid stain (Invitrogen Molecular Probes). All sections were analyzed under a Leica DM2000 optical microscope (Leica Microsystems) and microphotographs were collected using a Leica DFC320 digital camera (Leica).

4. Quantitative PCR Analysis

Lung samples of mice bearing B16 melanoma cells treated with dacarbazine or untreated were cut into small pieces and homogenized with QIAzol Lysis Reagent (QIAGEN). Total RNA was isolated according to the manufacturer’s instruction and reverse transcription was performed using SuperScript III First-Strand (Invitrogen). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) with a StepOne Real-Time PCR System (Applied Biosystems), using the following primers: RAE1 rev: 5’-CCC TCC TCT GGC CTC TCC TT -3’; RAE1 for: 5’-CCCAGTATCACCCA GCT TAC AT-3’; MULT1 rev: 5’-CAT CCA AGA GAG GTG GTG GT -3’; MULT1 for: 5’-AGCTCA TGT TGC ACT GGA AA-3’. Expression of the gene was normalized to GAPDH.

Lungs from healthy or tumor-bearing mice untreated or treated were digested in DMEM medium containing collagenase and hyaluronidase as described above, while naïve macrophages were isolated from healthy mice and co-cultured for 24 hours at a 1:1 ratio with non-adherent cells from tumor-bearing lungs of mice that were treated with or without aerosolized CpG-ODN/poly(I:C). At the end of the co-incubation, macrophages and lung cell suspensions were incubated with QIAzol Reagent (QIAGEN) and total RNA was isolated according to the manufacturer’s instructions. Reverse transcription was performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems), and real-time PCR was performed using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and SDS 2.4 on a 7900HT Fast Real-Time PCR System (Applied Biosystems), with the following TaqMan® gene expression assays (Applied Biosystems): Arg1 (Mm00475988_m1), Nos2 (Mm00440502_m1), IL-10 (Mm01288386_m1), IL-12 (Mm00434169_m1), IRF-4 (Mm00516431_m1), IRF-5 (Mm00496477_m1), ALOX15 (Mm00507789_m1), IL-6 (Mm00446190_m1), STAT-1 (Mm00439531_m1), STAT-6 (Mm01160477_m1), IFNγ (Mm01168134_m1), IL15 (Mm00434210_m1), and IL15Ra (Mm04336046_m1). Expression of each gene was normalized to ActB (Mm00607939_s1) and B2M (Mm00437762_m1).
5. Multi-parameter flow cytometry

Cell suspensions were stained for 30 min at 4°C with the following directly conjugated antibodies: CD45APCeFluor780 (eBioscience, 30-F11); CD3ePE (Miltenyi, 145-2C11); CD49bFITC (Miltenyi, DX5); CD69APC (Miltenyi, H1.2F3); CD314PeVio770 (NKG2D, Miltenyi, CX5); CD11bPE (BD, M1/70); CD11cPeCy7 (eBioscience, N418); F4/80PeCy5.5 (eBioscience, BM8); IL15Ra APC (R&D System, #888220); Ly6G PERCPVio700 (Miltenyi, 1A8); Ly6C APC (eBioscience, HK1.4); CD4PEVio770 (Miltenyi, REA604); CD8PeCy5 (eBioscience, 53-6.7). Purified rat anti-mouse CD16/CD32 monoclonal (eBiosciences, 93) was used to prevent nonspecific binding to mouse Fc receptors. Cells were examined using a FACSCanto flow cytometer (BD) and data were analyzed using FlowJo software (TreeStar). All analyses were performed gating on CD45+ cells that fell within the lymphocyte population (FSClowSSClow) after doublet exclusion to detect NK cell infiltrate. To detect expression of NKG2D ligands, B16 melanoma cells cultured for 24 hr in complete medium supplemented or not with different concentrations of dacarbazine (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 g/l) were washed, stained for 30 min at 4°C with RAE1γPE (eBioscience, CX1) or MULT1PE (eBioscience, 5D10) or Isotype control (eBioscience, 14-4031 and 14-4888) and analyzed by FACScanto as above.

6. In vitro NK degranulation and cytotoxic assays

To evaluate NK cell degranulation, CD107a mobilization was assayed. Briefly, lungs from mice injected with tumor cells and treated for 2 weeks with aerosolized CpG-ODN/Poly(I:C) or with dacarbazine alone or combined with aerosol CpG-ODN/Poly(I:C) (4 mice/group) or left untreated were digested in DMEM medium containing collagenase/hyaluronidase and lung suspensions were re-stimulated in vitro by co-culture with B16 melanoma cells. After 24 hr, non-adherent cells were collected and incubated in FACS tubes with B16 tumor cells (60:1) and CD107a-APC LAMP1 antibody (Miltenyi, 1D4B) for 1 hr at 37°C. Monensin (eBiosciences) was added to a final concentration of 6 µg/ml and cells were incubated for an additional 3 h at 37°C. Fifteen minutes before the end of the incubation, cells were stained with CD3FITC (Miltenyi, 17A2), CD49PE (Miltenyi, DX5) and CD45APCeFluor780 (eBiosciences, 30-F11) antibodies. After washing, cells were resuspended in FACS Buffer, 7AAD (BD) was added to each tube and cells were analyzed by flow cytometry as described above. For in vitro cytotoxicity assay, lung suspensions were plated in 24 wells plate for 2 h at 37°C; non-adherent cells were then recovered and co-cultured in 96 wells U-bottom plate for 12 hr with 2×10⁴ B16 tumor cells (5:1) labelled with CFSE (Life Technologies) according to manufactured protocol. After incubation, cells were harvested, 7AAD was added to each sample and cytotoxicity evaluated by flow cytometry assessing the percentage of CFSE+ 7AAD+ cells. NK cell-mediated cytotoxicity and the ability of adherent cells from lung immune infiltrates of mice to modulate activity of splenic naïve NK cells was evaluated by measuring cytotoxic activity.
on \textsuperscript{51}Cr-YAC-1 target cells. In the first experiment, spleen-derived NK cells were treated in vitro with or without poly(I:C) (100 µg/ml) and CpG-ODN (1 µM) for 24 hours. In a second experimental setting, BAL-derived macrophages were treated \textit{in vitro} with or without CpG-ODN/poly(I:C), as before. Twenty-four hours later, treated macrophages were washed extensively or not to eliminate TLR agonists from the cell culture. NK cells were then added to macrophages and cocultured for another 24 hours. At the end of each experiment, NK cells were harvested and used for the cytotoxicity assay. Specifically, YAC-1 target cells were labeled with 100 µCi \textsuperscript{51}Cr (PerkinElmer) for 1 hr at 37°C, washed 3 times with PBS-5% FCS, and resuspended in RPMI1640, containing 10% FBS. NK cells were then coincubated with YAC-1 target cells at an effector:target ratio of 50:1 in triplicate 96-well U-bottomed plates for 4 hours at 37°C.

Lungs from tumor cell-injected mice untreated or treated were digested in DMEM medium containing collagenase/hyaluronidase and suspensions were plated for 2 hr at 37°C to separate adherent cells containing mostly macrophages from non-adherent cells containing effector cells. Adherent cells were washed and cultured at a 1:1 cell ratio with naive NK cells purified from spleen of C57BL/6 mice by NK Cell Isolation Kit II mouse (Miltenyi). After 24 hr, non-adherent cells were collected and incubated for 4 hr at 37°C at an effector:target ratio 50:1 in triplicate 96-well U-bottomed plates with YAC-1 target cells labeled with 100 µCi \textsuperscript{51}Cr (PerkinElmer) for 1 hr at 37°C and washed 3 times with PBS-5% FCS.

To assess cytotoxic activity of effector cells of untreated or treated mice, lungs were enzymatically digested as above and lung suspensions were plated for 2 hr at 37°C to collect non-adherent cells containing effector cells. B16 melanoma and YAC-1 target cells were labeled with \textsuperscript{51}Cr as above and incubated for 4 hr at 37°C with non-adherent cells at an effector:target ratio of 50:1. Radioactivity of the supernatant (80 µl) was measured with a Trilux Beta Scintillation Counter (PerkinElmer). Percent specific lysis was calculated as: 100 × (experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm).

