Università degli Studi del Piemonte Orientale
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Department of Pharmaceutical Sciences

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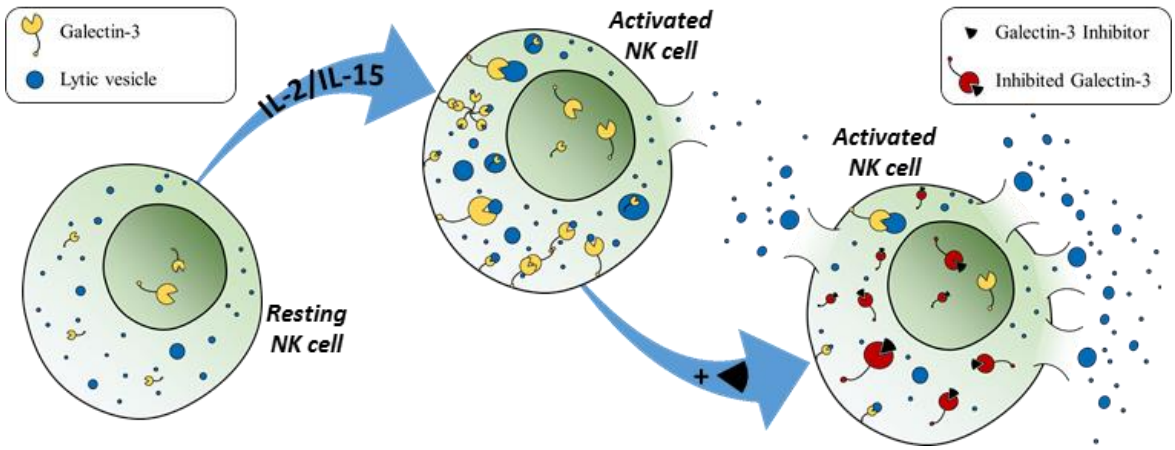
"In vitro" and "in vivo" studies on innate immune cells



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“Nothing is more damaging to the adventurous spirit within a man than a secure future. The very basic core of a man's living spirit is his passion for adventure. The joy of life comes from our encounters with new experiences, and hence there is no greater joy than to have an endlessly changing horizon, for each day to have a new and different sun.”

Christopher Johnson McCandless

To my grandfather

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List of abbreviations

aa amino acids

ADCC antibody-dependent cell-mediated cytotoxicity

AdCD40L CD40L-expressing recombinant adenovirus

AGE advanced glycation end products

AIP ALG-2 interacting protein

Alix ALG-2 linked protein x

AP activator protein

APC antigen presenting cell

Apo apoptosis antigen

ATF activating transcription factor

Bcl B-cell lymphoma

CCL C-C motif chemokine ligand

CCR C-C motif chemokine receptor

CD40/CD40R/CD40(+) CD40 receptor

CD40L CD40 ligand

CDC complement-dependent cytotoxicity

CEA carcinoembryonic antigen

CM complete medium

CRD carbohydrate recognition domain

CRE cAMP-dependent response element

CREB cAMP-response element-binding protein

CRM1 chromosome maintenance region 1

CXCL C-X-C motif chemokine ligand

CXCR C-X-C motif chemokine receptor

DAMPs damage-associated molecular patterns

DCs dendritic cells

DISC cell death-inducing signaling complex

EGF epidermal growth factor

ERK extracellular signal-regulated kinase

ESCRT endosomal sorting complex required for transport

Fc fragment crystallizable

FcεRI high-affinity IgE receptors

Fuc fucose

Gal galactose

Gal Galectin

Gal-1 galectin-1

Gal-3 galectin-2

GI gastrointestinal

Glc glucose

GM-CSF granulocyte-macrophage colony-stimulating factor

GPI glycosylphosphatidylinositol

GRIFIN galectin-related interfibre protein

GSK glycogen synthase kinase

H&E hematoxylin and eosin

HCC hepatocellular carcinoma

HIPK homeodomain-interacting protein kinase

HLA human Leucocyte Antigens

HLA-DR human leukocyte antigen-antigen D Related

hnRPN heterogeneous nuclear ribonucleoproteins

IFN interferon

Ig Immunoglobulin

IL interleukin

iNOS inducible nitric oxide synthase

IRF interferon regulatory factor

IS the immunological synapse

JNK c-Jun N-terminal kinase

KIRs killer cell Ig-like receptors

LacNAc N-acetyllactosamine

LAK lymphokine-activated killer cell

LAMPs lysosomal-membrane-associated glycoproteins

LcK lymphocyte-specific protein tyrosine kinase

LDL low density lipoproteins

LPS lipopolysaccharide

mAb monoclonal antibody

Mac macrophage antigen

MAPK mitogen activated protein kinase

mCD40L transmembrane form of CD40 ligand

M-CSF macrophage colony-stimulating factor

MDSC myeloid derived suppressor cells

MEK mitogen-activated protein

MHC major histocompatibility complex

MIC MHC class I polypeptide-related sequence

MOI multiplicity of infection

NAc N-acetyl

NCRs natural cytotoxicity receptors

Neu neuraminic acid

NF-IL6 nuclear factor for interleukin-6

NF-kB nuclear facto kappa B

NK natural killer

NO nitric oxide

NORE1a novel Ras effector 1a

NSCLC non-small-cell lung cancer

OC ovarian cancer

PAMPs pathogen associated molecular patterns

PB peripheral blood

PBMC peripheral blood mononuclear cells

PDA pancreatic ductal adenocarcinoma

PI propidium iodide

PI3K phosphoinositide 3-kinase

PLCγ phospholipase Cγ

Ras rat sarcoma

Rassf5 Ras association domain-containing protein 5

RCC renal cell carcinoma

RNI reactive nitrogen intermediates

ROI reactive oxygen intermediates

rVV40L recombinant vaccinia virus encoding for CD40L

rVVMART-1 FG MelanA/MART-1 full gene

rVVs recombinant vaccinia viruses

sCD40L soluble form of CD40 ligand

sCD40L/s40L soluble form of CD40 ligand

SIE sis-inducible element

Sp specificity protein

STAT signal transducer and activator of transcription

TAMs tumor-associated macrophages

Tcf T cell factor

TCM T central memory cell

TEM T effector memory cell

TEMRA T effector memory RA

TGF transforming growth factor

Th T helper lymphocyte

TLR toll like receptor

TMA Tissue microarray

TNF tumor necrosis factor

TNFR tumor necrosis factor receptor

TRAFs TNFR-associated factors

TRAIL necrosis factor-related apoptosis-inducing ligand

Tregs regulatory T cells

TTF thyroid-specific transcription factor

VCAM vascular cell adhesion molecule

VEGF vascular endothelial growth factor

VV Vaccinia Virus

WT wild type

M2 alternatively activated

macrophages

M1 classically activated macrophages

Preface

During the three years of my Ph.D. course, I was involved in two different research projects, both concerning cellular components of the innate immune system: natural killer cells and macrophages.

In particular, my principal project (University of Eastern Piedmont, Italy) aims to clarify the expression and role of a protein, galectin-3, on natural killer cells.

The other one (University of Basel, Switzerland) deals with the use of a viral vector to boost the antitumor activity of macrophages.

Chapter I

"In vitro" studies on galectin-3 in human natural killer cells

1. Introduction

1.1 The Sugar Code

An enormous amount of events take place among cells, either intra-, inter-, or extra-cellularly and the whole apparatus of proteins and nucleic acids is not sufficient to adequately perform all of them. A third category of bio-molecules with the ability to transmit a multitude of different messages seems to be required for assuring fine cellular processes, such as cell adhesion and communication.

Sugars, in the form of mono-, oligo-, or poly-saccharides, as well as glycoconjugates, are a perfect example of such type of bio-molecules. These compounds are, in fact, endowed with a large capacity in information-storing and a great ability to transfer information through protein bio-recognition.

As reported by Gabius (2000), oligo-saccharides surpass peptides by more than seven orders of magnitude in the theoretical ability to build isomers, when the total of conceivable hexamers is calculated. Most of the carbohydrates, in fact, contain at least one asymmetric carbon atom with a number of possible stereoisomers that grow rapidly with molecular weight. In addition, each carbohydrate has approximately four functional groups that can be used to establish connections with other sugar units in a linear and/or in a nonlinear/branched fashion. All the complex information stored in the glycostructures decorating the surface of a cell, protein, or lipid molecule can be deciphered by a protein having the ability to bind sugar, such as, for example, the lectins (Gabius 2000).

1.2 Lectins

The name lectin derives from the latin word *legere*, whose meaning is to pick/choose or select. Lectins represent a group of structurally diverse proteins able to bind and recognize simple/complex sugar moieties protruding from glycolipids or glycoproteins (Ghazarian et al. 2011). Carbohydrate-binding proteins can be roughly

classified into two main groups: lectins and sulfated glycosaminoglycan-binding proteins. The lectin superfamily phylogenetically comprises ancient proteins, defined by their ability to bind either soluble carbohydrates or glycoconjugates, mediating a large array of biological processes, including cell to cell interactions, communications at both inner and outer side of the cellular membrane and modulation of immune responses.

The broad range of functions carried out by this heterogeneous group of proteins are possible thanks to their ability to bind many different types of carbohydrates, which are present in the extracellular matrix, at the cell surfaces, or into secreted glycoproteins. Lectins can read the sugar code, recognizing even slight difference in the complex structure of oligosaccharides and/or in simple monosaccharides, binding only to specific sugars.

Originally isolated from plants, they are widely distributed in nature, ranging from bacteria and virus to animals. Today, based on the structures of animal lectins at least 15 structural families of mammalian lectins exist, where galectins and C-type lectins are the largest families (Gupta 2012).

1.3 Galectins

Galectins are a family of highly conserved proteins whose members are characterized by a highly conserved carbohydrate recognition domain (CRD), consisting of approximately 130 amino acids (aa) (Barondes et al. 1994) which bind small β-galactosides/poly-N-acetyllactosamine (LacNAc)-enriched glycoconjugates (Gupta 2012). These proteins were initially classified as S-type lectins, where the S (sulphydryl or thiol) was used to indicate the dependence on reducing conditions for activity, a specific property of galectin-1 (Gal-1), the first studied galectin (Gupta 2012).

To date, up to 15 galectins have been identified in mammals and, based on their molecular architecture and structural differences in CRD presentation, they can be

classified into 3 main groups, as proposed by Hirabayashi and Kasai in 1993 (Fig. 1A).

The prototype group are galectins containing one CRD, that can exist as monomer (galectins -5, -7, -10) or homodimers with two polypeptides containing a CRD each (galectins -1, -2, -11, -13, -14, -15), and a short N-terminal sequence. It should be mentioned that classification of galectin-11, initially identified as GRIFIN (galectin-related interfibre protein), is still under debate, since this galectin misses two of the seven crucial aa conserved in the CRD, and it displays no binding activity trough sugar molecules (Rabinovich et al. 2007).

Tandem repeat galectins are monomers with two non-identical CRDs joined by a short peptide linker region (galectins -4, -6, -8, -9, -12).

By contrast, galectin-3 (Gal-3) is the only member discovered so far of the chimera-type with a unique structure, characterized by one CRD connected to a long N-terminal domain. This latter is involved in Gal-3 oligomerization which results in the formation of Gal-3 multimers exposing several CRDs, enabling bivalent or multivalent binding of carbohydrate ligands (Dumic et al. 2006; Sato et al. 2009; Fortuna-Costa et al. 2014).

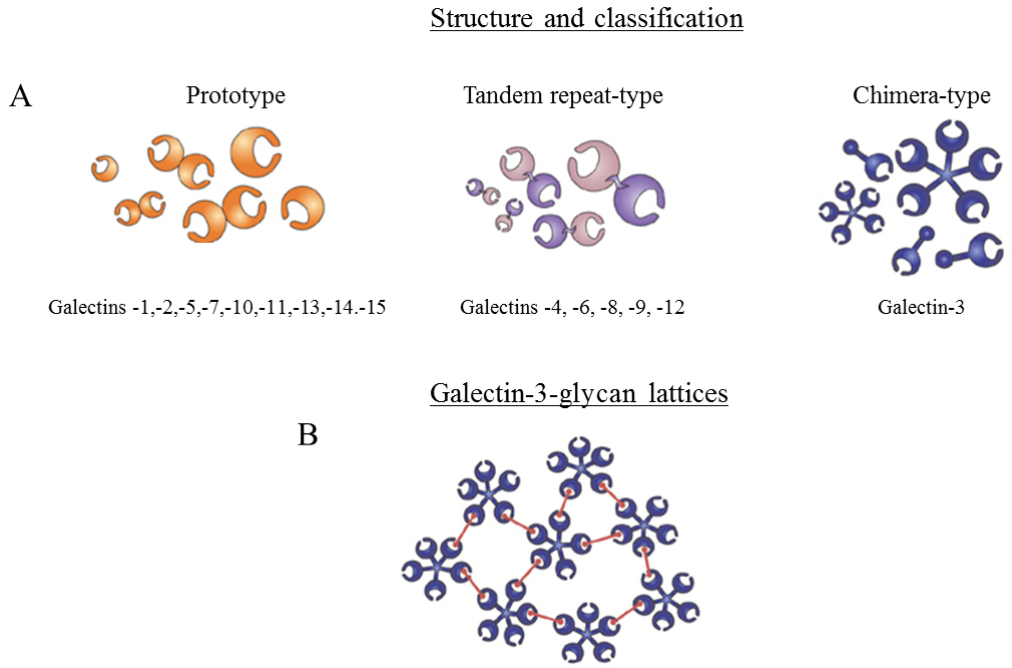

Figure. 1. Schematic representation of the structure of different members of the galectin family. (A) prototype galectins containing one or two homodimeric CRD, tandem repeats containing two different CRD connected by an aminoacidic linker and the unique chimera-type Gal-3 composed by a CRD and a collagen-like sequence. (B) schematic representation of Gal-3 oligomerization into pentamers through the N-terminal domain allowing the multivalent binding of glycans forming a lattice structure (adapted from Rabinovich and Toscano 2009).

1.4 Galectin-3

Gal-3 is the currently accepted name for a protein, initially identified as Mac-2, a surface protein expressed on murine macrophages (Ho and Springer 1982), formerly also known as CBP-35, LBP, L-34, L-29, or εBP (Gupta 2012). Human Gal-3, is a 31 kDa protein encoded by a single gene (*LGALS3*), which is located on chromosome 14, locus q21–q22 and spans on a total of 17 Kb. The gene has an open reading frame of 750pb and consists of 6 exons and 5 introns that are translated into a protein of 250 aa. In particular, the transition start site is found within the exon II, which contains also the initial methionine, the first six aa and part of the 5' untranslated sequence. The exon III contain entirely the long and flexible, collagen-like N-

terminal domain, while exon IV-VI codify for the C-terminal domain (Argüeso and Panjwani 2011; Gupta 2012; Díaz-Alvarez and Ortega 2017).

1.4.1 The carbohydrate recognition domain

Gal-3 shares whit Gal-1 and Gal-2 ~25% sequence identity and their CRD have a similar three-dimensional structure and identical topology (Seetharaman et al. 1998). This latter, is composed of two anti-parallel β-sheets with five and six β-strands (F1-5 and S1-6) (Seetharaman et al. 1998), forming a globular structure that accommodates the carbohydrate-binding site, composed by the side chains of the aa forming the 6-stranded β-sheet (Hughes 2001). This structure is responsible for the lectin activity of Gal-3. In particular, the two β-sheets composing the CRD are arranged in a convex side (F1-5) and a concave side (S1-6) with this latter forming a groove able to accommodate a linear tetrasaccharide (Fig. 2) (Leffler et al. 2002). The CRD can be schematically divided into four sub-binding sites: A, B, C, D, where the C subsite is made of approximately 6 conserved aa and is responsible for the β-galactoside binding. The other sites participate in the binding activity, either by increasing or decreasing the protein affinity, depending on the saccharide moiety linked to the galactose bound in the C subsite (Salomonsson et al. 2010). Beside carbohydrate recognition/binding, the CRD is also involved in the anti-apoptotic activity associated with Gal-3 (for further details see paragraph 1.8.1). This domain, in fact, presents the NWGR aa sequence, which is also highly conserved within the BH1 domain of the anti-apoptotic B-cell lymphoma (Bcl)-2 family, and the interaction with this latter is responsible for the resistance to apoptotic stimuli (Yang et al. 1996). The importance of this sequence for Gal-3 is demonstrated by the fact that it is highly conserved among different species, and that a single amino acid substitution of Gly to Ala in the NWGR sequence abrogates the anti-apoptotic properties (Akahani et al. 1997). The NWGR motif is also essential for the carbohydrate recognition activity since the tryptophan (W) amino acid is directly involved in the sugar binding (Yang et al. 1998).

Finally, despite Gal-3 oligomerization has been proposed to occur through the N-terminal domain (type-N model), recently a new model (named type-C self-association) has been proposed. Accordingly, oligomerization would be mediated by the carbohydrate recognition site: the carbohydrate-binding site of one CRD would bind at the other side of the next CRD, and the N-terminal domain would participate by enhancing this association. Both models remain equally probable, and whether and when each model occurs in a cellular system remain to be demonstrated (Lepur et al. 2012).

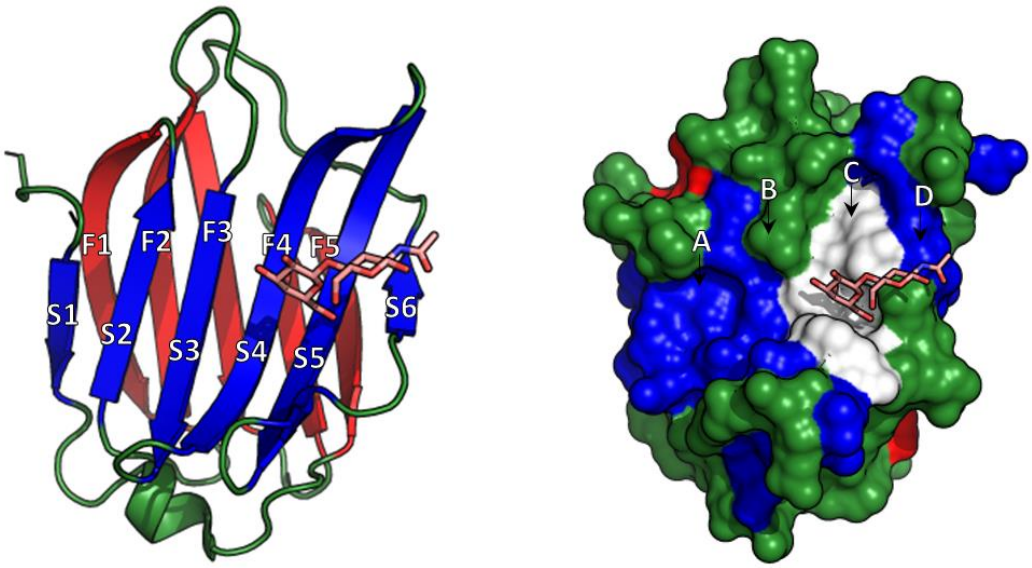

Figure 2. High Resolution X-Ray Structure of Human Gal-3 in complex with LacNAc visualized as stick model (PDB: 1KJL). (A) secondary structure of the Gal-3 CRD; (B) The solvent accessible surface of Gal-3 CRD with the position of A-D binding site shown above the binding groove, the C binding site was shown in white (adapted from Salomonsson et al. 2010; Hsieh et al. 2015).

1.4.2 The N-terminal domain

The N-terminal domain is a relatively flexible structure, consisting of two distinct portions: a collagenase sensitive, proline, glycine, alanine, and tyrosine rich motif, followed by 12 aa sequence also called the small N-terminal domain, containing a casein kinase I serine phosphorylation site. The former, consisting of 100–150 aa

residues, has homologous repeats (8 to 13 in vertebrates) (Ippel et al. 2016) of a consensus sequence Pro-Gly-Ala-Tyr-Pro-Gly, followed by three additional aa with a number of repetitions that vary among different species and lack of charged or large side-chain hydrophobic residues. This part of the N-terminus domain has 35.5% identity with collagen α1 (II) chain of bovine cartilage, and this explains the designed name of this domain as collagen-like N-terminal domain (Raz et al. 1989; Gupta 2012). In addition to collagen, this portion of the N-terminal domain has a 25% homology with some heterogeneous nuclear ribonucleoproteins (hnRPN) (Dumic et al. 2006).

At least four functional properties can be ascribed to the N-terminal domain:

i) Upon binding of specific glycoconjugates, Gal-3 changes its conformation, resulting in N-terminal domain self-assembly and consequent protein oligomerization (Sato et al. 2009). This process leads to the formation of a multimer (such as dimer or pentamer) (Gong et al. 1999; Ahmad et al. 2004), which possesses multivalent CRD (Sato and Nieminen 2002) even under non-reducing conditions or in the presence of sodium dodecyl sulfate (Henderson and Sethi 2009), leading to cross-linking of cell receptors;

ii) The N-terminal portion of the protein (small N-terminal domain) is involved in its secretion. In fact, the deletion of the initial 12 aa, completely abrogates secretion of this protein through a new mechanism independent from the classical secretory pathways (ER and Golgi system) (for further details see paragraph 1.6) (Dumic et al. 2006);

iii) Despite the carbohydrate binding is largely N-terminal independent (Dumic et al. 2006), molecular modeling and mutagenesis studies demonstrated that the aa present in the N-terminal domain can participate in sugar binding (Barboni et al. 2000). Particularly, the N-terminal domain would contribute to carbohydrate binding through the stabilization of the substrate-bound lectin complex (Barboni et al. 2000). These studies were performed with lipopolysaccharide (LPS) as binding partner,

demonstrating that Gal-3 is able to bind the side chain of the O-antigen of LPS, which contains polymers of LacNAc, a known ligand for the CRD of this lectin. Alternatively, for LPS devoid of beta-galactosides, Gal-3 can interact through the N-terminal domain, with the lipid A core region of LPS (Mey et al. 1996). The use of a monoclonal antibody (mAb) that recognizes an epitope within the N-terminal domain of Gal-3 completely abrogates the binding of this lectin to LPS (Fowler et al. 2006);

iv) Post-translational phosphorylation of a serine residue (Ser^{6}) in the small N-terminal domain of the protein, has been associated with several Gal-3 functions: a) this modification acts as an "on/off" switch for its sugar-binding capability controlling downstream biological effects (Mazurek et al. 2000) b) similarly to Bcl-2, whose phosphorylation at Ser^{70} appears to be critical for its anti-apoptotic function, non-phosphorylated Gal-3 mutants result in loss of Gal-3 anti-apoptotic activity and fail to induce cell cycle arrest (Yoshii et al. 2002).

1.4.3 Galectin-3 ligand specificity

The minimal sugar unit recognized by galectins is represented by a galactose residue linked to an adjacent monosaccharide in the β configuration (β-galactoside), such as that found in the LacNAc residues (Galactose [Gal]β1-4N-acetylglucosamine [GlcNAc]). Each member of the galectin family presents slightly, yet significant, differences in the CRD that can tune the affinity of each galectin for its ligands (Sato et al. 2009).

Gal-3 can bind both N- and O-glycans (Sundblad et al. 2011) and it was originally identified to bind preferentially to Galβ1→3(4)GlcNAc (Cardoso et al. 2016); specific modifications of the β-galactoside core, however, can enhance or reduce the affinity towards Gal-3. α-N-acetylgalactosamine modifications of the Gal residue in the β-galactoside core, indeed, significantly increase the affinity for Gal-3, while modifications with α2-6- sialic acid reduce the affinity for the same protein (Hirabayashi et al. 2002). Moreover, the extension of the non-reducing end of the

disaccharide with 2- o 3- substituents on the outer galactose, such as N-acetylneuraminic acid (NeuNAc)α2,3 or GalNAcα1,3 and fucose (Fuc)α1,2 substituents greatly enhances the affinity for Gal-3 (Krześlak and Lipińska 2004).
In addition, also galactomannans and polymannans can be bound by Gal-3 (Díaz-Alvarez and Ortega 2017). Usually the affinity of Gal-3 toward its glycan ligands is lower (approximately 10^{-6} M) than those typically observed for protein-protein interactions (approximately 10^{-8} M); however, the affinity increases for branched glycans and/or polylactosamine structures over simple saccharides. In fact, as previously mentioned, upon ligand binding Gal-3 can oligomerize through the N-terminal domain resulting in multiple CRD presentation. The multivalent binding of carbohydrate-containing glycoproteins or glycolipids by Gal-3 increases the avidity of the protein for its ligand, promoting the formation of organized glycan-galectin clusters, termed "lattices", and mediating cross-linking of glycosylated molecules (Fortuna-Costa et al. 2014) (Fig. 1B).
Further, since Gal-3 possesses an extended binding site, when compared to Gal-1 (Sato and Nieminen 2002), this protein displays a high affinity for glycan presenting repeated LacNAc residues, as found in polylactosaminoglycans (polylactosamine effect). Amniotic fibronectin, which contains a tetra antennary complex composed of four parallel lactosamine residues presented by a mannose core structure, is an example (Sato and Hughes 1992). Interestingly, no effect is displayed against plasma fibronectin, that presents only two bi-antennary chains.

