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Role of PD-1/PD-L1 axis in regulating functions of innate immune cells in the treatment of cancer

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ABSTRACT

Background. Programmed cell death-1 (PD-1), a member of the CD28 receptor family, is reported to be expressed by activated T lymphocytes and upon the interaction with its ligands (PD-L1 and -2) determines T cells exhaustion. PD-1/PD-Ls axis is crucial in controlling T cell activity in order to maintain peripheral tolerance and to prevent autoimmunity. For this reason, tumor cells, up-regulating PD-L1, exploit this pathway to escape immune elimination.

Although deeply investigated in T lymphocytes, PD-1 can be also expressed by innate immune cells (NK cells, monocytes, macrophages, dendritic cells) where its function seems to be similar to that described for T cells. In recent years, the discovery of immune checkpoint inhibitors (ICIs), antibodies directed against PD-1 or PD-L1, revolutionized the paradigm of tumors treatment. Clinical studies demonstrated the efficacy of PD-1 blockade in different types of cancer, even in those considered non-responsive to adaptive immunotherapy, suggesting that also innate immune system can be involved in the final anti-tumor activity of PD-1 blockade. Despite the enthusiasm of the beginning, only a fraction of patients really benefits from this treatment, while all the others remain unresponsive. Therefore, efforts are required to improve the efficacy of ICIs treatment.

Rationale. Since innate immune cells may influence the efficacy of PD-1 blockade therapy, the therapeutic stimulation of the innate immune system might improve PD-1 antibody efficacy. Among innate cells immunostimulants, Toll-like receptors (TLRs) agonists are the best known for their ability to strongly activate innate immune cells and for their anti-tumor activity.

Aim. The present thesis is aimed to evaluate the role of innate immune system in the context of PD-1 blockade therapy and to explore novel combination therapies.

Results. Immunodeficient mice xenografted with human ovarian cancer cells IGROV-1 were treated with CpG-ODN, TLR9 agonist, and anti-PD-1 antibody alone or in combination. Surprisingly, we observed a drastic reduction in CpG-ODN antitumor activity when combined with anti-PD-1 blocking antibody. Subsequent experiments revealed that anti-PD-1 antibody

alone produces an acceleration of tumor growth paralleled by an increase of $ArgI^+$ myeloid cells in the tumor microenvironment. These results suggested that myeloid cells can play a role in determining the deleterious effect of anti-PD-1 antibody on CpG-ODN activity. This idea was also supported by data showing that *in vivo* depletion of this innate immune cell population abrogated the negative effect of anti-PD-1 antibody.

Similar experiments were performed in another mouse xenograft model. Animals were injected with NCI-H460 human non-small cell lung cancer (NSCLC) cell line and treated as above. Again, we found that PD-1 blockade determined an acceleration of tumor growth together with an increase in intratumoral macrophages and $ArgI^+$ cells. Since blocking PD-1 in macrophages is reported to promote their anti-tumoricidal effector functions, it is possible to speculate that the detrimental effect of anti-PD-1 antibody may be not due to such blockade but instead to an interaction of the Fc portion of the antibody with macrophages/myeloid cells. Indeed, when H460 xenograft model was treated with anti-PD-1 $F(ab)_2$, lacking the Fc domain, no increase in tumors growth was detected.

Several publications reported that there is a fraction of patients experiencing hyperprogression, a particular aggressive and unpredictable spread of the disease following anti-PD-1 administration. This condition may resemble our preclinical models. By immunohistochemistry, we analyzed tumor biopsies of NSCLC patients treated with ICI. We found a peculiar cluster of $CD163^+$ $CD33^+$ $PD\text{-}L1^+$ macrophages with epithelioid morphology in all patients that experienced hyperprogression, highlighting the role of myeloid cells in inducing the detrimental effect of anti-PD-1 antibody.

Conclusion. Our findings, describing myeloid cells as crucial player in causing tumor acceleration after ICI therapy, may allow the identification of patients not to be treated with this type of immunotherapy.

INTRODUCTION

IMMUNE SYSTEM AND TOLL-LIKE RECEPTORS

The immune system has the capability to defend the human body against external attack, as traumatic or infective insults, by the detection and elimination of pathogens through different mechanisms and it is divided into innate and adaptive compartments. The first niche, composed by natural killer (NK) cells, dendritic (DC) cells, monocytes and granulocytes, is the first line of defense which rapidly detects invading pathogens and tumors in a non-specific manner and subsequently respond to them. Conversely, cytolytic T cells (CTL), T helper cells (T_H), and B cells form the adaptive side of the immune compartment, which is activated by antigen presentation and it is able to eliminate pathogens mounting an antigen-specific response *(Abbas AK et al, 1996, Nature)*. The innate immune system is evolutionarily more ancient than the adaptive counterpart and to protect the host it must accomplish four fundamental tasks. At first, it must rapidly detect and categorize infectious agents and recognize where they are localized (extracellularly or intracellularly). Based on the pathogen class, innate immune defences are activated to eradicate and/or contain temporarily the infection *(Krieg AM, 2006, Nat Rev Drug Discov).* Finally, innate effectors able to activate dendritic cells representing an important point of connection between the innate and adaptive immune responses. In this way, specific humoral and cellular immune responses are enhanced *(Murad YM, Clay TM, 2009, BioDrugs).*

Innate immune cells can sense and recognize infectious agents by pattern-recognition receptors (PRRs), which bind common molecular structures expressed across broad classes of pathogens, but absent or poorly present in vertebrates. PRRs recognize pathogen-associated molecular patterns (PAMPs), highly conserved components which are essential for the survival of microorganisms, as LPS, peptidoglycan and lipoproteins. Other PRRs can recognize endogenous stress signals, the so called danger-associated molecular patterns (DAMPs). Mannose receptors

(MR), Toll-like receptors (TLRs), NOD-like receptors (NLRs) represent different classes of PRRs which recognize different microbial structures.

The best known PRRs are the Toll-like receptors (TLRs), which are a family of pathogen recognition receptors evolutionarily conserved. TLRs are the mammalian homologues of Drosophila Toll protein and belong to the interleukin-1 receptor (IL-1R) superfamily (*Medzhitov R et al, 1997, Nature; Takeda K et al, 2003, Annu Rev Immunol*). In Drosophila, Toll protein is responsible for the embryonic development of the dorsal-ventral axis and it is important during the adult life of the insect for antifungal protection *(Hoffmann JA, 2003, Nature)*. In mammals, 13 TLR genes were described, 10 in humans (TLR1-10) and 12 in mice (TLR1-9 and 11-13) and some of them are homologous *(Akira S, Takeda K, 2004, Nat Rev Immunol; Albiger B et al, 2007, J Intern Med)*. TLRs are considered sensors for microbial infections and other "danger signals" and they work as a bridge between the innate and the adaptive immune responses.

TLRs are integral membrane type I glycoproteins that are ubiquitously expressed, from epithelial to immune cells. Different type of immune cells express these receptors and, as the other PRRs, they recognize and bind molecules which are specifically expressed by microorganisms. These receptors can be expressed on the cell surface or inside endocytic vescicles or other intracellular organelles, according to the specificity to different pathogens ***(Figure 1)*** *(Akira S, Takeda K, 2004, Nat Rev Immunol; Akira S, 2006, Curr Top Microbiol Immunol)*.

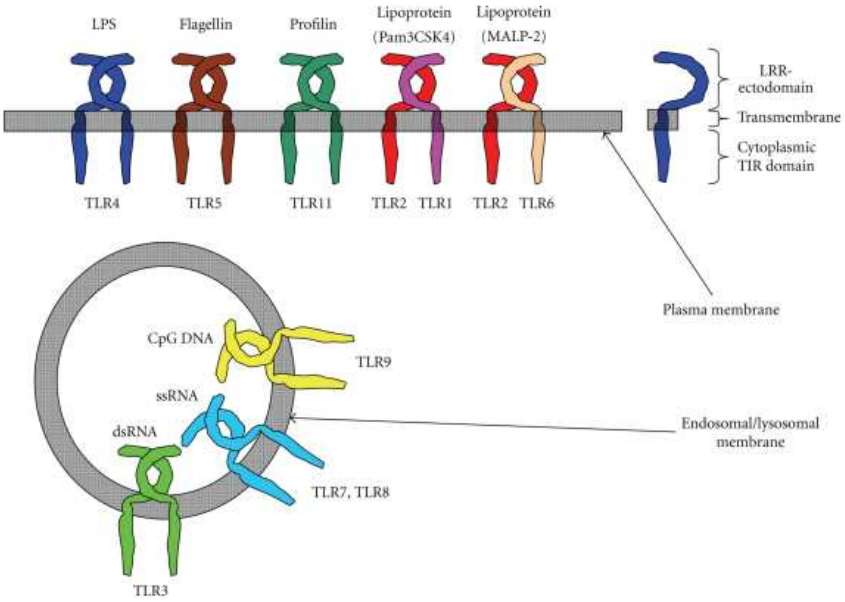

Figure 1. TLRs can be located extracellularly or intracellularly. TLRs 1, 2, 4, 5, 6, and 11 recognize their ligands on the cell surface, whereas TLRs 3, 7, 8, and 9 are localized inside the cells. Most TLRs form homodimers, while TLR2 heterodimerize with either TLR1 or TLR6 (Yamamoto M, Takeda K, 2010, Gastroenterol Res Pract)

TLRs 3, 7, 8 and 9 are located inside the cell mainly in endosomes. In particular, TLR3 binds to double-stranded RNA *(Alexopoulou L et al, 2001, Nature)*, single-stranded viral RNAs are ligands for TLRs 7 and 8 *(Heil F et al, 2004, Science)* and unmethylated CpG sequences in DNA molecules are recognized by TLR9. The other TLRs are expressed on the cell surface; bacterial, fungal and viral PAMPs activate TLRs 1, 2, 5, 6, 10 *(Takeuchi O et al, 2001, Int Immunol; Takeuchi O et al, 2002 J Immunol; Hayashi F et al, 2001, Nature)*, while lipopolysaccharide (LPS) is the specific ligand for TLR4 *(Yang H et al, 2000, J Biol Chem)*. TLRs agonists bind their receptor causing activation of innate immune cells through two major signaling pathways *(Spaner DE, Masellis A, 2007, Leukemia)*. The first pathway, which is MyD88-indipendent, leads to the production of type I interferons (IFNα or β), whereas the second pathway requires MyD88 protein to activate nuclear factor-kappa B (NF-κB), JUN kinase (JNK) and p38, leading to the production of TNFα, IL-12, IL-1 and other proinflammatory cytokines and induces typical mechanisms of innate immunity *(Iwasaki A, Medzhitov R, 2004, Nat Immunol)*. MyD88 is recruited at the TIR domain of the receptors in all TLRs, with the exception of TLR3, which

signals through MyD88-independent pathway. TLR4 can use both pathways. TLR triggering induces maturation of dendritic cells (DCs), leading to the up-regulation of costimulatory molecules, CD40, CD80, CD86, and to the secretion of immune modulatory cytokines and chemokines. Moreover, TLRs can stimulate the proliferation of $CD4^+$ and $CD8^+$ T cells and can reverse the suppressive function of Treg cells *(Peng G et al, 2005, Science; Crellin NK et al, 2005, J Immunol; Tabiasco J et al, 2006, J Immunol).* Additionally, $CD8^+$ cytotoxic T cells were demonstrated to be activated by TLRs 3, 4, 7 and 9 with increased production of IFNγ and other cytokines *(Hamdy S et al, 2008, Vaccine; Ramakrishna V et al, 2007, J Transl Med).*

TLR9 EXPRESSION AND LOCALIZATION

In humans, bone marrow derived cells, memory B cells *(Bernasconi NL et al, 2003, Blood)* and plasmacytoid dendritic cells (pDCs) express TLR9 *(Kadowaki N et al, 2001, J Exp Med).* This receptor was also shown to be expressed by activated human neutrophils and by non-hematopoietic cells, for example in epithelial cells of the respiratory and the gastrointestinal tract *(Parker LC et al, 2007, Clin Exp Immunol; Hopkins PA, Sriskandan S, 2005, Clin Exp Immunol).* TLR9 expression profile is not the same among different species. Indeed, in mice TLR9 was also detected in monocytes, macrophages and conventional dendritic cells *(Edwards AD et al, 2003, Eur J Immunol; Hemmi H et al, 2000, Nature).* In immune cells, TLR9 was found to be located in the endoplasmic reticulum (ER) and after engagement by CpG-DNA moves to endolysosomes *(Leifer CA et al, 2006, J Biol Chem).* The expression of TLR9 in epithelial cells is different. In these cells, it is located on the apical or basolateral membrane and the signal transduction varies in a tissue-specific manner. In the gut, NF-κB pathway is activated by the stimulation of basolateral TLR9, whereas apical TLR9 prevents NF-κB activation and promotes the production of inhibitory protein I kappa B-alpha (IκBα), which is NF-κB inhibitory protein. Moreover, when

apical TLR9 is stimulated, tolerance to subsequent TLR9 challenges is gained, suggesting that it is involved in the control of intestinal inflammation *(Lee J et al, 2006, Nat Cell Biol)*.

CELLULAR SIGNALING MEDIATED BY TLR9

TLR9 binds particular sequences of nucleic acids present in bacterial and viral DNA, characterized by non-methylated CG (CpG) dinucleotides. In vertebrates, CpG motifs are almost always methylated and are less represented compared to their frequency in bacterial and virus genomes, which generally contain CpG dinucleotide every 16 bases *(Krieg AM, 2007, Proc Am Thorac Soc)*. After the endocytosis or phagocytosis of pathogens or the infection by viruses, when the microbial DNA is in the endosomal or lysosomal compartment, TLR9 can interact with CpG-DNA at acidic pH, essential condition for DNA recognition. In fact, drugs that prevent acidification of the endosomal compartment, such as chloroquine and bafilomycin A1, interfere with the TLR9 activation. TLR9 signaling cascade involves mitogen-activated protein kinases (MAPKs), c-Jun NH2-terminal kinase (JNK), extracellular receptor kinase (ERK) and NF-κB-inducing kinase (NIK)-IKK-IκB pathways and it ends with the activation of several transcription factors, such as NF-κB, activating protein-1 (AP-1), cAMP-responsive element-binding protein (CREB), and CCAAT/enhancer binding protein (C/EBP), inducing cytokines and chemokines gene expression ***(Figure 2)*** *(Häcker H et al, 1999, EMBO J; Yi AK et al, 2002, J Immunol; Hartmann G, Krieg AM, 2000, J Immunol)*.

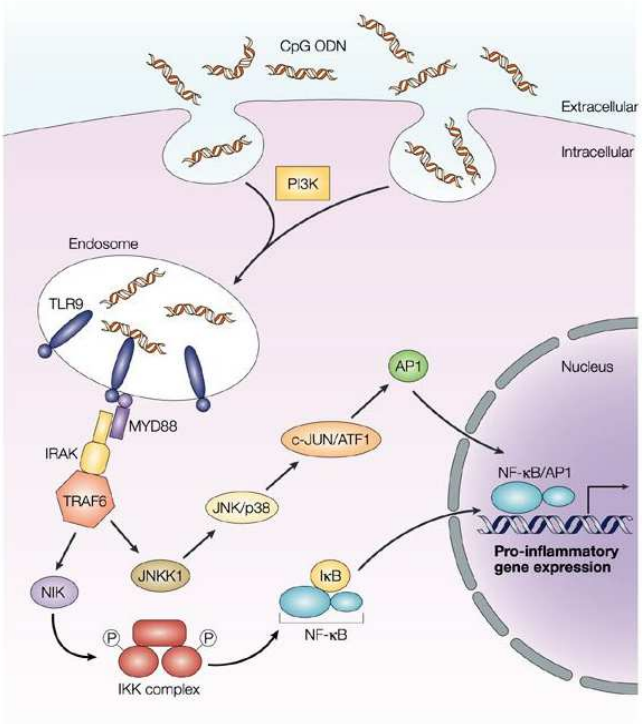

Figure 2. Summary scheme of the intracellular signaling mediated by TLR9/CpG-DNA (Klinman DM, 2004, Nat rev. Immunol)

SYNTHETIC CpG OLIGODEOXYNUCLEOTIDES

Several synthetic molecules that mimic CpG-unmethylated DNA sequences were generated in order to obtain TLR9 activation without using bacterial products. Modifications of nucleotide bases, in backbone or in secondary and tertiary structures, were introduced to influence the stimulatory properties of these synthetic compounds and made them able to better stimulate an immune response ***(Figure 3)***.

The B-Class ODN stimulates human B cells and the maturation of human pDCs and monocytes and one or more 6mer CpG motif with the general formula "purine-pyrimidine-C-G-pyrimidine-pyrimidine" are present *(Krieg AM et al, 1995, Nature)*. The optimal human CpG motif is the 6mer motif 5′-GTCGTT-3′ *(Hartmann G, Krieg AM, 2000, J Immunol)*, whereas the optimal CpG motif in mice is 5′-GACGTT-3′ *(Hartmann G et al, 2000, J Immunol)*. Chemical modifications that affected the sugar moiety, the backbone or the heterocyclic nucleobase, such as 2'-Omethyl

or 2'-O-methoxyethyl sugar, phosphorothioate modifications, locked nucleic acid (LNA), can modify the activity of B-Class ODN. Moreover, the length, number, spacing, position and type of bases surrounding CpG motifs can influence their immunostimulatory activity. G runs with PS linkages at the 5' and 3' ends surrounding a phosphodiester palindromic CpG-containing sequence define the A-Class CpG-ODN *(Krug A et al, 2001, Eur J Immunol)*. This class of synthetic CpG oligodeoxynucleotides strongly stimulates IFNα and IFNβ production, but it is weak in inducing other TLR9-dependent effects. As reported for B-Class, length, modifications of the base, sugar or backbone influence A-Class activity.