7. ELISA Assay
Concentrations of cytokines in the culture supernatants were measured using ELISA kits for IL-6 (eBioscience), IL-10 (eBioscience), and IL-12 (R&D System).

8. Statistical analysis
PCR data were analyzed using the $2^{\Delta\Delta Ct}$ method. In all experiments, differences between groups were compared using two-tailed unpaired student’s t-test and were considered to be significant at $p < 0.05$. 
RESULTS

1. Association of TLR3 agonist with aerosolized CpG-ODN reduce the number of Arg+ IL-10+ tumor associated macrophages and increases recruitment of NK cells

In order to evaluate whether TLR3 agonist, Poly(I:C), was able to reach the alveoli and recruit immune cells, C57BL/6 mice were treated with Poly(I:C) administered by aerosol (15 mg dissolved in 5 ml of PBS to treat up to 10 mice in the same aerosol box). Cytofluorimetric analysis of lung infiltrate obtained after enzymatic digestion from mice treated with Poly(I:C) aerosol showed a significant increase of macrophages and dendritic cells, as compared to saline-treated mice (figure 9).

Studies previously performed in our laboratory to evaluate aerosol delivery of CpG-ODN, agonist of TLR9, as a strategy for local administration of immunostimulators, have showed that aerosolized CpG-ODN reached the bronchoalveolar space, locally activated an immune response, and was more efficacious than systemic administration against lung metastases of N202.1A mammary carcinoma cells. In contrast, aerosol delivery of CpG-ODN was minimally effective against metastases of B16 melanoma cells (Sfondrini L, 2013, Int J Cancer), which selectively recruit CD68+ macrophages with an M2 phenotype and induce an immunosuppressive environment in the lung (Gil-Bernabe A, Blood, 2012).

Therefore, we evaluated whether combination of Poly(I:C), reported able to convert tumor-associated macrophages from tumor supporters (M2) to those with tumoricidal properties (M1) (Shime H, 2012, PNAS; Shime H, 2014, J Innate Immun), was able to improve the CpG-ODN-induced antitumor response in B16 lung metastases model.

Figure 9.
Percentage and representative dot plots of macrophages (CD11c-F4/80+ cells among CD45+ cells) (A), and dendritic cells (CD11c+F4/80- cells gated on CD11b+ cells) (B), obtained by enzymatic digestion of lungs from mice (4 mice/group) treated 3 times at 24-h intervals with aerosolized TLR3 agonist poly(I:C) (15 mg) or saline. *p<0.05.
To assess if aerosolized Poly(I:C)/CpG-ODN combination could induce modification of lung immune infiltrates and affect tumor-induced recruitment of TAMs, C57BL/6 mice were i.v. injected with 5×10⁵ B16 melanoma cells and treated twice weekly starting 4 days after tumor cell injection with aerosolized Poly(I:C) combined to CpG-ODN or with aerosolized CpG-ODN alone. Immunohistochemical analysis on lung sections obtained 3 weeks after tumor injection from mice treated with two aerosolized agonists highlight a significant reduction of arginase-positive macrophages number, as compared to untreated mice (16.4 ± 2.1 versus 24.7 ± 2.2; p=0.015), while not significant reduction of arginase-positive macrophages was detectable in the lungs of mice treated with CpG-ODN alone (20.6 ±2.6) (figure 10a).

Double immunofluorescence analysis revealed a reduction of CD68+ IL10+ macrophage mostly in mice treated with the combination of the two TLR agonists confirming the reduction of M2 macrophages (figure 10b).

Figure 10.
Immunohistochemical staining for arginase I positive and CD163 positive (A) macrophages in formalin-fixed, paraffin-embedded lung tissue collected after i.v. injection of 5×10⁵ B16 melanoma cells from mice treated with aerosolized CpG-ODN alone or combined with Poly(I:C) or left untreated (3-4 mice/group). Original magnification X400; Inset in upper right panel is a higher-magnification (X630) showing the cell morphology of macrophages infiltrating the lungs of untreated mice. Histograms in the bottom show the mean number of arginase I-expressing or CD163-expressing macrophages in lung tissue evaluated on 10 fields/group. *p<0.05; **p<0.001. Representative immunofluorescence images of lung samples showing M2-polarized macrophages as CD68 (red)-positive cells also expressing IL-10 (green) (B). Original magnification X400.
We therefore assessed whether this combination could result in an increased recruitment of effector cells in the lung tumor microenvironment, particularly focusing on NK cells, since their pivotal role in protection against B16 tumor (Sfondrini L, 2004, Cancer Immunol Immunother; Glasner A, 2012, J Immunol; Zheng S, 2012, Oncol Lett). Aerosolized CpG-ODN alone or Poly(I:C) alone failed to increase the percentage of NK cells in lung (Sfondrini L, 2013, Int J Cancer), while the combination of Poly(I:C)/CpG-ODN induced the expansion of NK cells, as showed by flow cytometric analysis of tumor infiltrating immune cells in lungs of mice i.v. injected with B16 melanoma cells and treated with aerosolized combined CpG-ODN/Poly(I:C) or with each agonist alone (figure 11.) (Mean % DX5+CD3−/CD45+ ± S.D.: 22.8 ± 1.5 % in untreated; 23.9 ± 2.5 % in CpG-ODN-treated; 25.5 ± 1.6 in Poly(I:C)-treated; 32.7 ± 2.1% in CpG-ODN/Poly(I:C)-treated; 4 mice/group; p=0.036 CpG-ODN/Poly(I:C) versus CpG-ODN; p=0.0094 CpG-ODN/Poly(I:C) versus untreated).

Analysis of the percentage of CD3 T lymphocyte did not reveal any expansion of these cells in lung of mice treated with aerosolized combined CpG-ODN/Poly(I:C) (Mean % CD3+/CD45+ ± S.D.: 19.96 ± 1.30 % in untreated; 16.76 ± 2.27% in CpG-ODN/Poly(I:C)-treated; 4 mice/group) These results suggest that the association of two TLR agonist administered by aerosol into the bronchoalveolar space is able to reduce the percentage of macrophages with M2 phenotype in B16 tumor bearing lungs allowing an increase of tumor infiltrating NK cells. Since the combined treatment with the two TLR ligands might lead inflammation in lung parenchima, histopathological examination of hematoxylin and eosin-stained sections of lung tissues was performed to evaluate the possible toxic effect of repeated combined treatment with Poly(I:C)/CpG-ODN aerosol. Analysis on healthy mice treated twice weekly with Poly(I:C)/CpG-ODN for 3 weeks showed focal areas of mononuclear and granulocytic infiltrate of the interstitium and absence of damage of bronchial-bronchiolar structures and of the alveolar walls in all lungs of mice treated with TLR3 and TLR9 agonists. Moreover, no overt signs of toxicity, such as weight loss, hunching, ruffled fur or difficulty breathing were observed (figure 12).
2. Improved antitumor efficacy of CpG-ODN aerosol against B16 experimental lung metastases through the association of aerosolized Poly(I:C)

Thus, to evaluate a possible cooperative effect on antitumor activity of the two agonists simultaneously administered, compared to antitumor effect of treatment with each agonist alone, 3 groups of mice were i.v. injected with B16 melanoma cells and treated twice weekly for 3 weeks starting 4 days after tumor injection. A fourth group of mice injected with tumor cells was left untreated and used as control. At 3 wk after tumor injection, the number of lung colonies was significantly lower in mice treated with both aerosolized CpG-ODN and Poly(I:C), as compared to mice treated with each aerosolized ligand alone (**p<0.001 CpG-ODN/Poly(I:C) vs CpG-ODN; *p<0.05 CpG-ODN/Poly(I:C) vs Poly(I:C)) (figure 13).
Therefore, our data show that the association of Poly(I:C) to CpG-ODN locally administered increase the antitumor activity of aerosolized CpG-ODN against B16 experimental lung metastases, without apparent signs of toxicity.