Mutational and structural studies of the human Gal-3 carbohydrate binding site (formed by β-strands S4–S6) in complex with LacNAc molecule revealed the aa directly implied in sugar binding. In particular, the galactose C-4 hydroxyl group interacts with His-158, Arg-162, Asn-160 and a water molecule (W1). The C-6 hydroxyl group interacts directly with Glu-184, Asn-174 and W3, while Arg144 can interact with the monomer linked to O-3 of the terminal galactose. Other favorable

stacking interactions are provided by Trp-181 with the hydrophobic galactose surface.

The N-acetylglucosamine moiety is more exposed to the solvent; therefore, only the C-3 hydroxyl group can interact with Glu-184 and Arg-162. The other interactions involving the GlcNAc moiety are mediated through its N-acetyl group bonded to Arg 186 and through a water molecule (W2) to Glu-165 and this probably accounts for the approximately 5-fold higher binding affinity of human Gal-3 for LacNAc over lactose (Seetharaman et al. 1998; Krześlak and Lipińska 2004).

Since position 4 and 6 of the galactose in the β-galactoside present key interactions with the protein, modifications of these positions are not tolerated. On the contrary, appropriate modifications of position 2 and 3 are well tolerated and can enhance the affinity of this protein for its ligand (Leffler et al. 2002; Salomonsson et al. 2010).

This chimera-type galectin not only interacts with glycan, but also with peptide motifs. Despite Gal-3 carbohydrate-binding activity appears to be critical for Gal-3 function, this lectin presents also many different molecular interactions that are largely carbohydrate independent, some of them involving the carbohydrate binding groove (Wang et al. 1995; Paron et al. 2003; Elad-Sfadia et al. 2004; Shimura et al. 2004; Shimura et al. 2005; Gupta 2012). For example, Gal-3 is able to interact with Bcl-2, an anti-apoptotic protein, which is not a glycoprotein (Yang et al. 1996).

1.5 Galectin-3 expression and distribution in human tissues

Data on Gal-3 expression in tissues of human adults and during embryonic stages are more limited if compared to mice. However, different studies (see below) have provided evidences about the expression pattern and distribution of this lectin in non-hematopoietic and hematopoietic human tissues.

1.5.1 Galectin-3 expression in human non-hematopoietic tissues

Gal-3 is broadly expressed in several human tissues and cells in a spatio-temporal fashion (Sundblad et al. 2011). During human embryogenesis, Gal-3 expression is mainly confined in epithelial cells of the skin, respiratory and digestive tract, excretory tubes of the kidney and to the uroepithelium. In the adult, Gal-3 expression resembles that found in embryogenesis, with protein expression restricted to the epithelial cells of different organs and to myeloid cells infiltrating the tissue. In particular, robust expression of this lectin was found during lung analysis in epithelial cells of the bronchus, chondrocytes of the bronchial cartilage, and in alveolar macrophages (Mathieu et al. 2005). Gal-3 expression is also described in the gastric epithelial cells, the colon, the colonic epithelial cells, and in the intestinal macrophages (Lippert et al. 2008). In the gut, Gal-3 expression is restricted to the crypt cells and macrophages of lamina propria (Mercer et al. 2009). Interestingly, the expression of this protein in endometrial and decidual tissues is tightly regulated through the menstrual cycle. Since Gal-3 has many immunomodulatory properties (for further details see paragraph 1.9), this observation led to hypothesize that Gal-3 might contribute not only to the regulation of the endometrial function, but also to the modulation of the endometrial immune system during the implantation process (von Wolff et al. 2005; Sundblad et al. 2011). Finally, Gal-3 expression has been detected also in prostate glands, in the cytoplasm of luminal cells, and intratubular fibroblasts of the breast, in the cytosolic and membrane-enriched fraction of chondrocytes, as well as in the coronary artery smooth muscle cells (Sundblad et al. 2011).

Under physiologic conditions, due to its ability to shape the immune response, Gal-3 expression in epithelia of the digestive tract and lungs might be involved in mucosal defense, modulating both host-pathogen interactions and immune attacks (Sundblad et al. 2011).

1.5.2 Galectin-3 expression and cellular localization in human hematopoietic cells

Gal-3 has been reported to be ubiquitously expressed in many immune cells, where it contributes to regulate both the innate and adaptive immune responses (Chen et al. 2005; Liu 2005; Rabinovich et al. 2007). First identified in macrophages (Ho and Springer 1982), Gal-3 was found in monocytes (Liu et al. 1995), dendritic cells (DCs) (van Stijn et al. 2009), mast cells and basophils (Craig et al. 1995), eosinophils (Rao et al. 2007), neutrophils (Wu et al. 2017), T and B cells (Craig et al. 1995; Chen et al. 2005), with a level of relative expression which is tightly regulated by the cellular activation state.

In human monocytes, this lectin is constitutively expressed at low levels, both intracellularly and at the cell surface; however, its expression increases dramatically at both mRNA and protein levels, as monocytes differentiate into macrophages (Nangia-Makker et al. 1993; van Stijn et al. 2009). In this latter, Gal-3 was found in the cytoplasm/nucleus and at the plasma membrane surface. Interestingly, alternative macrophage polarization (M2) is associated whit higher levels of Gal-3 when compared to classically (M1) polarized macrophages, with a pattern of expression that follows the subsequent scheme: monocyte << M1 < M2 (Novak et al. 2012). Despite the difference in the levels of expression, cells of the monocyte/macrophage lineages are able to secrete this lectin in the extracellular media when properly stimulated (Liu et al. 1995)

In a similar manner, differentiation of human monocytes into DCs leads to an increment in the Gal-3 protein levels with only a moderate increase in Gal-3 mRNA. Moreover, during cellular differentiation, the monocyte surface expression of this lectin slowly disappears, with Gal-3 protein that mainly localizes intracellularly in immature DCs (van Stijn et al. 2009). Conversely, in activated DCs, this lectin is principally restricted to lipid raft domains of membrane ruffles and lamellipodia, where it contributes to regulate cell motility (Hsu et al. 2009). Similarly to monocytes

and macrophages, Gal-3 secretion was reported also for DCs. Proteomic analysis of DC-derived exosomes demonstrates, in fact, that these cells can release in the extracellular microenvironment, membrane vesicles containing Gal-3 (Théry et al. 2001).

Through light microscopy immunohistochemistry and ultrastructural immunogold labeling, Gal-3 expression and localization were studied in human mast cells and basophils. In particular, in mast cells, this lectin was detected in the nucleus (over heterochromatin whereas euchromatin was unlabeled) and/or the cytoplasm. Of note, cytoplasmic labeling was principally concentrated over secretory granules. Gal-3 localization in basophils was similar to that found in mast cells, but with a general low intensity of staining (Craig et al. 1995).

Finally, Gal-3 expression was also reported for two other populations of innate immune cells: neutrophils and eosinophils. In the former, Gal-3 localize mainly intracellularly, despite at a lower level compared to other innate cells (Wu et al. 2017). In addition, it and can be secreted by these cells upon treatment with mannan structure derived from microbial pathogens (Linden et al. 2013). Conversely, in human eosinophils, this chimeric protein is confined to the cells surface, with a level of expression that increases in allergic subjects (Rao et al. 2007).

In sharp contrast with the constitutive expression of Gal-3 in innate immune cells, this lectin is absent, or only scarcely expressed, in lymphocytes and lymphoid cell lines. Resting T and B cells, for example, do not express Gal-3, but this protein can be induced following interleukin (IL)-4 stimulation or CD40 cross-linking in B cells (Acosta-Rodríguez et al. 2004), and by TCR engagement, or mitogen exposure, in T cells (Joo et al. 2001; Dumic et al. 2006). Likewise, also virus and/or parasite infections are able to induce Gal-3 expression in these cells (Dumic et al. 2006).

1.5.3 Regulation of galectin-3 gene expression

Despite the amount of data available on Gal-3 expression in hematopoietic/non-hematopoietic tissues, the regulation of gene transcription remains poorly understood.

The analysis of the promoter sequence of *LGALS3* revealed that this region contains, among others, two nuclear factor kappa B (NF-kB) and three putative activator protein (AP)-1 sites (Kadrofske et al. 1998). Particularly, in glioblastoma cells, the expression of this protein is regulated by Jun (a component of AP-1 transcription factor) and NF-kB, where Jun seems to be important for the basal expression of Gal-3, while NF-kB is implicated in the gene induction following cell damage (Dumic et al. 2000).

The presence in the promoter of cAMP-dependent response element (CRE) implies that cAMP-response element-binding protein (CREB) might participate in the Gal-3 gene regulation.

Particularly, the implication of CREB and NF-kB in Gal-3 gene regulation was studied in human T lymphotropic virus-I infected T cells (Hsu et al. 1996). In the latter, Gal-3 is under the regulation of both the CREB/activating transcription factor (ATF) and, to a lesser extent, the NF-kB transcription factor. Curiously, NF-kB regulation of Gal-3 expression is controlled by nuclin, an apoptosis-associated protein, which can interfere with NF-kB in order to inhibit the expression of Gal-3 (Liu et al. 2004). On the contrary, in macrophage cells, Gal-3 expression seems to be principally regulated by the rat sarcoma (Ras)/mitogen activated protein kinase (MAPK) signal transduction pathway (Kim et al. 2003).

Further analysis revealed that the promoter contains also numerous GC box motifs, which can be bound by the ubiquitously expressed specificity protein (Sp)1 transcription factor, a feature which is commonly observed in the promoters of the housekeeping genes (Fogel et al. 1999). Nevertheless, Gal-3 expression at both

mRNA and protein levels, following serum addition to serum-starved fibroblasts, represents a feature of early genes, and sis-inducible element (SIE) was suggested to be a potential candidate for the growth-induced activation of *LGALS3* expression after serum addition (Dumic et al. 2006)

Finally, epigenetic mechanisms could also be involved in Gal-3 expression. The gene promoter and the first exon, in fact, present a high content of GcP island and the methylation status seems to contribute to Gal-3 expression in pituitary tumors (Ruebel et al. 2005).

As evidenced by the different regulatory elements found in the Gal-3 promoter and the different transcription factors involved in LGALS-3 transcription, Gal-3 expression results to be a complicated process probably depending on cell type, environmental stimuli and malignant transformation.

1.6 Cellular localization

Gal-3 is a structurally unique protein expressed in many normal tissues and immune cells with a cellular localization that strongly depends on the cell type and its proliferation status. In addition, the cellular distribution of this protein can change during differentiation or neoplastic transformation (Dumic et al. 2006) (Fig. 3).

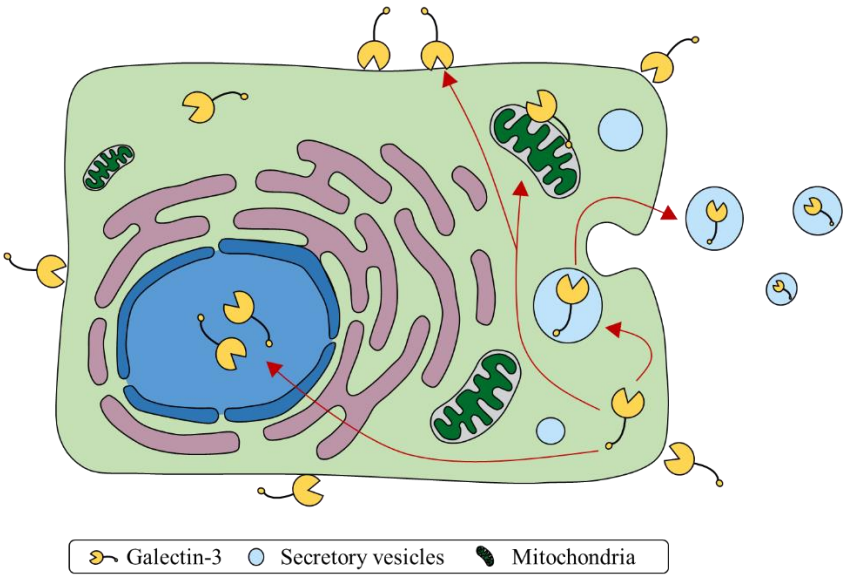

Figure 3. **Intracellular trafficking of Gal-3 based on experimental evidence.** Gal-3 can be either found the extracellular space, attached to cell surface, in the cytoplasm, in secretory vesicles or in the nucleus. Moreover, it can be secreted from cells in a manner that is independent from classical secretory pathway (adapted from Hughes 2001).

Initially synthesized on free ribosomes in the cytoplasm (Mehul and Hughes 1997), Gal-3 is able to shuttle between the nucleus and the cytoplasm in a manner that is still unclear. Using a truncated form of Gal-3 was discovered that mutants containing truncations of the amino-terminal half of this protein, were dispensable for nuclear import (Davidson et al. 2006). On the contrary, mutants of the same construct, containing truncations from the carboxyl terminus, showed loss of nuclear localization. Then, site-directed mutagenesis of this latter portion of the polypeptide suggested that nuclear import was dependent on the ITLT sequence (residues 253-256). However, this sequence, by itself, is not sufficient to guarantee a correct nuclear localization: its activity, in fact, is strongly regulated by a neighboring leucine-rich nuclear export signal.

Conversely, another study suggested that Gal-3 could be either imported into the nucleus via active transport and/or passive diffusion (Nakahara et al. 2006). Concerning the active transport, the Authors of this study identified a nuclear localization signal (NLS)-like motif in its protein sequence, HRVKKL (residue 223-

228), similar to those of p53 and c-Myc, respectively. Moreover, they suggest that nuclear localization of Gal-3 is, at least in part, due to the importin-α/β, with the Arg224 amino acid residue of human Gal-3 essential for its active nuclear translocation and molecular stability.

In contrast with nuclear import, nuclear export of this protein seems to be mediated by chromosome maintenance region 1 (CRM1), a nuclear export receptor. Candidate nuclear export signals, recognized by CRM1, can be found between residues 240 and 255 of murine Gal-3. Direct mutagenesis studies identified residues 240-255 of the Gal-3 protein, as containing fundamental aa for nuclear exportation, and partially overlaps with the region (residues 252-258), previously identified as important for nuclear localization (Li et al. 2006).

As previously reported, Gal-3 can undergo to post-translation modifications (see paragraph 1.4.2). In particular, this protein can be phosphorylated at both Ser^{6} and Ser^{12}, of the NH2-terminal, even though the majority of phosphate is present at Ser^{6}, while less than 10% at position Ser^{12} (Dumic et al. 2006). The protein responsible for Gal-3 phosphorylation seems to be casein kinase I and/or II, since the major acidic residues on both sides of Ser^{6} make it a good substrate for this kinase. The presence of both unphosphorylated and phosphorylated Gal-3 has been reported in mouse 3T3 fibroblasts; where phosphorylated Gal-3 was found in both nucleus and cytoplasm, whereas unphosphorylated form was detected only in the cytoplasm. This observation suggests that the phosphorylation is a mechanism required for Gal-3 nuclear import, however, mutagenesis studies of Ser^{6} show that it is not essential for protein localization in this cellular compartment (Dumic et al. 2006).

Similarly to nuclear import/export, Gal-3 secretion mechanism is still under debating. Gal-3 lacks any recognizable canonical secretion signals able to direct this protein in the classical secretory pathways through the endoplasmic reticulum and Golgi system (Hughes 1999). Nevertheless, it has been extensively demonstrated that

this protein can be secreted by immune (Théry et al. 2001; van Stijn et al. 2009; Linden et al. 2013) and many other cells (Hughes 1999; Menon and Hughes 1999). It has been proposed that Ga-3 can be released by cells through an incompletely understood mechanism, called ectocytosis (Menon and Hughes 1999), in which the protein forms aggregates underlying the plasma membrane and the release takes place through membrane blebbing.

In particular, the first step in the Gal-3 secretion consists in protein accumulation and capture at the cytoplasmic side of the plasma membrane. This is a rate-limiting step and seems to be mediated by molecular chaperones and/or heat shock proteins.

Essential requirements for this retention appear to be the acylation by the lymphocyte-specific protein tyrosine kinase (LcK). In particular, Cos cells transfected with a fusion protein containing within the N-terminal domain a Lck acylation sequence were efficiently acylated, retained at the cytoplasmic level and released in higher amount when compared to wild type lectin.

The following step consists in the evagination of the plasma membrane and secretion of the Gal-3-containing extracellular vesicles, in which the protein is protected against proteolysis. Under culture conditions, the Gal-3 release from these extracellular vesicles is quite fast, with a half-life of about 1 h. Conversely, isolated vesicles were more stable and this suggests that vesicles breakdown and cargo release requires specific factor(s) secreted by cells (Krześlak and Lipińska 2004).

The N-terminal domain of the protein seems to be fundamental for secretion and localization into secretory vesicles (Menon and Hughes 1999). The addition of the N-terminal domain to a cytosolic protein, the chloramphenicol acetyltransferase, leads to efficient export in transfected Cos cells. The 89–96 YP(90)SAP(93)GAY short sequence was identified as fundamental for Gal-3 secretion; however, this short segment alone is insufficient to induce the secretion of fusion proteins and results active only when larger portions of the Gal-3 N-terminal sequence are present.

1.7 Function of extracellular and intracellular galectin-3 in regulating cellular homeostasis

The many intra- and extra-cellular localizations of this protein, together with the ability to interact with numerous glycosylated or unglycosylated binding partners (Fig. 4 and 5), allow Gal-3 to modulate several physiological processes, such as cellular homeostasis, apoptosis, cell activation, cell adhesion, organogenesis, angiogenesis, immune response, and to act as chemoattractant for many immune cells (Sundblad et al. 2011).

1.7.1 Function of extracellular galectin-3

On cell surfaces, carbohydrates due to their structural and conformational diversity represent an incredible reservoir of biological information, even greater than DNA or peptides (Gabius 2000; Cardoso et al. 2016). Gal-3, as sugar binding proteins, can decipher this "sugar code" orchestrating a variety of biological processes (Sundblad et al. 2011).

In particular, once in the extracellular spaces, this protein can flow into the circulation, interact with the extracellular matrix or be associated with the cell surfaces, exhibiting numerous autocrine and paracrine effects (Sundblad et al. 2011). In particular, Gal-3 can modulate biological functions by forming ordered galectin-glycan arrays (lattices structure), cross-linking different cell surface glycoconjugates, or, alternatively, Gal-3 can engage specific cell surface ligands through the classical ligand-receptors interaction (Fig. 4) (Rabinovich and Toscano 2009; Sundblad et al. 2011).

1.7.2 Gal-3 function in endocytosis

In breast carcinoma cells, Gal-3 can participate in cellular endocytosis, through a caveolae-like pathway by means of which this protein promotes the endocytosis of CD29 (beta-1 integrin) in a lactose/temperature-dependent manner (Furtak et al.

2001). Interestingly, by the same pathway tumor cells can internalize from the extracellular spaces Gal-3 itself and this process is completely blocked by lactose (Furtak et al. 2001). In a similar manner, Gal-3 is involved in advanced glycation end products (AGE) and modified low density lipoproteins (LDL) endocytosis (Zhu et al. 2001).

In sharp contrast, Gal-3 can also interfere with endocytosis, extending the expression on cell surface of specific receptors and therefore altering cell responses during tumor progression. Gal-3 affinity for β-1,6-N-acetylglucosamine, in fact, mediates its binding to many glycoproteins expressed on cell surface, including lysosomal-membrane-associated glycoproteins (LAMPs)-1 and -2, carcinoembryonic antigen (CEA), mucin-1, macrophage antigen (Mac) -1/-3, transferrin receptor protein 1 (CD-71), protein tyrosine phosphatase receptor type C (CD45), and the glycosylated receptors for the vascular endothelial growth factor (VEGF), the epidermal growth factor (EGF), and the transforming growth factor (TGF)-β (Fortuna-Costa et al. 2014). Carcinoma transformation is often associated with the upregulation of the beta-1,6 N-acetylglucosaminyltransferase a Golgi enzyme which promotes the substitution of N-glycan with poly LacNac, the main ligand for Gal-3. This leads to Gal-3 cross-linking of N-glycans and lattice formation on EGF and TGF-β receptors at the cell surface, delaying their removal by constitutive endocytosis and prolonging cell activation (Partridge et al. 2004).

Of note, while extracellular Gal-3 plays a pivotal role in the balance of surface retention of glycosylated receptors against endocytosis, intracellular Gal-3 is equally important for proteins trafficking from the cytoplasm to the plasma membrane.
In a physiologic contest, cytosolic Gal-3 controls the expression of EGF receptors in mouse keratinocytes. In particular, in the absence of Gal-3, EGF receptor expression results dramatically reduced with the protein that accumulates intracellularly. Of note, in these cells, Gal-3 can regulate this process through the interaction and

modulation of ALG-2 linked protein x (Alix), a member of the endosomal sorting complex required for transport (ESCRT) apparatus (Liu et al. 2012).

1.7.3 Cell adhesion

Recognition of extracellular glycoproteins and glycosylated components of the extracellular matrix by Gal-3 plays a central role in cell adhesion/de-adhesion. Gal-3 was demonstrated to bind laminin, fibronectin, hensin, elastin, collagen IV, and tenascin-C/R (Dumic et al. 2006). Overexpression of this chimera-type protein in a human breast carcinoma cell line significantly enhanced adhesion to vitronectin, fibronectin and laminin (Materasse et al. 2000). Consistently, Gal-3 binding of Lamp-1/2, colon cancer mucin, the carcinoembryonic antigen, and the glycosylphosphatidylinositol (GPI)-anchored glycoprotein C4.4A were suggested to participate in cancer cell adhesion to extracellular matrix (Dumic et al. 2006).

In addition, Gal-3 was found to interact with integrins, molecules involved in cell adhesion. Particularly, Gal-3 has been documented to associates with α1β1 integrin in a lactose-dependent manner (Ochieng et al. 1998). Moreover, through a Gal-3 affinity column purification of binding partners it was found that Gal-3 is able to interact with the alpha-subunit (CD11b) of the CD11b/CD18 integrin (CR3, Mac-1 antigen), and with the heavy chain of CD98, an integrin-associated protein (Dong and Hughes 1997). This latter is a cell surface heterodimeric glycoprotein that participates in cell-cell and cell-substratum interactions, expressed on monocytes/macrophages and in activated lymphocyte (T and B cells) (Cantor and Ginsberg 2012). Gal-3 appears to be able to induce cross-linking of CD98, leading to activation of integrin-mediated adhesion (Hughes 2001).

Besides the previously reported data, also a negative effect mediated by Gal-3 on cell adhesion was reported. In vitro, Gal-3-enriched media were demonstrated to inhibit thymocyte interactions with thymic microenvironmental cells (Villa-Verde et al. 2002).