The CpG C-Class has a stimulatory hexameric CpG motif at or near the 5′ end and is linked by a T spacer to a GC-rich palindromic sequence *(Vollmer J et al, 2004, Eur J Immunol)* and combines characteristics of the A- and B-Classes.

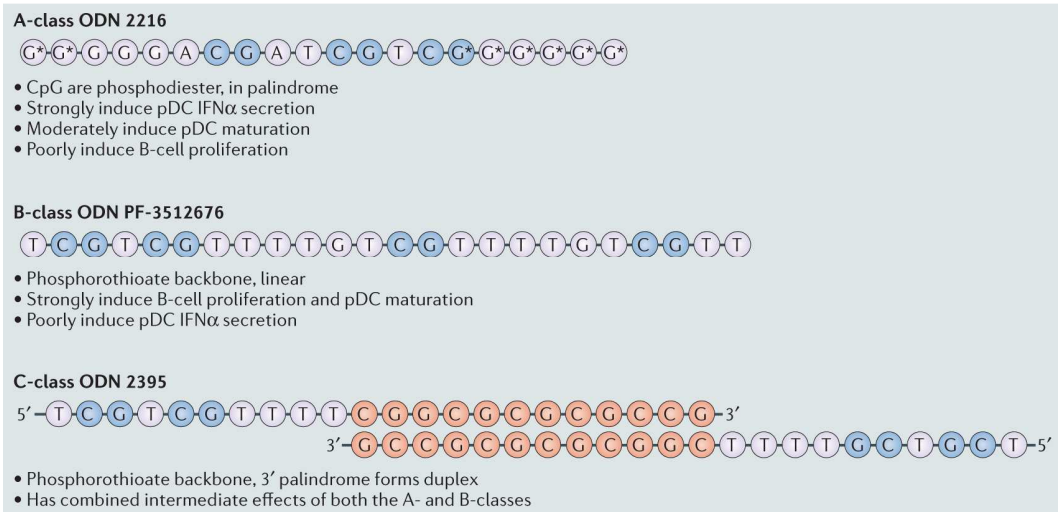

Figure 3. Three major classes of CpG-ODN (Krieg AM, 2006, Nat Rev Drug Discov)

STIMULATION OF INNATE AND ADAPTIVE IMMUNITY BY CpG-ODN

Immune cells, which constitutively express TLR9, are activated by CpG-ODN. CpG-ODN can be administered in saline and is taken up spontaneously by most immune cells, in particular B and pDCs cells, but its uptake is not restricted to cells expressing TLR9.

There are two types of immune responses. TH1 response which mounts in order to fight intracellular infections and involves NK cells and CTLs with the lysis of infected cells and TH2 immune activation which is directed to the secretion of specific antibodies. Between these two responses, the first is the most desired for cancer therapy since it will be directed to kill tumor cells and it is the one triggered by TLR9 activation. TLRs engagement stimulates antigen-nonspecific immunity and subsequently activates antigen-specific adaptive immunity ***(Figure 4)***. Different classes of TLR9 agonists activate innate immunity with a predominantly TH1 response and the production of cytokine and chemokine by B cells and pDCs, and in a second time also by other immune cells. Furthermore, B and pDC cells can up-regulate costimulatory molecules, such as CD80 and CD86, TNF-related apoptosis-inducing ligand (TRAIL) and CC chemokine receptor 7 (CCR7). TRAIL can induce tumor cell death, whereas CCR7 can induce the migration to the lymph nodes in the T cell zone, with augment in apoptosis resistance *(Iwasaki A, Medzhitov R, 2004, Nat Immunol)*. In this way the activation of TLR9 can promote tumor regression through the production of antitumor factors, as IFNα and TRAIL, and the activation of NK cell-mediated tumor killing *(Krieg AM, 2006, Nat Rev Drug Discov)*. In addition, antigen-specific antibody and T cell immune responses followed pDC and B cell maturation and pDCs are able to induce effective $CD4^+$ and $CD8^+$ T cells responses. Since TLR9 stimulation enhances the production of antigen specific antibodies, CpG-ODN might be used as vaccine adjuvant in order to induce a strong TH1-biased immunity.

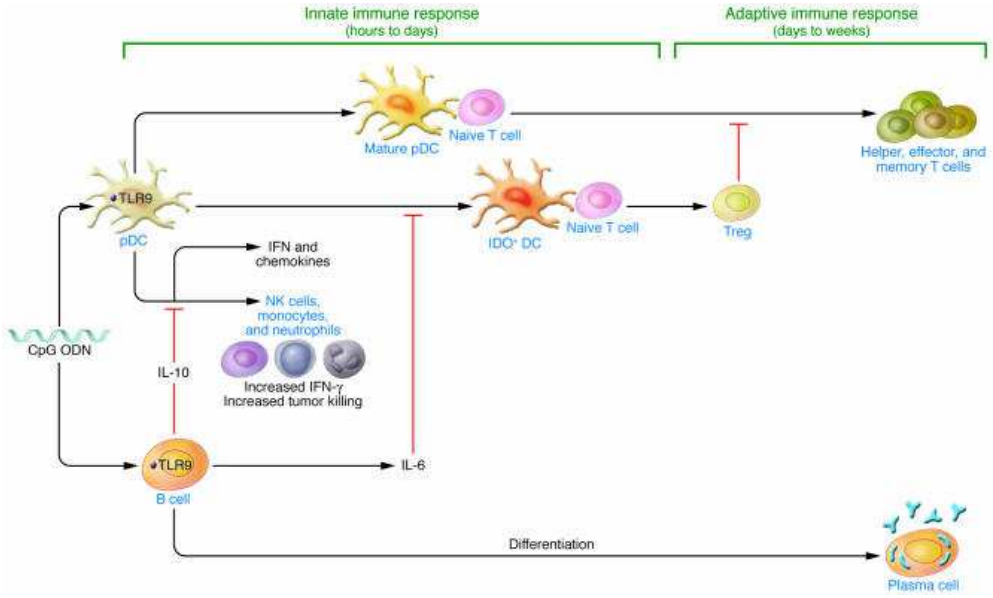

Figure 4. Activation of innate and adaptive immunity by TLR9 activation (Krieg AM, 2007, J Clin Invest)

PRECLINICAL STUDIES AND CLINICAL TRIALS OF TLR9 AGONISTS

CpG-ODN have shown moderate success in several mouse models in inducing the reduction of established tumors, either when used alone or in combination with other antitumor treatments *(Krieg AM, 2004, Curr Oncol Rep).* The antitumor efficacy of CpG-ODN as monotherapy can vary and it depends on tumor type. Several factors may impact on its mechanism of action: the susceptibility of tumors to immune effectors, tumor MHC expression, and even TLR9 expression by tumor cells *(Ballas ZK et al, 2001, J Immunol; Carpentier AF et al, 1999, Cancer Res; Lonsdorf AS et al, 2003, J Immunol).* An important factor that influence the efficacy of CpG-ODN treatment is represented by the route of administration. Systemical administration was seen to be less efficacious, whereas peritumoral or intratumoral injection is very effective in treating different tumors, as reported by several publications *(Heckelsmiller K et al, 2002, J Immunol; Kawarada Y et al, 2001, J Immunol).*

Promising results obtained in several preclinical models paved the way for the development of clinical trials to test the efficacy of CpG-ODN in different cancer types. For example, a phase I

clinical trial in Non-Hodgkin's lymphomas (NHL) patients, previously treated with standard therapy, demonstrated the safety of CpG-ODN Agatolimod, which produces immunomodulatory effects *(Link BK et al, 2006, J Immunother).* Furthermore, Agatolimod was used in combination with Rituximab in NHL patients, either subcutaneously or intravenously, without increasing the toxicity of Rituximab alone *(Leonard JP et al, 2007, Clin Cancer Res).* Agatolimod was also used in a multicenter phase I study in patients with advanced renal cell carcinoma, who showed biological responses after treatment, consistent with TLR9 agonists mechanism of action *(Thompson JA et al, 2004, J Clin Oncol, (July 15 supplement; abstract 4644)).* Different protocols tested CpG-ODN alone or in combination with other drugs also in melanoma. Pashenkov and his group showed that Agatolimod treatment may stimulate an innate immune response in melanoma patients. Moreover, it was identified a panel of biomarkers associated with tumor regression in response to TLR9 activation *(Pashenkov M et al, 2006, J Clin Oncol).*

The results of the published clinical trials demonstrated that CpG-ODN anti-tumor activity is not very impressive as monotherapy, but it can successfully be used in combination with other anti-tumor agents.

PROGRAMMED CELL DEATH-1

Programmed cell death-1 (PD-1) is a receptor which belongs to the CD28 receptor family. It is reported to be expressed by T lymphocytes, but it is also described to be present on innate immune cells. After the interaction with its ligands, PD-L1 and PD-L2, it is involved in the inhibition of T cell activation; indeed, PD-1/PD-Ls pathway is important in maintaining peripheral tolerance and preventing autoimmunity.

STRUCTURE OF PD-1 AND ITS LIGANDS

PD-1, also known as CD279, is a type I transmembrane glycoprotein of 50-55 kDa (288 amino acid). This protein is composed of an immunoglobulin variable region (IgV)-type extracellular domain, a stalk separating the IgV domain from the plasma membrane made of approximately 20 amino acids, a transmembrane domain, and an intracellular domain containing tyrosine-based signaling motifs. The membrane proximal cysteine residue which is necessary for homodimerization is not present in PD-1, so it probably exist as a monomer *(Zhang X et al, 2004, Immunity).* In the cytoplasmic tail, PD-1 contains two tyrosine residues: the membrane proximal one with the N-terminal sequence VDYGEL which forms an immunoreceptor tyrosine-based inhibition motif (ITIM) known to recruit SH2 domain-containing phosphatases *(Neel BG et al, 2003, Trends Biochem Sci)* and the second one which contains the immunoreceptor tyrosine-based switch motif (ITSM) with the C-terminal sequence TEYATI. PD-1 is encoded by the *Pdcd1* gene on human chromosome 2, while in mice it is on chromosome 1 *(Keir ME et al, 2008, Annu Rev Immunol.). Pdcd1* is constituted by 5 exons in both species, each of which encodes a specific part of the protein. PD-1 has different splice variants which have been cloned from activated human T cells *(Nielsen C et al, 2005, Cell Immunol)* and all of these are expressed in

resting peripheral blood mononuclear cells (PBMCs) at a level similar to the one of the full-length protein.

The two identified ligands for PD-1 are programmed death-1 ligand-1 (PD-L1 or B7-H1 or CD274) and the programmed death-1 ligand-2 (PD-L2 or B7-DC or CD273). Both of these two ligands are type I transmembrane glycoproteins with IgC- and IgV-type extracellular domains. PD-L1 and PD-L2 share 40% amino acid identity with each other and 20% with B7 molecules *(Okazaki T, Honjo T, 2007, Int Immunol.)*. PD-L1 is encoded by the *Cd274* gene on chromosome 9 in human and on chromosome 19 in mouse and it is adjacent to the *Pdcd1lg2* gene which encoded for PD-L2. A crystal structure analysis reveals how PD-1 interacts with PD-L1 or PD-L2. As shown in ***Figure 5***, the front β-face of PD-1 binds to the β-face of its ligands *(Lin DY et al, Proc Natl Acad Sci U S A. 2008)*.

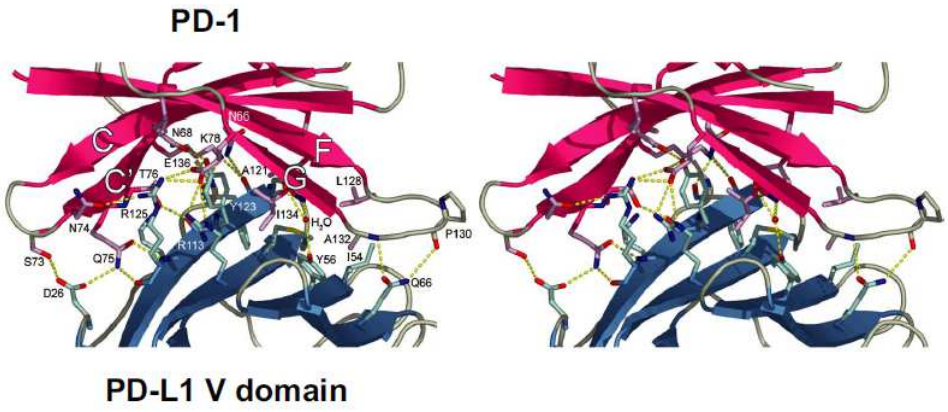

Figure 5. The PD-1/PD-L1 interface (Lin DY et al, 2008, Proc Natl Acad Sci U S A.)

EXPRESSION OF PD-1, PD-L1 AND PD-L2

PD-1. Programmed cell death-1 is expressed on activated CD4 and CD8 T cells, B cells, activated monocytes, natural killer (NK) cells, dendritic cells (DCs), and on $CD4^-$ $CD8^-$ thymocytes. When was discovered, PD-1 was thought to be preferentially expressed in apoptotic cells *(Ishida Y et al, 1992, EMBO J.*), but it was later understood that its important role is related to the maintenance of peripheral tolerance and not to cell death.

PD-1 expression is induced after T cells activation through the T-cell antigen receptor and also through some cytokines, (i.e. IL-2, IL-7, IL-15 and IL-21) *(Kinter AL et al, 2008, J Immunol.).* Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) is a transcription factor required for PD-1 transcription in T cells after its binding to the *PDCD1* promoter *(Oestreich KJ et al, 2008, J Immunol.).* Forkhead box O1 (FoxO1), Notch, and interferon regulatory factor 9 (IRF9) are other transcription factor able to promote PD-1 transcription. In particular, FoxO1, in addition to upregulating PD-1, it defines a positive feedback loop, since PD-1 signaling prevents FoxO1 degradation. On the other hand, T-box transcription factor TBX21 (T-bet) directly and actively represses PD-1 transcription *(Terawaki S et al, 2011, J Immunol.; Staron MM et al, 2014, Immunity; Mathieu M et al, 2013, Immunol Cell Biol.; Kao C et al, 2011, Nat Immunol.).* Further epigenetic modifications and several microRNAs are involved in controlling PD-1 expression. During $CD8^+$ T cells differentiation, a transient DNA demethylation at *Pdcd1* locus in naïve T cells is followed by a new DNA methylation to become functional memory T cells. In contrast, there is a dramatic demethylation in PD-1 promoter of exhausted $CD8^+$ T cells and a subsequent imprinting during the effector phase of CD8 T cell exhaustion *(Youngblood B et al, 2011, Immunity; Ahn E et al, 2016, J Virol).* Histone methylation/acetylation are also involved in the control of *Pdcd1* transcription. Different activation markers, such as histone H3 lysine 4 monomethylation (H3K4me1), H3K27 acetylation, H3K9ac and H3K27ac are enriched in PD-1

promoter to induce the transcription of PD-1 on CD8 T cells *in vitro (McPherson RC et al, 2014, Elife; Lu P et al, 2014, J Exp Med.).*

PD-1 ligands. T and B cells, DCs, macrophages, bone marrow-derived mast cells constitutively express PD-L1 *(Yamazaki T et al, 2002, J Immunol.).* PD-L1 is not only expressed on hematopoietic cells, but also on other cell types after activation, such as on lung cells, fibroblastic reticular and mesenchymal stem cells, liver non-parenchymal, vascular endothelium, and also on pancreatic islets, astrocytes and keratinocytes *(Keir ME et al, 2008, Annu Rev Immunol.).* PD-L1, and to a lesser extent PD-L2, are often overexpressed on tumor cells as a mechanism exploited by cancers to avoid immune surveillance *(Iwai Y et al, 2002, Proc Natl Acad Sci U S A).* PD-L1 has a broader expression than PD-L2, which is inducibly expressed on DCs, macrophages, and bone marrow-derived mast cells and on 50% to 70% of resting peritoneal B1 cells *(Zhong X et al, 2007, Eur J Immunol.).* Different mechanisms are involved in the regulation of PD-Ls expression. IL-2, IL-7 and IL-15 induce PD-L1 expression on T cells, monocytes and macrophages and also IFNγ controls the expression of this ligand. Whereas IL-10 stimulates PD-L1 expression on monocytes, IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) upregulate PD-L2 on DCs *(Chinai JM et al, 2015, Trends Pharmacol Sci.).* Different pathways disregulated in cancer cells can control and modify PD-L1 expression in tumor. Loss of PTEN and the subsequent over-activation of the PI3K/Akt pathway *(Parsa AT et al, 2007, Nat Med.),* EGFR mutation *(Akbay EA et al, 2013, Cancer Discov.)* and NRAS mutation *(Johnson DB et al, 2015, Cancer Immunol Res.),* the induction of the transcription factor hypoxia-inducible factor-1α (HIF-1α) *(Noman MZ et al, 2014, J Exp Med.)* and also microRNAs can play a role in PD-L1 regulation.

PD-L1 and PD-L2 are the only known ligands for PD-1. However, these two ligands have other binding partners. PD-L1 can also interact with B7-1 expressed on T cells *(Butte MJ et al, 2007, Immunity),* whereas PD-L2 on dendritic lung cells can interact with repulsive guidance molecule

B (RGMB) expressed on alveolar macrophages, interstitial macrophages and alveolar epithelial cells *(Xiao Y et al, 2014, J Exp Med).* These interactions seem to be generally inhibitory.