3. Combination of dacarbazine increase the anti-tumor activity of CpG-ODN/Poly(I:C) aerosolization in mice bearing B16 lung metastases

Dacarbazine (DTIC) is an alkylating agent currently used in patients with metastatic melanoma. This chemotherapeutic agent is able to enhance the expression of NKG2D ligands on tumor cells, favouring NK cells activity (Hervieu A, 2013, J Invest Dermatol). Since antitumor activity of TLR agonists against B16 tumor is mostly mediated by NK cells, so it is possible to suppose that DTIC could increase antitumor activity of CpG-ODN/Poly(I:C) aerosol treatment.

The DTIC-induced up-modulation of NKG2D ligands on B16 tumor cells was confirmed by cytofluorimetric analysis that revealed upregulation of Rae1 and Mult1 (figure 14a) after in vitro treatment of B16 melanoma cells with dacarbazine. Moreover, Real Time PCR analysis of NKG2D ligands in mRNA derivated from lungs of mice bearing experimental metastases of B16 melanoma cells showed that in vivo treatment for 3 weeks with DTIC (80 mg/kg administered i.p. 5 days/wk) induced an increase of Rae1 and Mult1 (figure 14b).

**Figure 14.**
A) MULT1 and RAE1 expression on the cell surface of B16 melanoma cells analyzed by flow cytometry after 24-h culture in complete medium alone (PBS) or supplemented with dacarbazine (DTIC) at different concentrations. The mean fluorescence intensity (MFI) was normalized to the isotype control. One representative experiment of 2 conducted is shown. B) Increase in Mult1 and Rae1 mRNA expression in lungs of mice injected i.v. with B16 melanoma cells and untreated or treated for 3 weeks with DTIC (80 mg/kg administered i.p. 5 days/week) (3 mice/group). Data represent mean relative expression (normalized to GAPDH) ± S.D. from 3 independent real-time PCR analyses. ***p <0.001.

Thus, we have evaluated the efficacy of the combination of DTIC to aerosol therapy with the two TLRs ligands in countering the growth of B16 lung metastases. To this aim, we performed two different experiments, starting treatment 4 or 7 days after tumor injection, to determine the activity of this combination administered at an early or more advantage stage of tumor growth,
respectively. In both experiments, mice were injected i.v. with B16 melanoma cells and divided into 4 groups. Three groups were treated with dacarbazin alone (80 mg/kg administered i.p. 5 days/wk), or with Poly(I:C) and CpG-ODN aerosolization alone (15 mg and 1.5 mg respectively twice weekly), or with dacarbazin combined to Poly(I:C)/CpG-ODN aerosolization. A fourth group of mice injected with tumor cells was left untreated and used as control.

Experiment in which the results of treatment was started 4 days after tumor injection revealed a significant reduction in the number of lung metastases 3 wks after tumor injection in mice receiving dacarbazin or Poly(I:C)/CpG-ODN aerosolization alone, as compared to untreated mice. However, the combination of Poly(I:C)/CpG-ODN aerosol with dacarbazin resulted more efficacious, as compared to Poly(I:C)/CpG-ODN aerosol or dacarbazin alone, and this combination resulted in 3 out of 8 mice completely cured at the end of the experiment (figure 15a).

Also in the experiment conducted treating mice one week after tumor injection, the aerosolized CpG-ODN/Poly(I:C) significant increased antitumor activity of dacarbazin (Mean number of lung metastases ± S.D.: Untreated mice 23.9 ± 7.3; Dacarbazin-treated mice 7.9 ± 3.8; Dacarbazin plus CpG-ODN/Poly(I:C) aerosol-treated 4.0 ± 3.1; p = 0.018 by t Student’s test Dacarbazin plus CpG-ODN/Poly(I:C) versus Dacarbazin alone) (figure 15b).

Figure 15.

Number of macroscopic B16 melanoma lung metastases in mice untreated (7 mice) or treated with aerosol CpG-ODN/Poly(I:C) (8 mice), DTIC (8 mice), or aerosol CpG-ODN/Poly(I:C) plus DTIC (8 mice) starting 4 days after tumor injection (A), and in mice untreated (7 mice) or treated with aerosol CpG-ODN/Poly(I:C) (8 mice), DTIC (7 mice), or aerosol CpG-ODN/Poly(I:C) plus DTIC (16 mice) starting 1 week after tumor injection (B). *p <0.05; ** p<0.01 ***p <0.001.
4. Treatment with CpG-ODN/Poly(I:C) aerosol in combination with dacarbazine increases NK cells recruitment and activity against B16 lungs metastases bearing mice

We next evaluated whether the improved anti-tumor activity induced by combination of DTIC with aerosolized CpG-ODN/Poly(I:C) correspond to an increased immune activation. Mice were i.v. injected with B16 melanoma cells and treated with Poly(I:C)/CpG-ODN aerosolization alone, or with dacarbazine alone, or with dacarbazine combined to Poly(I:C)/CpG-ODN aerosolization, as above. After two weeks of treatment, no significant difference was observed in the percentage of immune infiltrate detected as CD45+ cells in lung suspensions obtained from mice of the different groups. CpG-ODN/Poly(I:C) aerosol treatment induced a significant increase of the frequency of NK cells (CD49+CD3-), as compared to untreated mice; a stronger increase in the percentage of NK cells was observed in mice treated with dacarbazine combined to aerosolized CpG-ODN/Poly(I:C), as compared to treatments with CpG-ODN/Poly(I:C) or dacarbazine alone (figure 16a). Combination of DTIC with CpG-ODN/Poly(I:C) aerosol also induced the highest expression of the activation marker CD69 on NK cells surface (figure 16b). Even if not significant, NK cells obtained from lungs of mice treated with this combination also showed a trend increase in the level of NKG2D expression, the activating receptor essential in NK-mediated elimination of tumor cells (figure 16c) (Zhang C, 2008, Hum Immunol).

We then analyze whether the increased percentage and maturation of NK cells induced by combined treatment of DTIC and CpG-ODN/Poly(I:C) aerosol correspond to an increased NK cells cytotoxic activity against B16 tumor cells by a degranulation assay. Four groups of mice were injected i.v. with B16 melanoma cells and treated as above; after two weeks of treatments, suspension of cells obtained by enzymatic digestion of lung tissue were 24-hour co-cultured with B16 melanoma cells. An increase in the percentage of degranulating NK cells was induced by CpG-ODN/Poly(I:C) aerosol or by dacarbazine alone, but, the highest percentage of degranulating NK cells was observed in suspensions obtained from lungs of mice treated with the combination of CpG-ODN/Poly(I:C) aerosol and dacarbazine (Increase of the percentage of degranulating NK cells versus untreated: 2.4 fold in CpG-ODN/Poly(I:C) + DTIC, 1.8 fold in CpG-ODN/Poly(I:C) and 1.5 fold in DTIC) (figure 16d).

Moreover, accordingly to data obtained in degranulation assay, evaluation of cytotoxic activity of lung immune cells from mice untreated or treated as above, against CFSE-labelled B16 target cells revealed a significant increase in the percentage of dead B16 cells after co-culture with lung suspensions from mice treated with combination of DTIC with CpG-ODN/Poly(I:C) aerosol(p=0.028) (figure 16e).
Altogether these results demonstrate that dacarbazine, through its ability to up-regulate NKG2DL on cancer cells, increases the anti-tumor activity of the two aerosolized TLR agonists, enhancing the cytotoxic activity of NK cells.

Figure 16.
Percentage and representative dot plots of NK cells (DX5+ cells gated on FSClowSSCslowCD45+CD3- cells) (A), and of CD69+ (B) and NKG2D+ (C) NK cells, gated on DX5+ cells, obtained by enzymatic digestion of B16 metastases-bearing lungs of mice (4 mice/group) treated with aerosol CpG-ODN/Poly(I:C), DTIC, aerosol CpG-ODN/Poly(I:C)
plus DTIC or left untreated. Percentage and representative dot plots of degranulating NK cells (CD107a+ cells gated on CD45+CD3-DX5+ cells), after co-culture with B16 melanoma cells (D), and of CFSE-labeled B16 dead cells (7AAD+ cells gated on CFSE+ cells) after co-culture with cells obtained from lung enzymatic digestion (E). *p<0.05, **p <0.001.