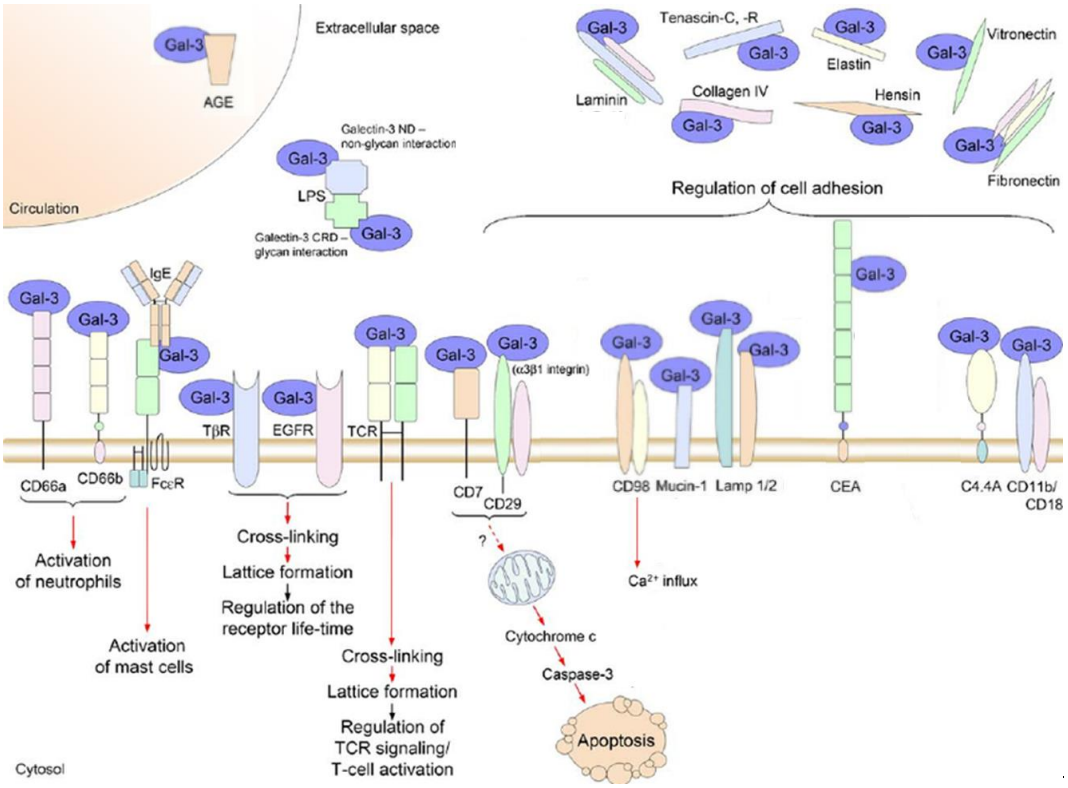

Figure 4. A cartoon schematically representing some extracellular binding partners identified for Gal-3. Red arrows indicate positive effects (adapted from Dumic et al. 2006).

1.7.4 Function of nuclear and cytoplasmic galectin-3

Depending on the cell type, differentiation and development status, Gal-3 can be predominantly nuclear, exclusively cytoplasmic, or spreads over these two compartments (Haudek et al. 2010). In the cytoplasmic compartment, Gal-3 interacts with numerous partners, implying a role for Gal-3 in the regulation of numerous intra-cytoplasmic events (Fig. 5).

1.7.5 Regulation of cell cycle

Cell cycle regulation is a complex process that requires intricate interactions among different proteins. Gal-3 expression, which is regulated by cell cycle (Moutsatsos et al. 1987), is also able to affect the cell cycle itself. Following expression of this lectin in BT549, a cell line that normally undergoes apoptosis induced by the loss of cell

anchorage (anoikis), Gal-3 mediates G1 phase arrest without detectable cell death. The acquisition of this anti-anoikis activity is accompanied by cyclin A and E (cyclins important for progression and maintenance of phase S) level downregulations, and upregulation of their inhibitory protein levels p21 and p27, as well as cyclin D.

These two phenomena may help the cells to bypass the critical apoptosis-sensitive point in early G1, arresting cells at an anoikis-resistant point (late G1). Furthermore, also the retinoblastoma protein, which is hyper-phosphorylated during the S/G2 phase and most of the M phase, in Gal-3 overexpressing cells, is kept in a hypophosphorylated form, showing that these cells fail to enter S phase (Kim et al. 1999). Similarly, in the same cell line overexpressing Gal-3, genistein, a soy-derived isoflavonoid able to induce apoptosis without any cell cycle arrest, induces arrest in G2/M phase without any apoptosis induction. The different phase arrests of the cell cycle might be dependent on genistein-induced upregulation of P21, but not P27 upregulation, thus leading to G2/M arrest (Lin et al. 2000).

These studies suggest that Gal-3 can regulate critical decision points through cell cycle progression, phase arrest (depending on the apoptotic stimuli) and induction of apoptosis.

1.7.6 Galectin-3 in gene regulation

Nuclear Gal-3 is also involved in gene transcription/regulation (Lin et al. 2002; Paron et al. 2003; Dumic et al. 2006). In particular, in human breast epithelial cells, Gal-3 is able to induce cyclin D promoter activity through stabilization and enhancement of the transcription factor binding to Sp1 and CRE sites. Moreover, in the same cells, Gal-3 overexpression is associated with changes in the expression levels of other proteins, such as cyclin A/E and p21/27. Since these cycle regulators contain in their promoter CRE and SP1 sites, they also represent possible candidates whose expression is regulated by Gal-3 (Lin et al. 2002).

The involvement of gene transcription was further demonstrated in papillary thyroid cancer cells, where Gal-3 directly interacts with the thyroid-specific transcription factor (TTF)-1 causing stimulation of TTF-1 binding activity (Paron et al. 2003; Dumic et al. 2006).

Further evidence came from a study demonstrating that, in the nucleus, Gal-3 co-localizes and binds to β-catenin/ T cell factor (Tcf) complex in a lactose-depended manner (even if β-catenin is not a glycoprotein). This interaction induces the transcriptional activity of Tcf-4 regulating c-Myc expression and promoting cell growth and proliferation (Shimura et al. 2004). Moreover, analysis of the Gal-3 and β-catenin sequences revealed that these two protein share structural similarity. In particular, both proteins contain the $S^{92}XXXS^{96}$ consensus sequence for glycogen synthase kinase GSK-3β phosphorylation. Axin, a regulatory protein of the Wnt signaling pathway, can complex with both proteins promoting their GSK-3β phosphorylation. The Authors of this study, therefore, suggest that Gal-3 is a key regulator of the Wnt/ β-catenin signaling pathway and of its target gene expression (Shimura et al. 2005).

1.7.7 Galectin-3 as a factor in pre-mRNA splicing

Nuclear Gal-3 can directly participate to the splicing machinery. It was found that Gal-3 can associate, in the nucleus, with ribonucleoprotein complexes and be involved in spliceosome assembly, acting as a pre-mRNA splicing factor (Wang et al. 1995). Despite in the previous study was showed that Gal-3 can interact directly with single-stranded DNA (ssDNA) and with RNA, more recently it has been demonstrated that Gal-3 might interact, in a weak protein-protein manner, with another splicing component, identified in Gemin-4, rather than through the direct binding to the splicing substrate (Wang et al. 2006).

1.8 Galectin-3 at the edge between life and death

An increasing number of literature data have provided evidence about a role of Gal-3 in the regulation of apoptosis (Hsu and Liu 2002). Despite it remains largely unclear how this protein is able to modulate this process, the mechanism seems to involve both the extracellular and the intracellular side of the membrane. In particular, Gal-3 can act in a dual manner either protecting or inducing cell apoptosis, depending on whether this protein is localized intracellularly (generally anti-apoptotic) or extracellularly (mainly pro-apoptotic) (Dumic et al. 2006).

1.8.1 The pro/anti-apoptotic effect of galectin-3

Yang and colleagues (1996) demonstrated that transfection of the T-cell line, Jurkat, with the Gal-3 gene promotes the growth of the cell line in suboptimal culture conditions (culture medium containing only 1% bovine serum), when compared to untransfected cells. Further, intracellular expression of the protein was able to confer protection to apoptosis induced by CD95 (apoptosis antigen [Apo]-1/Fas) receptor ligation or by staurosporine. The intracellular anti-apoptotic role of Gal-3 was also demonstrated in large B-cell lymphomas, where transfection with a Gal-3 expressing plasmids resulted in a markedly increased resistance to anti-Fas-induced cell death (Hoyer et al. 2004). Similarly, targeted disruption of Gal-3 gene in peritoneal macrophages resulted in cells more prone to undergo apoptosis than those from Gal-$3^{(+/+)}$ mice when treated with apoptotic stimuli, suggesting that expression of Gal-3 in these cells may lead to longer cell survival (Hsu et al. 2000).

Gal-3 expression in the breast carcinoma cell line BT-549 and Evsa-T, which do not or slightly express Gal-3 respectively, protects cells from apoptotic insults (UV/anticancer drugs/tumor necrosis factor [TNF]-α). Interestingly, in BT-549 cells this latter phenomenon is also associated with phosphorylated Gal-3 transport from the nucleus to the cytoplasm (Matarrese et al. 2000; Takenaka et al. 2004).

The underlying mechanisms regulating the intracellular anti-apoptotic effect of Gal-3 are still under debate; many Authors identified several cytoplasmic binding partners and suggested that regulatory pathways are involved (Dumic et al. 2006). A possible mechanism seems to be the translocation of Gal-3, upon different apoptotic stimuli, from the cytoplasm/nucleus to the mitochondria, where it can interact with other apoptosis regulators and block the changes in mitochondrial membrane potential, the release of cytochrome C and therefore preventing apoptosis (Nangia-Makker et al. 2007).

In fact, Gal-3 can interact with Bcl-2 (Yang et al. 1996), a death suppressor, probably through the NWGR sequence, designated as the anti-death domain, present in both proteins, which it is fundamental for Bcl-2/Bax heterodimerization, Bcl-2/Bcl-2 homodimerization (Hanada et al. 1995) and Gal-3 anti-apoptotic effect (Akahani et al. 1997).

Besides Bcl-2, Gal-3 prevents the mitochondrial damage following cytochrome C release through the interaction with synexin, a Ca^{2+}/phospilipid binding protein. Synexin can bind and mediate Gal-3 translocation to the perinuclear membrane of mitochondria. Downregulation of this protein impairs the ability of Gal-3 to exert its anti-apoptotic effect (Yu et al. 2002).

In response to cellular stress, P53, a master regulator of apoptosis, can induce cell death through Gal-3 repression. In particular, Gal-3 is downregulated by the specific cooperation of the homeodomain-interacting protein kinase (HIPK)2 and P53, and the expression of a non-repressible Gal-3 prevents HIPK2 and p53-induced apoptosis (Cecchinelli et al. 2006). Similarly, it was demonstrated that Nuclin, an apoptosis-associated protein, can interact with Gal-3 (Dumic et al. 2006) in the cytoplasm and interfere with NF-kB in order to inhibit expression of Gal-3 at both mRNA and protein levels (Liu et al. 2004).

Another important partner for Gal-3 is the activated form of K-Ras, a small GTPase protein able to respond to different extracellular stimuli, modulating different biological consequences, such as senescence/proliferation, death/survival. Gal-3 acts

as a specific binding partner of activated K-Ras and depending on cellular contest can stimulate anchorage independent cell growth, cellular proliferation, and inhibition of apoptosis via K-Ras-mediated Raf/mitogen-activated protein (MEK)/ extracellular signal-regulated kinase (ERK) activation (Shalom-Feuerstein et al. 2005; Levy et al. 2011), as well as phosphoinositide 3-kinase (PI3K) (Elad-Sfadia et al. 2004).

Finally, Alix and ALG-2 interacting protein (AIP)-1 might be implied in the anti-apoptotic effect of Gal-3. These proteins are able to interact with ALG-2, a protein inhibiting paraptosis, a form of programmed cell death. Similarly, Alix and AIP-1, are also able to interact with Gal-3, as demonstrated in Jurkat cells, probably through the N-terminal of these proteins, containing a proline glycine alanine and tyrosine-rich sequence, homologous to the N-terminal part of Gal-3 (Nangia-Makker et al. 2007).

In sharp contrast, exogenously added Gal-3 has been shown to induce apoptosis in human T cells, neutrophils, peripheral blood mononuclear cells (PBMC) (Fukumori et al. 2003; Fernández et al. 2005; Stillman et al. 2006) and mouse activated T cells (Fukumori et al. 2003), while at the moment there is no evidence for an anti-apoptotic activity of exogenously added Gal-3 (Dumic et al. 2006). Concerning the mechanisms involved in Gal-3-induced apoptosis, at least in T cells, cell death seems to pass through the interaction with CD95, a member of the TNF superfamily. Activation of CD95 by apoptotic stimuli, can determine two different apoptotic signal pathways, one requires a large amount of activated caspase 8 at the cell death-inducing signaling complex (DISC) (mitochondria independent, type I cells), the other (mitochondria dependent, type II cells) depends on the apoptogenic activity at the mitochondria. It was demonstrated that the major difference among cells which respond to the apoptotic insult with the type I (such as SKW6.4 or H9) or type II (such as Jurkat or CEM) pathway is the intracellular level of Gal-3. Transfection of Gal-3 in Gal-3 null cells results in binding of this lectin to CD95, converting their

phenotype from type II in type I apoptotic cells (Fukumori et al. 2004). This suggests that Gal-3 can modulate the signaling through CD95 determining which apoptotic signaling pathway a cell will follow.

The differences in the intracellular levels between type I/II cells also reflect how these cells will react after extracellular Gal-3 exposure. In particular, exogenous Gal-3 can induce apoptosis in type II cells with activation of intracellular signaling leading to cytochrome C release and caspase 3 activation, while type I are more resistant. This is probably due to the high level of endogenous Gal-3 in type I cells that can balance the pro-apoptotic effect of extracellular Gal-3 with an anti-apoptotic effect of endogenous Gal-3. Interestingly, the pro-apoptotic effect of Gal-3 passes through the carbohydrate-dependent interaction with surface glycosylated receptors, since lactose is able to reduce this effect in a dose-dependent manner. The receptors responsible for this effect were identified in C29 and CD7 (Fukumori et al. 2003). In particular, these two receptors may work as oligomers while delivering the Gal-3 death signal, through the clustering ability of Gal-3 (already observed for T cell; Hsu et al. 2009). This would be in line with the observation that oligomerization of Gal-3 is important for the pro-apoptotic effect, since the NH2 terminus removal abrogates this effect (Fukumori et al. 2003).

The behavior of Gal-3 in response to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is still elusive. This protein is a member of the TNF family of cytokines that promotes apoptosis, in a wide variety of tumor cells, but not in normal cells. Transfection of the TRAIL insensitive breast carcinoma cell line, BT-549, with Gal-3 gene, renders these cells sensitive to apoptosis suppressing the activity of Akt, a serine/threonine kinase, involved in resistance to TRAIL (Lee et al. 2003). Surprisingly, in J8 cells, a line of human bladder carcinoma, the levels of Gal-3 are elevated, and consistently the levels of constitutively activated Akt, rendering the cells resistant to apoptosis. This opposite result might be due to the use

of two different cell lines, containing different Gal-3 associated proteins (Oka et al. 2005).

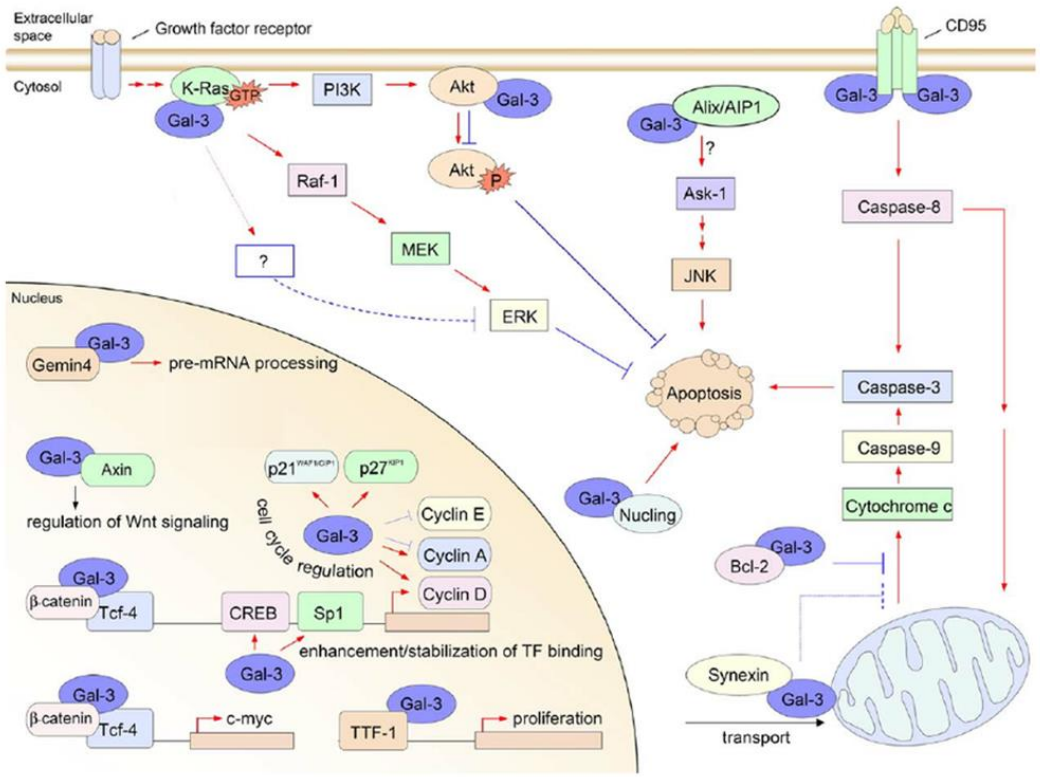

Figure 5., **A cartoon schematically representing some intracellular binding partners identified for Gal-3**. Blue lines indicate negative effects, red arrows indicate positive effects (adapted from Dumic et al. 2006).

1.8.2 Regulation of cell growth and differentiation by endogenous and exogenous galectin-3

Besides the previously reported implications of Gal-3 in life/death regulation, this protein can be a crucial mediator for cell growth and proliferation. In particular, incubation of human lung fibroblasts with recombinant Gal-3 leads to cell proliferation and DNA synthesis, in a dose-dependent manner (Inohara et al. 1998). Similarly, the addition of exogenous Gal-3 to mesangial cells cultured without serum is able to prolong cell survival, protect the cells from the negative effect of TGF-β,

and to induce the synthesis and secretion of collagen type IV (Sasaki et al. 1999). In neural mouse cells, extracellular Gal-3 can serve as adhesion molecule, while it is also able to induce the outgrowth of neurites from dorsal ganglia explants (Pesheva et al. 1998). Still, Gal-3 stimulates not only the capillary tube formation of umbilical vein endothelial cells *in vitro,* but also angiogenesis *in vivo* (Nangia-Makker et al. 2000).

A role of endogenous Gal-3 in cell growth was also demonstrated for different cancer and normal cells with the use of antisense oligonucleotide or cDNA strategy (Hsu and Liu 2002). The blockage of Gal-3 expression in human breast cancer cells leads to a reversion of the transformed phenotype and a suppression of tumor growth (Vandenbrule et al. 1997; Honjo et al. 2001), while in T cells significantly decreases their proliferation (Joo et al. 2001). Similarly, the overexpression of Gal-3 in thyroid papillary carcinoma cells is necessary for the maintenance of anchorage independent growth (Yoshii et al. 2001).

Interestingly, opposite Gal-3 effects were observed for proliferation. In a cancer prostate cell line (LNCaP) transfection of Gal-3 gene reduces the proliferation rate in vitro compared to the vector control-transfected cell lines or to the parental LNCaP (Ellerhorst et al. 2002).
Finally, Gal-3 is able to inhibit the positive effect on proliferation of bone marrow cells given by recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (Krugluger et al. 1997).

1.9. Function in immune system

During the last decades, compelling evidence has been accumulated regarding the ability of Gal-3 to affect both innate and adaptive immune system, modulating the course of immune responses, inflammation, cell growth, signaling and chemotaxis (Rabinovich et al. 2002; Dumic et al. 2006; Sundblad et al. 2011). This effect can be

mediated by either direct binding of extracellular Gal-3 to surface membrane receptors or by intracellular Gal-3 modulation of signaling pathways (Fig. 6) (Díaz-Alvarez and Ortega 2017).

1.9.1 T cells and B cells

As previously mentioned, Gal-3 expression in T lymphocyte is generally absent until induced by strong activating agents; in particular, a combination of receptor cross-linking and proliferative stimuli are required for high levels of Gal-3 expression. In these cells, the intracellular presence of this lectin is associated with an increase in cell proliferation, while cell growth is interrupted when Gal-3 expression is suppressed (Hsu et al. 2009 [A]).

Moreover, in activated T cells, Gal-3 is recruited along with Alix, one of its intracellular binding partner, to the cytoplasmic side of the immunological synapse (IS) (Chen et al. 2009). Alix is a protein involved in protein transport and regulation of cell surface expression of certain receptors, and, as demonstrated by Chen et al. (2009), Gal-3 and Alix recruitment to the IS is associated with low levels of early signaling events, attenuated signal transduction, and TCR downregulation in T cells. Therefore Gal-3, through the modulating of Alix's function at the IS can dampen T-cell activation and function.

The pivotal role of Gal-3 in T cells regulation is not only confined to the intracellular side of the membrane. Extracellularly delivered Gal-3 was reported to play a fundamental role in the regulation TCR signaling, modulating the required number of receptors at the site of antigen presentation. This process is dependent on the formation of multivalent complexes with the N-glycans exposed by TCR, that reduce the lateral mobility and recruitment to the site of antigen presentation, raising the threshold for T cell activation and preventing uncontrolled activation of these cells (Demetriou et al. 2001). The formation of this lattice structure seems to be important

for T cell homeostasis, since disruption of this Gal-3-N-glycan interaction leads to hyperactive T cells and autoimmunity (Sundblad et al. 2011).
Finally, in T cells Gal-3 plays an important role in cell life and death; either inducing (when extracellularly added) or preventing (when endogenously expressed) apoptosis of T cells (for further details see paragraph 1.8.1).

Interestingly, it has been suggested that in the intestinal mucosa, Gal-3 might play an immunomodulatory activity on these lymphocytes. In particular, the basal expression of Gal-3 and its release by epithelial cells of the intestinal mucosa may help to prevent inappropriate immune responses against luminal antigens of food or commensal bacteria. On the contrary, Gal-3 downregulation during tissue inflammation may reflect a desired event, in order to promote an adequate immune response by supporting activated T cell proliferation and reducing activation-induced T cell death (Müller et al 2006).

The function of Gal-3 in the regulation of B cell physiology is far from being completely understood and only a few literature reports explore the role of this chimeric protein in B cell activation and differentiation. Similarly to T cells, also B cells express Gal-3 only after a strong stimulation, such as IL-4 and CD40 cross-linking. In these cells Gal-3 was demonstrated to be a critical mediator of cell differentiation and survival. In particular, endogenous expression of this protein promotes B cells survival and blocks their final differentiation into plasma cells, allowing the rising of a memory B cell phenotype (Acosta-Rodríguez et al. 2004).
In addition, Gal-3, can be secreted by diffuse large B-cell lymphoma and binds back, in a carbohydrate-dependent manner, to the transmembrane tyrosine phosphatase CD45, promoting apoptosis resistance. Removal of cell-surface Gal-3 from CD45 renders these cells susceptible to chemotherapeutic agents (Clark et al. 2012).

1.9.2 Monocytes/macrophages

Macrophages are a heterogeneous population of highly plastic cells that can adapt their functional phenotypes in response to stimuli present in the environment (Díaz-Alvarez and Ortega 2017).

In particular, macrophages can differentiate into M1 macrophages in response to toll like receptor (TLR) and interferon (IFN)-γ stimulation. These cells produce high levels of pro-inflammatory cytokines and reactive nitrogen/oxygen intermediates and promote a Th1-type response. In addition, they have strong microbicidal and tumoricidal activities. Alternatively, under the influence of IL-4/IL-13 or IL-10, they can differentiate into M2/M2-like macrophage, which in turn are more involved in tissue remodeling, parasite containment, tumor progression, and immunoregulatory functions (MacKinnon et al. 2008; Díaz-Alvarez and Ortega 2017).

Increased Gal-3 expression and secretion is a feature of M2 macrophage activation, while in M1 macrophage activation, Gal-3 expression and release are lower (MacKinnon et al. 2008). With the use of a specific inhibitor, it has been demonstrated that Gal-3, released by alternative activate M2 macrophages in response to IL-4, is able to sustain this alternative activation, and that Gal-3 deficient mice display an impaired M2, IL-4-mediated, polarization (MacKinnon et al. 2008).