PD-1 SIGNALING PATHWAY

PD-1 signaling was mainly studied in activated T cells ***(Figure 6)***. Upon ligand engagement, the two intracellular tyrosine motifs of PD-1 are phosphorilated, and then the binding of phosphatases downregulates antigen receptor signaling by the dephosphorylation of signalling intermediates. In particular, two phosphatases SH2-domain containing tyrosine phosphates 1 (SHP-1) and SHP-2 bind to the ITIM and ITSM motifs of PD-1 *(Sheppard KA et al, 2004, FEBS Lett)* and trigger this inhibitory signal. Between the two inhibitory motifs, ITSM appears to play the main role. Indeed, when ITSM is mutated PD-1 loses its inhibitory function *(Chemnitz JM et al, 2004, J Immunol).* Moreover, SHP-1 interacts more weakly with PD-1 compared to SHP-2, although both of them are recruited to the antigen receptor signaling complex *(Okazaki T et al, 2001, Proc Natl Acad Sci U S A).*

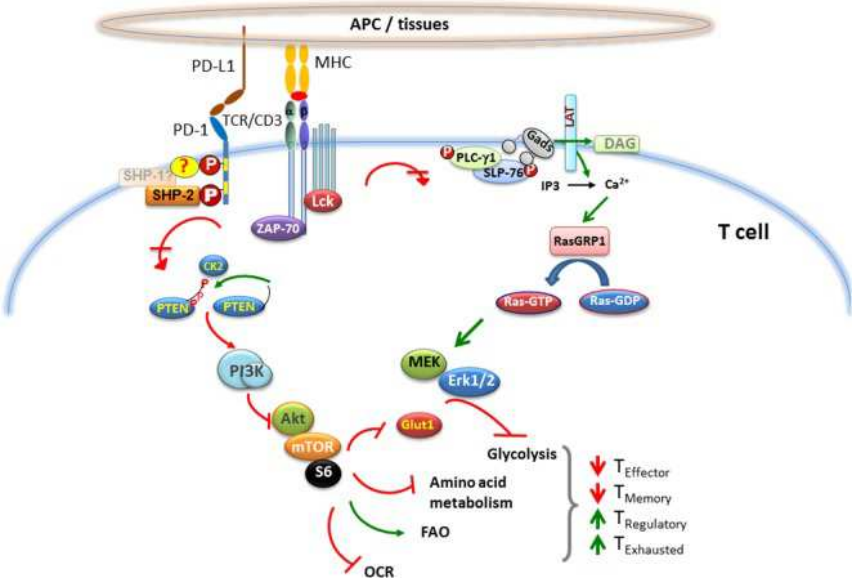

Figure 6. Effect of PD-1 on major signaling pathways and subsequent metabolic reprogramming in T cells (Bardhan K et al, 2016, Front Immunol)

PD-1 have to be close to the antigen receptor to inhibit its signaling. During T cell activation, PD-1 changes its distribution and translocates to dynamic T cell receptor microclusters, where it exerts its inhibitory effects by the recruitment of SHP-2 into the synapse, proximal to the T cell receptor. The ligation of PD-1 induces the inhibition of the activation of T cell receptor proximal kinases and it attenuates Lck-mediated phosphorylation of ZAP-70 and initiation of downstream events *(Sheppard KA et al, 2004, FEBS Lett).*

One important target of PD-1-mediated inhibition is the PI3K-Akt pathway *(Patsoukis N et al, 2012, Sci Signal.).* The recruitment of SHP-2 can block the activation of PI3K *(Parry RV et al, 2005, Mol Cell Biol.).* PTEN, a serine-threonine phosphatase, opposes the activation of PI3K and suppresses the PI3K-Akt pathway. During T cells activation, CK2 phosphorilates PTEN in the S380-T382-T383 cluster which promotes PTEN stability, reducing PTEN phosphates activity. PD-1 inhibits CK2-mediated PTEN phosphorylation and so PTEN phosphates activity is induced and represses PI3K-Akt signaling *(Vazquez F et al, 2000, Mol Cell Biol.; Torres J, Pulido R.***,** *2001, J Biol Chem.; Patsoukis N et al, 2013, Mol Cell Biol.).*

Another signaling pathway regulated by PD-1 is the Ras/MEK/ERK pathway. In T cells, activated RasGRP1 plays an important role for the activation of Ras and its downstream signal, and calcium and diacylglycerol downstream of PLC-μ activate RasGRP1 *(Bivona TG et al, 2003, Nature).* PD-1 inhibits the activation of PLC-μ and Ras, leading to a diminished activation of the MEK/ERK pathway *(Patsoukis N et al, 2012, Sci Signal).*

PD-1 has also an inhibitory effect on the B cell receptor (BCR) signaling. After PD-1 engagement, SHP-2 is recruited to the ITSM tyrosine and BCR-mediated Ca^{2+} mobilization and tyrosine phosphorilation of effector molecules are inhibited *(Okazaki T et al, 2001, Proc Natl Acad Sci U S A).*

PD-Ls ligands not only may influence responses after the engagement of PD-1, but also may deliver signals into cells which express PD-L1 or PD-L2 ligands. Growing evidences support this

“reverse” signaling, although PD-Ls ligands have only a short cytoplasmic tail which do not contain canonical conserved signaling motifs. PD-L1 expression on cancer cells leads to expression of glycolisis genes and enhanced glycolitic metabolism, through PI3K-Akt pathway, and this state is reversed after treatment with anti-PD-L1 antibody *(Chang CH et al, 2015, Cell).*

There is increasing evidence that PD-1 signaling also influence the metabolic reprogramming in T cells ***(Figure 6)***. During their activation, T cells switch their metabolism from a quiescent phenotype, when oxidative phosphorylation is the dominant metabolic program, to aerobic glycolysis, important to support growth, proliferation and effector functions *(O'Sullivan D, Pearce EL, 2015, Trends Immunol.)* PD-1 signaling can interfere with this process, inhibiting the up-regulation of glucose and glutamine metabolism driven by TCR and CD28 signaling. Moreover, PD-1 pathway can promote lipolysis and fatty acid oxidation. Thus, PD-1 may alter T cell differentiation acting on T cell metabolism and this modification establish a more oxidative environment, as indicated by diminished levels of reduced glutathione *(Patsoukis N, et al, 2015, Nat Commun.)*. This altered T cell metabolic program induced by PD-1 engagement may have an active role in T cell dysfunction during chronic infections and cancer, making this pathway of more therapeutic importance.

BIOLOGICAL SIGNIFICANCE OF PD-1

PD-1/PD-Ls pathway is crucial for the regulation of normal host physiology. Its inhibitory signal plays a crucial role in central and peripheral tolerance and it is important to protect self tissue from autoimmune responses. PD-1 pathway is important for its role in regulating T cell exhaustion, but it has also a crucial role in maturation and evolution of T cells, since it is involved in shaping the magnitude of T cell response, in regulate T cell differentiation and effector T cell fate and for the development of immunological memory. Indeed, PD-1 and its ligands are

involved in the maturation of thymocytes and can contribute either to positive and negative selection. Moreover, PD-1 is also important in inhibiting response of self-reactive T cells during peripheral tolerance since it controls the interaction between naïve self-reactive T cells and DCs *(Keir ME et al, 2008, Annu Rev Immunol.)*.

PD-1 in autoimmunity

PD-1 pathway is critical in the maintenance of tolerance and, as a consequence, it is implicated in autoimmunity. The first evidences of this role came from studies in PD-1 deficient mice. C57BL/6 *Pdcd1* KO mice developed a mild glomerulonephritis *(Nishimura H et al, 1999, Immunity)*, whereas *Pdcd1*$^{-/-}$ Balb/c mice were reported to manifest a dilated cardiomyopathy *(Nishimura H et al, 2001, Science)*. Other studies performed in different mice autoimmune models explored the role of PD-1/PD-Ls in autoimmunity.

In non-obese diabetic (NOD) mice, PD-L1 was found to be up-regulated on pancreatic islet cells and loss or blockade of PD-1 or PD-L1 resulted in a rapid and exacerbated diabetes. Moreover, there was a severe insulitis and T cells produced proinflammatory cytokines *(Liang SC et al, 2003, Eur J Immunol; Ansari MJ et al, 2003, J Exp Med)*.

PD-1, PD-L1 and PD-L2 play also influential roles in experimental autoimmune encephalomyelitis (EAE) pathogenesis of human multiple sclerosis *(Liang SC et al, 2003, Eur J Immunol)*. Neutralizing antibody used to block PD-1 or PD-L2 during EAE accelerated disease onset and severity and it was associated with increased myelin oligodendrocyte glycoprotein (MOG)-reactive T cell and antibody and with more CNS inflammatory infiltrates *(Salama AD et al, 2003, J Exp Med)*. It has also been reported that PD-L1^{+} myeloid DCs reduced EAE severity in CCR6-deficient mice *(Elhofy A et al, 2009, J Neuroimmunol)*.

Regulatory T (Treg) cells are important in the maintenance of peripheral tolerance, since they can suppress activated T cells. Tregs are reported to express PD-1 and PD-L1, which are important

for their regulatory functions *(Baecher-Allan C et al, 2003, Novartis Found Symp)*. Indeed, in colitis models it was identified a $CD4^+$ $CD25^+$ $PD\text{-}1^+$ regulatory subpopulation able to inhibit the development of colitis *(Totsuka T et al, 2005, Eur J Immunol)*. Moreover, PD-L1 expression is important in $CD4^+$ $DX5^+$ T cells suppressive population for their inhibitory activity *(Hornung M et al, 2006, Eur J Immunol)*.

Several PDCD1 single nucleotide polymorphisms (SNPs) have been reported in association with human autoimmune disease *(Keir ME et al, 2008, Annu Rev Immunol.)*, but it is not yet clear their mutual relation and the effects that these SNPs might have in PD-1 engagement.

PD-1 in viral infections

PD-1 has also an important role in the immune response in infections. During chronic infections, the sustained high levels of antigen exposure lead $CD8^+$ T cells to exhaustion *(Wherry EJ, 2011, Nat Immunol)*. The importance of PD-1 during this process was reported in a murine model of chronic LCMV infection, where there was described the PD-1 up-regulation on virus specific CD8 T cells in the acute period, which remains high in exhausted cells till the infection turns into its chronic phase. However, PD-1 rapidly increases in the acute phase and it is then followed by a sharp decrease *(Barber DL et al, 2006, Nature)*. A similar phenomenon was subsequent reported for simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) infections *(Velu V et al, 2009, Nature; Day CL et al, 2006, Nature; Boettler T et al, 2006, J Virol; Urbani S et al, 2006, J Virol)*. Moreover, the group of Iwai showed that PD-1 KO mice infected with experimental adenovirus eliminated the virus more rapidly than wild-type mice *(Iwai Y et al, 2003, J Exp Med)*.

Therefore, blocking PD-1 pathway might be of interest since it can reverse the exhausted phenotype in chronic infections, inducing the clearance of the virus. It was reported that blocking PD-1/PD-L1 interaction improves virus specific T cells functionality *(Ha SJ et al, 2008, J Exp*

Med). Thus, PD-1 is important for the pathogen clearance because either it fine tunes the lymphocyte reaction and at the same time it prevents a strong T cells attack which can destroy tissues.

PD-1 in anti-tumor immunity

Tumors are able to evade host immune response, a phenomenon called “cancer immune editing”. Subsequent to an initial immune surveillance phase, during which tumors are supposed to be recognized by T cells through their “neo-antigens” presented on MHC molecules, a tolerogenic microenvironment is created in order to establish tumor tolerance. It is clear that PD-1/PD-Ls pathway is involved in this process, since this pathway is exploited by cancer cells to escape immune surveillance.

PD-L1 and PD-L2, although to a lesser extent, are expressed on a wide variety of tumor cells and correlate with adverse prognosis *(Thompson RH et al, 2004, Proc Natl Acad Sci U S A; Hamanishi J et al, 2007, Proc Natl Acad Sci U S A; Hino R et al, 2010, Cancer)*. Other components of the tumor microenvironment were found to express PD-1 ligands, such as macrophages, preferentially M2, myeloid cells, myeloid suppressor cells (MDSC), stromal fibroblasts and endothelial cells.

The interaction between PD-1 and PD-L1 promotes cancer cells survival and inhibits the activation of pathways important for survival, expansion and T cells differentiation, leading to tumor tolerance because memory and effector T cells generation is inhibited and, at the same time, the differentiation of Treg and exhausted T cells is promoted *(**Figure 7**, left side)*.

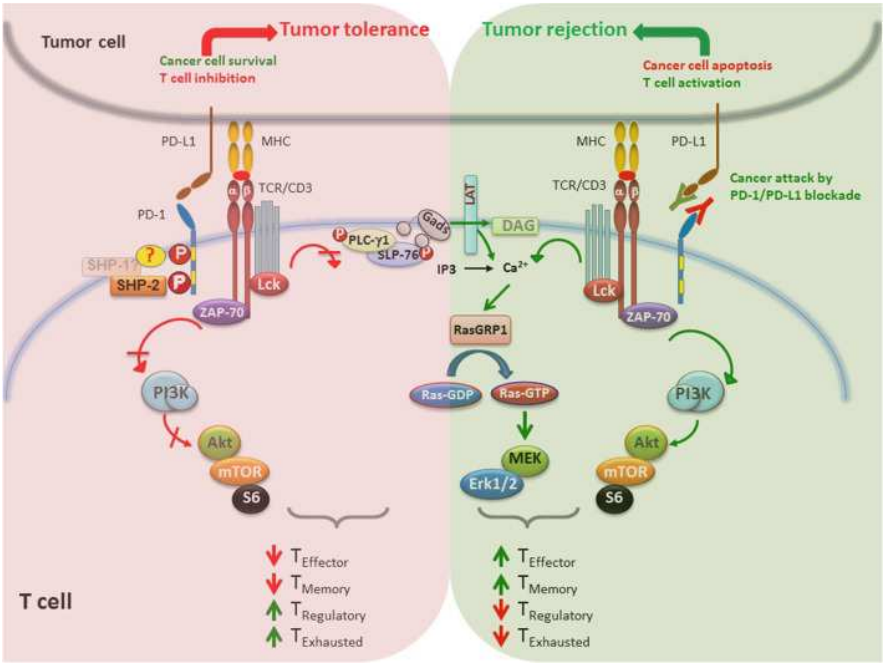

Figure 7. (*Left*) Binding of PD-1 to its ligands activates antiapoptotic signals which promote cancer cells survival. (*Right*) Anti-PD-1 or anti-PD-L1 antibodies that block this immune checkpoint enhance the antitumor T cell responses and cancer cells death (Bardhan K et al, 2016, Front Immunol)

Furthermore, PD-1 levels increased on tumor infiltrating T cells, which has impaired effector functions and an exhausted phenotype *(Ahmadzadeh M et al, 2009, Blood)*. Even though PD-Ls expression is a worse prognostic element, on the other hand their up-regulation correlates with a better clinical response to PD-1 or PD-L1 blockade, which leads to tumor regression and rejection, enhancing antitumor T cell responses ***(Figure 7**, right side).*

It is important to take into account the heterogeneity of the tumor microenvironment, which varies with tumor types and might influence the efficacy of therapy based on PD-1 blockade.

PD-1/PD-L1 BLOCKADE IN CANCER THERAPY

Cancer cells exploit PD-1 pathway to promote immune evasion. Therefore, blocking the interaction between PD-1 and its ligands can improve T cell functions and can induce a

regression in tumor growth. Clinical trials demonstrated the success of treatment with antibodies against PD-1 and/or PD-L1 in different kind of tumors. The first immune checkpoint inhibitor approved by the U.S. Food and Drug Administration (FDA) was Ipilimumab in 2010, a CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) inhibitor, for the treatment of melanoma *(Hodi FS et al, 2010, N Engl J Med)*. Then, antibodies against PD-1/PD-L1 pathway were developed with remarkable response, showing better clinical benefits and better toxicity profile in comparison to antibodies targeting CTLA-4 *(Larkin J et al, 2015, N Engl J Med)*. Indeed, immune-related adverse events (irAEs) associated with these antibodies include colitis, diarrhea, pancreatic, neurologic and hematologic adverse effects, but these toxicities are less common and less severe than the ones observed after anti-CTLA-4 antibody treatment. To date, a rapidly growing number of PD-1 signal inhibitors are emerging and used for the treatment of different types of solid and hematological malignant tumors (***Table 1***).

Table 1. CTLA-4-, PD-1-, and PD-L1-Blocking Agents in Clinical Development

Target	Agent	Antibody Class	Developer	Stage of Development
CTLA-4	ipilimumab	human IgG1	Bristol-Myers Squibb	FDA approved (melanoma)
	tremelimumab	human IgG2	Medimmune and Pfizer	phase III
PD-1	nivolumab (BMS-936558)	human IgG4	Bristol-Myers Squibb	FDA approved (melanoma, NSCLC, RCC)
	pembrolizumab (MK-3475)	humanized IgG4	Merck	FDA approved (melanoma, NSCLC)
	MEDI0680 (AMP-514)	humanized IgG4	Medimmune	phase I
	pidilizumab (CT-011)	human IgG1	CureTech	phase II
PD-L1	durvalumab (MEDI4736)	human IgG4	Medimmune	phase III
	atezolizumab (MPDL-3280A)	human IgG1	Genentech	phase III
	AMP-224[a]	NA	Amplimmune	phase I
	MDX-1105/BMS-936559	human IgG4	Bristol-Myers Squibb	phase I
	avelumab (MSB0010718C)	human IgG1	Merck Serono	phase II

NA, not applicable.
[a]PD-L2 fusion protein.