5. CpG-ODN/Poly(I:C)-activated lung macrophages stimulate NK cells cytotoxicity

Our data demonstrated that aerosolized CpG-ODN/Poly(I:C) induced a strong anti-tumor effect related to stimulation of NK cells activity. Experiments were then performed to investigate the mechanism by which the two combined agonists could induce the activation of this effector cells populations. Although NK cells have been recently reported to express some TLRs, able to sense viral or bacterial PAMPs, controversies remain regarding a direct responsiveness of NK cells to these agonists (Adib-Conquy M, 2014, Immunol Cell Biol). Since different studies have suggested an involvement of macrophages in the activation of NK cells induced by TLR agonists. (Zhou Z, 2012, PloS One; Nedvetzki S, 2007, Blood) Recently, the complex network of interaction between NK cells and polarized M1 that promotes NK cells activation has been recently demonstrated by in vitro experiments in human setting (Mattiola I, 2015, J Immunol). We therefore evaluate whether NK cells activation by combination of CpG-ODN/Poly(I:C) was induce by a direct stimulation or require macrophage activity.

To assess whether association of two TLR agonists was able to directly induce IFN-γ secretion and NK cells cytotoxicity, purified splenic NK cells were in vitro stimulated with CpG-ODN/Poly(I:C). Twenty-four hours after, a strong secretion of IFN-γ was observed, while no cytotoxic activity against YAC-1 cells was observed (figure 17).

![Figure 17.

IFN-γ levels evaluated by ELISA in supernatant of NK cells pre-incubated with combined CpG-ODN/Poly(I:C) for 24 hrs. Percent specific lysis of YAC-1 target cells by purified splenic naive NK cells pre-incubated with combined CpG-ODN/Poly(I:C) for 24 hrs. Box and whiskers: min to max.]
To evaluate whether activation of NK cells cytotoxicity by aerosolized CpG-ODN/Poly(I:C) in the lung might be promoted by activation of resident macrophages, AMs obtained from bronchoalveolar lavages of healthy mice were pre-treated or not with two agonists. After 24 hr, pre-treated lung macrophages were extensively washed to completely remove TLR agonists or not washed and co-cultured with purified splenic NK cells. Co-culture of NK cells with TLR agonists-pretreated lung macrophages significantly increased the percentage of lysis of YAC-1 cells, as compared to NK cells co-cultured with untreated lung macrophages (figure 18), even when macrophages were washed to remove TLR agonists, suggesting that activation of NK cells was mediated by signals induced by TLR agonists on macrophages.

Consistent with a previous study published by Tross et al (Tross D, 2009, Mol Immunol) who investigated the genes expressed by RAW264.7 macrophage cell line in response to stimulation with combined CpG-DNA plus Poly(I:C), alveolar macrophages treated for 24 hrs with TLR agonists significantly increased expression of IL-6, IL-12 and IL-33 (figure 19). These in vitro results suggest that the stimulatory effect of TLR agonists on splenic NK cell cytotoxicity depend on activation of lung-derived macrophages induced by TLR agonists.

**Figure 18.**
Percent specific lysis of YAC-1 target cells by purified splenic naïve NK cells cultured for 24 hr with alveolar macrophage pre-incubated with combined CpG-ODN/Poly(I:C) for 24 hrs. Box and whiskers: min to max. *p ≤0.05; **p ≤0.01

**Figure 19.**
Increased IL6, IL12 and IL33 mRNA expression in alveolar macrophage in vitro treated for 24 hrs with CpG-ODN/Poly(I:C) Data are presented as 2^-ΔΔCt and represent mean relative expression ± SEM. from real-time PCR analyses. *p ≤0.05, ***p ≤0.001.
6. Crosstalk between CpG-ODN/Poly(I:C)-activated macrophages and NK cells in B16 melanoma tumor-bearing lung

To evaluate whether this is a physiological mechanism working to activate NK cells in lungs of tumor bearing mice treated with aerosolized TLR agonists, two groups of C57BL/6 mice were i.v. injected with B16 melanoma cells and treated at 72 hr-interval starting from day 7 with CpG-ODN/Poly(I:C), or left untreated. Three weeks after tumor injection, tumor infiltrating cells were obtained by enzymatic digestion of lungs, lung suspensions were then plated 2 hr at 37 °C to separate adherent cells, containing macrophages, from non-adherent cells, containing effector cells and then were analyzed by flow cytometry. Accordingly to our previous data, flow cytometry analysis revealed that TLR agonists induced an increased recruitment of NK cells in lungs, while no difference was detectable in the percentage of CD3 T cells (figure. 20).

![Figure 20](image)

Percentage (±SEM) of NK cell, evaluated as CD49b+ cells gated on CD45+ cells, and Lymphocyte T, evaluate as CD3+ cells gated on CD45+ cell, in lung of B16 bearing mice treated with aerosolized CpG-ODN/Poly(I:C) or untreated. P* ≤0.05

Cytotoxic assay performed using non-adherent cells were cultured with 51Cr-labelled YAC target cells revealed a significant increase in the percentage of lysis using lung suspension from mice treated with two agonists as compared to untreated mice, confirming the in vivo activation of NK cells by aerosol treatment with CpG-ODN/Poly(I:C) (figure. 21).

![Figure 21](image)

Percent specific lysis of YAC-1 target cells by non-adherent cells from lung infiltrate of untreated mice or CpG-ODN/Poly(I:C) treated mice. Box and whiskers: min to max.
To assess whether macrophages derived from lung of mice aerosolized with TLR agonists were able to activate NK cell cytotoxicity, adherent cells were cultured for 24 hr with naïve NK cells, purified from spleen of C57BL/6 mice and then their cytotoxic activity assessed by $^{51}$Cr-release assay on YAC-1 target cells. A significant increase in the percentage of lysis was observed in naïve NK cells cultured with adherent cells from lungs of mice treated with TLR agonists, as compared to cells co-cultured with adherent cells from untreated mice (figure 22).

Since a bidirectional crosstalk between NK cells and macrophages has been recently demonstrated by Mattiola et al. (Mattiola I, 2015, J Immunol), the ability of macrophages-activated NK cells to reciprocally influence macrophages polarization was assessed, incubating for 24 hr non-adherent cells from lungs of CpG-ODN/Poly(I:C) treated or untreated mice with naïve macrophages-containing adherent cells from lavages of C57BL/6 healthy mice. As reported in figure 23, real Time PCR analysis on RNA extracted from macrophages incubated with non-adherent cells from lungs of CpG-ODN/Poly(I:C) treated mice revealed a significant up-regulation of M1 markers (IL-12, IL-6, iNOS2) and reduction of M2 related genes (IL-10, Arg1, IRF4, Alox15, STAT6) as compared to macrophages incubated with non-adherent cells from lungs of untreated mice.
IL12, IL10, ARG1, NOS2, IRF4, IRF5, ALOX15, IL6, STAT1 and STAT6 mRNA expression in naïve alveolar macrophage incubated for 24 hrs with non-adherent cells from lung infiltrate of untreated mice or CpG-ODN/Poly(I:C) treated mice. Data are presented as $2^{-\Delta\Delta Ct}$ and represent mean relative expression ± SEM. from real-time PCR analyses. *p ≤0.05, **p ≤0.01.

Accordingly to the results obtained by Real Time PCR analysis, in the supernatants of naïve macrophages co-cultured with non-adherent cells from B16-bearing lungs of mice treated with CpG-ODN/Poly(I:C) a reduced secretion of IL-10 and an increased release of IL-12 and IL-6, as compared to macrophages co-cultured with cells from untreated mice was observed (figure 24).

Taken together, our results demonstrate that aerosolization with CpG-ODN/Poly(I:C) induce in the lung the activation of macrophages, which in turn promote NK cells cytotoxicity. Moreover, our data suggest that activated NK cells in the lung have the potential to increase the macrophage programming to M1-like phenotype.
We then focused our studies in the development of strategies to locally shape a lung microenvironment that further could favor macrophages-mediated NK cells activation against B16 melanoma lung metastases, by combined inhalation-based immunotherapies.