Gal-3 plays a critical role also in macrophage phagocytosis, as demonstrated by Sano et al. (2003). In particular, Gal-3-deficient cells exhibited reduced phagocytosis of IgG-opsonized erythrocytes and apoptotic thymocytes in vitro, while Gal-3 deficient mice showed attenuated phagocytic clearance of apoptotic thymocytes by peritoneal macrophages in vivo. Interestingly, extracellular Gal-3 does not contribute appreciably to phagocytosis, while the intracellular accumulation of Gal-3 around the phagosome seems to be responsible for this process.

In addition, Gal-3 has an important anti-apoptotic role in these cells since gene disruption leads to a lower number of macrophages, with altered morphology/phenotype and more prone to undergo apoptosis (Hsu et al. 2000).

In human monocytes, Gal-3, can binding to cell surface glycoconjugates inducing superoxide release and potentiating their IL-1 production, in a lactose-sensitive manner (Jeng et al. 1994; Liu et al. 1995). Finally, Gal-3 recognizes and binds the highly glycosylated CD13 molecule, expressed on monocyte surfaces, inducing homotypic aggregation of inflammatory monocytic cells (Mina-Osorio et al. 2007).

1.9.3 Neutrophils

Neutrophils are abundant specialized effector leukocytes, possessing high phagocytosis and microbicidal activities. They represent one of the first lines of defense to arrive at the site of tissue injury (Díaz-Alvarez and Ortega 2017)

In neutrophils, similarly to T cells, exogenously added and endogenous Gal-3 have distinct effects. Extracellular Gal-3 enhances the ability of neutrophils to migrate, phagocytize, produce IL-8 and reactive oxygen species (ROS), bind and induce IgE-dependent cellular activation, as well as NADPH-oxidase activation and oxidative burst. In particular, candidate receptors able to induce neutrophil activation, oxidative burst, and NADPH-oxidase activation are the CD66a and CD66b protein (Feuk-Lagerstedt et al. 1999). On the contrary, endogenous Gal-3 dampens their activation and their pro-inflammatory activity (Truong et al. 1993; Nieminen et al. 2005; Wu et al. 2017; [A]).

1.9.4 Mast cells and eosinophils

Gal-3 induces the cross-linking of cell surface glycoproteins, possibly high-affinity IgE receptors (FcεRI), that in turn trigger a signal transduction cascade, that activates and initiates degranulation in mast cells (Frigeri et al. 1993). Gal-3 function in these cells was studied by the use of transgenic mice. Gal-3 deficient mice showed reduced secretion of histamine, as well as IL-4, upon cell activation, and a general defect in mast cell responses (Chen et al. 2006).

Addition of Gal-3 induces a selective downregulation of IL-5 expression in human eosinophils, PBMC and in an Ag-specific T cell line (CD4+), in a lactose-dependent manner (Cortegano et al. 1998). These authors proposed that inhibition of IL-5 passes through IgG receptor (FcγRII or CD32), since incubation of PBMC from a FcγRII deficient mouse with Gal-3 leads to no changes in the expression of the IL-5 gene (Cortegano et al. 2000).

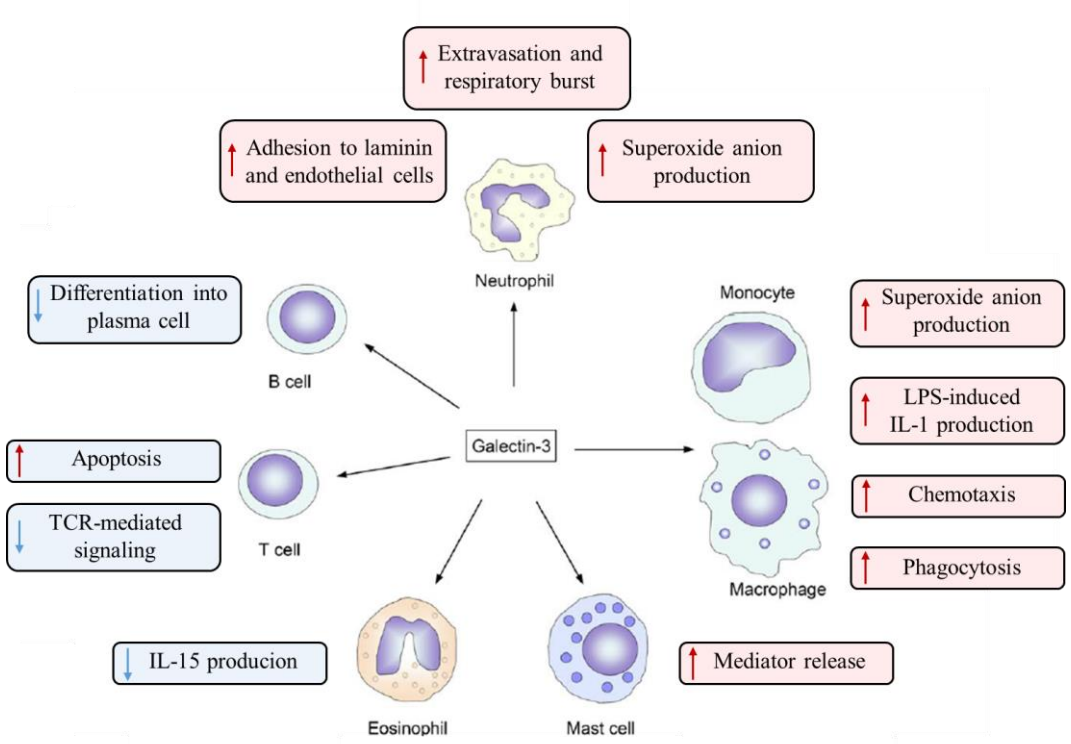

Figure 6. A cartoon summarizing the effect of Gal-3 on immune cells. Red arrows indicate a positive effect, blue arrows indicate a negative effect. Light red boxes indicate pro-inflammatory effects; light blue boxes indicate anti-inflammatory effects (adapted from Dumic et al. 2006).

1.9.5 Adhesion and migration of immune cells

Gal-3 is a fundamental protein in cell adhesion (by bridging cell to cell or cell to extracellular matrix) and migration of immune cells.

In neutrophils, this lectin is involved in neutrophil recruitment and extravasation to the infection site, adhesion to laminin in a carbohydrate- and amino terminal-dependent manner, and to endothelial cells through oligomerization of Gal-3 and cross-linking of neutrophils to the endothelium (Kuwabara and Liu 1996; Sato et al. 2002).

In a model of sepsis, Gal-3 deficient mice display a better survival rate and lower level of lung tissue damage compare to wild type animals, which display a high leucocyte infiltration (principally neutrophils), release of inflammatory cytokines/mediators, and vascular injury (Mishra et al. 2013; Díaz-Alvarez and Ortega 2017).

Interestingly, despite Gal-3 is not a chemoattractant for neutrophils in vitro (Baseras et al. 2012), and Gal-3 deficient mice present a decrease in the number of infiltrating neutrophils (Bhaumik et al. 2013), suggesting that Gal-3 can indirectly induce neutrophil migration (Díaz-Alvarez and Ortega 2017). Similarly, once in the extracellular media, Gal-3 can act as chemoattractant for monocytes and macrophages, in a dose- and lactose-dependent manner (Sano et al. 2000).

The expression of Gal-3 on the surface of human eosinophils favors the recruitment of these cells to the site of inflammation, mediated by rolling and adhesion to the vascular cell adhesion molecule (VCAM)-1 molecule (Rao et al. 2007), while it is also responsible for the IgE-dependent activation of these cells (Truong et al. 1993). These findings support a function for Gal-3 during inflammatory and infectious diseases.

Endogenous Gal-3 has been demonstrated to be important for T cell adhesion to DCs or macrophage cells. During naive lymphocyte recruitment from blood to secondary lymphoid organs, the expressed of L-selectin on these cells, plays an important role in the initial attachment of T cells to high endothelial venules in lymph nodes, and

in cell activation. Gal-3 can enhance and optimize the initial interaction between L-selectin activated lymphocytes and DCs (Swarte et al. 1998; Sundblad et al. 2011).

1.9.6 Galectin-3 at the interface between innate and adaptive immunity

DCs play a central role in the regulation of adaptive and innate immune responses. These cells patrol the peripheral tissues, capturing and processing pathogens, migrating to draining lymph nodes where they determine the polarization of the adaptive immune response, which in turn is reflected on innate immunity (Díaz-Alvarez and Ortega 2017).

Infection with *Trypanosoma cruzi* induces the upregulation of Gal-3 and Gal-3 ligands in splenic DCs. Since DCs migration is reduced on Gal-3 coated surfaces, the secretion of this lectin during *Trypanosoma cruzi* can impair the migration of these antigen presenting cells to lymphoid organs and therefore reduce T cell activation (Vray et al. 2004). In another study of infection, investigating the role of Gal-3 during *Toxoplasma gondii* infection in a model Gal-3 deficient mice, it was found that Gal-3 deficient mice develop a general reduced inflammatory response of leucocyte infiltrating the intestines, liver, and brain but not the lungs, when compare to wild type animals (Bernardes et al. in 2006). Brain from Gal-3 deficient mice showed a significantly reduced number of infiltrating monocytes/macrophages and CD8+ cells, therefore suffering a higher parasite burden. DCs from Gal-3 null mice produce higher amounts of IL-12, which reflects in a higher Th1 response. Similarly, DCs from Gal-3 deficient mice infected with histoplasmosis produce higher IL-23/IL-17 and lower IL-12/IFN-γ cytokines. This leads to an increase in Th17 and in a reduction in Th1 cells with a lower fungal burden (Wu et al. 2013). Finally, studies with the use of siRNA evidenced how Gal-3 can modulate cytokine secretion by human DCs. In particular, the use of Gal-3 siRNA in TLR stimulated (LPS) DCs, is able to downregulate the expression of IL-1β, IL-6 and IL-23, while it upregulates IL-12p35 and IL-10 (Chen et al. 2015; Díaz-Alvarez and Ortega 2017). Since the use of exogenous Gal-3 or neutralizing antibody are not able to revert this effect, the

Authors conclude that the change in DCs cytokine profile is due to the intracellular and not to the extracellular version of the protein (Chen et al. 2015). These studies underline, once again, the importance of Gal-3 expression in the regulation of the immune response, since changes in DC cytokine secretion deeply influence CD4+ T cells, which in turn affects the innate immune response.

1.9.7 Recognition of foreign pathogens

Unlike adaptive immunity, that can tailor the specificity of receptors for foreign antigens, innate immunity is programmed to recognize pathogen-associated molecular patterns (PAMPs) or host intracellular damage-associated molecular patterns (DAMPs), that are released from dying cells upon damage induced by microorganisms (such as heat shock protein, interleukin-1α and annexin) (Sato et al. 2009).

Cells of the immune system can be induced to release this DAMPs without dying, using a leaderless secretory pathway, in order to recruit and activate other innate immune cells and restore tissue homeostasis. As previously mentioned, Gal-3 can be passively released by dying cells, or actively secreted from cells upon specific stimuli, such as IFN-γ and LPS, though a leaderless pathway. The intriguing emerging idea is that Gal-3 can work as DAMP recognizing PAMP, recruiting in the site of infection immune and phagocytic cells (Sano et al. 2000; Bhaumik et al. 2013), potentiating their activities and thereby modulating the innate/adaptive immune responses (Jeng et al. 1994; Yamaoka et al. 1995; Sano et al. 2003; Fernández et al. 2005; Sato et al. 2009; Díaz-Alvarez and Ortega 2017).

During *Helicobacter pylori* colonization of gastric tissues, Gal-3 mRNA is upregulated and the protein is released by bacteria infected cells (Fowler et al. 2006). As previously reported (for further details see paragraph 1.4.2), Gal-3 has been demonstrated to recognize and bind two independent sites of LPS. This interaction, either through the CRD or the N-terminal domain, was demonstrated for *Neisseria*

gonorrhoea (John et al. 2002), *Helicobacter pylori* (Fowler et al. 2006), *Pseudomonas aeruginosa* (Gupta et al. 1997), *E.coli* (Fermino et al. 2011) and *Klebsiella pneumoniae* (Mey et al. 1996). Gal-3 binding to LPS results in oligomer formation, greatly enhancing neutrophil activation (Fermino et al. 2011). Interestingly, macrophages from Gal-3 deficient mice show an elevated production of pro-inflammatory cytokines upon LPS stimulation, when compared to wild type animal, and this prevents LPS-induced endotoxic shock, increasing both resistance to infection and Th1 response (Díaz-Alvarez and Ortega 2017). Further, evidences show the Gal-3 ability to recognize mycobacterial mycolic acids, the major constituents of *Mycobacterium tuberculosis* cell envelope. Despite this interaction inhibits the lectin self-association Gal-3 could be involved in the recognition of trafficking mycolic acids (Barboni et al. 2005).

In macrophages, Gal-3 and Dectin-1 (a receptor for β-glucans) strictly collaborate for the recognition of pathogen microorganisms. In particular, Gal-3 recognizes oligomannans, carbohydrates composing the wall of fungi such as *Candida albicans*, and both, Dectin-1 and Gal-3 must be presented in order to have a specific TNF-α secretion by these cells. Direct binding of Gal-3 to β-1,2-linked oligomannans can induce the death of *Candida albicans*, and this structure is important for discrimination of pathogenic (*Candida albican*) and non pathogenic (*Saccharomyces cerevisiae*) yeast (Díaz-Alvarez and Ortega 2017).

In addition, Gal-3 was shown to accumulate at the cytosolic face of the phagosome membrane in Mycobacterium-containing phagosomes during the course of *Mycobacterium tuberculosis* infection. Conversely, Gal-3 null mice revealed a reduced capacity to clear late *Mycobacterium* infection (Beatty et al. 2002)

This lectin plays also an important role in parasite recognition. Gal-3 was reported to bind to GalNAc1-4GlcNAc (LacdiNAc) units present on soluble egg of *Schistosoma mansoni*, a human helminth parasite. In particular, since Gal-3 was found accumulated in granuloma tissue around egg shells and due its ability to

mediate macrophage LacdiNAc-coated particle phagocytosis; van den Berg et al. (2004) proposed that Gal-3 can act as an opsonin to facilitate the uptake of LacdiNAc-containing glycoconjugates by leukocytes, contributing to the development of host immunity.

Finally, Gal-3 recognizes molecules associated with protozoan parasites *Trypanosoma cruzi* (Moody et al. 2000) and *Leishmania major*. Despite in the latter case Gal-3 recognition leads to the cleavage of Gal-3 (Pelletier and Sato 2002), it was proposed (Sato et al. 2014) that Gal-3, once released during *Leishmania major* infection, can facilitate the infiltration of neutrophils to the infected sites, reducing the initial parasite burden.

All these observations raise the possibility that Gal-3 is involved in the complex molecular mechanisms underlying the immune system recognition of infection agents to develop adequate immune responses.

1.9.8 Role in immune system regulation

As shown in the previous paragraphs, Gal-3 is a highly promiscuous protein, with different cellular localization within the tissue microenvironment. It can either control immune cells through the intracellular compartment or it can be released and directly interacts with foreign molecules/immune cells, either as monomer or oligomer. This great flexibility of Gal-3, in turn, reflects the different processes modulated by this protein.

An interesting hypothesis is that Gal-3, during homeostatic conditions, can bind to self-molecules expressed on cell surface of leukocyte, including macrophages, DCs, and T lymphocytes, lattices. Until Gal-3 keeps recognizing self-molecules the master switch of our innate immune system remains settled on ‘OFF’ (Sato and Nieminen 2002). If the protein is either cleaved or sequestrated by foreign pathogens, the immune system can be switched to ‘ON’, starting a proper immune response. As

previously reported, in fact, Gal-3 has the ability to form a lattice structure on T cell surface that raises their activation threshold, while following its absence the cells become hypersensitive for activation. Further, truncation of Gal-3 and therefore depletion of this protein, during *Leishmania major* infection, leads to an increment of immune responses, as observed during this parasite infestation (Sato and Nieminen 2002).

Importantly, Gal-3 would be implied not only in the beginning of immune responses, but also in the promotion of the inflammatory responses, through cell activation, cell migration, or inhibition of apoptosis, thus prolonging the survival of inflammatory cells (Hsu et al. 2000; Zuberi et al. 2004; Chen et al. 2005; Henderson and Sethi 2009)

Finally, Gal-3 can participate also in the resolution of inflammatory responses. In fact, Gal-3 plays an important role also in the removal of immune cells, an important mechanism to restore homeostasis after an inflammatory period. In particular, extracellular Gal-3 can lead to exposure of phosphatidylserine on activated leukocyte, a signal for macrophage phagocytosis (Stowell et al. 2008; van Stijn et al. 2009). Absence of Gal-3 shows reduced neutrophils apoptosis, and impaired clearance of these cells (Wright et al. 2017)

1.10 Natural killer cells

Natural killer cells (NK) are white blood, bone marrow-derived granular lymphocytes cells, which can exert direct cytotoxic activities against pathogen-infected or tumor cells with an activity similar to that of CD8+ cytotoxic T lymphocytes (Sun and Lanier 2011). These cells belong to the innate arm of the immune system and play a pivotal role in host defense against infections and malignancies, as demonstrated in the murine model and in cases of NK cells deficiencies (Smyth et al. 2005; Orange 2006).

Characterized as lymphocytes for their morphology, the expression of lymphoid markers and their origin from the common lymphoid progenitor, human NK cells are phenotypically identified by the absence of CD3 co-receptor and the expression of the neural cell adhesion molecule CD56.
In 2007, it was proposed by the Vivier's group that another marker able to specifically identified NK cells is the NKp46 receptor; however, this glycoprotein can also be found on T cells, while some subpopulations of NK do not express this surface marker (Walzer et al. 2007). Therefore, since there is still controversy on the use of this surface molecule for NK cells identification, the current phenotypic definition remain the $CD56^{+}CD3^{-}$ combination (Caligiuri 2008).

Human NK cells represent about the 10% of PBMC with a range that can vary between 5 to 20 % and about 2 billion of cell circuiting in the body of an adult at any given time, with an average turnover of two weeks (Caligiuri 2008; Vivier et al. 2008; Pampena and Levy 2015). These innate lymphoid cells are not a homogeneous population; on the contrary, they can be generally dived in two categories with very distinct phenotypic and functional characteristics: the $CD56^{bright}$ and $CD56^{dim}$ subsets. The $CD56^{brigh}$ population is generally found in lymph nodes, tonsils and secondary lymphoid organs while only a little fraction of these cells is found in the peripheral blood (PB) (10%). These cells do not, or only partly, express the CD16, the natural cytotoxicity receptors (NCRs) and lack killer cell Ig-like receptors (KIRs) expression (see next paragraph for further details on NK cells receptors) (Ferlazzo et al. 2004). Moreover, they display low amount of perforin and only little ability to kill target cells, while they can produce high amount of cytokines within minutes after activation (Ferlazzo et al. 2004; Caligiuri 2008; Vivier et al. 2008). In particular, $CD56^{brigh}$ represent the most vigorous producer of IFN-γ within the NK population. IFN-γ is a pro-inflammatory cytokine secreted during the early phase of the innate immune response, able to shape both, adaptive and innate immune response. This cytokine, in fact, induces CD8+ T cells to become cytotoxic lymphocytes and help

CD4+ T cells to differentiate toward a Th1 phenotype (Mocikat et al. 2003; Martín-Fontecha et al. 2004). In addition, IFN-γ regulates B cell functions, such as immunoglobulin production and class switching, macrophage antimicrobial/antitumor activities and upregulation of major histocompatibility complex (MHC) class I molecules, DC maturation, leukocyte attraction, growth, and maturation (Wallach et al. 1982; Schroder et al. 2004; Vivier et al. 2008). Moreover, IFN-γ impairs proliferation of transformed and virally infected cells (Maher et al. 2007).

In sharp contrast, $CD56^{dim}$ population presents an inverted ratio between secondary lymphoid tissue and bloodstream being relatively dominant in PB. These cells are characterized by a $CD56^{dim}CD16^{+}$ phenotype and express both KIRs and NCRs.
The $CD56^{dim}$ subset is composed of cytotoxic effector cells able to efficiently lyse target cells upon activation and to secrete, although to a lower extent, chemokines and cytokines (most notably IFN-γ, TNF-α, C-C motif chemokine ligand (CCL)3 and CCL5) (Moretta et al. 2014).

In addition, another important characteristic that distinguishes these two subsets consist in the IL-2 receptor composition. $CD56^{brigh}$ cells express the hetero-trimeric, high affinity IL-2 receptor (IL-2Rα[CD25]/IL-2Rβ[CD122]/IL-2Rγ[CD132]) which can sense picomolar concentration of this cytokine released from activated T cells (Caligiuri et al. 1990; Nagler et al. 1990). On the contrary, $CD56^{dim}$ cells only express the hetero-dimeric form of the receptor (IL-2Rβ[CD122]/IL-2Rγ[CD132]) which can bind both IL-2/IL-15 and display only intermediate affinity for IL-2. These variations in the IL-2 receptor composition are reflected in a higher capacity of the $CD56^{brigh}$ subset to proliferate when compared to the $CD56^{dim}$ subset (Caligiuri 2008; Vivier et al. 2008). Finally, $CD56^{brigh}$ and $CD56^{dim}$ cells differ also for their homing capacity.

The $CD56^{brigh}$ subset, in fact, expresses the C-C motif chemokine receptor (CCR)7 and C-X-C motif chemokine receptor (CXCR)4, which are likely to coordinate these cells into lymph nodes (Campbell et al. 2001; Fehniger et al. 2003; Hanna et al. 2003). On the contrary, the $CD56^{dim}$ expresses the CXCR1 and CX3CR1, which can recruit the cells into peripheral inflamed tissue (Campbell et al. 2001; Hanna et al. 2003).

Despite it remain largely unclear the different stages of maturation of NK cells, it was proposed that $CD56^{bright}$ population could represent a more immature stage/precursor in NK cells development, while the $CD56^{dim}$ represents the mature form of these innate lymphoid cells (Chan et al. 2007; Romagnani et al. 2007; Takahashi et al. 2007).
This was further supported by in vitro studies, demonstrating that cytokine stimulation is able to induce the expression of molecules (i.e., CD16, NCRs. KIRs and perforin), as well as functional characteristics (i.e., the cells become able to lyse sensitive target cells) similar to PB $CD56^{dim}$ NK cells (Ferlazzo et al. 2004; Caligiuri 2008; Moretta et al. 2008; Vivier et al. 2008).

This scenario is further complicated by the fact that PB NK cells not only present these subsets, but they can express a different combination of activating and inhibitory receptors. This feature makes NK cells an extremely heterogeneous population able to sense different environmental stimuli and easily adapt to them (Mandal and Viswanathan 2015).

1.10.1 Natural killer cells activation and functions

As mentioned in the previous paragraph, the largest amount of NK cells found in the PB are $CD56^{dim}CD16^{+}$ NK cells and that type of cells are responsible for the cytolytic activities.

This ability consists in the polarization and release by NK cells of lytic granules containing cytotoxic proteins, such as perforin and granzymes, which can generate a pore on the plasma membrane of target cell and therefore induce cell death (Moretta et al. 2008).

The name NK cells, derives from the observation that these cells can kill, as their name implies, their target without prior sensitization and without the need for target cell to present antigens on MHC molecules (Caligiuri 2008).
In sharp contrast with T and B cells, to fulfill their direct cytotoxicity on a specific target, these lymphocytes do not express clonal antigen receptors, produced by somatic recombination, instead, they are equipped with a wide array of germline-encoded activating and inhibitory receptors (Vivier et al. 2011).