Table 1. Anti-CTLA-4, anti-PD-1 and anti-PD-L1 antibodies in clinical trials (Callahan MK et al, 2016, Immunity)

For example, Nivolumab and Pembrolizumab, two anti-PD-1 antibodies deeper investigated in clinical trials, are fully human IgG4 and humanized IgG4 monoclonal antibodies, respectively. It was decided to use IgG4 isotype in order to decrease antibody dependent cell-mediating cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). The first blocking agent against PD-1 reported to have clinical activity in several types of cancer was Nivolumab. As

reported in ***Table 1***, now there is a number of additional PD-1- or PD-L1-blocking agents that have demonstrated clinical activity in different cancer types.

PD-1 blockade in melanoma

Melanoma is an immunogenic tumor. Melanocytes express PD-L1 and their interaction with tumor infiltrating lymphocytes (TILs) through PD-1 contributes to immune evasion *(Taube JM et al, 2012, Sci Transl Med)*. In December 2014 Nivolumab was approved for patients with melanoma previously treated with Ipilimumab or BRAF inhibitor, but whose disease state progressed anyway. In a phase I study of patients with refractory melanoma, there was an objective response rate (ORR) of 31%. Although in 22% of patients occurred serious adverse events (grade 3/4), the efficacy and acceptable safety of Nivolumab was demonstrated *(Topalian SL et al, 2014, J Clin Oncol)*. Subsequently, a phase III study compared Nivolumab with Dacarbazine (DTIC, a chemotherapy used in the treatment of melanoma) in patients with melanoma without B-Raf mutation. Nivolumab was associated with a better ORR (40% vs 14%) and survival benefit (73% vs 42%) when compared to Dacarbazine arm *(Robert C et al, 2015, N Engl J Med)*. Moreover, adverse effect were reduced and PD-L1 positive tumors had a better response than PD-L1 negative tumors (53% vs 33%). In another phase III trial, Nivolumab was compared to chemotherapy in patients with advanced melanoma who progressed after Ipilimumab treatment. The response rate in Nivolumab group was higher (32% vs 11%) with durable tumor regression *(Weber JS et al, 2015, Lancet Oncol)*.

Other promising clinical trials were conducted to test Pembrolizumab. A phase II dose escalation trial in patients with Ipilimumab-refractory melanoma compared two dose levels of Pembrolizumab to chemotherapy *(Hamid O et al, 2013, N Engl J Med.)*. Both groups which received Pembrolizumab had clear benefit with 6-month progression free survival (PFS) compared to chemotherapy group. Another phase III study in patients with advanced melanoma,

where again two different administration schedules were tested and compared to Ipilimumab, provided evidences that Pembrolizumab improved survival rate *(Robert C et al, 2015, N Engl J Med.).* Based on these results, Pembrolizumab was approved by FDA for the treatment of Ipilimumab- or BRAF inhibitor-refractory metastatic melanoma patients.

PD-1 blockade in NSCLC

It was surprising that PD-1 pathway blockade showed signals of activity against lung cancer since, unlike melanoma, this tumor type was generally considered not to be an immunogenic tumor. In non-small cell lung cancer (NSCLC) PD-1/PD-L1 axis is activated by oncogenic signaling through PI3K or EGFR pathway or cytokine secretion *(Pardoll DM, 2012, Nat Rev Cancer).* In 2015 FDA approved Nivolumab for the treatment of squamous NSCLC patients who progressing after platinum-based chemotherapy. A dose escalation phase I trial revealed that patients with squamous and non-squamous cancers had a well response, with a low grade of toxicities *(Topalian SL et al, 2012, N Engl J Med).* A phase III trial in patients with metastatic squamous NSCLC compared Nivolumab to Docetaxel. In the Nivolumab group there was a significant improvement in median overall survival (OS) *(Borghaei H et al, 2015, N Engl J Med).* Other clinical trial assessed the efficacy and safety of Pembrolizumab and based on these studies FDA approved this anti-PD-1 antibody also for the treatment of PD-L1-positive NSCLC *(Garon EB et al, 2015, N Engl J Med).*

PD-1 blockade in other cancers

Other clinical trials investigated the effective use of anti-PD-1 and/or PD-L1 agents in multiple tumor types.

For example, FDA approved the use of Nivolumab in the therapy for renal cell carcinoma (RCC), an immunogenic tumor, like melanoma. In a randomized phase II trial different doses of

Nivolumab, specifically 3 doses, were tested, and Nivolumab treatment showed long-lasting objective response in 22% of the patients *(Motzer RJ et al, 2015, J Clin Oncol)*. Pembrolizumab was also evaluated in the treatment of naïve metastatic RCC patients.

Hodgkin lymphoma was another tumor in which PD-1 blockade resulted very efficacious, maybe since the amplification of 9p24.1 results in an increase of gene dosage of PD-L1 and PD-L2. A phase I study on 23 patients treated with Nivolumab showed 87% of ORR with a 17% of complete response rate *(Ansell SM et al, 2015, N Engl J Med)*.

PD-1 blockade was also tested in ovarian cancer, in which there is a negative correlation between the expression of PD-L1 and the number of intraepithelial TILs, and therefore in which the high PD-L1 expression is associated with a poor prognosis *(Hamanishi J et al, 2007, Proc Natl Acad Sci U S A)*. In one study 20 patients with relapsed platinum-resistant ovarian cancer were treated with Nivolumab and the best overall response was 15%, with 2 patients with a durable complete response *(Hamanishi J et al, 2015, J Clin Oncol)*. Another non-randomized, multicohort phase Ib study evaluated the effect of Pembrolizumab in ovarian cancer *(Weiss L et al, 2016, Memo)*. The observed objective response rate was 11,5% and among the 26 patients treated with Pembrolizumab, there was 1 complete and 2 partial responses and 6 stable diseases.

Thus, silencing the function of PD-1 pathway with blocking antibodies might be a promising clinical strategy in the care of several kind of cancers, since it reverses immunosuppression. Even though PD-1 and PD-L1 antibodies showed their efficacy when used as monotherapy, there are several clinical trials investigating possible combinatorial approaches, for example with cancer vaccines, anti-tumor mAbs or chemotherapies. These therapies might be of interest for patients who respond poorly or are unresponsive to “standard” immunotherapies in order to improve their immune response.

PD-1 RESISTANCE

Several studies revealed that systemic blockade of PD-1 function using specific antibodies is a highly promising therapy and it has elicited durable antitumor responses and long-term remission in a subset of patients with a broad spectrum of cancers (*Chinai et al., 2015, Trends Pharmacol Sci*). Nevertheless, in some cases immune checkpoint inhibitor (ICI) treatments are not effective due to primary resistance; indeed, the efficacy of PD-1 blockade was rarely more than 40%, with a large number of partial responders *(Topalian SL et al, 2012, N Engl J Med).* Moreover, patients might also acquired resistance to PD-1 therapy, which leads to disease progression or relapse.

Several immune or tumor intrinsic mechanism of resistance to anti-PD-1 therapy have been proposed *(Pitt JM et al, 2016, Immunity).* For instance, the presence of non-functional T lymphocytes or their absence at the tumor lesion is linked to PD-1 blockade treatment failure. Moreover, within the tumor microenvironment there are other cells, such as Tregs, myeloid derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), that might be involved in impairing the activity of PD-1 antibody *(Bai J et al, 2017, Oncotarget).* Epigenetic modification, cellular signaling pathways are other factors associated to resistance to PD-1/PD-L1 blockade together with the accessibility of PD-1 ligands in the tumor.

Two general mechanisms of immune resistance related to the modulation of PD-L1 have emerged *(Pardoll DM, 2012, Nat Rev Cancer).* Independently of inflammatory signals, constitutive oncogenic signaling can regulate PD-1 expression on tumor cells. The deletion or silencing of PTEN, linked to PI3K-Akt activation, up-regulate PD-L1 in glioblastomas *(Parsa AT et al, 2007, Nat Med)*; similarly, anaplastic lymphoma kinase (ALK) through signal transducer and activator of transcription 3 (STAT3) signaling induce PD-L1 expression in certain lymphomas and lung cancers *(Marzec M et al, 2008, Proc Natl Acad Sci U S A).* These processes are examples of innate immune resistance, which is alternative to the adaptive immune resistance *(Pardoll DM,*

2012, Nat Rev Cancer), in which tumor uses the normal process occurring during an infection to protect a tissue from damage in order to evade the anti-tumor immune response.

Despite several clinical trials demonstrated that blocking the interaction between PD-1 and its ligands produces objective responses in a wide spectrum of solid and hematologic malignancies, it is now emerging that some patients treated with anti-PD-1/anti-PD-L1 mAbs developed "hyperprogressive disease" *(Berger KN, Pu JJ, 2018, Gene).* This hyperprogression (HP) is an acceleration in tumor growth subsequently to immune checkpoint inhibitors treatment, as the therapy with anti-PD-1 antibody, but none of the mechanisms related to the resistance to PD-1 blockade seems to be able to explain this phenomenon. In 2016 two groups described the first evidence of rapid disease progression after PD-1 blockade treatment *(Lahmar, J et al, 2016, J. Clin. Oncol. 34 (suppl; abstract 9034); Saada-Bouzid E et al, 2016, J. Clin. Oncol. 34 (suppl; abstract 6072)).* After, other studies investigating hyperprogression appeared in literature. The group of Champiat reported that in a trial of 131 patients which underwent anti-PD-1 therapies, 12 of them (9%) developed "hyperprogressive disease" and this status seems to be more common in elder patients (over 65 year old) *(Champiat S et al, 2017, Clin Cancer Res).* In another study, in which 155 patients were treated with anti-PD-1/anti-PD-L1, 6 patients had MDM2/MDM4 amplification and experienced time-to-treatment failure (TTF) of less than 2 months and 4 of them exhibited a hyperprogressive response. Moreover, the same TTF was experienced by 8 of 10 individuals with EGFR mutations and 2 demonstrated hyperprogression *(Kato S et al, 2017, Clin Cancer Res).*

These concerns point to the importance of identifying biomarkers not only able to predict positive clinical responses, but also in order to avoid hyperprogression in patients after treatments aimed to blocking PD-1 pathway.

AIM OF THE THESIS

The present thesis is aimed to evaluate the role of innate immune system in the context of PD-1 blockade therapy. Moreover, since innate immune cells may influence anti-PD-1 antibody efficacy, combination between agent that stimulates innate cells and PD-1 blockade therapy is investigated.

MATERIALS AND METHODS

CELL CULTURE

IGROV-1 human ovarian cancer cell line was a gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France, NCI-H460 (hereafter H460) human non small cell lung cancer (NSCLC) cell line, B16 mouse melanoma cell line and 4T1 mouse mammary tumor cell line were purchased from ATCC (American Type Culture Collection; Rockville, MD, USA). All cells were routinely maintained at 37°C in a 5% CO_2 atmosphere in RPMI medium 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.) and 2mM glutamine (Cambrex, East Rutherford, NJ). B16 cell line was maintained in DMEM medium (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.) and 2mM glutamine (Cambrex). Cell lines were authenticated by the Genomic Facility at Fondazione IRCCS - Istituto Nazionale dei Tumori (Milan, Italy) and regularly tested for Mycoplasma using a specific kit (mycoAlert Plus Kit; Lonza, Basel, Switzerland).

Bone marrow derived macrophages (BMDMs) were obtained from tibia and femur of mice, as described by Weischenfeldt *(Weischenfeldt J, Porse B, 2008, CSH Protoc)*. Briefly, tibia and femur of mice were removed and the ends of the bones cut. Bone marrow was flushed out and collected in Falcon tubes. Bone marrow cells were centrifuge at 1500 rpm for 5 minutes at 4˚C and red blood cell were lysed by incubation in ammonium chloride lysis buffer. Cells were seeded into 10mm Petri Dishes in IMDM medium (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 2mM glutamine (Cambrex) and 100 U/ml penicillin and 100 U/ml streptomycin (Lonza) at 37°C in a 5% CO_2 atmosphere. The day after, floating cells were collected from Petri Dish, centrifuged, resuspended in fresh medium and cell number was determined using trypan blue exclusion assay. 5×10^6 cells were seeded in 10 mm Petri Dish in complete medium supplemented with 20 ng/ml of murine M-CSF (PeproTech, London, UK). Three days after, medium containing murine M-CFS was replaced with fresh

medium. Four days after, BMDMs were ready for *in vitro* experiments.

MICE

All xenograft experiments were carried out using 8-weeks-old female athymic nude mice, C57BL/6 and Balb/c mice (Charles River Laboratories, Calco, Italy). Mice were housed in a thermostatically maintained animal house with a 12 hours light cycle, free food access and water *ad libitum*. Animal protocols were approved by the Ministry of Health and OPBA board (Organismo per il Benessere Animale) of Fondazione IRCCS - Istituto Nazionale Tumori of Milan. Animal experiments were performed in accordance with National law (D.lgs 26/2014) and Guidelines for the Welfare of Animals in Experimental Neoplasia *(Workman P et al, 2010, Br J Cancer)*.

REAGENTS AND ANTIBODIES

Purified phosphorothioated TLR-9 agonist ODN1826 (5'-TCCATGACGTTCCTGACGTT-3'), containing CpG motifs (CpG-ODN), was synthesized by TriLink Biotechnologies (San Diego, CA, USA). Susceptibility to DNase digestion was diminished by phosphorothioate modification, prolonging *in vivo* half-life. Rat anti-mouse PD-1 (clone RMP1-14) and hamster anti-mouse PD-1 (clone J43) antibodies were purchased from BioXCell (West Lebanon, NH, USA). Liposomal clodronate were purchased from ClodronateLiposomes.com (Haarlem, The Netherlands).

Anti-mouse PD-1 $F(ab)_2$ was generated using Immobilized Pepsin (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Enzymatic digestion was performed at 37°C for 7 hours. Antibody concentration was determined by BCA Protein Assay Kit (Thermo Fisher

Scientific Inc.). Antibody digestion was verified by SDS-PAGE under non-reducing conditions and visualized by Coomassie Blue staining (Thermo Fisher Scientific Inc.).

The following primary antibodies were utilized for immunohistochemical analyses: purified rat anti-mouse CD45 (BD Biosciences, 30-F11), rat anti-mouse F4/80 (BioRad, Cl:A3-1,), purified rat anti-mouse Ly-6G and Ly-6C (Gr-1) (BD Pharmingen, RB6-8C5), purified rat anti-mouse CD45R (BD Pharmingen, clone RA3-6B2), mouse NKp46/NCR1 antibody (R&D systems) and anti-Arginase I (Genetex International Corp).

IN VIVO STUDIES

Athymic nude mice were injected intraperitoneally (i.p.) with 2.5×10^6 IGROV-1 cells in 0.2 ml of saline. Mice were treated with CpG-ODN delivered i.p. at a dose of 20 μg/mouse 5 days/week, with monoclonal antibody anti-mouse PD-1 delivered i.p. at a dose of 200 μg/mouse twice a week or with their combination. Control mice were left untreated. Mice were inspected daily and weighed three times/week. All mice were anesthetized and euthanized upon evidence of ascites appearance. The day of sacrifice was considered day of death. To deplete macrophages, IGROV-1 xenografted mice were treated i.p. with 200 μl of liposomal clodronate three days after tumor cells injection. Mice were then treated with CpG-ODN and anti-PD-1 antibody, as described above. Percent of disease-free mice in each treatment group was estimated by the Kaplan-Meier product limit method and compared with the log-rank test.

For *in vivo* studies with lung cancer xenograft model, 2.5×10^6 H460 cells were injected subcutaneously (s.c.) in mice right flank in 0.2 ml of saline. Mice were i.p. or peritumorally (p.t.) treated twice a week with 200 μg/mouse of anti-mouse PD-1 (clone RMP1-14) antibody starting 6 or 3 days after tumor injection, respectively, with CpG-ODN delivered p.t. at a dose of 20 μg/mouse 5 days/week, alone or in combination. Control group was left untreated. For anti-PD-1

$F(ab)_2$ *in vivo* experiment, mice injected with H460 cells were treated with 200 μg of anti-mouse PD-1 $F(ab)_2$ fragments, as described above. Tumor mass (mm^3) was measured with a caliper twice a week and volume was calculated as [long diameter × (short diameter)^2/2].

For *in vivo* studies in immunocompetent models, C57BL/6 and Balb/c mice were s.c. injected with 10^6 B16 melanoma cells or 4T1 breast cancer cells, respectively. Mice were treated as described for H460 experiments.

FLOW CYTOMETRY

Healthy athymic nude mice were treated with CpG-ODN for 4 days or left untreated. In other experimental setting, nude mice were xenografted with IGROV-1 cells. At the end of treatment or seven days after tumor cells injection, flow cytometry analysis was performed on splenocytes in order to evaluate the expression of PD-1. Splenocytes were obtained as described *(Calcaterra C et al, 2008, J Immunol)*. Cell suspensions (10^6 cells) were incubated for 30 minutes at 4°C in staining solution (PBS containing 1% BSA) with directly-conjugated antibodies mix [CD45 FITC (eBioscience, 30-F11), CD49b PE (Miltenyi, DX5), PD-1 APC (eBioscience, RMP1-30) for the identification of NK cells; CD45 FITC (eBioscience, 30-F11), CD11b PE (BD, M1/70), F4/80 PerCp (BioLegend, BM8), PD-1 APC (eBioscience, RMP1-30) for the identification of macrophages].