7. Increases CpG-ODN/Poly(I:C)-induced NK cells activity depleting locally MDSC by nebulized RB6-8C5 antibody

B16 murine melanoma has been reported to strongly lead to the recruitment of MDSC in tumor microenvironment (Sceneay J, 2012, Cancer Res; Schlecker E, 2012, J Immunol; Youn J, 2008, J Immunol). Several studies showed a suppressive activity of MDSC on NK cells mainly through cell-cell contact mechanisms involving membrane-bound TGF-beta and recognition of NKG2D, activatory receptor of NK cells (Elkabets M, 2010, Eur J Immunol; Hoechst B, 2009, Hepatology; Li H, 2009, J Immunol; Liu C, 2007, Blood). Moreover, since a cross-talk between MDSCs and macrophages through soluble mediators and cell contact has been demonstrated to exacerbate a M2 phenotype shift of macrophages, resulting in increased IL10 production, reduction of IL12 and IL6 production and increased immunosuppressive activity (Ostrand-Rosenberg S, 2012, Semin Cancer Biol) is possible that the presence of MDSCs on lung microenvironment hinder the macrophages activation by TLR agonist.

Thus, in order to increase NK cells activation by aerosolized TLR agonists, creating a more permissive microenvironment, we assess the possibility to deplete MDSCs in the lung by nebulized RB6-8C5 antibody. C57BL/6 mice were i.v. injected with B16 melanoma cells and treated 7 days later with RB6-8C5 antibody or saline at 72-hr intervals. Cytofluorimetric analysis of enzymatically digested lungs revealed that nebulized RB6-8C5 antibody was able to reach the lung and significantly deplete both granulocytic (Ly6G-highLy6C-low cells) and monocytic MDSC (Ly6G-lowLy6C-high cells) (Figure 25).

Figure 25.
Representative dot plots and percentage (± SEM) of MDSC, identified as granulocytic (Ly6G-highLy6C-low) and monocytic (Ly6G-lowLy6C-high) MDSC (gated on CD11b+ cells among CD45+ cells) obtained by enzymatic digestion of lungs of mice injected i.v. with B16 melanoma cells and treated 7 days later with nebulized saline or RB6-8C5 antibody (25 μg) (3 mice/group) at 72-hr intervals for 2 weeks *p≤0.05, ***p≤0.001.
Moreover, since our experiments revealed that granulocytic and monocytic MDSC repopulate completely the lungs at 96 hr after RB6-8C3 nebulization (Ly6G+Ly6Clow cells ± SEM: 57.1 ± 8.3% in treated versus 56.4 ± 1.7% in untreated; Ly6GlowLy6C+ cells ± SEM: 3.6 ± 0.8% in treated versus 4.2 ± 0.3% in untreated; 2 mice/group), thus to maintain a prolonged depletion of these cells, we decided to repeat nebulized RB6-8C3 treatments at 72-96 hr intervals.

The effect of MDSC depletion on recruitment and activation of NK cells induced by CpG-ODN/Poly(I:C) aerosolization was evaluated in mice i.v. injected with B16 cells and treated with aerosolized CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody, as above described. Flow cytometry analysis of enzymatically digested lungs revealed that depletion of MDSC allowed an enhanced expression of CD69 activation marker on NK cells, without an increase in the recruitment of these cells (figure 28A).

Although RB6-8C5 antibody is not only MDSCs specific, but can also target other Ly6G- or Ly6C-expressing cells, no significant difference in the frequency of macrophages, DCs, monocytes and T lymphocytes was detectable in lung of mice treated with aerosolized CpG-ODN/Poly(I:C) and RB6-8C5 antibody compared to mice treated with TLR agonists alone (figure 26).

![Figure 26.](image)

*Figure 26.* Percentage (± SEM) of macrophages (CD11b+CD11c+F4/80+ and CD11b+CD11c-F4/80+), dendritic cells (CD11b+CD11c+F4/80+), monocytes (CD11b+CD11c-F4/80-Ly6C+). CD8+ (CD3+CD8+) and CD4+ (CD3+CD4+) T lymphocytes in lung of mice i.v. injected with B16 melanoma cells and treated, starting 1 week later for 2 weeks at 72-hr intervals, with aerosolized CpG-ODN/Poly(I:C) alone or combined with RB6-8C5 (25 μg). Three weeks after tumor injection lung were processed for flow cytometric analysis (4-5 mice/group).
Experiments were then performed to evaluate the effect of MDSC depletion on the expression of immunosuppressive marker in the lung. A strong reduction in expression of immunosuppressive markers strongly associated with MDSC suppressive activity (Keskinov A, 2015, Immunobiology) was observed in lungs of mice bearing B16 melanoma lung metastases treated with combined RB6-85 and CpG-ODN/Poly(I:C) aerosol, compared to mice treated with CpG-ODN/Poly(I:C) alone; as showed by analysis of Il-10, Arg-1 and Nos transcript levels in immune infiltrates obtained from lungs enzymatically digested (figure. 28B).

Since we observed that macrophages are essential for activation of NK cell cytotoxicity also in the lung and their central role in responding to stimulation with TLR ligands, we assess whether MDSC depletion might increase the ability of CpG-ODN/Poly(I:C)-activated macrophages to promote the cytotoxic activity of NK cells. Mice were i.v. injected with B16 tumor cells and treated with aerosolized CpG-ODN/Poly(I:C) alone, or combined with nebulized RB6-8C5 antibody, or with RB6-8C5 antibody alone, or left untreated. Three weeks after tumor cells injection, lung suspensions obtained from enzymatic digestion were plated 2 hr at 37 °C to separate adherent cells, containing macrophages, from non-adherent cells, containing effector cells. Adherent cells were then cultured with splenic naïve NK cells obtained from healthy mice and 24 hr later cytotoxic activity of NK cells was assessed on YAC target cells. In vivo combination of nebulized RB6-8C5 to TLR agonists significantly increased the ability of lung-derived immune adherent cells to activate NK cell cytotoxicity (figure. 28C), increasing the stimulatory effect of CpG-ODN/Poly(I:C). No increase of NK cell cytotoxic activity was induced by treatment with RB6-8C5 antibody alone, suggesting that depletion of MDSC alone is not enough to promote macrophage-mediated NK cell activation.
Figure 28.

A) Representative dot plots and percentage (± SEM) of NK cells (CD49b+ cells gated on FSC<sub>low</sub>SSC<sub>low</sub>CD45+CD3- cells); of CD69+ NK cells (gated on CD49b+ cells) and NKG2D+ NK cells (gated on CD49b cells), in mice untreated or treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody (5 mice/group). **p≤0.01, ***p≤0.001. B) mRNA levels (mean relative expression ± SEM) of IL10, Arg-1 and NOS2 evaluated by real-time PCR in mice treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5 antibody from 2 independent experiments (4 mice/group in each experiment). Results are presented as 2<sup>ΔΔCt</sup>; *p≤0.05. C) Percent specific lysis of YAC-1 target cells by purified splenic naïve NK cells cultured for 24 hr with adherent cells obtained by plating lung suspensions from mice untreated or treated with CpG-ODN/Poly(I:C) alone or combined with nebulized RB6-8C5
antibody or with RB6-8C5 antibody alone. Box and whiskers: min to max represent data pooled from 2 independent experiments (3-4 mice/group). *p≤0.05; **p≤0.01.

Altogether, these results indicate that airway delivered RB6-8C5 antibody reaches the bronchoalveolar space, efficiently depletes MDSC from the tumor microenvironment and this depletion allowed an increased NK cell activation by aerosolized CpG-ODN/Poly(I:C). Then, with the aim to directly promote effector cells activation, we assessed another inhalation-based immunotherapy using TLR agonists combined with aerosolized IFNa, essential for NK cell priming in response to cancer (Trinchieri G, 1978, J Exp Med; Karimi K, 2015, Innate Immun) and able to induce the M2 to M1 phenotypic shift in the lung microenvironment (Zhuang PY, 2013, PloS One).