Although NK cells can sense a lower number of antigens compared to the T and B cells, the broad range of receptors expression allow these cells to respond faster to danger signals, in contrast with a more delayed response of adaptive immunity (Montaldo et al. 2013).
The major activating receptors include the CD16 or low affinity FcγIIIA receptor, that is able to recognize the crystallizable fragment (Fc) region of antibodies blocked on cell surface. CD16 interaction with Fc leads to NK cells activation and degranulation, in a process defined antibody-dependent cell-mediated cytotoxicity (ADCC) (Vivier et al. 2008). However, NK cells can recognize target cells also in the absence of antibody coated cells, through many other activating receptors.
These include NKG2D, DNAM-1, and the NKp30/NKp44/NKp46 receptors, collectively named natural cytotoxic receptors.
Among the most characterized ones, NKG2D is a C-type lectin-like receptor whose ligands (the MHC class I polypeptide-related sequence [MIC]A and MICB) are normally not expressed in healthy and normal tissues, but they can be upregulated in virally infected cells or during malignant transformation. Similarly, Nkp30

recognizes the B7-H6 molecule expressed by certain tumors (Vivier et al. 2011) and absent on non transformed cells. Notably, while NKG2D, NKp30 and NKp46 are already present on unstimulated NK cells, NKp44 is upregulated only after cytokine stimulation and with NKp46 recognize hemagglutinins on virus infected cells (Mandelboim et al. 2001; Montaldo et al. 2013).
Furthermore, DNAM-1 is a surface glycoprotein that belongs to the Ig-like family, which is implicated in the recognition of cancer/virally infected cells. This adhesion molecule, in fact, recognize CD112 and CD155, two nectin molecules upregulated on aberrant or stressed cells (de Andrade et al. 2014).

All the receptors presented here can recognize cell surface antigens, expressed under pathological conditions, killing the dangerous cells.
Despite the great cytotoxic potential of NK cells, healthy cells are generally spared by their activity. Besides activating receptors, in fact, NK cells are decorated with a broad range of inhibitory receptors. Dozens of these molecules, collectively called killer cell Ig-like receptors, have been identified. These include receptors that recognize MHC class I molecule (human leucocyte antigens [HLA]-A,-B and -C), and the heterodimer C-type lectin-like CD94-NKG2A, which recognize the non classical HLA-E molecule. Once engaged, a KIR delivers to the NK cell a signal able to prevent cellular activation and to overcome a low level of activating signals.

Therefore, in order to fulfill their function, NK cells must encounter cells which present downregulation of MHC class I molecule (the missing self hypothesis), however, this signal alone is not sufficient to guarantee target killing, there must be simultaneously present also activating signals.
As a result, NK cell activation is a complex and sophisticated process tightly regulated by the integration of the positive and negative signals, generated by both activating and inhibiting receptors, which determine whether or not they have to kill

a specific target (Moretta et al. 2014). These mechanisms allow the tolerance to self and to specifically kill only potentially dangerous cells (Fig. 7).

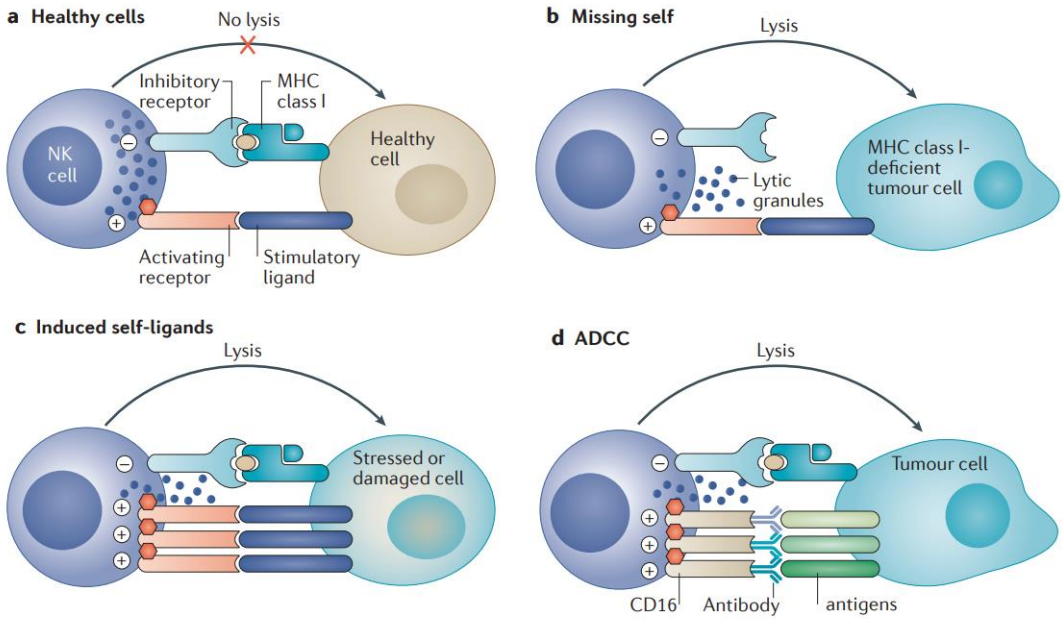

Figure 7. Schematic representation of NK cells activation. A) balance of signals delivered by activating and inhibitory receptors regulates the recognition of healthy cells by NK cells. B) Cells that downregulate MHC class I molecules are detected as 'missing self' and are lysed by NK cells. C) Cells can overexpress induced stress ligands recognized by activating NK cell receptors, which override the inhibitory signals and elicit target cell lysis. D) antigen-specific antibodies bind to CD16 and elicit antibody-dependent NK cell-mediated cytotoxicity (Morvan and Lanier 2016).

The use of mouse models as studies on NK cells from human PBMC, helped to further clarify the mechanism underlying NK cells regulation (Bryceson et al. 2006; Fehniger et al. 2007). In particular, it was demonstrated that although NK cells are already equipped with activating and inhibitory receptors, similar to T cells, they require "priming" in order to be fully activated. Despite priming mechanisms remain to be fully characterized, cytokines produced by other immune cells, including IFN, IL-12, IL-2, IL-15 and IL-18, are strong activators of NK cell functions. Particularly, IL-2 and IL-15 cytokines have been demonstrated to transform resting NK cells into lymphokine-activated killer cell (LAK), which develop effector cytotoxic functions

against cancer cells, otherwise resistant (Becknell and Caligiuri 2005; Zwirner and Domaica 2010).

As previously stated, NK cells found in the PB are not merely effector cells, but they also possess the ability to release, upon activation, cytokines (such as IFN-γ, TNF-α, GM-CSF, M-CSF) and chemokines (such as CCL2, CCL4 and CCL5) which, in turn, can recall and shape the activity of other immune cells. In particular, once in the peripheral inflamed tissues NK cells can start a cross-talk with other immune cells, determining, not only the initiation of the innate immune response, but also the polarization and amplification of the adaptive one(Caligiuri 2008; Moretta et al. 2008; Montaldo et al. 2013; Moretta 2014).

Through TNF and IFN-γ production, NK cells can promote DC maturation and kill immature DCs in order to remove myeloid cells, which are unresponsive to specific infectious stimuli paving the way for mature DCs.

NK cell-mediated lysis of pathogen-infected cells might lead to the release of microbial products, such as LPS, that along whit IFN-γ can promote macrophage polarization to a pro-inflammatory phenotype. NK cells-macrophages interaction goes beyond M1 polarization, in fact, NK cells can preferentially kill M2 macrophages, further sustaining a pro-inflammatory response. NK cell regulatory activity is not only exerted on myeloid cells, but also on T cells; in fact, as previously reported, NK cells can promote Th1 repose. Moreover, antigenic peptide released during NK cell cytotoxic activity against dangerous cells can promote the cross-presentation by DCs to CD8+ T cells therefore specifically addressing the adaptive immune response (Cooper et al. 2004; Biswas and Mantovani 2010; Malhotra and Shanker 2011; Deauvieau et al. 2015).

The presence on the same subset of NK cells of effector and cytokine producing functions allow a rapid and effective (around 4h) immune intervention without the

need for coordination with other cell subsets localized in a different anatomic compartment.

The high grade of plasticity, the ability to respond directly to potentially dangerous cells combined with their regulatory activity on innate and adaptive immune cells make of NK cells a major player during the immune response.

2. Outline of the thesis

In spite of the overall weight of evidences on the Gal-3 expression/function in human immune cells, data on NK cells are still very scarce and all observations are derived from mouse models.

In summary, from literature data:

- In 2002, Crider-Pirkle et al. found that during mouse pregnancy (from day 12) uterine NK (uNK) cells start to express Gal-3 at both mRNA and protein levels. uNK cells are a subset of NK lymphocytes that accumulate in large numbers in the decidua basalis and the metrial gland of the rodent implantation site. These cells influence the development of placental vasculature by production of proteases/matrix/biologically active factors and are less cytotoxic than peripheral NK cells;
- In 2005, Kang et al. performed a serial analysis of gene expression (SAGE) at four different stages of NK cell differentiation, founding that Gal-3 expression deeply changes during development;
- In 2011, Radosavljevic et al. showed that Gal-3 null transgenic mice have a general reduction in the number of NK cells, which exhibit a higher cytotoxic activity than cells from wild type mice;
- In 2015, Chaudhari et al. reported that Gal-3 null transgenic mice present a severe dysregulation in NK cell-mediated immune responses, highlighting the importance of Gal-3 in NK cell functions.

The overall data strongly support the importance of Gal-3 in immune-mediated responses, but they also clearly suggest that the role of Gal-3 in NK cell functions has not yet been fully defined.

On this basis, the aim of my Ph.D. research was to:

- examine the expression and localization of Gal-3 in human resting/activated NK cells;
- analyze if changes in Gal-3 expression functionally correlate with human NK cell degranulation.

3. Materials and methods

3.1 Human NK cell isolation and cell cultures

Human NK cells were isolated from buffy coats of healthy donors ($n \geq 6$), after their informed consensus, as previously described (Fallarini et al. 2017). After cell separation through negative selection (Miltenyi Biotec, Calderara di Reno, Italy), the percentage of $CD3^-CD56^+$ cells was routinely analyzed by FACS (S3 flow cytometer, Biorad, Segrate, Italy) using FITC anti-human CD56 (NCAM) Antibody [Clone: MEM-188 (BioLegend, San Diego, CA, USA)], PerCP anti-human CD3 [Clone: BW264/56 (Miltenyi Biotec)] and always resulted ≥ 97%. Approximately 6 x 10^6 NK cells were routinely obtained from each patient.

Human NK cells, human erythroleukemia K562 cells (a generous gift from Prof. M.C. Mingari, Department of Experimental Medicine, IRCCS AOU San Martino-IST, Genova, Italy), human monocytic leukemia THP-1 cells (ATCC TIB-202; American Type culture Collection, Manassas, VA, USA) and lymphoblastic leukemia Jurkat cells (ATCC TIB-152) were cultured (1×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Carlo Erba, Cornaredo, Italy), 2mM L-glutamine, 100 µg/ml kanamycin, 1 mM sodium pyruvate e 1% MEM amino acid solution (Sigma-Aldrich, Milan, Italy) in a humidified incubator at 5% CO_2 and 37°C. Recombinant human (rh) IL-2 (100 U/ml) and rh IL-15 (20 ng/ml) (PeproTech, London, UK) were added, if required, to the NK cells only at the beginning of the culture.

3.2 PCR and real-time PCR

Total RNA was isolated from 5 x 10^5/ml unstimulated/stimulated NK, THP-1 (positive control) and Jurkat (negative control) cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, and reverse transcribed into cDNA (Manuelli et al. 2014).

For PCR amplification, 3 µl of cDNA were added to GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) in 25 µl reactions, containing 0.5 µM of forward and reverse primers. Amplification products were visualized on 1% agarose gel, containing 1 µg/ml ethidium bromide (Sigma–Aldrich). For real-time PCR, cDNA samples were equally diluted for subsequent PCR amplification with Maxima SYBR Green qPCR Master Mix (Fermentas, Milan, Italy) and a final volume of 20 µL, which contained 1µL of template cDNA, was used. Real-time PCR analysis was performed in triplicate for each sample in a CFX96 Real-Time PCR system (Bio Rad). The level of 18S RNA was measured and used for normalization of the target gene abundance. The primer sequences used for PCR and real-time PCR are listed in Table I.

Template	**Primers**
Galectin-3	forward 5'-GCAGACAATTTTTCGCTCCATG-3' reverse 5'-CTGTTGTTCTCATTGAAGCGTG-3'
GAPDH	forward 5'-GGTCGGAGTCAACAACGGATTTGG-3' reverse 5'-ACCACCCTGTTGCTGTAGCCA-3'
S18	forward 5'-TGCGAGTACTCAACACCAACA-3' reverse 5'-CTGCTTTCCTCAACACCACA-3'

Table I. Oligonucleotide primers used for PCR and real-time PCR

3.3 Western blot

Proteins were extracted from 1 x 10^6/ml human unstimulated/stimulated (24 h) NK cells, THP-1 (taken as positive control for Gal-3 expression), K-562 (taken as positive control for Gal-1 expression), and Jurkat (negative control) cells (Fallarini et al. 2012).

Equal amounts of proteins (35 µg) were separated on 12% polyacrylamide gel and electro-blotted on nitrocellulose membrane. The membranes were blocked and then incubated with the following primary antibodies: rabbit anti-human Gal-3 antibody

[Clone: EP2275Y (Abcam, Cambridge, UK); 1:5000], mouse anti-human Gal-1 antibody [Clone: C-8 (Santa Cruz Biotechnology, Heidelberg, Germany); 1:500], or mouse anti-human β-Actin antibody [Clone: AC15 (Sigma-Aldrich); 1:5000] with gentle shaking overnight at 4 °C or for 1 h at room temperature (RT) for β-Actin. The blots were, then, incubated with a specific anti-rabbit horseradish peroxidase (HRP) labeled secondary antibody (Ge Healthcare Life Sciences; 1:5000) or a specific anti-mouse HRP labeled secondary antibody (Sigma–Aldrich; 1:5000) for 1 h at RT.

Proteins were visualized with an enzyme linked chemiluminescence (ECL) detection kit, according to the manufacturer's instructions (Ge Healthcare Life Sciences). The protein signals were detected using an enhanced chemiluminescence system (Biorad). Band intensities were quantified by the computer program Image Lab from Biorad. All samples were analyzed independently and the final increase in Gal-3 protein was expressed as mean ± SEM of at least six independent experiments.

3.4 Flow cytometry

5 x 10^5/ml human NK cells were seeded in 24 multiwell plates and unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml). THP-1 (positive control) and Jurkat (negative control) cells were always included in the experiments. Cells were harvested and washed with a FACS wash solution (0,5 % BSA-supplemented PBS and 1% FBS). For the intracellular staining, the samples were fixed with cold paraformaldehyde (4% PFA) for 15 min at RT and permeabilized with 100% cold acetone for 10 min at -20°C. All samples were, then, incubated with a blocking solution (1% BSA-supplemented PBS and 2% FBS) for 30 min at RT. Afterwards, the samples were incubated for 30 min with the following primary antibodies: anti-human Gal-3 antibody [Clone: EPR2774 (Abcam); 1:65] or isotype control [Clone: EPR25A (Abcam); 1:65]. After washing, the cells were labeled with the Alexa 555-conjugated secondary antibody (Abcam; 1:1000). Samples were acquired and analyzed using a S3 flow cytometer (Biorad). All

samples were analyzed independently and the final increase in Gal-3 protein was expressed as mean ± SEM of at least six independent experiments.

3.5 Confocal microscopy

5 x 10^5/ml human NK cells, unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control), and Jurkat (negative control) cells were harvested, washed with PBS and fixed with cold 4% PFA for 15 min at RT. For intracellular staining, the cells were permeabilized with 100% cold acetone for 10 min at -20°C; for extracellular staining, the permeabilization occurred before nuclear staining. Fixed cells were blocked with blocking solution for 1 h at RT, incubated with anti-human Gal-3 [Clone: EP2275Y (Abcam); 1:65] or isotype primary antibody [Clone: EPR25A (Abcam); 1:65] for 30 min at RT and subsequently with Alexa 555-conjugated secondary antibody (Abcam; 1:1000) for 30 min at RT. To localize Gal-3 in exocytic vesicles NK cells were also stained with FITC-conjugated anti-perforin antibody [Clone: HCD56 (BioLegend)] (positive control) for 15 min at RT. After washing, nuclei were counterstained with To-pro-3 iodide (Thermo Fisher Scientific; 1:500) for 15 min at RT and cells centrifuged onto cytospin slides at 1200 rpm for 5 min (Shandon Cytospin 4, Thermo Fisher Scientific). Prolong Gold anti-fade kit (Thermo Fisher Scientific) was used as mounting fluid on the cells. Images were collected using a Leica TCS SP2 confocal microscope with 63X oil immersion objective and by sequential scanning. Images were acquired at a scanning speed of 400 Hz, 1024 x 1024 pixel resolution and a line average of 4. A 488 nm laser was used for IgG-FITC, 543 nm for IgG-Alexa 555 and 633 nm for To-Pro 3-iodide.

3.6 NK cell degranulation assay

NK cell degranulation was measured as previously described (Aktas et al. 2009), by determining the expression of CD107a, the lysosome-associated membrane protein-1 (LAMP-1) in absence/presence of a Gal-3 inhibitor (van Hattum et al. 2013). The used thiodigalactoside-based inhibitor (Bis-{3-deoxy-3-[4-(phenyl)-1H-1,2,3-

triazol-1-yl]-β-D-galactopyranosyl} sulfane) was a generous gift by Prof. Roland J. Pieters (Department of Medicinal Chemistry and Chemical Biology, Utrecht University, Netherlands). For the experiments, a stock solution in 40 % DMSO was prepared and diluted in RPMI-1640 before cell treatment (DMSO concentration did not exceed 0.1 % v/v). NK cells unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), were untreated/treated with increasing concentrations (1–30 μM) of Gal-3 inhibitor for 1 h at 37°C. To induce NK cell degranulation, the cells were incubated with K562 cells (target cells) at an effector/tumor cell ratio of 1:1 for 1 h at 37 °C in presence of anti-CD107a-Phycoerythrin (PE) (Miltenyi Biotec). Monensin (BD GolgiStop™ reagent, BD Bioscences, Milan, Italy) was added and cells incubated for 3 h at 37 °C. Cells were washed, stained using FITC anti-human CD56 (NCAM) Antibody [Clone: MEM-188 (BioLegend)], and CD107a expression on $CD56^+$ cells evaluated by FACS (Fallarini et al. 2017). To detect spontaneous NK cell degranulation, a negative control (NK cells without K562 target cells) was always included, while to measure the basal response, NK cells were stimulated only with IL-2 (100 U/ml) and IL-15 (20 ng/ ml) (positive controls). All control samples were treated with 0.1 % DMSO.

3.7 Statistical analysis

Results were expressed as means ± SEM of at least four independent experiments always run in triplicate. Statistical significance was evaluated by the one-way ANOVA followed by Student's t-test for unpaired populations (Graph Pad Software, Inc., San Diego, USA). Differences were considered statistically significant when $P \leq 0.05$.

4. Results

4.1 Galectin-3 gene expression in human resting NK cells

To determine whether human NK cells express the gene coding for Gal-3 we, first, performed PCR studies on resting NK cells. Human THP-1 cells were always taken as positive control.

Figure 8A. clearly demonstrates that human resting NK cells express Gal-3 mRNA.

4.2 Galectin-3 mRNA in human activated NK cells

Since the expression of Gal-3 gene in a variety of immune cells can be modulated by cellular activations and environmental stimuli (Dietz et al. 2000; Acosta-Rodríguez et al. 2004; Hsu et al. 2009 [A]; Novak et al. 2012), we measured the levels of Gal-3 mRNA in NK cells unstimulated/stimulated with IL-2 (100 U/mL) and IL-15 (20 ng/mL) for different periods of time (1-24 h) by real-time PCR. Figure 8B shows that the expression of Gal-3 mRNA significantly ($P \leq 0.01$) decrease (-92 ± 2% in comparison to resting NK cells) after 1 h of cytokine stimulation, and this decrease lasts for at least 6 h. Afterwards (12-24 h), the mRNA expression level progressively restores to the basal level.

The overall data demonstrate that human NK cells can regulate the gene expression of Gal-3 following specific stimuli for an appropriate period of time.

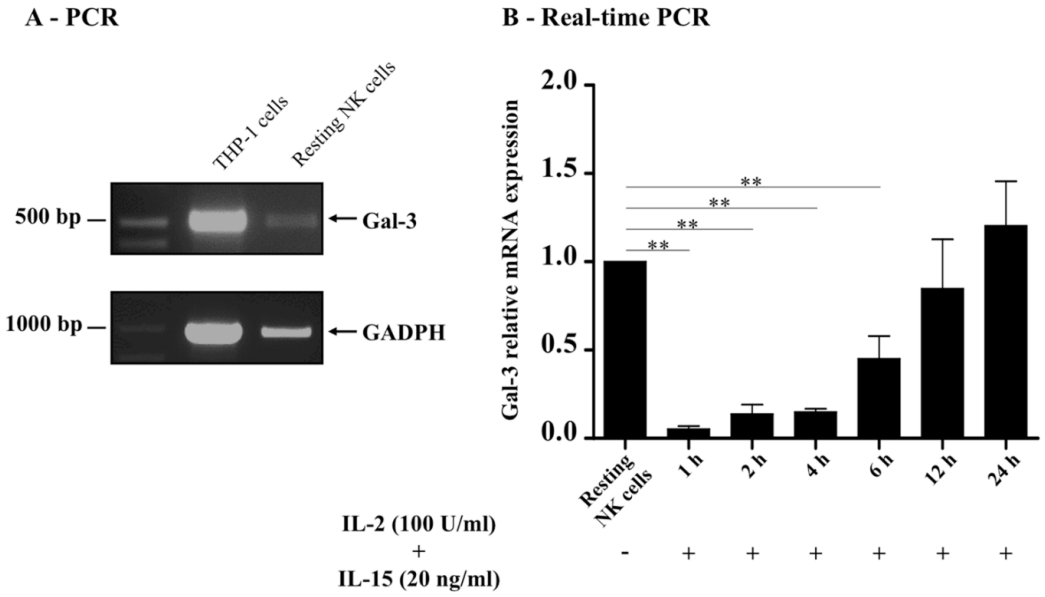

Figure 8. Gal-3 mRNA is expressed in human NK cells and its expression is modulated by cell activation. (A) Gal-3 mRNA expression was analyzed by PCR in NK and THP-1 (positive control) cells. The total extracted mRNA was reverse transcribed to cDNA and Gal-3 cDNA amplified using gene-specific primers (see Table I). PCR products were electrophoresed on 1% agarose gel and PCR fragments sized using a 50 -10,000 bp ladder. The expected fragment size for Gal-3 is 500 bp. GAPDH was amplified as a loading control with a fragment size of 1000 bp. (B) Time course of Gal-3 gene expression in activated NK cells measured by real-time PCR. Total mRNA was extracted from NK cells unstimulated/stimulated with IL-2 (100 U/ml) and IL-15 (20 ng/ml) for 1-24 h, converted to cDNA, and analyzed by real-time PCR for Gal-3 expression. The mRNA levels are expressed relative to 18S mRNA, and the value of the resting cells was set at 1. The results represent the mean ± SEM of at least six independent experiments. $^{**}P \leq 0.01$ vs. unstimulated NK cells.

4.3 Galectin-3 protein in human activated NK cells

Then, to determine whether the modulation of Gal-3 mRNA levels, measured in activated NK cells (see Results, 4.2), correlates with changes at protein levels, the same cells were analyzed by western blot. The estimated total Gal-3 protein levels were always normalized against the corresponding β-Actin level and compared to those measured from resting cells.

As shown in Figure 9, we determined resting NK cells with high (Fig. 9A) or low (Fig. 9B) basal level of Gal-3 protein. This variability was consistently measured in all experiments and seems related to the individual donor analyzed. Following cytokine stimulation for 24 h the Gal-3 levels resulted significantly ($P \leq 0.05$) increased in all samples analyzed (+116 ± 32% in comparison to resting NK cells) (Fig. 9C).

In THP-1 cells (positive control) Gal-3 protein was always detectable, while no expression was determined in Jurkat cells (negative control).

These data indicate that upon IL-2 and IL-15 stimulation human NK cells can increase the level of Gal-3 protein.