To verify that RMP1-14 clone does not cross-react with human cell line, H460 cells (10^6 cells) were incubated with 10 μg/ml of anti-mouse PD-1 clone RMP1-14 in staining solution for 30 minutes at 4°C. After washing, cells were incubated with FITC-conjugated Anti-Rat IgG (H+L) Antibody, Mouse Serum Adsorbed (KPL, SeraCare Life Sciences, Milford, MA, USA) for 30 minutes at 4°C.

To test PD-1 expression on BMDMs, macrophages were incubated for 30 minutes at 4°C in

staining solution with directly-conjugated antibodies mix [CD45 FITC (eBioscience, 30-F11), CD11b PE (BD, M1/70), F4/80 PerCp (BioLegend, BM8), PD-1 APC (eBioscience, RMP1-30)].

The ability of PD-1 $F(ab)_2$ fragment to bind mouse PD-1 was assessed by flow cytometry analysis using EL-4 murine T lymphoblast cell line constitutively over-expressing PD-1 *(Oestreich KJ et al, 2008, J Immunol)*. EL-4 cells were incubated with 10 µg/ml of anti-mouse PD-1 clone RMP1-14, anti-mouse PD-1 $F(ab)_2$ or rat IgG2a isotype control for 30 minutes at 4°C. After washing, cells were incubated with FITC-conjugated Anti-Rat IgG (H+L) Antibody, Mouse Serum Adsorbed (KPL, SeraCare Life Sciences) for 30 minutes at 4°C.

To prevent non-specific binding to mouse Fc receptors, cells were also stained with purified rat anti-mouse CD16/CD32 monoclonal antibody (eBioscience). Samples were then acquired with BD LSRII Fortessa™ (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (FlowJo LCC, Ashland, OR, USA).

REAL-TIME PCR

To test if anti-PD-1 antibody has a direct effect on macrophages, bone marrow derived macrophages (6×10^5 BMDMs) were obtained from healthy nude mice, as described above. BMDMs were exposed to 10 µg/ml of anti-PD-1 antibody or isotype control for 24 hours. For co-culture experiment, macrophages were seeded in Transwell inserts (0.4 µm pores), which consists of a membrane permeable for liquids but not for cells. The Transwells were inserted into a 6-well plate previously coated with H460 cells. After 24 hours of co-culture, anti-PD-1 antibody or isotype control were added and the co-culture was maintained for other 24 hours. At the end of each treatment, mRNA from macrophages was extracted with Direct-zol RNA Kits (Zymo Research), 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) with a StepOne Real-Time PCR System (Applied Biosystems), with the Arg1 (Mm00475988_m1) TaqMan® gene expression assays (Applied Biosystems). Gene expression was normalized to B2M (Mm00437762_m1).

PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENCE ANALYSIS

Immunohistochemical and immunofluorescence analyses were performed according to the protocols described in Lo Russo et al. *(Lo Russo G et al, 2018, Clin Cancer Res).*

CLINICAL SERIES

Patient's characteristics are described in Lo Russo et al. *(Lo Russo G et al, 2018, Clin Cancer Res).*

STATISTICAL ANALYSIS

Statistical analyses of the results were performed using either unpaired t-test or Mann-Whitney U test, depending on data distribution, and were considered to be significant at p-values<0.05. Analyses were performed using Graph Pad Prism (GraphPad Software, San Diego, CA, USA).

RESULTS

1. Tumor cells and TLR9 agonist enhance PD-1 expression on NK cells but not on macrophages

Athymic nude mice lack T lymphocytes, therefore the only cells able to mediate cytotoxic activity against tumors are NK cells and macrophages *(Belizário JE., 2009, The Open Immunology Journal).* Increased PD-1 expression on NK cells and macrophages has been observed in cancer patients and it is correlated with stage of disease *(Liu Y et al, 2017, Oncogene; Gordon SR et al, 2017, Nature; Pesce S et al, 2017, J Allergy Clin Immunol).* To evaluate if we could made the same observation in our preclinical model, athymic nude mice were intraperitoneally (i.p.) xenografted with IGROV-1 human ovarian cell line and, seven days after tumor cell injection, mice were sacrificed and flow cytometry analysis was performed. We found that the frequency of splenic PD-1^+ NK cells (identified as $CD49b^+$ cells) of tumor-bearing mice was higher compared to control mice [PD-1^+ $CD49b^+$ NK cells: 48.2 ± 8.0% (mean±SEM) in tumor-free mice vs 79.8 ± 5.97% in IGROV-1 tumor-bearing mice, p=0.0366 by unpaired t-test] with a significant up-regulation of PD-1 expression on NK cells of tumor bearing mice [PD-1 MFI: 1176.9 ± 226.1 (mean±SD) in tumor-free mice vs 2459.5 ± 183.5 in IGROV-1 tumor-bearing mice, p=0.0141 by unpaired t-test] ***(Figure 8A)***. No significant modulation of PD-1 expression was detected in peritoneal macrophages.

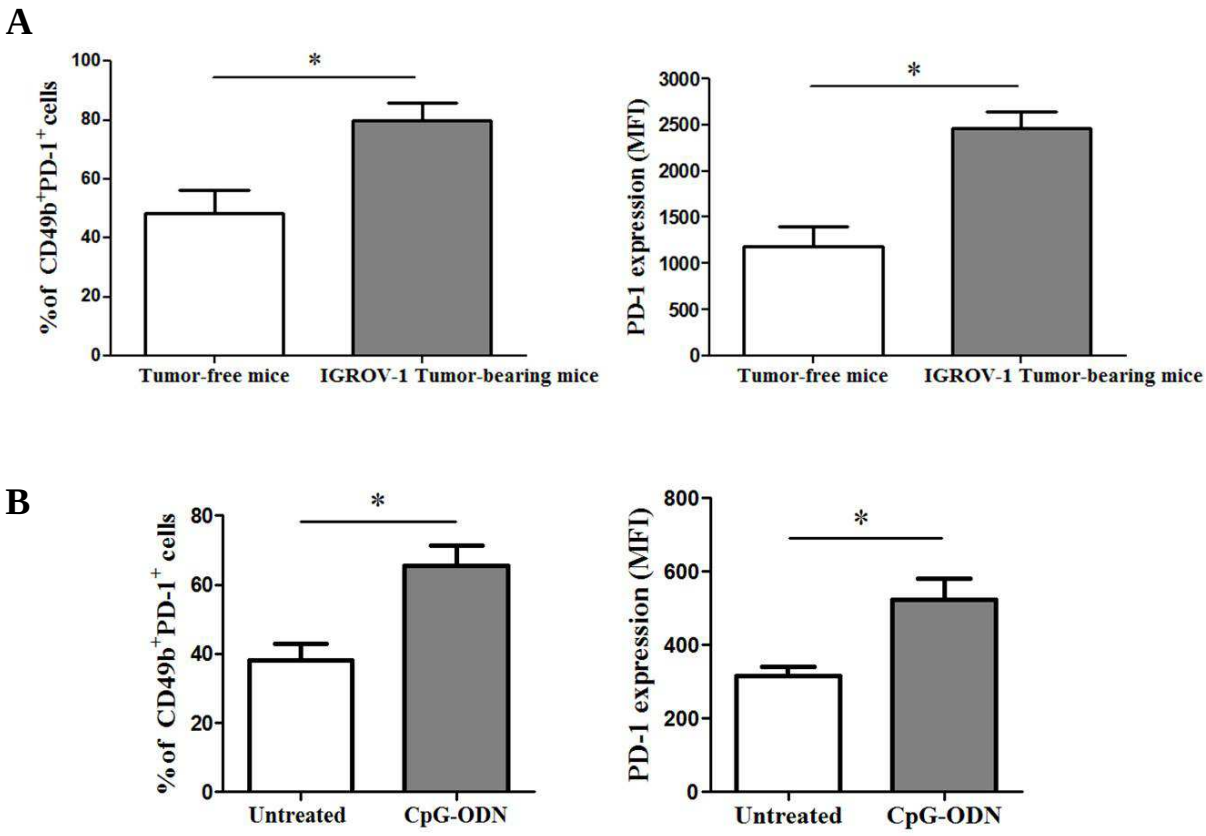

Figure 8. Percentage and MFI of splenic PD-1$^+$ NK cells (identified as CD49b$^+$ cells) of athymic mice IGROV-1 xenografted or tumor-free mice (**A**). Percentage and MFI of splenic PD-1$^+$ NK cells (identified as CD49b$^+$ cells) of healthy athymic mice treated with CpG-ODN for 4 days (20 µg/mouse) or left untreated (**B**). *p<0.05 by unpaired t-test.

Although CpG-ODN, agonist of TLR9, is reported to exert anti-tumor activity *(Mikulandra M et al, 2017, Curr Med Chem)*, it is also known that *in vitro* TLR9 engagement on NK cells and macrophages can induce PD-1 up-modulation as negative feedback mechanism *(Terme M et al, 2011, Cancer Res; Said EA et al, 2010, Nat Med)*. We therefore investigated if CpG-ODN treatment can determine the same effect *in vivo*. Flow cytometry analysis of spleen NK cells obtained from athymic mice i.p. treated for 4 days with CpG-ODN (20 µg/mouse) revealed an increased percentage of PD-1$^+$ NK cells (identified as above), as compared to spleen NK cells from untreated mice [PD-1$^+$ CD49b$^+$ NK cells: 38.2 ± 4.77% (mean±SEM) in untreated mice vs 65.57 ± 5.87% in CpG-ODN treated mice, p=0.0224 by unpaired t-test]. The average of MFI of PD-1 expression was 315.0 ± 25.2 from control mice and 523.7 ± 58.7 on NK cells from spleens

of CpG-ODN treated mice (p=0.0309) ***(Figure 8B)***. No significant modulation of PD-1 expression was detected in macrophage subset.

These results indicate that the presence of a growing tumor as well as the CpG-ODN treatment induce an increase expression of PD-1 on NK cells.

2. CpG-ODN anti-tumor activity decreases when combined with anti-PD-1 antibody in ovarian cancer xenograft model

In order to evaluate the contribution of the innate immune system during immune checkpoint inhibition therapy, IGROV-1 human ovarian cancer cell line was injected i.p. in athymic nude mice. IGROV-1 tumor-bearing mice were subsequently divided into 5 groups and treated starting 7 days after tumor cells injection with CpG-ODN alone for 5 days per week or in combination with anti-PD-1 antibody (clone RMP1-14) at different time points (starting at day 7 or at day 13, twice weekly) or with anti-PD-1 antibody alone starting at day 7. The treatment lasted 3 weeks. Untreated mice represented the control group ***(Figure 9)***. Since IGROV-1 cells grow either as floating cells in the peritoneum and as small tumor masses adhering to the peritoneal wall and since these cells eventually induce the formation of ascitic fluid, we monitored ascites development as indicator of tumor growth.

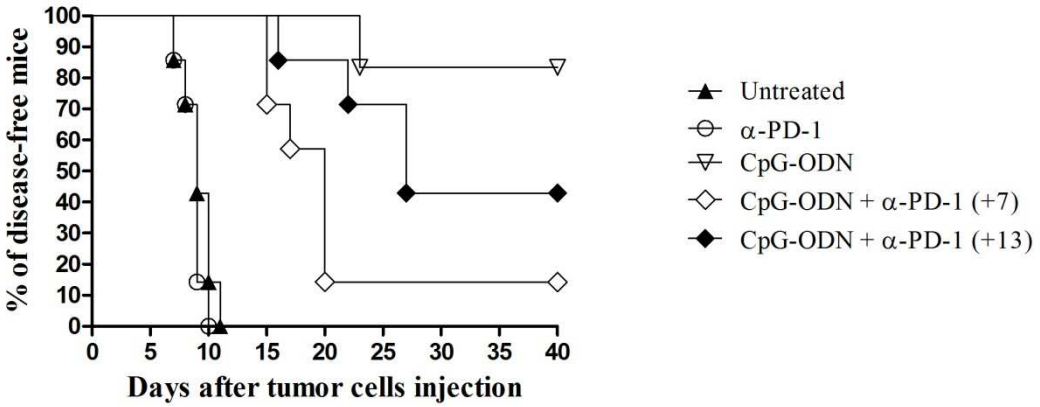

Figure 9. Kaplan-Meier plot of percent of disease-free mice over time among IGROV-1 ovarian tumor-bearing athymic mice. Mice were treated i.p. with CpG-ODN (starting at day 7; 20 µg /mouse, 5 days/week; n=6 mice/group) or with anti-mouse PD-1 antibody (starting at day 7; 200 µg/mouse, 2 days/week; n=6 mice/group) as single agents or with their combination, starting anti-PD-1 antibody at day 7 (n=7 mice/group) or at day 13 (n=7 mice/group). Control group were left untreated (n=7 mice/group).

As expected, CpG-ODN treatment alone significantly delayed ascites appearance compared to control mice *(De Cesare M et al, 2010, J Immunother; Sommariva M et al, 2011, Cancer Res; Sommariva M et al, 2013, J Transl Med).* Anti-PD-1 antibody as single agent did not produce any therapeutic effect compared to control group. Surprisingly, when CpG-ODN was combined with anti-PD-1 antibody, a strong reduction in TLR9 agonist anti-tumor activity was observed [CpG-ODN vs CpG-ODN+α-PD-1(+7), p=0.0079 by log-rank test]. Moreover, we obtained similar results even when we modified the schedule treatment starting CpG-ODN treatment 5 days after tumor cells injection alone or in combination with anti-PD-1 antibody, starting at day +8 ***(Figure 10A)***. To exclude that the inhibitory effect of anti-PD-1 antibody on CpG-ODN activity was not due to a non-specific biological activity of the RMP1-14 clone, we performed an additional experiment, similar to those described above, using another commercial anti-mouse PD-1 blocking antibody (hamster anti-mouse PD-1, clone J43) ***(Figure 10B)***. Again, anti-PD-1 antibody clone J43 alone did not impact on ascites appearance and, most importantly, reduced CpG-ODN anti-tumor activity.

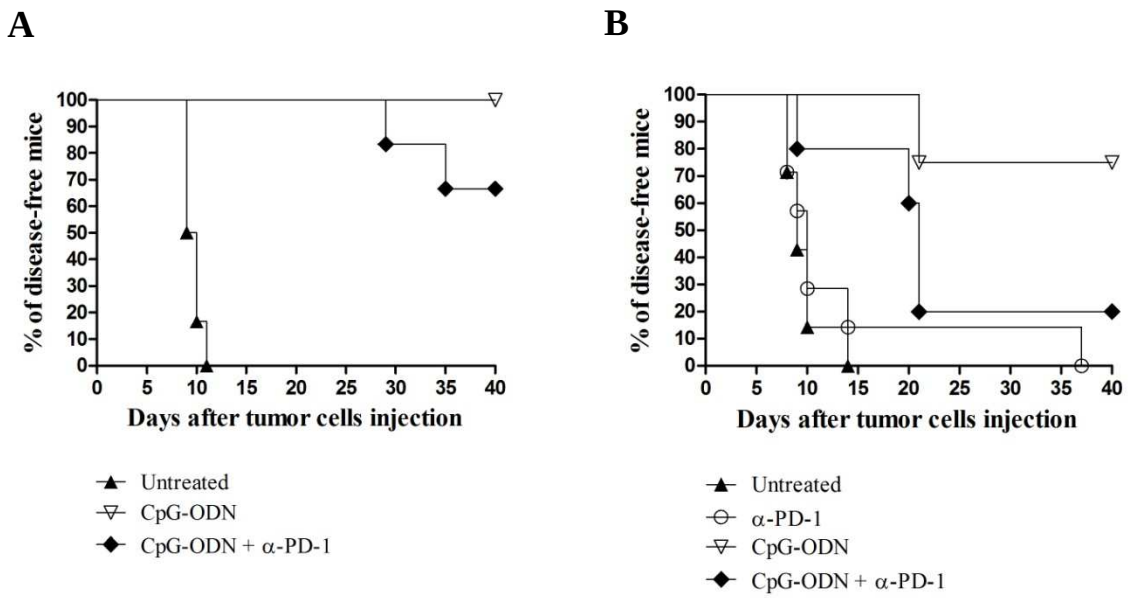

Figure 10. Kaplan-Meier plot of percent of disease-free mice over time among IGROV-1 ovarian tumor-bearing mice treated i.p. with anti-PD-1 antibody, clone RMP1-14 (**A**) or with anti-PD-1 antibody, clone J43 (**B**). Mice were treated with CpG-ODN (20 µg/mouse, 5 days/week), with anti-PD-1 antibody, clone RMP1-14 or clone J43 (200 µg/mouse, 2 days/week) or with the combination of the two agents. Control group were left untreated.

These results suggest that in mice, where the adaptive immune cells are not present, anti-PD-1 antibody not only did not synergize with TLR9 agonist but also exert a negative effect on CpG-ODN therapeutic efficacy.