8. NK cell activation in lung by aerosolized IFN-α

IFN-I are essential elements for priming and activation of NK cells, since its ability to enhance their cytotoxicity and IFN-γ production. IFN-I also represent the principal inducers of IL-15 production, essential for NK cells accumulation and survival. In addition to its direct effects on NK cells, IFN-α is known to stimulate other cell types critical for NK effector activation, such as macrophages and DC, and to promote a shift of macrophages to tumoricidal effectors (Zhuang PY, 2013, PloS One). We therefore evaluate the possibility to deliver IFN-α to the lung by aerosolization, as strategy to increase its concentration locally and limit the possible toxicity associated with systemic administration (Trinchieri G, 2010, J Exp Med) and to improve immune activation in the lung induced by aerosol CpG-ODN/Poly(I:C). Healthy mice were treated at intervals of 72 hrs with aerosolized IFN-α for 2 wk. Flow cytometry analysis of immune cells obtained from enzymatic digestion of lungs showed a significant increase in the percentage of NK cells and up-modulation of CD69 and NKG2D activation markers on their cell surface, as compared to untreated mice (figure. 29A). Moreover, an increased expression of IL-15Ra was also observed on NK cells surface (figure. 29A) and in RNA extracted from lung immune infiltrates of IFN-α-treated mice (figure. 29B). Analysis of transcript levels in RNA extracted from lung immune infiltrates of these mice also revealed a significant up-regulation of IL-15 and IFN-γ (figure. 29B).
Figure 29.
A) Percentage (± SEM) of NK cells evaluated by flow cytometry as CD49b+ cells gated on FSC<sup>low</sup>SSC<sup>low</sup>CD45<sup>-</sup>CD3<sup>-</sup>cells, of CD69+ NK cells gated on CD49b+ cells; NKG2D+ NK cells gated on CD49b cells and IL-15Ra+ NK cells gated on CD49b+ cells in healthy mice untreated or treated with aerosolized IFNα at 72-hr intervals for 2 weeks (4 mice/group); *p≤0.05. B) Percent (± SEM) IL15Ra, IL15 and IFNγ mRNA levels evaluated by real-time PCR and given as mean relative expression from 2 independent experiments (4 mice/group in each experiment). Results are presented as 2<sup>-ΔΔCt</sup>; *p≤0.05. C) Percent specific lysis of YAC-1 target cells by purified splenic naïve NK cells cultured for 24 hr with adherent cells obtained by plating lung suspensions from mice treated with CpG-ODN/Poly(I:C) alone or combined with aerosolized IFNα. Box and whiskers: min to max represent data obtained from 4 mice/group; *p≤0.05.

Moreover, our experiments indicated that IFN-α aerosol therapy was well-tolerated, since mice did not reveal overt signs of toxicity, such as weight loss, hunching, ruffled fur or difficulty breathing. Furthermore, histopathological examination of hematoxylin and eosin-stained sections of lung tissue revealed no histological changes in the structure of lungs (figure, 30).

Figure 30.
Body weight and representative histopathological evaluation of hematoxylin and eosin-stained lung tissue sections from mice treated with aerosolized IFNα or saline (4 mice/group). No effects on body weight or lung histology were observed in mice exposed to IFNα aerosolization. Magnification X200.
In a model of lung metastases induced by hepatocellular carcinoma cells, it was been reported that IFN-α promote macrophage anti-tumor activity through their shift to M-1 phenotype (Zhuang PY, 2013, PloS One; Hervas-Stubbs S, 2011, Clin Cancer Res). Thus, we assessed whether association of IFN-α to CpG-ODN/Poly(I:C) aerosol therapy in mice bearing B16 melanoma metastases increases the ability of lung-derived macrophages to promote the cytotoxic activity of NK cells. Adherent cells obtained from lung suspensions of mice i.v. injected with B16 cells and treated with aerosolized IFN-α plus CpG-ODN/Poly(I:C) induced a significantly increased cytotoxic activity of naive NK cells against YAC target cells as compared to adherent cells from lungs of mice treated with aerosolized CpG-ODN/Poly(I:C) alone (figure 29C).

Together, these data show that aerosolized IFN-α reach the bronchoalveolar space in the lung and locally activate an innate immune response without apparent signs of toxicity, improving the macrophages-mediated activation of NK cells by aerosolized CpG-ODN/Poly(I:C).

9. Anti-tumor efficacy of CpG-ODN/Poly(I:C) aerosol therapy improved by the association of Nebulized RB6-8C5 antibody and IFN-α aerosolization

The local depletion of MDSC from lung microenvironment by RB6-8C5 nebulization and the local delivery of IFN-α has proved to be able to increase NK cells activation induced by CpG-ODN/Poly(I:C) aerosol treatment. In order to evaluate which of the two combination, was more effective in increasing the anti-tumor effect induced by CpG-ODN/Poly(I:C) aerosolization in the treatment of B16 melanoma lung metastases, different combined treatments were started one week after i.v. injection of B16 tumor cells. At this time, indeed, the presence of multiple microscopic metastatic foci was clearly detectable in the lung (figure 31A).

B16 tumor bearing mice were divided in six groups and treated for 2 weeks at 72 hr interval with aerosolized CpG-ODN/Poly(I:C) alone or combined to RB6-8C5 or IFN-α, or with RB6-8C5 or IFN-α alone, or left untreated. Both combinations of CpG-ODN/Poly(I:C) aerosol with RB6-8C5 or IFN-α induced a significant increase of antitumor activity, as compared to Poly(I:C)/CpG-ODN aerosol alone (figure 31B). No significant anti-tumor effect was observed by treatment with RB6-8C5 or IFN-α alone.

To investigate whether the observed reduction in the number of lung metastases was related to an increased ability of NK cells to promote anti-tumor activity, lungs were enzymatically digested and cytotoxic activity of non-adherent immune cells was evaluated using B16 tumor cells as target. The result of this experiment revealed a significant increase in the percentage of lysis by lung suspensions obtained from mice treated with CpG-ODN/Poly(I:C) combined to RB6-8C5 antibody or IFN-α, as compared to those from mice treated with CpG-ODN/Poly(I:C) alone (figure. 31C).
To assess a possible synergistic effect in combining CpG-ODN/Poly(I:C) aerosol with RB6-8C5 nebulization and IFN-α aerosolization, an experiment was performed in mice i.v. injected with B16 melanoma cells and treated starting one week after tumor injection, as above, with aerosolized CpG-ODN/Poly(I:C) combined to RB6-8C5 or to IFN-α or to RB6-8C5 plus IFN-α. A control group was left untreated. The results of this experiment confirmed the strong reduction in the number of lung metastases obtained combining CpG-ODN/Poly(I:C) with RB6-8C5 antibody or IFN-α, but no synergistic effect was observed in mice treated with CpG-ODN/Poly(I:C) combined to both reagents (Figure 31D).

Altogether, these results indicate that an immunotherapeutic strategy based on direct delivery in the lung of CpG-ODN/Poly(I:C) by aerosol combined with local nebulization of an antibody to deplete MDSC or with aerosolized IFN-α significantly increases the antitumor activity of the two TLR agonists, allowing an increased activation of anti-tumor effector cells.

Figure 31
A) Representative images of multiple metastatic foci in hematoxylin and eosin-stained lung tissue sections from mice at day 7 after i.v. injection of B16 melanoma cells. Magnification X200. B) Mean number of B16 melanoma lung metastases in mice injected i.v. with B16 melanoma cells and untreated (7 mice) or treated starting at day 7 at 72-hr intervals for 2 weeks with nebulized RB6-8C5 alone (6 mice), aerosolized IFN-α alone (6 mice), aerosolized CpG-ODN/Poly(I:C) alone (8 mice), aerosolized CpG-ODN/Poly(I:C) combined with RB6-8C5 (8 mice) or IFNα aerosol (7
mice). *p≤0.05; ***p≤0.001. C) Percent specific lysis of B16 target cells by non-adherent cells obtained by plating suspensions of digested lungs from untreated or treated mice after 24 hr co-culture. Box and whiskers: min to max represent data pooled from 2 independent experiments (3-4 mice/group) *p≤0.05; **p≤0.01. D) Mean number of B16 melanoma lung metastases in mice injected i.v. with B16 melanoma cells untreated (6 mice) or treated starting at day 7 at 72-hr interval for 2 weeks with aerosolized CpG-ODN/Poly(I:C) combined with RB6-8C5 (9 mice) or with IFNα (9 mice) or with RB6-8C5 and IFNα (9 mice).
DISCUSSION

Locoregional therapy with drugs administered by aerosols represents an option for treating asthma and chronic obstructive pulmonary disease but could be a promising pathway to combat also metastatic lung tumors. Only a minority of patients with primary lung cancer are eligible for curative surgical resection. Similarly, surgical treatment of lung metastases is often contraindicated due to the number or site of the lesions and the patient’s respiratory and general status. Chemotherapy is therefore widely used in both primary and secondary lung carcinomas, but its clinical efficacy remains limited. Several studies have demonstrated that after systemic administration the drug concentration in lung tumors is low. Thus, the poor clinical outcome often observed in lung cancer patients has been partly attributed to the inability to achieve therapeutic concentrations at the tumor site (Sharma S, 2001, J Clin Oncol). Because bronchial and bronchoalveolar tumors are accessible via the endobronchial space, lung cancers are candidate tumors for taking advantage of local delivery. Inhalation of aerosolized drugs, allowing increased comfort to patients and self-administration, could be a convenient and simple approach for repeated application to treat primary lung cancer and to treat and/or prevent metastases derived from different types of primary tumors.