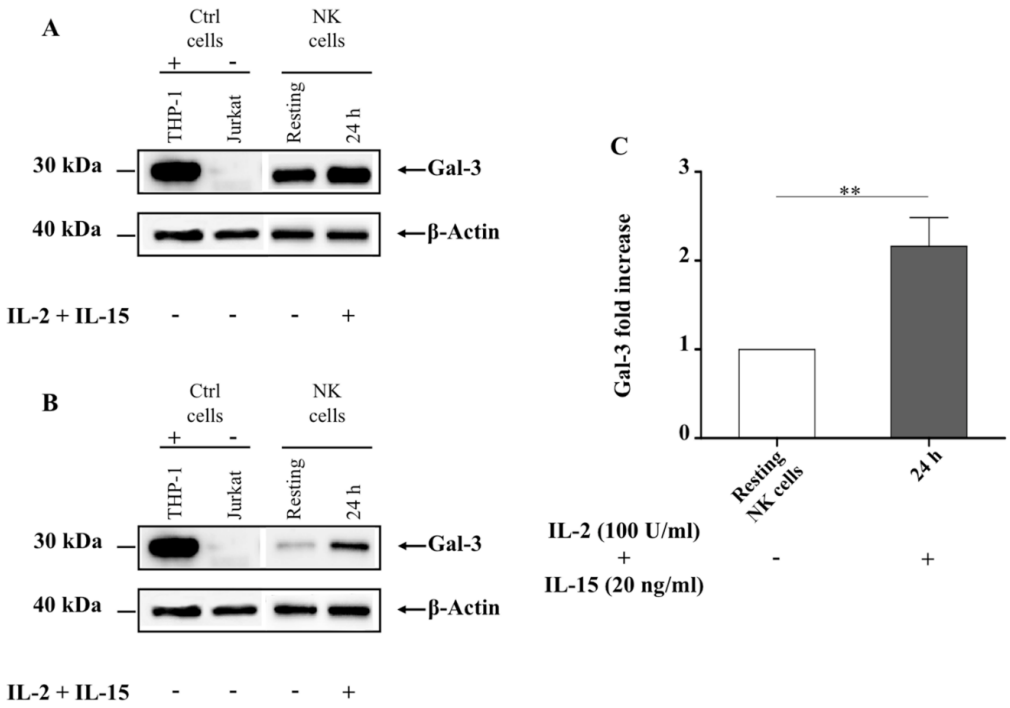

Figure 9. Gal-3 protein is expressed in human NK cells and its expression increases upon cell activation. Gal-3 protein expression was evaluated by western blot analysis in NK cells, unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells. Total cellular proteins (35 µg) were resolved by 12% polyacrylamide gel and immunoblotted with a specific anti-human galectin-3 antibody. β-Actin was used as internal control. Left panels are representative of typical immunoblottings from NK cells expressing high (A) or low (B) basal levels of Gal-

3. The western blot signals were densitometrically analyzed by Image Lab software and results expressed as fold increase of Gal-3 signal in stimulated cells compared to unstimulated cells set at 1 (C). The results represent the mean ± SEM of at least six independent experiments. $^{**}P \leq 0.01$ vs. unstimulated cells.

4.4 Galectin-3 protein localization in human NK cells

While being predominantly a cytosolic protein, Gal-3 can be detected also at the cellular surface (Krześlak and Lipińska 2004). To study the Gal-3 localization in human NK cells the Gal-3 levels were measured in resting cells by flow cytometry (FACS). Median fluorescence intensity (MFI) measurements confirm previous data from the western blot analysis (see Results, 4.3): resting NK cells express high or low basal levels of Gal-3 protein, as clearly shown by the distinct separation of each histogram from the isotype control (Fig. 10Aa and 10Ab). These data confirm that human NK cells can express different levels of Gal-3 depending on the individual donor analyzed.

IL-2 (100 U/mL) and IL-15 (20 ng/mL) stimulation for 24 h, significantly ($P \leq 0.01$) increased (+30 ± 9% in comparison to resting cells) (Fig. 10B) the levels of Gal-3 expression in NK cells.

Then, to specifically localize this lectin, NK cells were incubated with the primary antibody before (extracellular) or after (intracellular) the permeabilization step (see Methods, 3.5). In all samples analyzed, Gal-3 did not localize at the membrane cell surface, neither in resting nor in activated cells, indicating that human NK cells do not express this protein at the membrane level. In THP-1 cells, taken as positive control, Gal-3 was both extracellular and intracellular detectable (Fig. 10A and 11A), while no expression was determined in Jurkat cells (negative control).

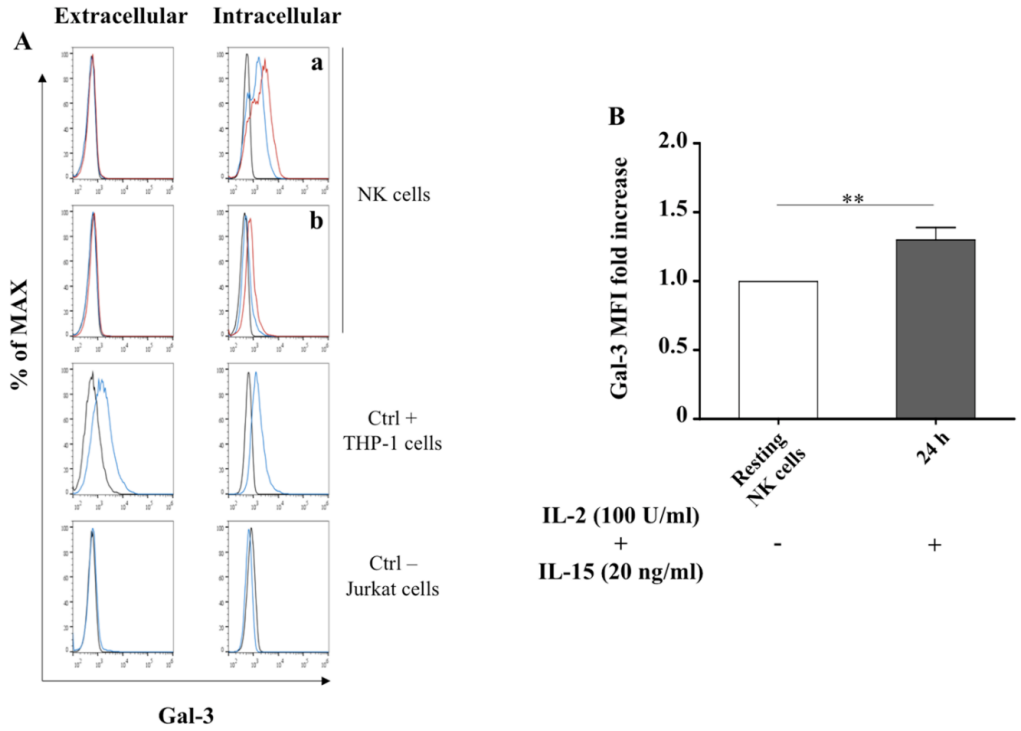

Figure 10. Gal-3 is intracellularly expressed in human NK cells and its expression increases upon cell activation. NK cells, unstimulated/stimulated for 24 h with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells were harvested, stained with anti-human Gal-3 and analyzed by FACS. (A) Representative histograms of extracellular (left panels) and intracellular (right panels) Gal-3 expression in human NK with high (a) and low basal (b) Gal-3 expression, THP-1 and Jurkat cells. Resting cells are shown as blue histograms, stimulated NK cells as red, while the isotype controls as black histograms. (B) Mean Fluorescence Intensity (MFI) levels of Gal-3 in human NK cells expressed as fold increase in stimulated cells compared to unstimulated cells set at 1. The results represent the mean ± SEM of at least three independent experiments. $^{**}P \leq 0.01$ vs. unstimulated cells.

Since Gal-3 differently localize depending on the immune cell type (Craig et al. 1995; van Stijn et al. 2009; Novak et al. 2012) to investigate the subcellular localization of Gal-3 in human NK cells immunofluorescence staining studies were performed in cells, unstimulated/stimulated with IL-2 (100 U/mL) and IL-15 (20 ng/mL) for 24h (Fig. 11A), before (extracellular) or after (intracellular) the permeabilization step. At the membrane level, confocal microscopy analysis revealed an extracellular diffuse staining in THP-1 cells (positive control: Fig.

11Aa), while all NK cell samples analyzed, either resting or activated (Fig. 11Ab and 4Ac, respectively), resulted negative. These data demonstrated that human NK cells do not express membrane-bound Gal-3 protein, confirming the previous results obtained by FACS. Conversely, all NK cell samples resulted positive for the intracellular staining (Fig. 11Ae-f and 11Ah-i), where Gal-3 was visualized as scattered dots within the cells. Interestingly, we observed positive (Fig. 11Af and h, close arrows) and negative (Fig. 11Af and h, open arrows) cells in both resting (Fig. 11Ae and f) and activated (Fig. 11Ah and i) samples from the same individual. In positive cells, the intracellular localization of Gal-3 resulted heterogeneous, as some cells mainly localize the protein in cytoplasm and nucleus (Fig. 11Ba and b, open arrows), while others showed fluorescent dots only in cytoplasm (Fig. 11Ba and b, close arrows). These results were independent from the cellular activation status. Moreover, overlapping the perforin and Gal-3 stains, shown as yellow dots (Fig. 11Bc), we observed that Gal-3 can occur within perforin in exocytic vesicles. Finally, the analysis of positive cells confirm the high variability of Gal-3 expression in NK cells: some samples having high levels (Fig. 11Ae and h), while others low levels of Gal-3 (Fig. 11Af and i). Jurkat cells, taken as negative control, resulted always unstained (Fig. 11Ag).

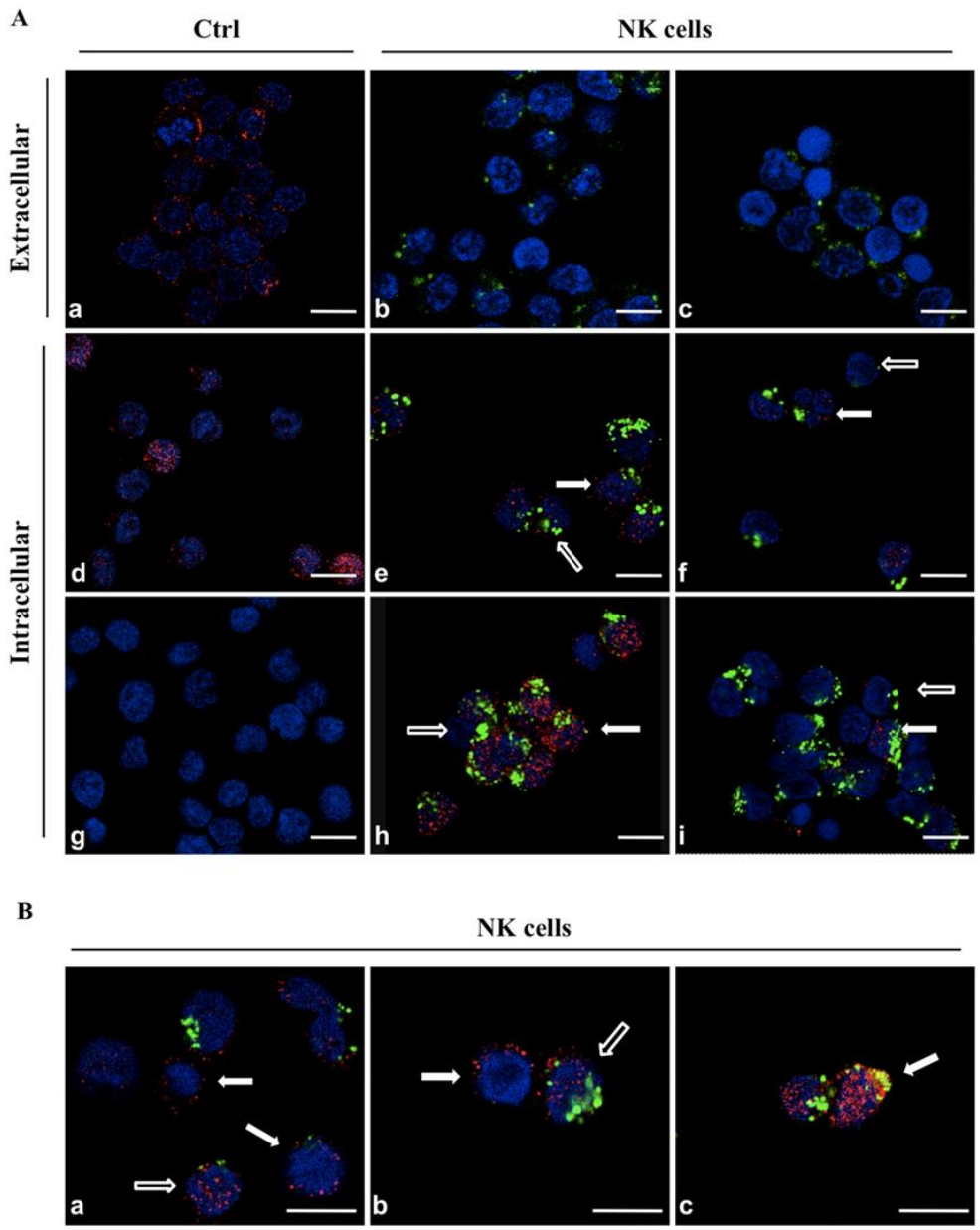

Figure 11. Confocal microscopy analysis of cytoplasmic and nuclear Gal-3 subcellular distribution in human NK cells. (A) NK cells, unstimulated/stimulated for 24 h with IL-2 (100 U/ml) and IL-15 (20 ng/ml), THP-1 (positive control) and Jurkat (negative control) cells were harvested, stained with anti-human Gal-3, and analyzed by confocal microscopy. Representative images of Gal-3 expression in: THP-1 cells (positive control; a and d), Jurkat cells (negative control; g), resting NK cells (b, e and f), stimulated NK cells (c, h and i). Gal-3 is shown as red dots, perforin as green dots. Nuclei are shown in blue. Scale bar, 10 µm. (B). Representative images of Gal-3 localization in the nucleus and/or in the cytoplasm (a-

b) or co-localization of Gal-3 with perforin (c). Gal-3 is shown as red, perforin as green dots. Yellow dots represent co-localization of Gal-3 with perforin. Nuclei are shown in blue. Scale bar, 10 µm.

4.5 Galectin-3 functional studies in human NK cells

NK-cell toxicity is mediated by exocytosis of cytosolic vesicles, containing perforin, granzymes, and other lytic proteins (Caligiuri 2008). Since Gal-3 expression can be upregulated upon cytokine stimulation and accumulated within vesicles (see Results, 4.4), to determine whether these data have functional correlations, NK cell responses were studied in the absence/presence of a thiodigalactoside-based Gal-3 inhibitor (Kd = 44 nM) (see Materials, 3.6) in a typical degranulation assay (see Methods, 3.6).

As shown in Figure 12Aa (representative contour plots), unstimulated NK cells present a small percentage of degranulating cells (dashed gate); among these cells, only a little percentage releases a high number of granules (solid gate). Cytokine stimulation (Fig. 12Ab) induced an increase in NK cell degranulation (dashed gate) and the number of cells expressing high levels of CD107a (solid gate) resulted larger than in resting cells. When cytokine-stimulated cells were treated with increasing concentrations (1-30 µM) of the Gal-3 inhibitor, the number of degranulating cells slightly increased (data not shown). The maximum increase was measured at 30 µM Gal-3 inhibitor (+15 ± 1% vs. inhibitor-untreated cells, Fig. 12Ac, dashed gate). Of note, a significant ($P \leq 0.05$) concentration-dependent increase was determined (Fig. 12B) in NK cells releasing high amount of cytotoxic granules (high level of CD107a expression, Fig. 12A, solid gates).

The maximum increase was measured at 30 µM Gal-3 inhibitor (+ 36 ± 3% vs. inhibitor-untreated cells at 30 µM; Fig. 12B and Fig. 12Ac, solid gate). No changes in the CD107a levels were observed in resting NK cells incubated with K-562 cells, even in the presence of 30 µM of Gal-3 inhibitor (data not shown).

Finally, since the Gal-3 inhibitor we used has binding affinity also for the Gal-1 lectin with similar Kd values (49 *vs.* 44 nM) (Delaine et al. 2016) we determined whether

unstimulated/stimulated NK cells express Gal-1. Results show that human NK cells do not express Gal-1 protein at least in our experimental conditions (Fig. 12C). These data demonstrate that the Gal-3 expression functionally correlates with NK cell degranulation.

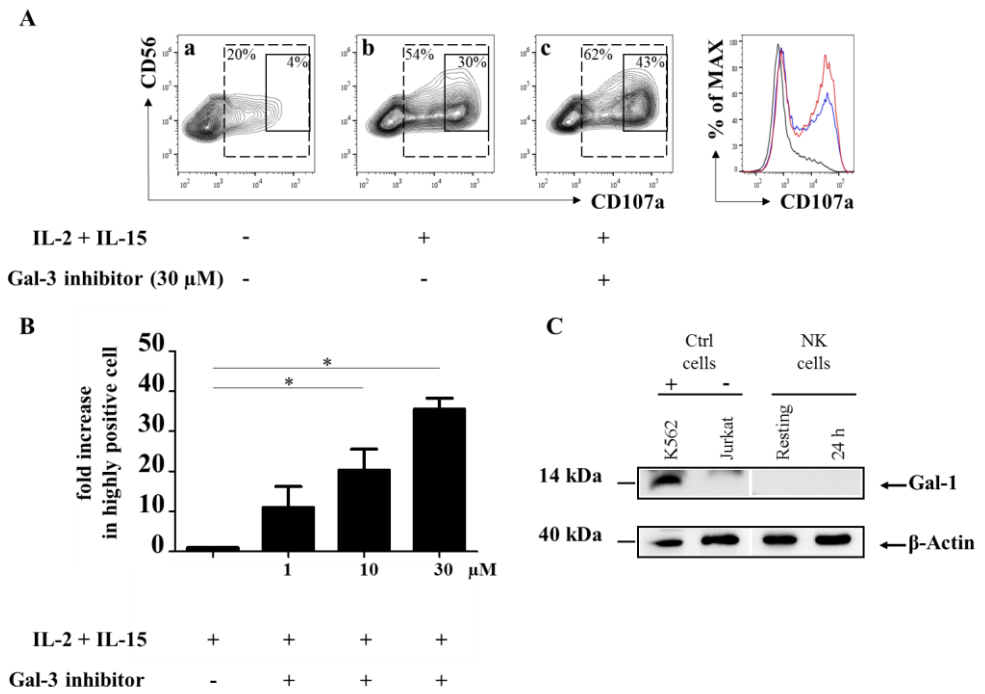

Figure 12. Gal-3 inhibition increases the degree of degranulation in human activated NK cells. A classical degranulation assay was used to measure NK cell activity (see Methods, 3.6). (A) The first three panels are representative contour plots of resting and cytokine-stimulated NK cells in the absence/presence of the 30 µM Gal-3 inhibitor (dashed gates represent the percentage of total CD56/CD107a positive cells, while solid gates those highly positive) incubated (4h) with K562 cells. The fourth panel on the right represents overlapped histograms of CD107a expression in resting (black histogram, 5Aa), cytokine-stimulated (blue histogram, 5Ab), cytokine-stimulated plus Gal-3 inhibitor (red histogram, 5Ac) NK cells incubated (4h) with K562 cells. (B) Percentage of highly positive CD107a cells in activated NK cells untreated/treated with increasing concentrations (1-30 µM) of the Gal-3 inhibitor. The percentage increases in CD107a highly positive cells are calculated vs the value of inhibitor-untreated cells set at 1. The results represent the mean ± SEM of at least four independent experiments. *P $\leq$ 0.05 vs. inhibitor-untreated cells. (C) Representative Gal-1 protein expression evaluated by western blot analysis in NK cells, unstimulated/stimulated (24 h) with IL-2 (100 U/ml) and IL-15 (20 ng/ml), K562 (positive control) and Jurkat (negative control) cells. Total cellular proteins (35 µg) were resolved by

12% polyacrylamide gel and immunoblotted with specific anti-human galectin-1 antibody. β-Actin was used as internal control.

5. Discussion

In this study we demonstrate, for the first time, that: i) human resting NK cells express Gal-3 at both gene and protein levels; ii) resting NK cells can express high or low basal levels of Gal-3 protein, at least in our experimental conditions, depending on the individual donor analyzed; iii) cytokine stimulation is able to modulate either Gal-3 mRNA and protein levels; iv) Gal-3 localizes in both cytoplasm and nucleus, while it was not detected at the cell membrane surface, neither in resting nor stimulated cells; v) Gal-3 can occur within perforin in exocytic vesicles; vi) Gal-3 inhibition leads to an increase in NK cell degranulation.

Several galectins can regulate the homeostasis and functions of immune cells, influencing both innate and adaptive immune responses (Chen et al. 2005; Liu 2005; Rabinovich et al. 2007). In NK cells, particularly, the expression of Gal-1 has been described in the third trimester of normal pregnancy (Molvarec et al. 2011), while that of Gal-3 remains to be investigated. Scanty literature data focus only on profiles of gene expression in mouse NK cells (Kang et al. 2005) and on counter-receptor isolation for Gal-3 from tissues of the murine utero-placental complex (Crider-Pirkle et al. 2002). Using Gal-3 null transgenic mice, finally, a dysregulation of NK cell activity has been observed (Radosavljevic et al. 2011; Chaudhari et al. 2015), when compared to the wild type mice. If this effect is due to the absence of Gal-3 in NK cells or mediated by its absence in other cells, remains to be clarified.
To fill the research gap in this field we, here, decided to fully characterize Gal-3 in human NK cells.

We, first, concentrate on human resting NK cells, because a downregulation of the transcription of genes involved in cellular quiescence and an upregulation of genes

associated with cell proliferation (Dybkaer et al. 2007) can occur in activated NK cells.

From our results, resting NK cells express Gal-3 at both mRNA and protein levels with a highly variable relative expression, depending on the individual donor analyzed. In particular, NK cells, expressing low or high levels of Gal-3 protein, were identified. In our opinion, this is not a surprising result because NK cells are a highly heterogeneous population. These cells not only can be roughly categorized in two major subsets ($CD56^{dim}$ and $CD56^{bright}$), based on different expression level of surface CD56 molecule (Moretta et al. 2014), but they can also exert effector functions through a wide array of activating or inhibitory receptors (see Introduction, 1.10.2), which are finely controlled (Vivier et al. 2011; Thielens et al. 2012). NK cells can be thus considered as plastic cells, that can adapt their responsiveness and different receptor pattern, according to environmental stimuli, aging, and diseases (Vivier et al. 2011; Hazeldine and Lord 2013; Terra Junior et al. 2016). Consistently, Lavin et al. (2017), analyzing the distribution of NK cells in lung tumor lesions, showed that the specific microenvironment can shape NK cell protein expression leading to cells that are unique to tumors. The clinical implications of these findings may be of great importance, because mapping the immune cells around the tumors could aid successful immunotherapies.

Collectively, our data complete those on Gal-3 expression on mammalian immune cells, because Gal-3 has been previously demonstrated in macrophages (Liu et al. 1995), DCs (van Stijn et al. 2009), neutrophils (Wu et al. 2017), monocytes (Liu et al. 1995), eosinophils (Rao et al. 2007), mast cells and basophils (Craig et al. 1995), but not in human NK cells.

IL-2 and IL-15 have been here used for activating cells, because these two cytokines, more than any others, are implicated in NK cell survival and cytotoxic function (Becknell and Caligiuri 2005).

Following cytokine stimulation, the intracellular levels of Gal-3 mRNA resulted significantly reduced at 1-6 h, while it returned to the basal levels after 12-24 h. A two fold increase in Gal-3 protein was also measured after 24 h cytokine stimulation, compared to unstimulated cells (see Fig. 8-9). At present, we ignore the molecular bases of the drastic drop in the mRNA levels that we observed after 1 h of stimulation. However, these results are consistent with other similar transcriptional/posttranscriptional gene regulation in NK cells. Resting murine cells are "pre-armed" with a high amount of specific mRNA that upon cell activation can be rapidly translated into protein, allowing the rapid production of effector proteins without new gene transcription and with minimal or absent change in mRNA levels (Fehniger et al. 2007). In addition, human NK cells, have a number of genes, involved in cytolytic functions, already expressed that are rapidly downregulated upon cytokine stimulation (Dybkaer et al. 2007; Mah and Cooper 2016).