3. Anti-mouse PD-1 antibody does not have any detrimental effect on CpG-ODN anti-tumor activity in immunocompetent mice

In immunocompetent mice, it has been proposed that CpG-ODNs-based immunotherapy may benefit from combination with immune checkpoints blockers *(Sato-Kaneko F et al, 2017, JCI Insight*; *Wang S et al, 2016, Proc Natl Acad Sci U S A; Wang C et al, 2016, Adv Mater).* However, we observed that in immunodeficient mice the ability of TLR9 agonist to exert antitumor effects is affected by the combination with anti-PD-1 antibody. To investigate if the

same phenomenon can occur even in the presence of adaptive immunity, C57BL/6 and Balb/c mice were injected subcutaneously (s.c.) with B16 melanoma ***(Figure 11A)*** and 4T1 breast carcinoma cell lines ***(Figure 11B)*** respectively, and treated with peritumoral (p.t.) CpG-ODN alone or with anti-PD-1 antibody. Even if no additive effect was observed when TLR9 agonist were combined with anti-PD-1 antibody, the combination group did not show an increase in tumor growth, indicating that the innate-immunity related detrimental effect might be hidden when the adaptive immune system is present.

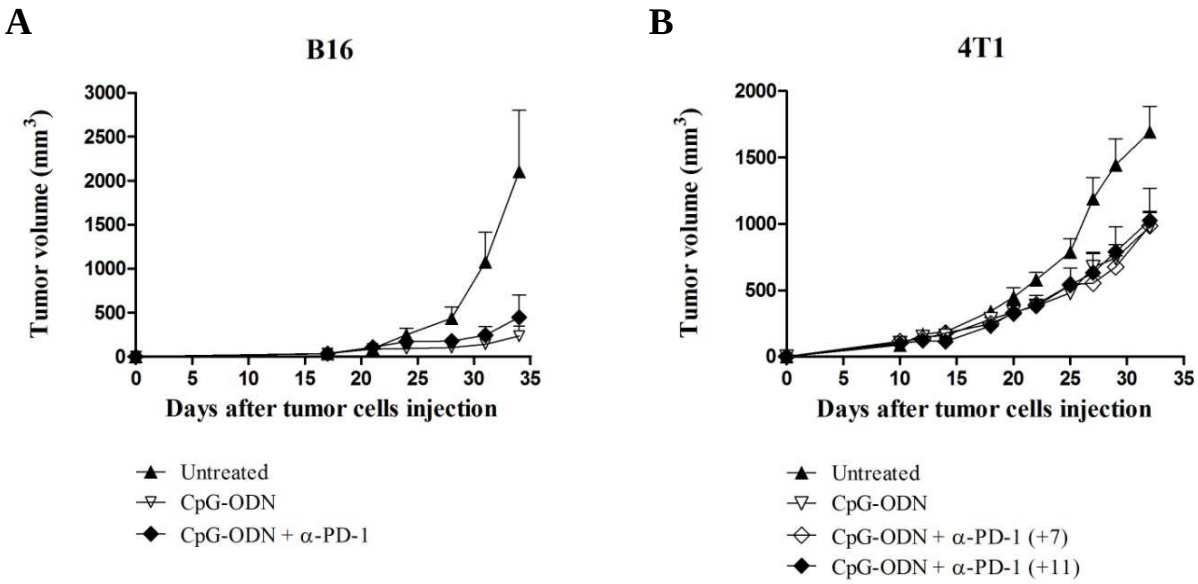

Figure 11.
(**A**) C57BL/6 mice xenografted s.c. with B16 melanoma cells and treated with CpG-ODN (20 µg/mouse, 5 days/week; n=4 mice/group) alone or with anti-PD-1 antibody (200 µg/mouse, 2 days/week; n=4 mice/group) starting 21 days after tumor cells injection. Control group were left untreated (n=4 mice/group). Dots represent mean±SEM of tumor volume for each group.
(**B**) Balb/c mice xenografted s.c. with 4T1 breast carcinoma cells and treated with CpG-ODN alone (starting at day 10; 20 µg/mouse, 5 days/week; n=7 mice/group) or with anti-PD-1 antibody (200 µg/mouse, 2 days/week; n=7mice/group), starting at day 7 or day 11 after tumor cells injection. Control group were left untreated (n=6 mice/group). Dots represent mean±SEM of tumor volume for each group.

4. Anti-mouse PD-1 antibody induces early ascites appearance in IGROV-1 tumor bearing mice and myeloid cells depletion is able to abrogate anti-PD-1 antibody inhibitory activity on CpG-ODN anti-tumor effect

Since it has been recently reported that PD-1 blockade can sustain immunosuppression in ovarian cancer upon interaction with cells of myeloid origin *(Lamichhane P et al, 2017, Cancer Res)*, the negative effect of anti-PD-1 antibody on CpG-ODN anti-tumor activity might be due to an enhancement of an immunosuppressive microenvironment that may restrain CpG-ODN efficacy. To understand whether anti-PD-1 antibody might exert a detrimental effect *per se*, athymic nude mice xenografted with IGROV-1 were treated with anti-PD-1 antibody the day immediately after tumor cells injection when the ascitic fluid has not appeared yet. Even if not statistically significant**,** we observed that in anti-PD-1 treated group tumor ascites developed faster compared to control group ***(Figure 12)***.

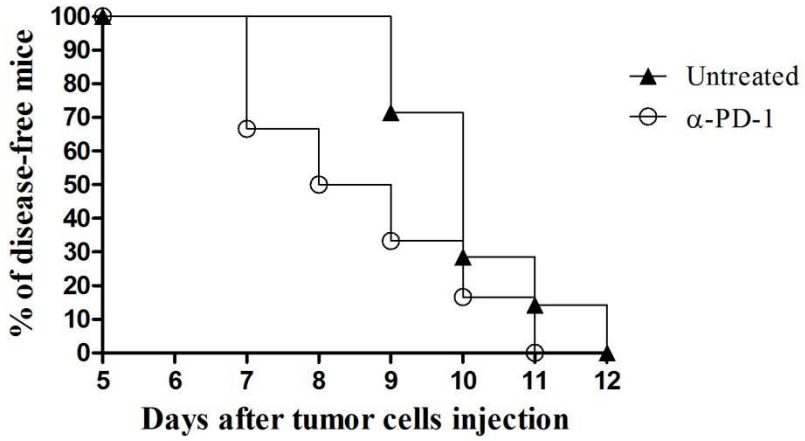

Figure 12. IGROV-1 xenograft mice were treated with anti-PD-1 antibody (200 µg/mouse, 2 days/week) starting the day after tumor cells injection. Control group were left untreated (n=4-5 mice/group).

We then performed an immunohistochemical analysis to detect the expression of Arginase I (ArgI), a marker of myeloid cells with immunoregulatory activity *(Murray PJ et al, 2014,*

Immunity), on tumor masses obtained from mice treated with anti-PD-1 antibody or control mice ***(Figure 13)***. Quantification analysis revealed a statistically significant increase in the number of $ArgI^+$ cells in the stroma of anti-PD-1 treated tumor, suggesting that the interaction of the antibody with myeloid cells may boost tumor growth.

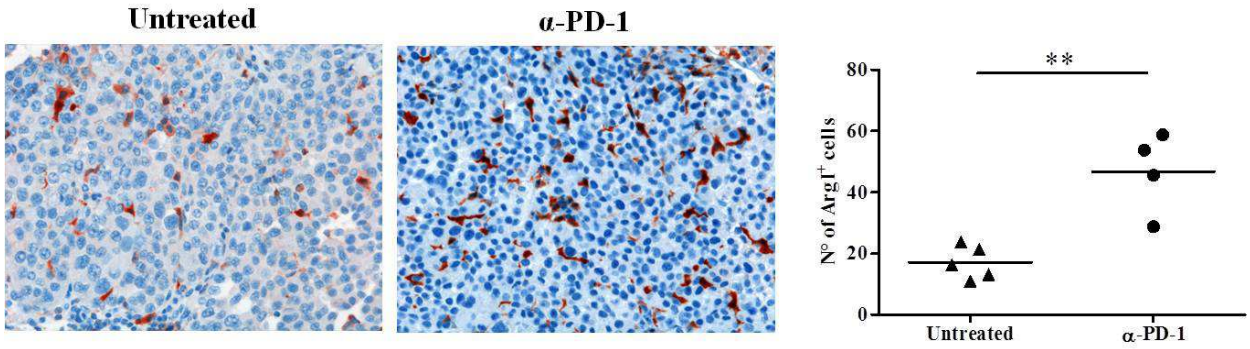

Figure 13. Representative histopathological evaluation and quantification of Arginase I-stained IGROV-1 tumor samples from mice treated with anti-PD-1 antibody or untreated mice. Original magnification X20. **p<0.01 by unpaired t-test.

To further investigate the involvement of myeloid cells in worsening CpG-ODN activity, we *in vivo* depleted this immune cell population using liposomes containing clodronate, reported to deplete cells with phagocytic activity such as myeloid cells *(Van Rooijen N, Sanders A. 1994, J Immunol Methods)*, in IGROV-1 xenograft model ***(Figure 14)***. Three days after tumor cells injection, IGROV-1 tumor bearing mice were treated with clodronate and three days after clodronate injection we treated mice with CpG-ODN or anti-PD-1 antibody or with their combination, as above. While there were no differences between anti-PD-1 treated group and control mice, as observed in previous experiments, depletion of myeloid cells abrogated the inhibitory activity of anti-PD-1 antibody on CpG-ODN antitumor effect. These results may suggest that the interaction between anti-PD-1 antibody and myeloid cells could be involved in the dampening of CpG-ODN therapeutic activity.

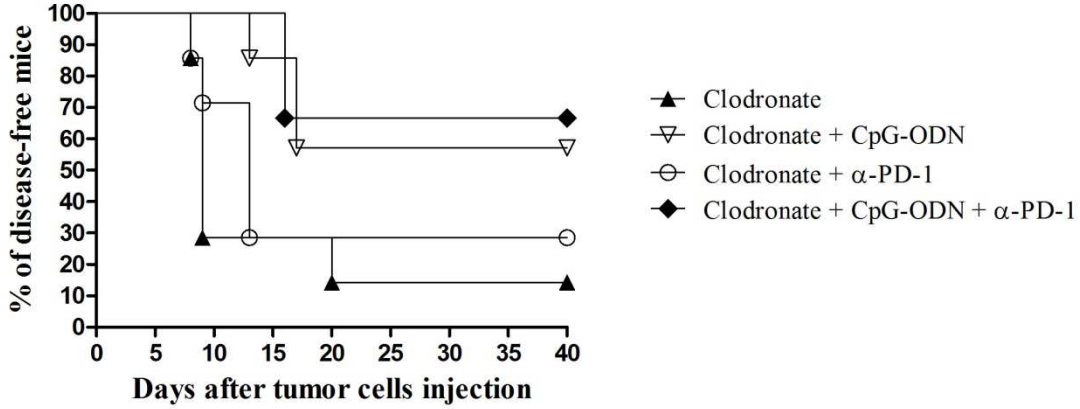

Figure 14. Kaplan-Meier plot of percent of disease-free mice over time among IGROV-1 ovarian tumor-bearing mice treated with clodronate 3 days after tumor cells injection and subsequently with CpG-ODN (starting at day 6; 20 µg/mouse, 5 days/week) in combination with anti-PD-1 antibody (starting at day 6; 200 µg/mouse, 2 days/week) (n=6 mice/group) or with each reagent alone (n=7 mice/group). Control group received only clodronate liposomes treatment (n=7 mice/group).

5. Anti-mouse PD-1 antibody induces tumor progression in non-small cell lung cancer (NSCLC) xenografts

Since our results suggest a possible involvement of innate immune cells in mediating the negative effect of anti-PD-1 antibody, we tested our hypothesis in another xenograft model different in term of tumor hystotype and site of tumor implantation. NCI-H460 human NSCLC cell line was injected s.c. in athymic nude mice and treated using two different schedules of treatment and two routes of administration. Specifically, H460 tumor-bearing mice were treated with anti-PD-1 antibody either i.p. ***(Figure 15A)*** or p.t. ***(Figure 15B)***, starting at day +6 and +3 after tumor cells injection respectively, alone or in combination with CpG-ODN. Single agents were also included and control mice were left untreated.

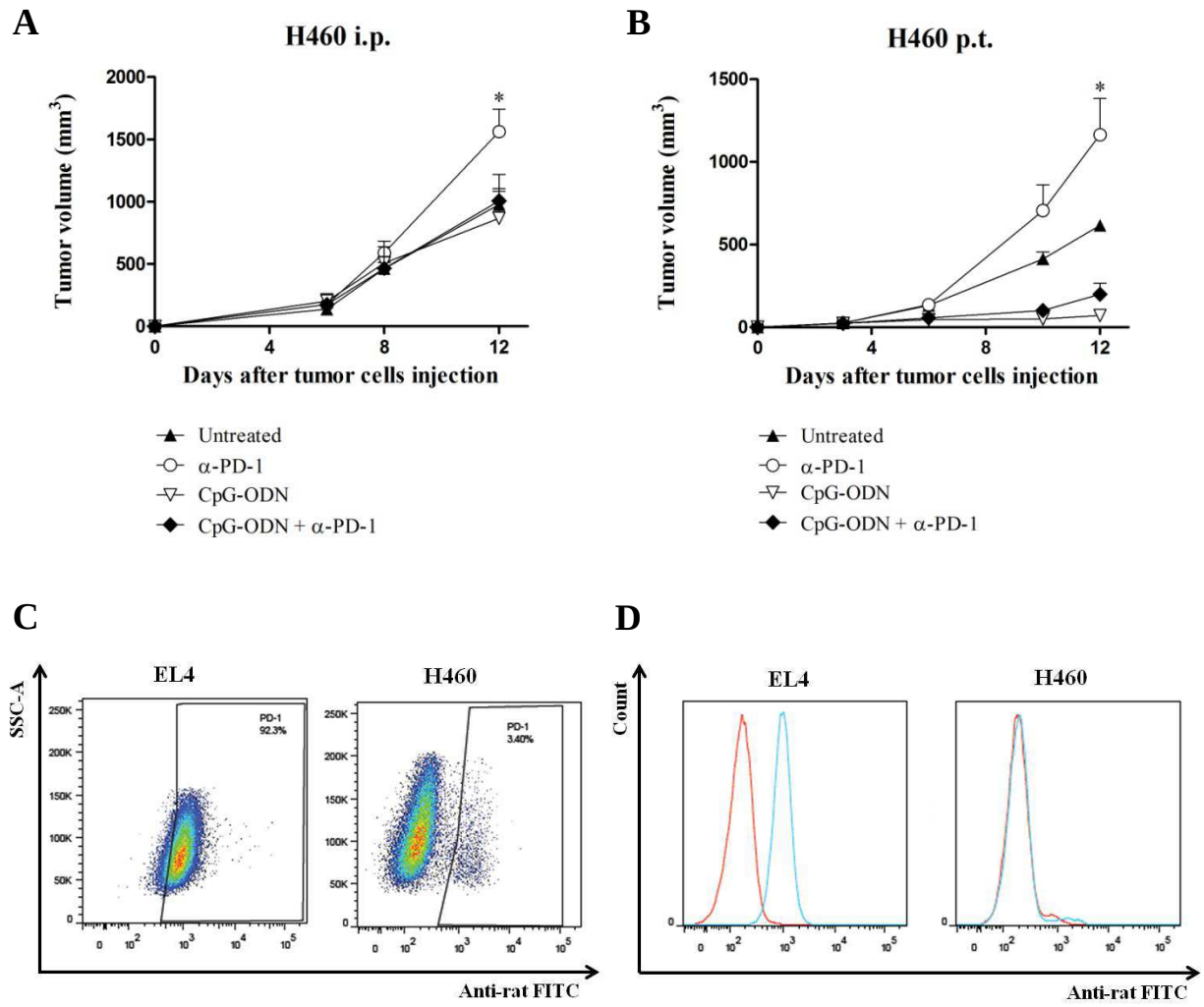

Figure 15. Mice were xenografted s.c. with H460 lung cancer cell line and treated i.p., starting from 6 days after tumor cells injection (n=6 mice/group) (**A**) or p.t., starting from 3 days after tumor cells injection (n=5 mice/group) (**B**) with anti-mouse PD-1 antibody (200 µg/mouse, 2 days/week), CpG-ODN (20 µg/mouse, 5 days/week) or both. Control group were left untreated. Dots represent mean±SEM of tumor volume for each group. Untreated vs α-PD-1, *p<0.05 by unpaired t-test.
Dot plots (**C**) and MFI (**D**) of EL-4- and H460-PD-1 positive cells stained with anti-rat FITC secondary antibody (red line) after anti-PD-1 staining with anti-PD-1 antibody clone RMP1-14 (blu line).

The results obtained were similar to those obtained in IGROV-1 model. Indeed, the treatment with anti-PD-1 antibody slightly reduced the antitumor effect of TLR9 agonist and, notably, anti-PD-1 treatment alone induced a marked increase in tumor growth compared to all the other groups regardless of route and schedule of treatment.

It has been recently reported that PD-1 can be expressed by NSCLC cells and that PD-1 blockade accelerated tumor growth of M109 (mouse lung cancer cell line) xenograft model in immunodeficient mice *(Du S et al, 2018, Oncoimmunology)*. To verify whether the increase in tumor mass following anti-PD-1 antibody administration could be due to the interaction between our antibody and PD-1 expressed by tumor cells, we tested the cross-reactivity of the rat anti-mouse clone RMP1-14 with human lung cancer cells H460 ***(Figure 15C and 15D)***. Flow cytometry analysis revealed that anti-mouse PD-1 antibody does not react with H460 cell line but specifically recognizes murine PD-1 (using EL-4 mouse lymphoma T cells as positive control) indicating an involvement of innate immune system in determining the deleterious effect associated with PD-1 treatment.