Several therapeutic agents have been explored for inhalation delivery in malignancies, including chemotherapeutic agents (Lemarie E, 2011, J Aerosol Med Pulm Drug Deliv; Reed MD, 2013, Br J Cancer), cytokines (Esteban-Gonzalez E, 2007, Anticancer Drugs; Guma SR, 2014, Pediatr Blood Cancer), antisense oligonucleotides (Mainelis G, 2013, J Aerosol Med Pulm Drug Deliv), and monoclonal antibodies (Maillet A, 2011, Pharm Res), demonstrating the feasibility of aerosol delivery, the potential anti-tumor effects and the reduced side-effects compared to systemic treatment, allowing to reach with a higher local concentration of drugs.

Previously studies of aerosol delivery of TLR9 agonist performed in the laboratory where I conducted my thesis showed that aerosolized CpG-ODN was able to reach the bronchoalveolar space, locally activate an immune response without any apparent signs of toxicity and was more efficacious than systemic administration against lung metastases of N202.1A mammary carcinoma cells. In contrast, aerosol delivery of CpG-ODN was not very effective against metastases of B16 melanoma cells, which selectively recruit CD68+ macrophages with an M2 phenotype and induce an immunosuppressive environment that hinder the recruitment of NK cells (Sfondrini L, 2013, Int J Cancer).

The first aim of this thesis was to explore the aerosol administration of Poly(I:C), reported able to convert TAMs to those with tumoricidal properties, for a possible combination with CpG-ODN to simultaneously reduce immunosuppressive microenvironment while activating tumor-infiltrating immune cells, as a novel approach for the treatment of lung metastases. Because lungs are in contact with the external environment, the airways are occupied by a significant population of

We highlighted that the addition of Poly(I:C) to aerosolized CpG-ODN resulted in increased anti-tumor activity in mice bearing B16 lung metastases as compared to mice treated with either aerosolized TLR agonists alone. The enhanced anti-tumor effect of the CpG-ODN/Poly(I:C) aerosol combination correlated with a significant reduction in the number of macrophages producing arginase and/or IL-10 and/or CD163 expressing macrophages, three different markers of the M2 phenotype, which are selectively recruited by B16 melanoma cells in the lung and responsible for the low efficacy of locally delivered CpG-ODN alone (Sfondrini L, 2013, Int J Cancer). Moreover, our results showed that aerosolization of the two TLR agonists into the bronchoalveolar space did not induce evident signs of toxicity on mice and/or signs of injury in the architecture and structure of lung parenchyma. Other immune populations can contribute to the tumor-induced immunosuppressive microenvironment. Myeloid-derived suppressor cells (MDSC) represent one of the most relevant of these populations. Besides blocking conversion of tumor-associated macrophages to the M2 phenotype, Poly(I:C) treatment was shown to promote the maturation of myeloid-derived suppressive cells (MDSC), immature cells often elevated in tumor-bearing hosts, rendering them competent for NK cell activation (Shime H, 2014, J Innate Immun). Our results indicated that the combination of Poly(I:C) with aerosolized CpG-ODN significantly expanded the frequency of CD49b+ cells in lung tumor infiltrates and increased their cytotoxic activity against B16 tumor cells. Thus, activation of NK cells might depend on an effect of aerosolized CpG-ODN on NK cells promoted by both the blocking of suppressive macrophages by Poly(I:C), and also on the induction by Poly(I:C) of NK cell-activating molecules on MDSC present in the tumor microenvironment.

Since a heightened immune response alone can rarely cure patients, in this thesis we also evaluated the combination of aerosolized CpG-ODN/Poly(I:C) with chemotherapy. Accumulating evidence indicates that some of them have immuno-therapeutic activities. Dacarbazine, for example, was recently shown to trigger the expression of NKG2D ligands on mouse tumor cells, thus indirectly promoting NK cell cytotoxicity (Ugurel S, 2013, J Invest Dermatol). Hervieu et al. (Hervieu A, 2013, J Invest Dermatol) demonstrated that coculture of different human NK cells with dacarbazine-treated human melanoma cell lines was shown to sensitize the melanoma cells to NK cell lysis and that the increase in mortality of tumour cells was prevented by addiction an anti-human NKG2D antibody. Accordingly, our results indicated that treatment with dacarbazine up-
modulated RAEI and MULTI expression in B16 melanoma cells both in vitro and in vivo, and that the combination of dacarbazine with aerosol CpG-ODN/Poly(I:C) induced a significant increase in the frequency and degranulation activity of CD49b+ cells, resulting in improved anti-tumor activity. Note that the induction of NKG2D ligand expression has also been observed for other genotoxic agents, such as 5-fluorouracil and cisplatin (Gasser S, 2005, Nature; Khallouf H, 2012, J Immunother), raising the possibility that other chemotherapeutic drugs might increase the anti-tumor activity of aerosol CpG-ODN/Poly(I:C).

Overall the results obtained in the first part of this thesis revealed that repeated aerosol delivery of immunostimulants, such as TLR9 and TLR3 agonists, might represent a convenient and simple approach to locally maintain activation of NK cells, while inhibiting polarization of tumor-infiltrating myeloid derived cells versus an immunosuppressive phenotype, minimizing their possible systemic side-effects. Moreover, this strategy might improve the response to standard treatments with chemotherapeutic agents by maintaining an immune microenvironment able to counteract tumor growth.

In the second part of project we focused to investigate the mechanism by which the two combined agonists could induce the activation of effector cells, investigating the interplay between NK cells and alveolar macrophages. AMs, resident innate immune cells in the lungs and characterized by an M2-like phenotype, are critical in maintaining immune homeostasis, preventing unwanted reactions against antigens that are inhaled during the respiratory process (Hussehl T, 2014, Nat Rev Immunol). These cells exert their immunomodulatory activity through the release of various factors, including PGE2, NO, and IL-10 (Bingisser RM, 2001, Swiss Med Wkly), or a cell-cell contact-dependent mechanism, switching off innate and adaptive immunity.

However, alveolar macrophages are an important component of host defense against invading microorganisms, and it has been proposed that TLR engagement on these cells attenuates their immunosuppressive activity through the downmodulation of IL-10 receptor (Fernandez S, 2004, J Immunol).