Of note, it was demonstrated that NK cells can transcribe and retained at nuclear level specific mRNA, such as IFN-γ, which upon stimulation can be transported into the cytoplasm, translated into the corresponding protein and rapidly degraded. Such type of mechanism allows a fast translation of protein involved in activated NK cell functions and tightly regulate the proper amount of protein inside the cell (Hodge et al. 2002; Kim et al. 2009).

Despite the molecular mechanism regulating Gal-3 expression in NK cells remain to be fully characterized, the drastic drop in mRNA observed immediately after stimulation and the increase in the corresponding protein at 24 h lead us to hypothesize for Gal-3 a similar mechanism of regulation and experiments are in progress in our laboratory to clarify this point.

Gal-3 cellular localization seems to be highly heterogeneous: the protein has the ability to space from nucleus to cytoplasm/cellular surface and to be released into extracellular compartments (Krześlak and Lipińska 2004). Depending on localization Gal-3 can modulate cellular homeostasis, organogenesis, apoptosis,

immune responses, cytokine secretion, cell migration, tumor invasion and metastasis (Dumic et al. 2006). A direct interaction of tumor-secreted Gal-3 with the NK cell surface activating receptor NKp30, having inhibitory activities on cytotoxicity, has been specifically reported by Wang et al. (2014).

In our cells, we never detected Gal-3 at cell surface membrane, even after cytokine stimulation, while in THP-1, a monocytic cell line, taken as positive control, we found Gal-3 also bound to the plasmatic membrane. This result is in agreement with data from Dabelic et al. (2012) on THP-1 cells and from van Stijn et al. (2009) on monocytes.

All samples analyzed were, on the contrary, positive for the intracellular localization of Gal-3 with different levels of protein expression, depending on the individual donor. Gal-3 was always detected in each sample analyzed, being found in the nucleus and/or in the cytoplasm in a heterogeneous manner.

From our results, Gal-3 co-localizes with perforin in exocytic vesicles, which represent one of the main mechanisms underlying NK cell functions. Gal-3 could, thus, directly participate to NK cell degranulation. When Gal-3 function was inhibited by a thiodigalactoside-based Gal-3 inhibitor (van Hattum et al. 2013), indeed, a significant increase in the degree of granules released by NK cells was measured (see Fig. 12B). This result suggests that the level of intracellular Gal-3 expression into NK cells may modulate the mobilization of secretory vesicles for releasing their cytotoxic contents.

Previously, Chen et al. (2009) demonstrated that endogenous Gal-3 negatively regulates T-cell activation by TCR downregulation through the interaction with Alix. Alix protein, in turn, can interact with the endocytic trafficking complexes (ESCRT), involved in exosome biogenesis and secretion (Hurley and Odorizzi 2012), as well as with other intracellular regulators, involved in signal transduction (Colombo et al. 2013). Similarly, endogenous Gal-3 might negatively regulate the release of cytotoxic granules from activated NK cells through the modulation of Alix (Liu et

al. 2012). The Gal-3 upregulation may be, consequently, a physiological mechanism specifically designed to protect healthy cells against toxic compounds released in the surrounding microenvironment. Our observations support also previous data from Radosavljevic et al. (2011), showing that splenic NK cells from Gal-3 null transgenic mice have higher cytotoxic activities than wild type animals.

The function of intracellular Gal-3 in NK cells remains, however, to be fully characterized. In addition to the above mentioned hypothesis it could: i) exert anti-apoptotic activities by binding to molecules involved in apoptosis (i.e., Bcl-2, CD95 (APO-1/Fas), Nucling, Alix/AIP1, synexin) (Dumic et al. 2006); ii) regulate cell proliferation, differentiation, and survival through the modulation of specific signal pathways (i.e., K-Ras/MEK, PI3K/Akt and Wnt (Shalom-Feuerstein et al. 2008; Song et al. 2009); iii) control the dynamics of membrane domains and subsequent signaling events, when localized at the inner leaflet of plasma membrane (Compagno et al. 2014).

Similarly, the nuclear Gal-3, associated with a ribonucleoprotein complex, could: i) act as a pre-mRNA splicing factor; ii) operate as a regulator of gene transcription, promoting the stabilization/transactivation of several transcription factors (i.e., CREB, Sp1, TTF-1, β-catenin) (Dumic et al. 2006; Funasaka et al. 2014).

The signal transduction cascades, initiated by IL-2/IL-5 stimulation, resulting in Gal-3 upregulation should be investigated. The mechanism underlying Gal-3 gene regulation is still poorly understood (Dumic et al. 2006): Jack/STAT (Becknell and Caligiuri 2005), NF-kB (Hsu et al. 1996), or the Ras/MEKK1/MKK1-dependent/AP-1 signal transduction pathway (Kim et al. 2003) may control the Gal-3 expression as in other cells, but this will be the topic of future research.

In conclusion, the present study provides a clear demonstration of Gal-3 expression in human NK cells. The presence of this lectin in resting cells supports its roles in NK cell homeostasis, while the protein upregulation after cytokine stimulation sustains roles in cell function. Finally, the Gal-3 co-localization with perforin in

granules, as well as the increase in NK cell degranulation following Gal-3 inhibition suggests functional roles in NK cytotoxicity.

6. Conclusion and outlook

The aim of the first part of my Ph.D. course was to evaluate the expression of Gal-3 in human NK cells. The data here reported clearly demonstrate that these cells express Gal-3, suggesting further studies.

In particular, we found that this protein is not equally expressed among the analyzed donors, ranging from individuals expressing high levels of Gal-3 to others that barely express this protein even after cytokine stimulation.

Here we always analyzed NK cells from the peripheral blood of healthy patients, but we are planning to determine the expression of Gal-3 also in patients suffering from allergy, infections, or other inflammatory diseases. Moreover, we did not analyze Gal-3 expression in the different subpopulations of NK cells (i.e., $CD56^{dim}$ and $CD56^{bright}$), but we believe that possible differences among NK cell subpopulations may help to clarify the role of Gal-3 in these cells.

Furthermore, the analysis of Gal-3 localization in NK cells clearly demonstrated this protein into the exocytic vesicles; we did not analyze at the moment the content of vesicles released during cell degranulation, but in our opinion this should be investigated in the near future, since literature data show that this protein composes the cargo of secreted vesicles. At this point, it is intriguing to speculate that once in the extracellular compartment Gal-3 can modulate other immune cells, recalling them in the correct site and regulating the progression/termination of the immune response.

Finally, we demonstrated that Gal-3 is implied in NK cell degranulation; in particular, the inhibition of this lectin led to a higher release of cytotoxic granules compared to controls. This finding may extend the biological application of NK cells in that fields in which is required an increase in NK cell activity, such as in cancer.

I am strongly aware that these results need to be completed and validated by "in vivo" studies, however I think that they can add a piece to the scientific knowledge on Gal-3 expression/function on innate immune cells.

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Chapter II

CD154/40L recombinant vaccinia virus induces direct and macrophage-mediated in vitro and in vivo antitumor effects

1. Introduction

1.1 Cancer and immune therapeutic strategy

Despite considerable achievements of the scientific community in the last decades, cancer still represents the second leading cause of death worldwide, as reported by the world health organization (http://www.who.int).

Recently, several immunotherapeutic strategies have been evaluated in order to reduce incidence and cancer progression. The rationale supporting implementation and clinical validation of different immunotherapeutic strategies relies on the theoretical ability of the immune system to eradicate cancer or to prevent its progression (Hanahan and Weinberg 2011). Furthermore, development of new immunotherapeutic approaches is also prompted by the necessity to bypass the limited efficacy of the present standard, chemo and radiotherapy, based anticancer treatments (Beatty et al. 2011; Mishra et al. 2013; Vonderheide et al. 2013; Miller et al. 2016; Mills et al. 2016).

Our current understanding of cancer immunotherapeutic strategies largely focuses on the pivotal role played by T lymphocytes, in particular CD8+ T cells, in the elimination of cancerous cells. In this regard, pioneering studies performed in mouse models have clearly demonstrated the potential therapeutic activity of tumor-reactive CD8 positive T cells (Schumacher and Schreiber 2015; Ward et al. 2016). These initial observations have been also confirmed in clinical settings (Galon et al. 2006; Fridman et al. 2012). Indeed, it has widely been demonstrated that high percentages of tumor-infiltrating CD8+ T cells within the tumor masses are associated to a significantly increased overall and/or progression free-survival in different tumor types including breast, melanoma, ovarian, bladder and colorectal cancer (Figure 1) (Galon et al. 2006; Fridman et al. 2012; Fridman et al. 2017).

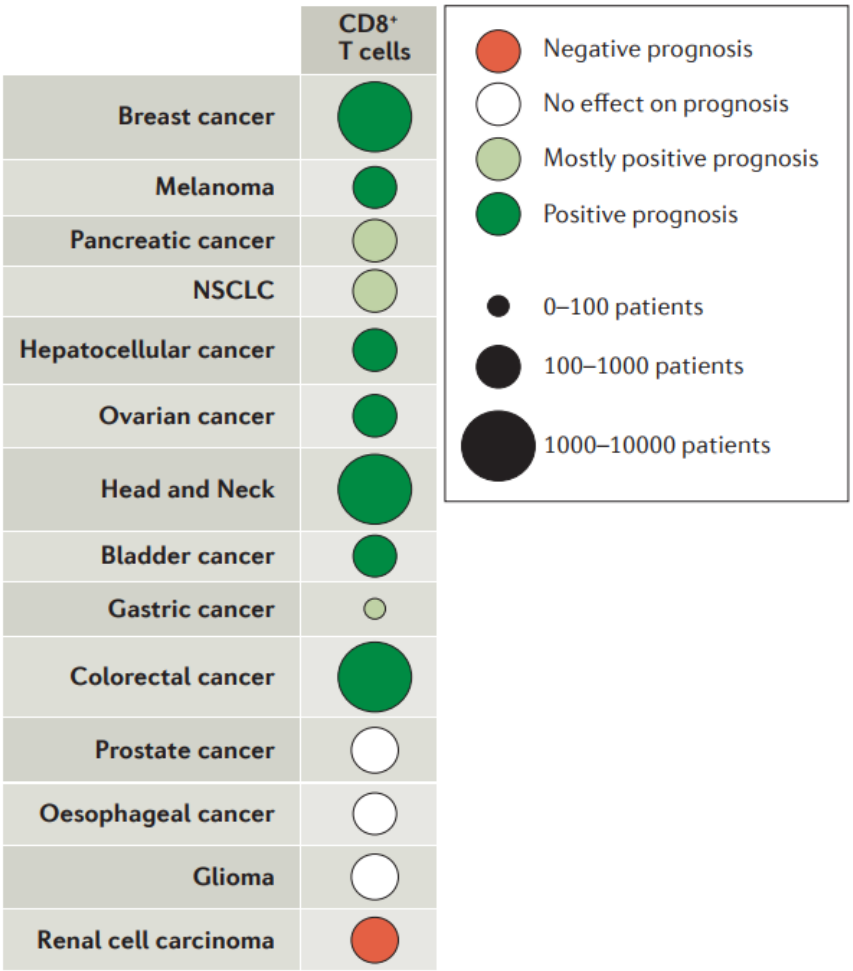

Figure 1. CD8+ T cells infiltration and clinical outcome of cancer patients. Bold colors indicate a positive (green) or a negative (red) prognostic association following analysis of all relevant studies; light green indicate a predominantly positive prognostic association in the majority of studies analyzed. White circles indicate no statistically significant correlation or that unclear prognostic associations were observed in a number of studies. The size of the circles relates to size of the cohort in clinical studies. Small circles indicate 0–100 patients, Medium-sized circles indicate 100–1,000 patients and Large circles indicate 1,000–10,000 patients NSCLC: non small cell lung carcinoma (adapted from Friedman et al. 2017).

Nevertheless, although these studies clearly underline the therapeutic potential of CD8+ T cell; cumulative results obtained in animal models and clinical studies have also evidenced that transformed cells are able to actively orchestrate an immune resistance, preventing the generation and impairing the effectiveness of tumor-reactive CD8+ T cells (Schreiber et al. 2011; Ghirelli and Hagemann 2013). As

reported by Hanahan and Weinberg in 2011, in fact, a new hallmark of cancer, consists in its ability to evade destruction mediated by immune cells.
In the last decade, several studies have contributed to shed light on the mechanism underlying the process of immune evasion. In this respect, it has been observed how tumor cells can prevent the generation of an effective immunological synapse with CD8+ T lymphocytes through their downregulation of major histocompatibility complex (MHC) class I molecules (Dong et al. 2002; Bubeník 2004; Ahmadzadeh et al. 2009). Moreover, therapeutic potential of tumor-reactive CD8+ T cells is impaired by the activation in cancer cells of distinct mechanisms contributing to the establishment of cancer immunological tolerance. In this regard, a pivotal role can be ascribed to PD1 : PD-L1/PD-L2 pathway.

Animal studies and clinical observations have contributed to underline the relevance of this pathway in favoring cancer progression (Tran et al. 2008; Ahmadzadeh et al. 2009; Topalian et al. 2012). PD1 expression on T cell surfaces has been extensively indicated as a physiological step following CD4 and CD8 positive lymphocytes activation, whose engagement lead to the silencing of T cells response. This mechanism, which physiologically prevents the exacerbation of adaptive immune responses against pathogens and the onset of autoimmune diseases, can also prevent T lymphocytes from killing tumor cells (Tran et al. 2008; Pardoll 2012; Topalian et al. 2012).
In fact, expression of PD1 ligands (PD-L1/PD-L2) on surface of malignant cells, has been shown to be significantly associated with poor prognosis of cancer patients (Zitvogel and Kroemer 2012; Topalian et al. 2016). Remarkably, upregulation of PD-L1 on malignant cells appears to result from both intrinsic and adaptive immune resistance mechanism utilized by malignant cells (Gajewski et al. 2010; Spranger et al. 2013; Topalian et al. 2014). Intrinsic immune resistance is referred to the constitutive expression of PD-L1, as a consequence of the activation of specific signaling pathway during neoplastic transformation, as well as a consequence of

chromosomal rearrangement usually detectable in malignant cells. Most importantly, PD-L1 upregulation can also be detected in different solid tumor types and hematological malignancies as a result of an active adaptive immune resistance of transformed cells (Pardoll 2012; Topalian et al. 2016). Indeed, it has been reported that the expression of PD-L1 on cellular surface of cancer cells may be induced in response to interferon (IFN)-γ, produced by tumor-infiltrating immunocompetent cells (Kmieciak et al. 2011; Topalian et al. 2015). The latter observation paradoxically underlines, once again, the potential ability of the immune system to mediate cancer eradication and, most importantly, the ability of malignant cells to react and dampen immune-mediated threat.

These findings have prompted the development and evaluation of therapeutic efficacy of different monoclonal antibodies (mAbs) targeting negative regulators of tumor-reactive immune responses (Sharon et al. 2014). So far, administration of these antibodies has proven of clinical effectiveness in around 30% of cancer patients enrolled in different clinical trials.

Despite these encouraging results, it must be underlined that cancer progression cannot be ascribed only to the capacity of malignant cells to evade immune recognition through the expression of inhibitory ligands on their cellular surfaces. In this regard, several studies have indicated that cancer progression may mostly be related to the generation and promotion by malignant cells of an immunosuppressive microenvironment, limiting the activity of immune cell types of proven clinical relevance. In particular, cancer cells can evade immune recognition through the activation of genes encoding for enzymes, cytokines and chemokines (Cremonesi et al. 2017) able to restrain the antitumor activity of T cells directly or through the recruitment of immune suppressive cells. In this respect, a selective recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MSDC) has been widely reported in different tumor types including colorectal, breast and pancreatic cancer (Gabrilovich and Nagaraj 2009; Facciabene et al. 2012).

Finally, In addition to selective recruitment of immune suppressive cell types, malignant cells can also steer the functional differentiation of immune cell with antitumor potential. In this regard, in the last years, an increased interest has been registered for tumor-associated macrophages (TAMs). Notably, macrophages have been repeatedly indicated as first line of defense against cancer (Imaizumi et al. 1999; Buhtoiarov et al. 2005; Lum et al. 2006 [A]; Beatty et al. 2011; Kang et al. 2011; Murray and Wynn 2011; Rakhmilevich et al. 2012). These myeloid cells are recruited into and infiltrate tumor mass as an early event during cancer development (Noy and Pollard 2014). Once in the tumor mass, macrophages have the potential to kill cancer cell, either through a direct activity against malignant cells or with an indirect activity that passes through the activation of adaptive immune system (Imaizumi et al. 1999; Lum et al. 2006 [A]; Biswas and Mantovani 2010; Mills et al. 2016). Nevertheless, in most cases, the tumor microenvironment is able to re-educate these cells steering their activity from antitumor to tumor promoting (Mantovani et al. 2002; Biswas and Mantovani 2010; Mills et al. 2016).

These observations, suggest that cancer immunotherapy reagents, rather than a direct cytotoxic activity on malignant cells, must display the capacity to sustain the activation of tumor infiltrating immunocompetent cells and to prevent their functional inactivation. Moreover, such type of reagents should ideally also be able to simultaneously target different immune cell types within the tumor mass in order to be clinically effective. In this scenario, a pivotal role is played by the CD40 molecule, a costimulatory protein, widely expressed on immune cells, able to activate both, innate and adaptive immunity (Elgueta et al. 2009; Beatty et al. 2017).

1.2 The CD40 receptor and CD40 ligand

The CD40 molecule or CD40 receptor (CD40R) belongs to the tumor necrosis factor receptor (TNFR) family (van Kooten and Banchereau 2000; Elgueta et al. 2009) and is encoded by a gene located on human chromosome 20q11-20q13-2. Notably, CD40R is an integral type I membrane glycoprotein of 45-50 kDa composed of 277 amino acid (aa). The mature form of the molecule includes a 193 aa extracellular domain, a 21 aa leader sequence, a 22 aa transmembrane domain and a 62 aa intracellular tail (with no similarity to any other characterized molecule) (Banchereau et al. 1994). Of note, human and murine CD40 molecule share 62% aa identity for the extracellular domain whereas 78% homology has been reported for the intracellular domain. In addition, 32 aa composing the C-terminal of the intracellular domain of CD40R and 22 cysteines located in the extracellular domain, are fully conserved between mouse and human. These observations suggest that both mouse and human CD40 molecules possess similar functional domains (Banchereau et al. 1994; van Kooten and Banchereau 2000).

CD40R was initially detected on the cellular surfaces of B lymphocytes. Later studies have documented the expression of CD40 on a wider range of cell types including myeloid immunocompetent cells (dendritic cells [DCs], monocytes and resident macrophages) but also endothelial cells, epithelial cells and stromal cells (e.g.: fibroblast and mesenchymal stromal cells) (van Kooten and Banchereau 2000; Elgueta et al. 2009). Remarkably, CD40 expression has been also observed in almost 70% of solid tumors and nearly all B cell malignancies (Vonderheide 2007).
So far, only one ligand for CD40R has been identified: CD154 also known as CD40 ligand (CD40L). CD40L belongs to the tumor necrosis factor (TNF) superfamily and it is a type II transmembrane glycoprotein, with a molecular weight ranging between 32 and 39 kDa. CD40L is encoded by a gene located on Xq26.3-Xq27.1. CD40L protein is composed of 261 aa. In particular, 22 aa compose the cytoplasmic domain, 24 aa the transmembrane domain and a 215 aa are included in the extracellular

domain. Similarly, to its cognate receptor, comparison between human and mouse CD40L revealed 75% of homology for the extracellular domain, 96% for the transmembrane region and 81% for the cytoplasmic domain.
In line with other members of the TNF superfamily, crystallography studies have indicated the ability also for CD40L molecule to form trimeric structures (van Kooten and Banchereau 2000; Elgueta et al. 2009).

In sharp contrast with CD40R, CD40L displays a highly selective pattern of expression. Indeed, CD40L is primarily detected on platelets, activated T lymphocytes and natural killer (NK) cells (van Kooten and Banchereau 2000). Most importantly, in addition to the transmembrane form (mCD40L), the TNF-like molecule CD40L was also described to have two additional isoforms (18 and 31 kDa), generated by proteolytic cleavage of the full-length protein. These shorter CD40L isoforms can be released as soluble proteins (sCD40L/s40L) by activated platelets and T cells (Huang et al. 2012).

1.2.1 The CD40 receptor : CD40 ligand (CD154) pathway

CD40R does not possess an intracellular domain with an intrinsic kinase activity. Ligation of CD40R by its cognate ligand results in the recruitment and activation of specific adaptor proteins termed as TNFR-associated factors (TRAFs).
TRAFs family of adaptor proteins is composed of six different members: TRAF-1, -2, -3, -4, -5 and TRAF-6. Members or TRAF family, except for TRAF-4, can be recruited to the cytoplasmic domain of CD40R, directly or through the formation of heterocomplexes (Bishop et al. 2007).
Recently, some reports have contributed to the identification of another member of TRAFs family named TRAF-7. However, biological role and pattern of expression of TRAF-7 are still unclear (Bishop et al. 2007; So et al. 2015).
CD40 triggering further tuned by specific ligand isoforms promotes the recruitment of different TRAF adaptor proteins resulting in the activation of complex signaling

cascades. These include the mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and the phospholipase Cγ (PLCγ) pathways (Elgueta et al. 2009). The signal cascade initiated by ligation of CD40R eventually results in the activation of transcription factors such as the nuclear factor kappa B (NF-kB), activator protein (AP)-1 and the nuclear factor for interleukin-6 (NF-IL6), leading, in turn, to the transcription of an array of different genes involved in the regulation of a wide spectrum of molecular and cellular process (Figure 2) (Bishop et al. 2007; Elgueta et al. 2009; Elmetwali et al. 2010; Huang et al. 2012).

Different studies have shed light on the critical relevance of the nature of CD40L stimuli in dictating different, even opposite, biological effects in targeted cells. In this respect, it has been shown that similarly to membrane-bound CD40L (mCD40L), also s40L can retain his trimeric structure resulting in the engagement of CD40R but culminating in a differential or reduced initiation of signaling cascade (Vonderheide 2007; Elmetwali et al. 2010; Huang et al. 2012; Elmetwali et al. 2016). In line with these observations, the s40L isoform was associated with survival pathway in carcinoma cells as well as increased immunosuppressive activity of MDSC and Tregs (Elmetwali et al. 2010; Huang et al. 2012; Jenabian et al. 2014). On the contrary, mCD40L isoform has been associated with apoptosis induction of CD40-expressing malignant cells and also to the activation of cellular and humoral responses (Elgueta et al. 2009).

In the last decade, molecular studies from several authors have contributed to an increased understanding of CD40-initiated signaling pathway. Although differential cellular responses induced by sCD40L or mCD40L in target cells still need to be fully characterized, it is now widely accepted that engagement of CD40R results in differential receptor conformations which in turn results in a selective TRAFs recruitment finally leading to either short-living signals or sustained stimuli

(Georgopoulos et al. 2006; Elgueta et al. 2009). In this regard, Elmetwali and colleagues in 2010 clearly demonstrated that apoptosis induction in CD40-expressing transformed cells, following stimulation with mCD40L, is associated with selective upregulation of TRAF-1 and TRAF-3 and a reduction of TRAF-6 intracellular levels. Notably, only the TRAF-1 protein was upregulated at transcriptional level while no changes were observed in TRAF-3/6 mRNA. These observations suggested that regulation of TRAF-3 and TRAF-6 intracellular levels might reflect protein stabilization and turnover of TRAF-3 and TRAF-6. Alteration of TRAF-1, -3 and -6 protein levels leads to JNK-dependent apoptosis as results of cytochrome c release and activation of caspases. In line with these observations, further studies also demonstrated that in CD40 positive cancerous cells, apoptosis induced by the membrane-bound CD40L, but not soluble isoforms, is related to the induction of the novel Ras effector 1A (NORE1A)/Ras association domain-containing protein 5 (RASSF5) molecule (Elmetwali et al. 2010; Elmetwali et al. 2016).