We then analyzed the tumor microenvironment of samples collected from anti-PD-1 antibody-treated mice and controls. We evaluated by immunohistochemistry the immune cell infiltrate considering the total leukocytes ($CD45^+$), macrophages ($F4/80^+$), B lymphocytes ($CD45R/B220^+$), granulocytes ($Gr\text{-}1^+$) and NK ($NKp46^+$) cells ***(Figure 16)***. There was a significant increase in $CD45^+$ leukocyte infiltration at the host–tumor interface in tumors of mice treated with anti-PD-1 antibody. While B lymphocytes, granulocytes, and NK cells density was comparable among the two groups, it has been observed a high number of intratumoral macrophages and $ArgI^+$-expressing cells in anti-PD-1 treated tumors explaining the increase in the $CD45^+$ population.

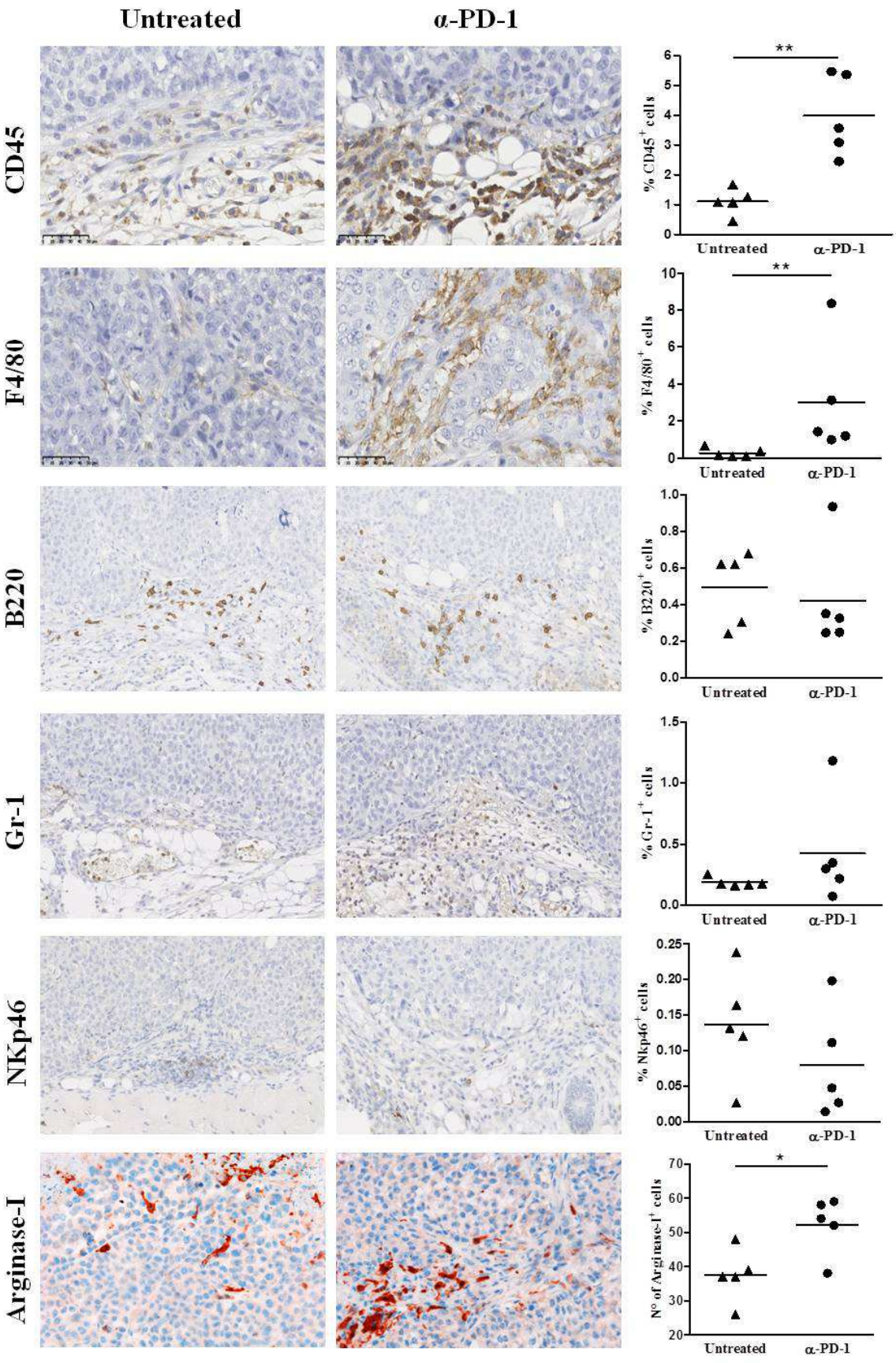
Untreated
α-PD-1
CD45
F4/80
B220
Gr-1
NKp46
Arginase-I
% CD45+ cells
% F4/80+ cells
% B220+ cells
% Gr-1+ cells
% Nkp46+ cells
N° of Arginase-I+ cells
**
**
*
Untreated
α-PD-1

Figure 16. Representative immunohistochemistry images and quantification of leukocytes ($CD45^+$), macrophages ($F4/80^+$), B lymphocytes ($CD45R/B220^+$), granulocytes ($Gr\text{-}1^+$), NK ($NKp46^+$) cells and Arginase-I^+ cells in the tumor microenvironment of H460 lung cancer xenograft mice (p.t. experiment). Original magnification X20. *p<0.05, **p<0.01 by Mann-Whitney U test.

6. FcR triggering on macrophages might induce tumor progression after anti-PD-1 treatment

The data showed before point to a role of anti-PD-1 antibody-myeloid cells interaction in the observed exacerbation of the disease. Tumor-associated macrophages (TAMs) and myeloid cells are reported to express PD-1 on their surface and blocking PD-1 on these cells can restore their antitumor functions *(Gordon SR et al, 2017, Nature; Liu Y et al, 2009, Cancer Immunol Immunother)*. Because we observed an increase in tumor growth after anti-PD-1 administration paralleled by an increase in myeloid cell infiltration, we may not ascribe anti-PD-1 detrimental effect to such receptor blockade but rather to the Fc domain of the antibody. To exclude a direct effect of anti-PD-1 antibody on myeloid cells, we treated bone marrow derived macrophages (BMDMs) *in vitro* with anti-PD-1 antibody. Flow cytometry analysis revealed that in our *in vitro* culture 5-10% of macrophages were positive for PD-1 ***(Figure 17A)***. BMDMs were exposed to 10 µg/ml of anti-PD-1 antibody or isotype control for 24 hours. At the end of treatment, mRNA from macrophages was extracted and real-time PCR was performed to assess the acquisition of a M2-like phenotype using the M2-related marker Arginase I, found to increase after anti-PD-1 administration *in vivo*. PD-1 treatment had no effect in inducing Arginase I mRNA in macrophages ***(Figure 17B)***. To better recapitulate the situation occurring *in vivo*, BMDMs were co-cultured with H460 cell line without direct cell-cell contact, a method to induce M2 phenotype *(Hagemann T et al, 2006, J Immunol)*. After 24 hours of co-culture, anti-PD-1 antibody or isotype control were added to the culture medium and mRNA from macrophages was extracted. Real-time PCR was performed as described above ***(Figure 17C)***. Again, no differences in terms

of Arginase I mRNA level were found between anti-PD-1- or isotype-control-treated macrophages, suggesting that the binding of the antibody to PD-1 on macrophages is not responsible for the reported anti-PD-1 detrimental effect.

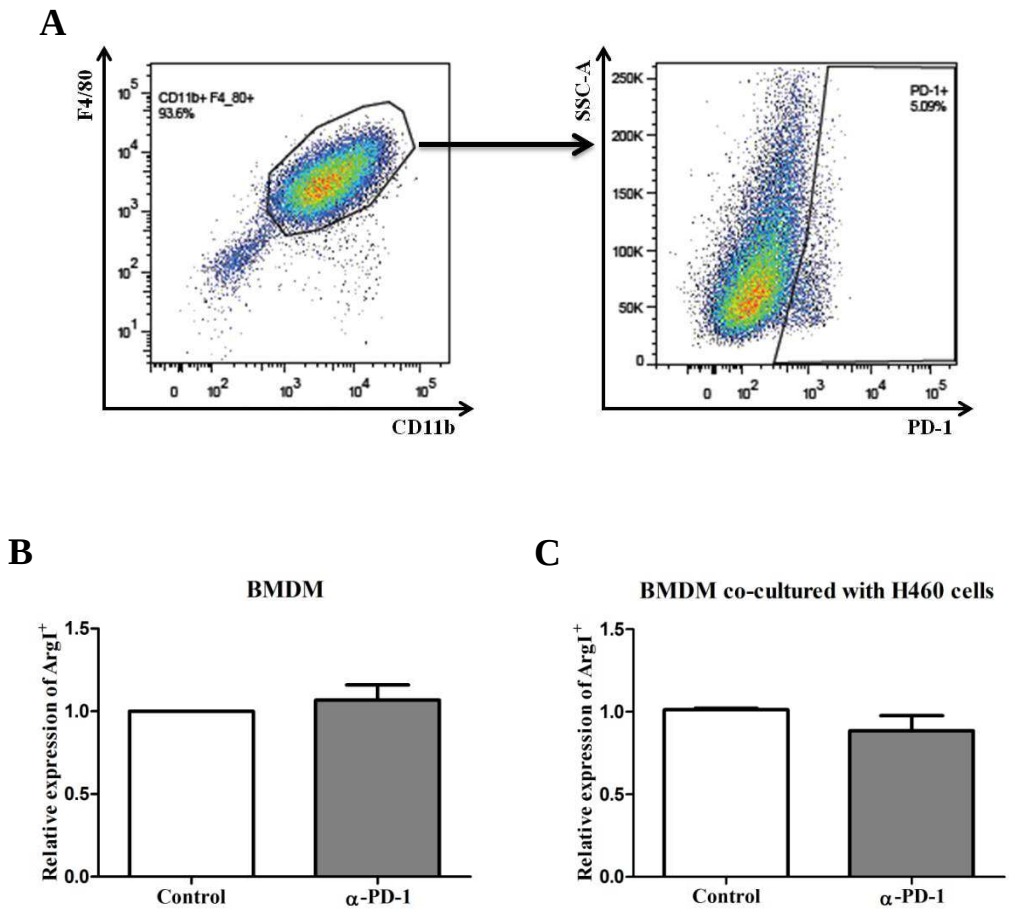

Figure 17. (**A**) Flow cytometry analysis of BMDM (gated on $CD11b^+$ $F4/80^+$ cells) analyzed for PD-1 expression (5,09%). mRNA levels of ArgI evaluated by real-time PCR in BMDM alone (**B**) or co-cultured with H460 cell line (**C**) treated with isotype control (10 µg/ml) or anti-PD-1 antibody (10 µg/ml) for 24 hours. Results are presented as $2^{-\Delta\Delta Ct}$.

Based on our previously described data, we can hypothesize that anti-PD-1 antibody may exert its biological activity through its Fc domain. Indeed, Dahan et collegues reported that the anti-PD-1 Fc domain modulates the anti-tumor activity of anti-PD-1 antibodies *(Dahan R et al, 2015, Cancer Cell)*. Therefore, we performed an *in vivo* experiment in H460 xenografted mice using anti-PD-1 $F(ab)_2$, instead of the whole antibody. $F(ab)_2$ is a portion of antibody that lacks the Fc

domain loosing the ability to bind any Fc receptors. We confirmed by FACS analysis that anti-PD-1 $F(ab)_2$ is able to efficiently bind PD-1 ***(Figure 18A)***. Then, H460 tumor bearing mice were treated two times/week with p.t. anti-PD-1 $F(ab)_2$ or left untreated starting 3 days after tumor cells injection ***(Figure 18B)***. The increase in tumor growth seen in our previous experiments is lost when using the anti-PD-1 $F(ab)_2$.

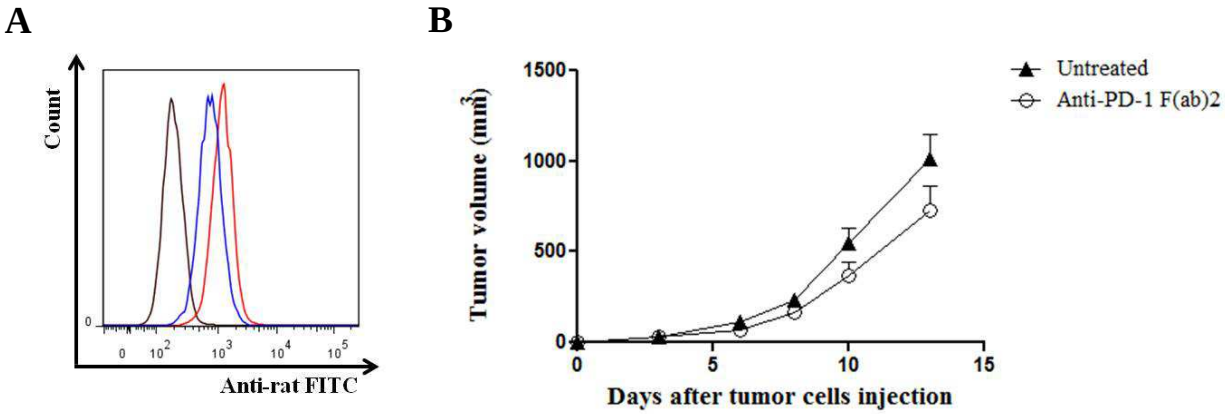

Figure 18. (**A**) Flow cytometry analysis of EL-4 cells, expressing high levels of PD-1, stained with the whole antibody (red line) or with anti-PD-1 $F(ab)_2$ (blue line). Rat IgG2a served as isotype control (black line).
(**B**) Mice were xenografted s.c. with H460 lung cancer cell line and treated p.t. with anti-PD-1 $F(ab)_2$ from 3 days after tumor cells injection (200 µg/mouse, 2 days/week; n=7 mice/group). Control group were left untreated. Dots represent mean±SEM of tumor volume for each group.

Collectively, these data indicate that the increased tumor growth observed after anti-PD-1 treatment in our experimental model may be due to a "non-conventional" response of myeloid cells to anti-PD-1 antibody through Fc-Fc receptor interaction causing their functional reprogramming toward a pro-tumorigenic phenotype.

7. ICI treatment of patients with advanced NSCLC induces acceleration of tumor growth

Our preclinical results recall data published in recent publication *(Champiat S et al, 2017, Clin Cancer Res; Saâda-Bouzid E et al, 2017, Ann Oncol; Kato S et al, 2017, Clin Cancer Res)* and case reports *(Davar D et al, 2015, Case Rep Oncol Med; Chubachi S et al, 2016, Case Rep Oncol Med)* describing a fraction of cancer patients experiencing hyperprogressive disease after immune checkpoint inhibitors therapy, such as anti-PD-1 antibody, with a particularly aggressive, unpredictable spread of the disease and clinical deterioration *(Champiat S et al, 2017, Clin Cancer Res; Saâda-Bouzid E et al, 2017, Ann Oncol; Kato S et al, 2017, Clin Cancer Res).* To date, a convincing explanation of this phenomenon has not been provided.

Thanks to a collaboration with the Department of Medical Oncology of Fondazione IRCCS - Istituto Nazionale dei Tumori (Milan, Italy), tumor samples of 35 patients with advanced NSCLC who received treatment with ICIs were analyzed by immunohistochemistry to evaluate the immune cell infiltrate, as performed on mouse tumor specimens. Patients were classified according to clinical and radiological criteria ***(Table 2)*** into 4 categories: responders (R, 7 patients), patients with stable disease (SD, 11 patients), progressors (P, 6 patients) and patients with hyperprogressive disease (HP, 11 patients).

HP CLINICAL RADIOLOGICAL CRITERIA
Time-to-treatment failure (TTF) less than 2 months
At the first radiological evaluation increase in sum of target lesions diameters of 50% or more
At the first radiological evaluation appearance of at least two new lesions in an organ already affected by the disease
At the first radiological evaluation spread of the disease to a new organ
Clinical deterioration with decrease in ECOG performance status ≥ 2 in the first 2 months of treatment

Table 2. Patients who fulfilled at least 3 of the clinical/radiological criteria were defined HP, while patients showing RECIST 1.1 progressive disease (PD) as best response without fulfilling at least 3 criteria were defined as progressive patients (P). Responders (R) and stable (SD) patients were classified according with RECIST 1.1 criteria.

The composition of tumor microenvironment and the distribution of tumor-infiltrating immune elements before ICI treatment were assessed. No significant differences were observed in the subsets of tumor-infiltrating T lymphocytes (TILs), considering $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes and $FOXP3^+$ regulatory T cells (Tregs). Moreover, there were no differences in the number of other immune subpopulations, $CD138^+$ plasma cells (PCs), $CD123^+$ plasmacytoid dendritic cells (pDCs), peritumoral and stromal myeloperoxidase $(MPO)^+$ myeloid cells, $CD163^+$ macrophages, $CD33^+$, PD-1 and PD-$L1^+$ immune cells.

Notably, within neoplastic foci dense clusters of macrophages with epithelioid morphology were identified. These cells were found positive for the simultaneous expression of CD163, CD33 and PD-L1 ***(Figure 19)***. This particular phenotype was defined "complete immunophenotype" and was observed in all patients with HP, with statistically significance if compared to patients without HP ($p<0.0001$) ***(Table 3)***.

These data point again to a role for myeloid cells in accelerating tumor growth in patients suffering hyperprogressive disease after anti-PD-1 treatment.