Consistent with several in vitro observations (Baratin M, 2005, PNAS; Bellora, 2014, Eur J Immunol; Eissmann P, 2010, J immunol; Tu Z, 2008 J Exp Med) our results support that alveolar macrophages are required to induce NK cell cytotoxicity stimulated by TLR agonists. The increased cytotoxicity of NK cells that is induced by TLR agonist-activated alveolar macrophages might be related to the secretion of soluble mediators by activated macrophages. Accordingly, we found that IL-6, IL-12 and IL-33 mRNA levels were raised in bronchoalveolar-derived adherent cells incubated for 24 hrs with TLR agonists. IL-12 (Bourgeois E, 2009, Eur J Immunol), a cytokine crucial in promoting NK cell cytotoxicity, has been indeed reported to synergize with IL-33 to promote NK cell activation and IFN-γ production. Moreover, it has been also reported that transgenic expression of IL-33 increases NK cell cytotoxic activity in vitro and in vivo against B16 melanoma cancer cells (Gao X, 2015, J Immunol).
If macrophages govern NK cell behavior, the latter might influence macrophage biology. Notably, our ex vivo experiments using NK cells from the lungs of mice that harbored B16 metastases treated with aerosolized TLR agonists, demonstrated that these cells induced a shift toward an M1-like phenotype in naive alveolar macrophages. After being cocultured with activated NK cells, naive alveolar macrophages indeed up-regulated M1 markers, such as NOS2, and increased their secretion of M1 cytokines, as IL-12 and IL-6. Concurrently, a decline in M2-associated molecules, such as IRF4 and Alox15, and IL-10 secretion in macrophages was observed. Accordingly with data obtained by Mattiola et al. (Mattiola I, 2015, J Immunol), these results indicate that the crosstalk between NK cells and macrophages favors the reprogramming of macrophages toward the M1 phenotype, thus sustaining the antitumor response that is induced by TLR agonists. Thus, therapeutic approaches, such as the local administration of TLR9 and TLR3 agonists, that re-educate tumor-associated macrophages toward the M1-like phenotype can also take exploit this positive feed-forward loop with NK cells to potentiate the antitumor M1 phenotype.

Therefore, the data yielded in the second part of this thesis has demonstrated that in the lung, NK cell activation by TLR agonists is mediated by macrophage activity. Given the increasingly appreciated function of NK cells in protective therapeutic strategies against cancer (Rezvani K, 2015, Front Immunol), our findings contribute to establishing the sequence of immune cell activation that triggers NK cells in vivo by immunotherapeutic agents.

The last aim of this thesis was to evaluate strategies to improve the efficacy of CpG-OND/Poly(I:C) aerosolization against lung cancer by combination with other aerosolized immunotherapeutic agents, to favor a lung microenvironment more permissive to an effective immune destruction of tumors by NK effector.

We evaluated the usefulness of RB6-8C5 nebulized antibody, to deplete MDSC cells and thereby create a more permissive microenvironment for NK cell activation by aerosolized TLR agonists (Saiwai H, 2013, J Neurochem). The feasibility of using nebulized therapeutic antibodies was demonstrated in a study showing that airway-delivered cetuximab, an anti-EGFR antibody, accumulated rapidly in cancerous lung tissue at concentrations twice those achieved after i.v. delivery (Guilleminault L, 2014, J Control Release) and in a study on an anti-influenza virus neutralizing MAb that revealed higher concentrations of the antibody in the bronchoalveolar lavage fluid (BAL), with low penetration into the bloodstream upon direct delivery to the respiratory tract (Leyva-Grado V, 2015, Antimicrob Agents Chemother). Moreover, local delivery of an anti-VEGF MAb to K-ras-induced adenocarcinoma-bearing lungs efficiently reduced tumor burden at ~100-fold lower serum concentration of MAb than that after systemic delivery (Herve V, 2014, MAbs)

Airway-delivered RB6-8C5 antibody, directed to both Ly6G and Ly6C, at 72-96 hr interval effectively depleted MDSC recruited in the lung tumor microenvironment at a dose 4- to 8-fold
lower than that used in systemic administration (Saiwai H, 2013, J Neurochem; Srivastava M K, 2012, PloS One), improving antitumor effects of aerosolized TLR agonists. Although RB6-8C5 antibody is not only MSDCs specific, but can also target other Ly6G- or Ly6C-expressing cells, at a late time point of the experiment no differences in the percentage of macrophages, DCs, monocytes and CD8+ T cells were observed in the lungs. Our data indicate that while MDSC depletion by the nebulized antibody did not enhance NK cell recruitment induced by CpG-ODN/Poly(I:C), depletion did lead to increased NK cell activation, possibly by minimizing direct inhibition of these effector cells and/or by enhancing the ability of aerosolized CpG-ODN/Poly(I:C)-activated macrophages to promote NK cell cytotoxicity. Accordingly, Mattiola et al. (Mattiola I, 2015, J immunol) have demonstrated in vitro that LPS/IFNγ-polarized M1 macrophages, but not M0 or IL4-polarized M2 macrophages, can prime resting autologous NK cells. Thus, the ex vivo results showed in this thesis indicating that nebulized RB6-8C5 combined with TLR agonists significantly increases the ability of lung-derived immune adherent cells to activate cytotoxicity of naïve NK cells suggest that MDSC depletion also favors reprogramming of macrophages to the M1 phenotype. Consistent with our results, systemic MDSC depletion by RB6-8C5 antibody in a model of murine Lewis lung carcinoma induced increased anti-tumor NK and T cell activity that was related to enhanced functional activity of antigen-presenting cells, such as macrophages and dendritic cells, in the tumor (Srivastava MK, 2012, PloS One). Moreover, we evaluated the possibility to directly increase the potency of NK cells, acting directly on these cells. To this aim we aerosolized CpG-ODN/Poly(I:C) together with IFNα, a proinflammatory cytokine has been used successfully to treat infections and several types of cancer, based on its ability to promote NK cell activation. For example, IFNα is used as an adjuvant therapy after surgery to prevent recurrence of melanoma, although side-effects upon systemic treatment are substantial (Jonasch E, 2001, Oncologist). Our results indicate that aerosol-delivered IFNα reaches lung NK cells, promotes their recruitment and activation, and upregulates IL15/IL-15Ra expression necessary for NK cell priming. Notably, we detected no evident signs of toxicity on the structure of lung parenchyma or on the weight and behavior of mice, consistent with data indicating that aerosol-delivered IFNα in patients with pulmonary tuberculosis reaches the lower respiratory tract without systemic side-effects (Giosue S, 2000, Eur Cytokine Netw).

No significant anti-tumor effect was observed in mice treated with aerosolized IFNα alone, consistent with the limited anti-tumor effect reported in some pioneer studies in patients with advanced bronchioalveolar carcinoma treated with inhaled IFNα alone (Van Zandwijk N, 1990, Eur J Cancer; Kinnula V, 1990, Eur J Cancer). However, in combination with TLR9 and TLR3 agonists, which strongly activate an innate immune response while reducing M2 polarization, IFNα improved NK-mediated protective immunity, raising the possibility that TLR agonists create a microenvironment favorable for immune stimulation by IFNα. Additive immunostimulatory
effects by IFNα and CpG-ODN were observed in an intraperitoneal model of melanoma systemically treated with IFN-α and CpG-ODN (Brown L, 2006, Surgery), and a synergistic effect of CpG-ODN and IFNα on dendritic cell maturation was found in mice bearing MC38 colorectal cancer treated with dendritic cells pre-incubated with IFNα-secreting cells and CpG-ODN (Hiraide A, 2008, Cancer Sci). In addition to its direct effects on NK cells, IFNα is known to stimulate other cell types critical for NK effector activation, such as macrophages and DC (Hervas-Stubbs S, 2011, Clin Cancer Res), and to promote a shift of macrophages to tumoricidal effectors, as shown in a model of lung metastases induced by hepatocellular carcinoma cells, wherein systemic IFNα treatment directly transformed the lung microenvironment by modulating macrophage polarization (Zhuang PY, 2013, PloS One). Accordingly, we found that adherent cells from lung immune infiltrates of B16 metastases-bearing mice treated with CpG-ODN/Poly(I:C)/IFNα aerosol significantly increased cytotoxic activity of naïve NK cells, as compared to cells from mice treated with CpG-ODN/Poly(I:C) alone.

The two different inhaled combinations showed a similar level of anti-tumor effect, i.e., up to 79% reduction in mean number of lung metastases. Unexpectedly, no additive effect was observed in mice treated with CpG-ODN/Poly(I:C) combined with both IFNα and MDSC-depleting antibody. Further experiments are needed to assess a possible interference between IFNα and the MDSC-depleting antibody and to evaluate other doses or time frames of administration.

Together, the results of this thesis indicate that an effective anti-tumor response can be obtained against established lung multiple foci of a highly aggressive tumor using airway delivery as a convenient and non-invasive way to administer immunotherapy.

This proof-of-principle project of thesis points the promise of rational combinations of immunostimulants, cytokines and antibodies delivered locally by inhalation to reprogram the immune tumor microenvironment as a novel strategy to treat lung cancer patients.
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