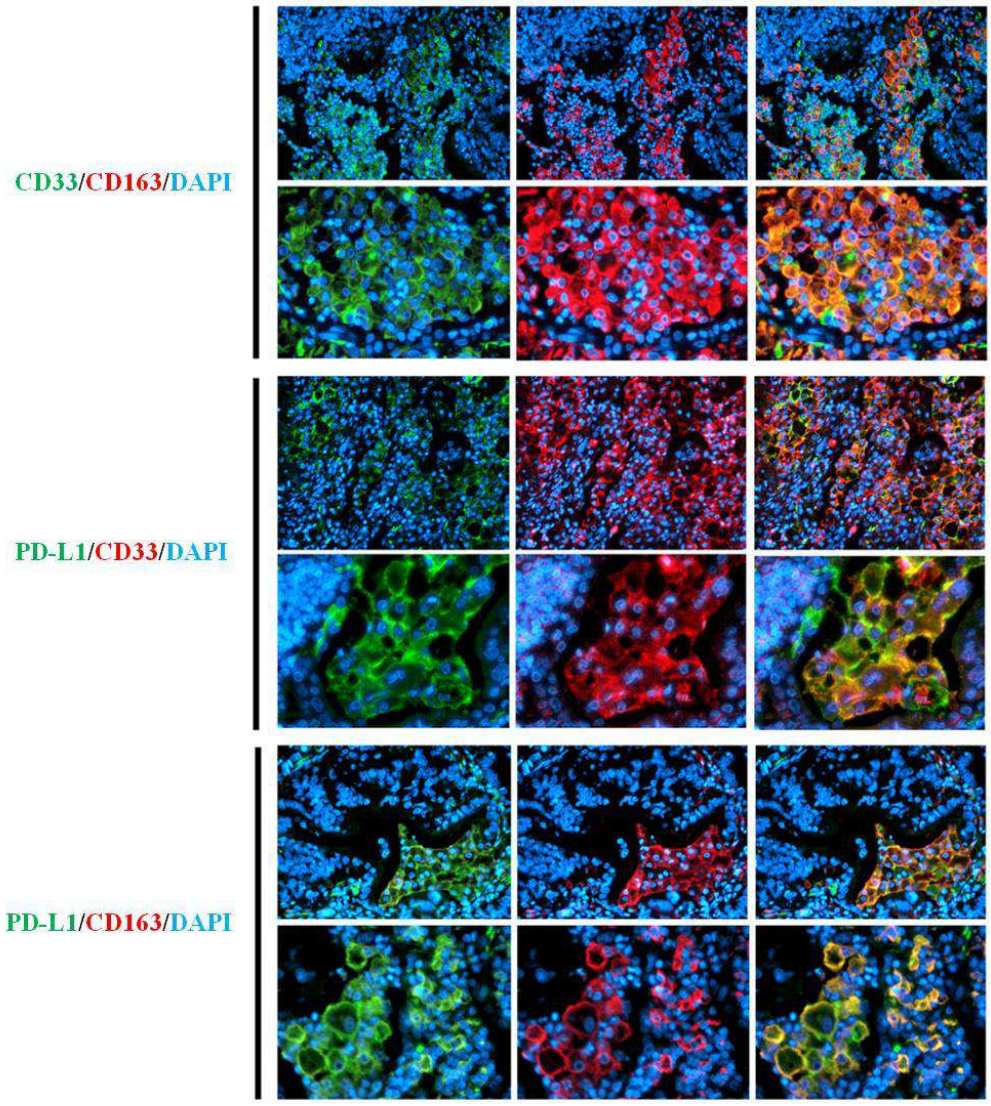

Figure 19. Representative images of prototypical hyperprogressive disease infiltrates showing the co-localization of CD33, CD163 and PD-L1 in clustered macrophages with epithelioid morphology. Immunofluorescence panels show three different combinations of double-marker stainings. Green and red signals correspond to Opal-520 and Opal-620 fluorophores; original magnifications X100 and X400.

Variables	TRUE +	FALSE +	LR+	95% CI	p-value
Epithelioid morphology	11/11	9/24	2.67	1.59- 4.47	0.0006
PD-L1	11/11	10/23	2.30	1.44- 3.66	0.0109
CD163	11/11	13/24	1.85	1.28- 2.67	0.0075
CD33	11/11	14/24	1.71	1.22- 2.40	0.0125
Complete phenotype	11/11	4/24	6.00	2.45-14.68	< 0.0001

Table 3. Macrophage markers and "complete phenotype" associate with HP patients (PD-L1: Programmed death-ligand 1; CI: Confidence Interval; LR: Likelihood ratio)

DISCUSSION

Cancer immunotherapy, enhancing the power of the immune system, is nowadays the most promising way to fight cancers because of its capability to achieve complete, long-lasting remission and, eventually, cure cancer.

Especially in the last years, the discovery of immune checkpoint inhibitors (ICIs) revolutionized the treatment of oncologic patients and profoundly changed the paradigm of care for different kind of tumors. Among ICIs, antibodies targeting PD-1/PD-L1 axis were the first approved by FDA for use in humans and results of past and ongoing clinical trials demonstrated anti-tumor activity in different clinical settings. Despite the enthusiasm of the beginning, it is now emerging that only a fraction of patients experienced durable antitumor responses and long-term remission *(Chinai JM et al, 2015, Trends Pharmacol Sci)*, while the majority remains unresponsive. Therefore, enhancing ICIs efficacy through the development of new immune-therapeutic interventions represents an unmet clinical need.

Programmed cell death-1 (PD-1) is a cell surface receptor that modulates the activity of effector T cells. Interaction of PD-1 with either of its two known ligands, PD-L1 and PD-L2, results in inhibition of T cell activation, proliferation, cytokine production and in T lymphocyte exhaustion. Since ICIs prevent immune cell exhaustion mainly acting on adaptive immune cells, it is reasonable to hypothesized that innate immunity stimulants would improve ICIs antitumor efficacy by activating innate immune cells that, in turn, are reported to promote anti-tumor T cell activity.

Toll-like receptors (TLRs) are best-known for their ability to activate innate immune cells recognizing pathogen- and danger-associated molecular patterns. TLR agonists are included in the NCI list of immunotherapeutic agents with the highest potential to cure cancer. Innate immune responses triggered by TLR agonists include the activation of NK cells, macrophages, neutrophils, monocytes and dendritic cells, most of which must be activated locally, unlike cells of the adaptive immune response, which can reach the antigen wherever they are activated.

Previous studies performed in the laboratory where I conducted my PhD thesis showed that intratumoral (i.t.) administration of the TLR9 agonist CpG-ODN was able to control tumor growth by activation of innate immune responses of IGROV-1 human ovarian carcinoma cell line orthotopically xenografted in athymic nude mice *(De Cesare M et al, 2010, J Immunother; Sommariva M et al, 2011, Cancer Res; Sommariva M et al, 2013, J Transl Med)*, pointing to the importance of innate effector cell activation at the site of tumor growth. Since we could not achieve a complete response and, eventually, mice succumbed, we explored the biological causes underlying this phenomenon. Interestingly, we found that *in vivo* CpG-ODN administration led to PD-1 up-regulation on NK cells ***(Figure 8)***. Our data are in line with other reports showing that PD-1 expression can be up-regulated by TLR ligands of bacterial origin in other innate immune cells such as monocytes *(Said EA et al, 2010, Nat Med)*, macrophages *(Bally AP et al, 2015, J Immunol)* and dendritic cells *(Yao S et al, 2009, Blood)*. Although the double ability of TLR ligand to stimulate immune response and, at the same time, to induce immune-suppressive mechanisms may appear counterintuitive, this may be probably due to a negative feedback mechanism necessary to maintain a correct balance during immune cell response to avoid immune cell over-activation and activation-induced cell death (AICD). Since it is reported that PD-1 can be expressed by innate immune cells and its expression is related to immune cell function impairment *(Terme M et al, 2011, Cancer Res; Said EA et al, 2010, Nat Med.)* and reduced immune-responsiveness, combinations of immunotherapies that augment the immune response, such as TLRs therapies, with anti-PD-1 antibody, are likely to be extremely effective. The blockade of PD-1 signaling may enhance the immune stimulatory effects of TLR agonists and, at the same time, TLR ligation may improve the effect of PD-1 blockade by stimulating immune cells. Our hypothesis is also supported by recent data that identified CpG-ODN as a potential candidate for combinatorial therapies with ICIs. For instance, preclinical studies demonstrated that CpG-ODNs in combination with anti-PD-1 antibody produce synergistic effect

in murine bladder cancer model compared to single agents *(Mangsbo SM et al, 2010, J Immunother)*. Moreover, it has been shown that anti-PD-1 treatment resistance can be reversed by intratumoral injection of TLR9 agonist *(Wang S et al, 2016, Proc Natl Acad Sci U S A)*. However, the role of innate immune system during PD-1 blockade therapy has never been properly evaluated and it remains matter of debate.

To investigate the contribution of innate immune system during PD-1 blockade therapy, athymic nude mice were injected with IGROV-1 human ovarian cell line and treated with CpG-ODN, a synthetic TLR9 agonist, anti-PD-1 antibody or their combination. The choice to utilize ovarian cancer bearing-athymic nude mice, instead of immunocompetent mice, relies on the fact that athymic mice are virtually devoid of T lymphocytes that may cloud the role played by PD-1/PD-L1 axis in modulating innate immune cells behavior and that ovarian cancer is generally poorly responsive to PD-1 blockade *(Gaillard SL et al, 2016, Gynecol Oncol Res Pract)* making it a good model to test new combination therapies. As expected, PD-1 monotherapy did not show any effect. When we combined anti-PD-1 antibody with CpG-ODN, we unexpectedly observed a reduction, instead of an improvement, of TLR9 agonist anti-tumor efficacy ***(Figure 9)***. Similar results were obtained using another clone of the anti-PD-1 antibody or changing the treatment schedules ***(Figure 10)***.

In our opinion, there are three possible scenarios that can explain our data: *i*) simultaneous TLR9 stimulation and PD-1 pathway blockade can lead to a NK cells exaggerated activation causing their apoptosis, *ii*) TLR9-primed macrophages induce the development of PD-1^+ NK cells where PD-1 blockade decreased NK cell effector molecules synthesis or *iii*) anti-PD-1 antibody can exert pro-tumor activity through Fc/Fc receptor interaction.

i) Immune cell homeostasis and activation are very tightly spatio-temporal regulated mechanisms. Since ICIs and immune-stimulants alter immune system biology, it is now emerging that timing and sequence of administration of these immune-drugs represent an important aspect to take into

consideration when ICIs are combined with immunotherapeutic agents. For instance, it has been demonstrated that concurrent administration of anti-PD-1 antibody with anti-OX40 *(Messenheimer DJ et , 2017, Clin Cancer Res; Shrimali RK et al, 2017, Cancer Immunol Res)* or with anti-41BB *(McKee SJ et al, 2017, Cancer Immunol Res)* reduced anti-OX40/anti-41BB therapeutic efficacy. Our experimental setting can be considered a "concurrent administration" because CpG-ODN and anti-PD-1 antibody were administered at the same day and, therefore, our study design resemble those described in the aforementioned papers. The detrimental effect exerted by PD-1 blockade was associated with a decrease of T cell proliferation and an increase of T cell apoptosis (*Messenheimer DJ et , 2017, Clin Cancer Res; Shrimali RK et al, 2017, Cancer Immunol Res; McKee SJ et al, 2017, Cancer Immunol Res)*. It is possible to hypothesize that, also in our experiments, the inhibition of a regulatory pathway together with a strong stimulation could have determined NK over-activation eventually inducing their death.

ii) PD-1 on NK cells is described as negative regulator of immune effector function and blocking this receptor enhances the immune response against tumors *(Hsu J et al, 2018, J Clin Invest; Liu Y et al, 2017, Oncogene)*. However, it has been reported that in the context of pathogen infection, PD-1 on NK cells was required for the synthesis of perforins and granzymes and that PD-1 genetic deletion or pharmacological inhibition determined an impairment of NK cell function *(Solaymani-Mohammadi S et al, 2016, J Leukoc Biol)*. The use of CpG-ODN in our models could have mimicked a bacterial infection and, based on this evidence, anti-PD-1 antibody could have negatively affected NK lytic activity.

It is unlikely that CpG-ODN can act directly on NK cells in inducing PD-1 expression as demonstrated by Beldi-Ferchiou and his group *(Beldi-Ferchiou A et al, 2016, Oncotarget)* and, indeed, sensing of TLR agonist by NK cells is still controversial. However, macrophages, that express functional TLRs, are able to trigger NK cell cytotoxicity upon TLR agonist stimulation through cytokine release and increased expression of activating NK cell ligands *(Hamerman JA*

et al, 2004, J Immunol; Le Noci V et al, 2015, Oncoimmunology; Sommariva M et al, 2017 Cell Immunol). NK cells sustained interaction with activating ligands promotes PD-1 induction on these cells *(Beldi-Ferchiou A et al, 2016, Oncotarget).* Our data show that the negative effect exerted by anti-PD-1 antibody on CpG-ODN anti-tumor activity is abrogated in absence of macrophage/myeloid cells. Therefore, it possible to speculate that macrophages may promote the development of PD-1^+ NK cells through CpG-ODN-induced NK-activating ligands upregulation and then anti-PD-1 antibody, inhibiting PD-1 pathway necessary for lytic molecules production, blocks NK cell anti-tumor activity.

iii) A third hypothesis that should be considered is that anti-PD-1 antibody can exert pro-tumor activity *per se*. Although anti-PD-1 monotherapy did not produce any effect in IGROV-1 advanced tumor model ***(Figure 9 and 10)***, we noticed an acceleration in ascites formation when we performed an early treatment with anti-PD-1 antibody starting the day immediately after tumor cell injection ***(Figure 12)***. This observation became extremely evident in H460-tumor bearing mice where PD-1 blockade induced an increase in tumor mass ***(Figure 15)***.

Evaluation of intra-tumoral immune cell contexture revealed an accumulation of macrophages with M2-like phenotype in anti-PD-1 treated tumors, indicating again the involvement of myeloid lineage in determining the detrimental effect associated with anti-PD-1 antibody. Regarding the role of PD-1 on myeloid cells, some reports described that PD-1 inhibition is able to promote a shift from pro-tumorigenic to anti-tumor phenotype *(Gordon SR et al, 2017, Nature)*, whereas other research groups observed that the binding of specific antibodies to PD-1 exacerbated immunosupression *(Lamichhane P et al, 2017, Cancer Res; Said EA et al, 2010, Nat Med).* Because our *in vitro* data did not reveal any particular activity of anti-PD-1 antibody on macrophages ***(Figure 17)***, it can be reasonably excluded that the detrimental effect observed in our models can be due to the blocking of PD-1 signaling in myeloid cells. It has been recently reported that macrophages can capture anti-PD-1 antibody from T cell surface through interaction

of the Fc fragment of the antibody and the FcμRIIb (Fc gamma receptor IIb) expressed by macrophages *(Arlauckas SP, 2017, Sci Transl Med)*. Although the Authors considered this phenomenon only a mechanism of resistance to anti-PD-1 therapy, it is not clear the biological consequences of such interaction. FcμRIIb is an inhibitory Fc receptor able to dampen immune response upon ligation with antibody Fc fragment *(Guilliams M et al, 2014, Nat Rev Immunol)*. Since anti-PD-1 antibody used in our *in vivo* experiments is reported to interact with FcμRIIb *(Dahan R et al, 2015, Cancer Cell)*, we might hypothesize that the inhibitory signal delivered by FcμRIIb can reprogram myeloid cells shaping their behavior toward a more pro-tumorigenic phenotype. This hypothesis was supported by anti-PD-1 $F(ab)_2$ *in vivo* experiment, showing that the treatment with the antibody lacking the Fc portion did not increase the growth of tumor ***(Figure 18)***.

Results obtained in our experimental models recall recently published clinical data *(Champiat S et al, 2017, Clin Cancer Res; Saâda-Bouzid E et al, 2017, Ann Oncol; Kato S et al, 2017, Clin Cancer Res)* describing a fraction of cancer patients experiencing particularly aggressive and unpredictable spread of the disease following ICI immunotherapy, a peculiar outcome defined hyperprogression *(Fuentes-Antrás J et al, 2018, Cancer Treat Rev)*. Based on our data, we performed an immunohistochemical analysis to evaluate the immune tumor microenvironment in a cohort of lung cancer patients treated with ICIs at Fondazione IRCCS - Istituto Nazionale dei Tumori di Milano. We found a cluster of macrophages characterized by the simultaneous expression of CD163, CD33, PD-L1 surface markers and epithelioid morphology in all tumors of patients that experienced hyperprogressive disease, highlighting again the central role of myeloid cells in this phenomenon. As in humans, not all the immunodeficient preclinical models show acceleration of tumor growth after PD-1 blockade therapy. Indeed, it has been reported that anti-PD-1 administration resulted in an inhibition of tumor growth in a murine model of colon, melanoma and head and neck carcinoma injected in non-immunocompentent mice *(Kleffel S et al,*

2015, Cell; Terme M et al, 2011, Cancer Res; Liu Y et al, 2017, Oncogene), suggesting that tumor cells of diverse origin may differently shape myeloid cell phenotype making them prone (or not) to interact in a “non-conventional” way with anti-PD-1 antibody.

Together our findings revealed that innate immune cells can affect the activity of anti-PD-1 antibody. The identification of a particular cluster of myeloid cells, which might be involved in the acceleration of tumor growth, might be of interest since it may allow to identify patients not to be treated with this type of immunotherapy.

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