Collectively, these observations, along with CD40 broad pattern of expression, suggest that different TRAFs adaptor proteins are associated to the initiation of different intracellular signaling pathways. Therefore, it is not surprising that the effects of CD40R stimulation are multifaceted. In particular, CD40R ligation on cancer cells may lead to apoptosis or to pro-survival effects. Similarly, engagement of CD40R on immune cells might result in the initiation and progression of both humoral and cellular immune response or establishment of immune tolerance (Vonderheide 2007).

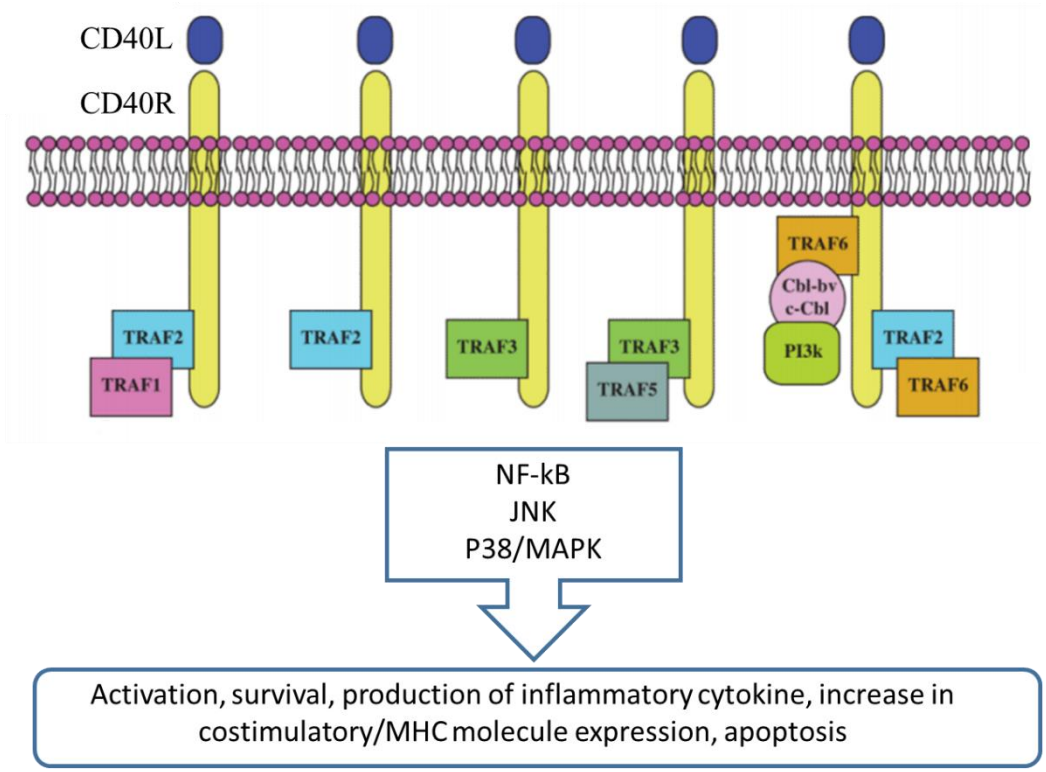

Figure 2. Multiple effects of CD40R : CD40L signaling. A cartoon summarizing TRAF-dependent activation of intracellular pathway in response to CD40R stimulation. TRAF molecules are recruited to the cytoplasmic tail of CD40R where they can mediate activation of NF-kB, JNK, p38/ MAPK. Depending on the different microenvironment, molecular characteristic of the ligand, and strength of CD40R stimulation, these molecules may activate the transcription of different genes implied in the induction of apoptosis or promotion of survival (adapted from Elgueta et al. 2009).

1.2.2 The CD40 receptor : CD40 ligand (CD154) in the immune system

A pivotal role for CD40R : CD40L pathway in the regulation of immune system has been extensively reported (Banchereau et al. 1994; Bishop et al. 2007; Vonderheide 2007; Elgueta et al. 2009; Vonderheide et al. 2013; Loskog et al. 2016). Indeed, ligation of CD40R expressed on the surface of immunocompetent cells has been associated to upregulation of a broad range of costimulatory/inhibitory receptors/ligands, production of antibodies and release of cytokines in the extracellular environment (Banchereau et al. 1994; Vonderheide 2007; Elgueta et al 2009; Vonderheide et al. 2013).

CD40 was initially described and functionally characterized as a key receptor expressed on cellular surfaces of B cells (van Kooten and Banchereau 2000). Stimulation of CD40R on surfaces of B cells, by CD40L-expressing CD4+ T cells, has been shown to play a pivotal role in the initiation and maintenance of effective humoral responses. Indeed, triggering of CD40R, results in B cells proliferation/expansion, inhibition of apoptosis and, most importantly, in their maturation as indicated by antibody isotype switching and differentiation into long-living plasma cells. Furthermore, CD40R-stimulated B cells display an increased expression of molecules mediating intracellular adhesion and co-stimulation of T cell (CD23 [FcεRII], CD80 [B7.1] and CD86 [B7.2], MHC class I/II molecule) as well as of cytokine production (IL-6, IL-10, TNF-α) (van Kooten and Banchereau 2000; Elgueta et al 2009).

Remarkably, mutations in the CD40L gene have been associated to hyper Immunoglobulin (Ig)M syndrome characterized by elevated levels of IgM, low levels of IgE, IgG and IgA, absence of germinal centers and inability to generate a protective humoral response (Grewal and Flavell 1998).

The identification of an increasing number of different immunocompetent cells expressing CD40 has expanded considerably the implication of this pathway in the control of immune responses. In particular, pioneering studies (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998) on DCs have underlined the critical relevance of CD40R : CD40L pathway not only in the generation of a humoral response but also in the generation of CD8-mediate immune responses.

Naïve CD8+ T lymphocytes require a full activation to produce antiviral and antitumor cytokines and to develop the capacity to eliminate target cells (Lanzavecchia 1998; Laidlaw et al. 2016). It is nowadays widely accepted that activation and generation of protective viruses/tumor-specific CD8+ T cells are achieved by the integration of three different signals. In particular, full activation of naïve CD8+ T cells requires, along to the recognition by the T cell receptor of the

MHC class I-peptide complex on the surface of DCs (Signal-1), also the delivery of additional signals such as costimulatory ligands (Signal-2) and exposure to pro-inflammatory cytokines (Signal-3) (Curtsinger et al. 2003; Obar and Lefrançois 2010). In this scenario, several studies have repeatedly reported a critical role for CD4+ T cells help in the generation of an efficient CD8+ T cell immune response (Keene and Forman 1982; Mitchison and O'Malley 1987; Cassell and Forman 1988; Husmann and Bevan 1988; Guerder and Matzinger 1992).

The initial interpretation of the "help" provided by CD4+ T cells was focused on the production of high amounts of IL-2 after the simultaneous recognition on the same antigen presenting cell (APC), of the cognate antigen (Mitchison and O'Malley 1987; Cassell and Forman 1988; Keene and Forman 1992). However, CD8+ T cells response could be also generated in IL-2 independent manner (Steiger et al. 1995). In addition, a potential limitation of this model was represented by the simultaneous interaction of two rare antigen-specific cells on the same APC (Lanzavecchia 1998).

Our current understanding of the interplay between DCs, CD8 and CD4 positive T cells largely relies on the APC-licensing model proposed by Matzinger (Matzinger 1994). According to this model, CD4+ T cells following antigen recognition on DCs can deliver a signal able to activate the APC to a state in which they can autonomously and properly prime CD8+ T cells (Matzinger 1994; Ridge et al. 1998). Thus, rather than being simple scaffold with the mere function to bring CD4+ and CD8+ T cells together, the DC play a pivotal role in the generation of the CD8+ T cell immune response (Ridge et al. 1998; Behrens et al. 2004).
The molecular basis accounting for CD4+ T cell-mediated activation of DCs were identified in the CD40R : CD40L interaction and currently, stimulation of CD40R-expressing DCs by CD40L+ CD4+ T cells is referred as antigen presenting cells licensing (APCs-licensing) (Lanzavecchia 1998).

Notably, APCs-licensing has been extensively indicated in animal studies and clinical studies as a critical step in the generation of protective CD8-mediated cellular responses (Di Rosa and Matzinger 1996; Vonderheide 2007). T-helper cells, through the expression on their cell surfaces of CD40L, promote the activation of CD8+ T cells by increasing the ability of CD40-licensed DCs to deliver the three activating signals to specific CD8+ T cells (Di Rosa and Matzinger 1996; Lanzavecchia 1998, Vonderheide 2007). Indeed, upon recognition of the cognate antigen in the context of MHC class II molecules, specific CD4+ T cells rapidly express on their cellular surfaces CD40L, which in turn results in an increased antigen presentation capacity of DCs to T cells as confirmed by upregulation of MHC class I/II molecules. Moreover, CD40L-stimulated DCs express high levels of molecules implicated in the formation of the immunological synapse such as the adhesion and costimulatory molecules CD58 (LFA-3), CD54 (ICAM-1), CD80 (B7.1) and CD86 (B7.2) (van Kooten and Banchereau 2000; Quezada et al 2004; Elgueta et al. 2009; Ma and Clark 2009; Kawabe et al. 2011).

Finally, CD40R-stimulated DCs release high amount of pro-inflammatory cytokines, most importantly, IL-12. This cytokine plays a crucial role in the initiation and maintenance of protective T helper (Th)1-responses (Hsieh et al. 1993; Hirohata 1999; Athie-Morales et al. 2004). Moreover, IL-12 is a strong amplifier of CD8+ response and was demonstrated to act as a third signal in CD8 priming (Curtsinger et al. 2003; Behrens et al. 2004).

This model has been further confirmed by later studies demonstrating that the crucial nature of CD4+ T cell help relies on the CD40-dependent licensing of APC (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998; Fransen et al. 2011). Indeed, neutralization of CD40L resulted in the abrogation of CD8+ T cell priming (Schoenberger et al. 1998). Most importantly, these studies demonstrated that specific agonistic monoclonal antibodies targeting CD40R can replace CD4+ T cells during CD8+ T cells priming.

1.2.3 Targeting CD40 receptor to harness the immune system against cancer

Generation of an effective CD8+ T cells response plays a pivotal role in protective antitumor immunity (Vonderheide 2007; Vonderheide and Glennie 2013). Several studies have shown that activation status of APCs (e.g.: DCs) represents a critical determinant for the induction of tumor-reactive CD8+ T cells. In this regard, ligation of CD40R on cellular surfaces of DCs leads to their activation. Indeed, CD40R-stimulated DCs display a high expression of MHC class I/II, co-stimulatory molecules and cytokines able to optimally prime tumor-reactive naïve CD8+ T cells. Based on the critical role of DCs in regulating antitumor CD8-mediated immune responses, initial therapeutic strategies were designed in order to promote, in a CD40-dependent manner, APC activation (Vonderheide 2007; Elgueta et al. 2009). Different reagents including agonistic CD40R antibodies, small molecules and viral vectors have been tested in animal models and also evaluated in cancer patients (Vonderheide 2007; Ullenhag and Loskog 2012).

In particular, animal studies have documented the antitumor efficacy of anti-CD40R agonistic antibodies. Intratumoral or intravenous administration of these type of antibodies resulted in the rapid generation, in a CD4+ T cell independent manner, of cytotoxic CD8+ T cells targeting syngeneic lymphoma (French et al. 1999). Furthermore, administration of anti-CD40R agonistic antibodies resulted in an increased induction of specific CD8+ T cells following peptide-based antitumor vaccination protocols (Diehl et al. 1999; French et al. 1999; Sotomayor et al. 1999). Most importantly, pre-clinical studies underlined therapeutic activity of CD40R mAbs for treatment of both solid and hematological cancers regardless the CD40 status of malignant cells (Todryk et al. 2001; Tutt et al. 2002). The latter observation prompted the development and clinical validation of similar reagents due to the broad range of tumors potentially treatable with anti-CD40R mAbs (Todryk et al. 2001; Tutt et al. 2002; van Mierlo et al. 2002; Vonderheide 2007).

Different anti-CD40R mAbs with activities ranging from antagonist to agonist have been used for clinical treatment of lymphoma and solid tumors. Results obtained in phase I/II clinical trials are consistent with disease stabilization in 20 to 60% of treated patients. Most importantly, clinical efficacy of CD40R mAbs based treatment has been correlated to activation of antitumor immune responses (Vonderheide 2007; Vonderheide and Glennie 2013; Remer et al. 2015). In particular, targeting CD40R with CP-870,893, a fully human IgG2 anti-CD40R agonistic mAb, resulted in objective tumor response against melanoma and pancreatic cancer. Patients receiving this mAb displayed activation of B cells, as antigen presenting cells, as evidenced by upregulation of CD86 costimulatory marker, MHC class I/II and CD54 adhesion molecule. To a similar extent, also ChiLob 7/4 mAb has been demonstrated to induce host APCs activation. This latter antibody is a chimeric IgG1 mAb that was tested in phase I clinical study with lymphoma and melanoma patients (Vonderheide 2013; Remer et al. 2015). Subjects enrolled in the study displayed DCs activation as indicated by CD83 expression and production of Th1 cytokines (Vonderheide 2007; Carpenter et al. 2009; Vonderheide and Glennie 2013; Remer et al. 2015).

The development of cancer immunotherapies targeting CD40R was prompted by the necessity to achieve a full activation of host APCs (especially DCs) in order to generate a powerful antitumor T cells response.

Nevertheless, targeting CD40R : CD40L pathway might also result in the activation of other immune mechanisms, which are not mutually exclusive, and can contribute to the elimination of cancerous cells (Vonderheide 2013). Indeed, engagement of CD40R on tumor endothelium can participate in the recruitment of immune effector cells in the tumor mass. Stimulation of CD40(+) endothelial cells, in fact, has been associated to the production of pro-inflammatory cytokines, such as the granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and with the upregulation adhesion molecules. The latter include vascular cell adhesion molecule (VCAM)-1, intracellular cell adhesion molecule (ICAM)-1 and E-selectin important for the

recruitment and adhesion of leucocyte in the inflamed area (Déchanet et al. 1997; Kotowicz et al. 2000; Ullenhag and Loskog 2012). However, once in the tumor mass, effector cells can then be impaired in their function by cells with immunosuppressive activity. The tumor microenvironment, in fact, is usually highly infiltrated by immunosuppressive cells belonging to the lymphocytic or myeloid lineage (Fridman et al. 2011; Fridman et al. 2012; Fridman et al. 2013; Fridman et al. 2017). These cells may impair antigen-presenting capacity of DCs through direct contact or by cytokine release, blocking the generation of tumor-specific effector clones and therefore establishing tumor tolerance (Ullenhag and Loskog 2012). In this scenario, CD40R engagement has been demonstrated to be associated with the reduction of immunosuppressive mechanism and elimination of immunosuppressive cell populations thereby concurring in overcoming the immune tolerance detectable in tumor microenvironment. Finally, CD40R-stimulation of cancerous cells might result in the upregulation of adhesion (CD54), costimulatory (CD80 and CD86) and MHC (class I/II) molecule on their cell membrane, promoting the formation of a productive immunological synapse between CD8+ T lymphocytes and malignant cells (de Charette et al. 2016).

In addition to these immune-mediated effects, CD40R engagement with agonistic mAbs can also have a direct effect on cancer cells, potentially resulting in their apoptosis (Vonderheide 2007; Loskog and Eliopoulos 2009; Ullenhag and Loskog 2012).

Interestingly, cell death induction in cancer cells has been associated with the intensity of CD40R stimulation and therefore with the specific TRAF molecule and signal transduction chain expressed by tumor cells. The most profound effect in carcinoma cells has been found to be induced by mCD40L rather than sCD40L (Elmetwali et al. 2010; Vonderheide 2007). Moreover, antibodies recognizing cancer cell markers also induce elimination of targeted cells through other, T cell independent, mechanisms, which include antibody-dependent cell-mediated

cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Vonderheide et al. 2013).

Based on the latter considerations, different mAbs have been developed in order to mediate direct cancer cell death, as consequence of direct CD40R engagement on target cell. In this regard, Dacetuzumab, a humanized IgG1 agonist was demonstrated to induce cancer cell death through various mechanism, including ADCC, CDC and direct apoptotic signaling. Similarly, Lucatumumab a fully humanized IgG1 blocking CD40L ligation was demonstrated to augment ADCC (Vonderheide et al. 2013; Suresh et al. 2014; Remer et al. 2015). Despite the remarkable antitumor activity of anti-CD40R mAbs, the use of this strategy in patients with cancer is associated with several side effects. In particular, the systemic administration of antibody targeting CD40R might result in activation of immune and normal cells leading to adverse reactions. Concerns are principally related to possible induction of cytokine release syndromes, thromboembolic syndrome, due to CD40 expression on platelets and endothelial cells and autoimmune reactions. Some of these effects were observed in patients after antibody treatment. In Particular, CP-870.893 and SGN-40 administration resulted in the development of cytokine release syndrome with serum increase of cytokines such as IL-6, TNF-α, IL-10, IL-1β, fever and chills. Moreover, transaminase elevation, a symptom of liver stress, was also observed immediately after antibody administration (Vonderheide and Glennie 2013; Remer et al. 2015; Beatty et al. 2017).

Importantly, although CD8+ T cells and DCs play key role in tumor cell eradication, data obtained from clinical studies as well as evidence from animal models suggest that tumor regression may also be obtained through T cell independent mechanisms. In this regard, a principal role has been described for myeloid cells. In particular, CD40R-stimulated macrophages were reported to display an efficient tumoricidal activity possibly based on the secretion of reactive nitrogen (RNI)/oxygen (ROI)

intermediates and/or effector cytokines such as TNF-α (Beatty et al. 2011; Ullenhag and Loskog 2012; Vonderheide et al. 2013).

1.3 Macrophages

Macrophages have been widely described as critical players of innate immune responses against cancer and pathogens (Murray and Wynn 2011). Traditionally, circulating CD14+ monocytes were indicated as precursors of tissue resident macrophages. This initial interpretation has recently been challenged. Indeed, origin of macrophages has been associated to specific tissue precursors seeded, in different anatomical district, during embryonic development (Geissmann et al. 2010; Ginhoux et al. 2016; Mantovani et al. 2017).

A peculiar feature of macrophages is represented by their high plasticity. Indeed, cells belonging to macrophage lineage display an enhanced ability to integrate different stimuli from the extracellular environment and to respond to them by acquiring specific phenotypic and functional profiles. In this regard, it was reported that IFN-γ and/or microbial products might promote the differentiation of classically activated (M1) CD14-derived macrophages with pro-inflammatory functional attributes. In contrast, Th-2 cytokines (IL-4/IL-13) promote the generation of alternatively activated (M2) CD14-derived macrophages defined by an immunosuppressive functional profile (Stein et al. 1992; Mantovani et al. 2002; Biswas and Mantovani 2010; Sica and Mantovani 2012; Martinez and Gordon 2014)

M1 macrophages are characterized by high expression of CD16 receptor and reduced expression of CD163 and CD204 on their cell surfaces (Nebiker et al. 2014). In addition, activated CD14-derived M1 macrophages display high levels of MHC class I/II and costimulatory molecules (CD80/CD86). Activation of M1 macrophages results also in the de-novo production of pro-inflammatory cytokines including IL-12, IL-23, TNF-α and RNI/ROI (Gordon and Taylor 2005; Mantovani et al. 2008;

Sica and Mantovani 2012; Mills 2015). Based on this functional profile, CD14-derived M1 macrophages have been indicated as sentinels of innate immune responses against cancer, viruses and bacterial infections (Mosser and Edwards 2008). In addition to their direct activity against target cells, CD14-derived M1 macrophages have been shown to have a critical role in regulating adaptive immune responses. In this regard, release of C-X-C motif chemokine ligand (CXCL)9 and CXCL10, efficient antigen presentation capacity and production of high amount of pro-inflammatory cytokines, including IL-12, have been described as crucial elements dictating the ability of M1 macrophages to recruit and promote the activation of Th1-mediated immune responses (Martinez et al. 2006; Mantovani et al. 2008; Biswas and Mantovani 2010).

In contrast, M2 macrophages display reduced expression on their cell surfaces of CD16 and high levels of CD163 and CD204 (Nebiker et al. 2014). Several studies support a critical role of CD14-derived M2 macrophages in the elimination of parasites and in tissue remodeling. Moreover, a pro-tumoral activity in different types of malignancies has also been described for M2 macrophages. These roles have been directly associated to the impaired ability of CD14-derived M2 macrophages to produce pro-inflammatory cytokines including IL-12 (Mantovani et al. 2002; Gordon and Taylor 2005; Mantovani et al. 2008; Sica and Mantovani 2012; Mills 2015). In addition, activation of M2 macrophages results in the secretion of C-C motif chemokine ligand (CCL) 17, CCL22 and CCL24 promoting the recruitment of Th2 lymphocytes (Martinez et al. 2006; Mantovani et al. 2008).

These two different forms of macrophage polarization are tightly regulated at the intracellular levels by specific transcription factors. In this regard, high levels of signal transducer and activator of transcription (STAT)1, NF-kB and interferon regulatory factor (IRF)5 have been associated to an M1-like functional profile as suggested by the increased expression of genes encoding pro-inflammatory cytokines such as IL-12, IL-23 and TNF-α. On the other hand, stimuli resulting in

increased levels of IRF4, STAT3/6 and cMyc have been associated to the skewing of CD14 monocytes toward the M2 profile (Sica and Mantovani 2012; Mantovani and Allavena 2015).

Notably, these M1 and M2 activation status of macrophages mirror the Th1/Th2 polarization of CD4+ T cells. Nevertheless, in contrast with T-helper cells differentiation, M1 and M2 macrophages have been extensively indicated as two extremes of a continuum of differentiation states (Mantovani et al. 2002; Mosser and Edwards 2008). Indeed, it has been observed that in different pathological or physiological contexts macrophages can assume a phenotype that does not overlap with M1 or M2 profiles but present features of both polarization states (Chan et al. 2008; Shaul et al. 2010). Moreover, due to their intrinsic plasticity, macrophage polarization can also be modified during the course of different disease such as cancer and obesity (Lumeng et al. 2007; Biswas et al. 2008).
Chemokines, including CCL2 and CXCL4, soluble factors such as macrophage colony-stimulating factor (M-CSF), IL-10, and transforming growth factor (TGF)-β have been indicated as critical mediators in the establishment of an M2 favorable immunosuppressive microenvironment. These molecules, in fact, can cooperate in recruitment and induction of macrophage polarization toward phenotype sharing distinct features with IL-4/IL-13 activated macrophages (M2) and often observed in TAMs (Roca et al. 2009; Biswas and Mantovani 2010; Gleissner et al. 2010)

1.3.1 Macrophages and cancer

Macrophages represent a significant fraction of tumor-infiltrating immunocompetent cells (Noy and Pollard 2014). Abundance of macrophages has been extensively reported in several solid tumor types, in both tumor center and invasive margins (Fridman et al. 2017). Most importantly, several studies have contributed to define the relationship occurring between “quantity” and “quality” of macrophages infiltration and clinical outcome in cancer patients (Figure 3).

In this regard, high infiltration of M1 macrophages defined using, among others, the human leukocyte antigen-antigen D related (HLA-DR) or the inducible nitric oxide synthase (iNOS) markers, has been correlated with a favorable prognosis in non-small-cell lung cancer (NSCLC) (Ohri et al. 2009; Ma et al. 2010; Ohri et al. 2011) (patient n=140), hepatocellular carcinoma (HCC) (Dong et al. 2016) (n=253), ovarian cancer (OC) (Zhang et al. 2014) (n=112) and gastric cancer (Pantano et al. 2013; Zhang et al. 2015) (n=232). In contrast, high infiltration by M2-like macrophages, as defined using, among others, CD163 and CD204 expression as marker, has been associated with poor prognosis in breast (Medrek et al. 2012) (n= 144), bladder (Ichimura et al. 2014) (n=171), ovarian (Lan et al. 2013) (n=110), renal cell carcinoma (RCC) (Dannenmann et al. 2013; Xu et al. 2014) (n=239), gastric (Kawahara et al. 2010; Zhang et al. 2015) (n=434) and in melanoma (Jensen et al. 2009) (n=227) (Fridman et al. 2017).

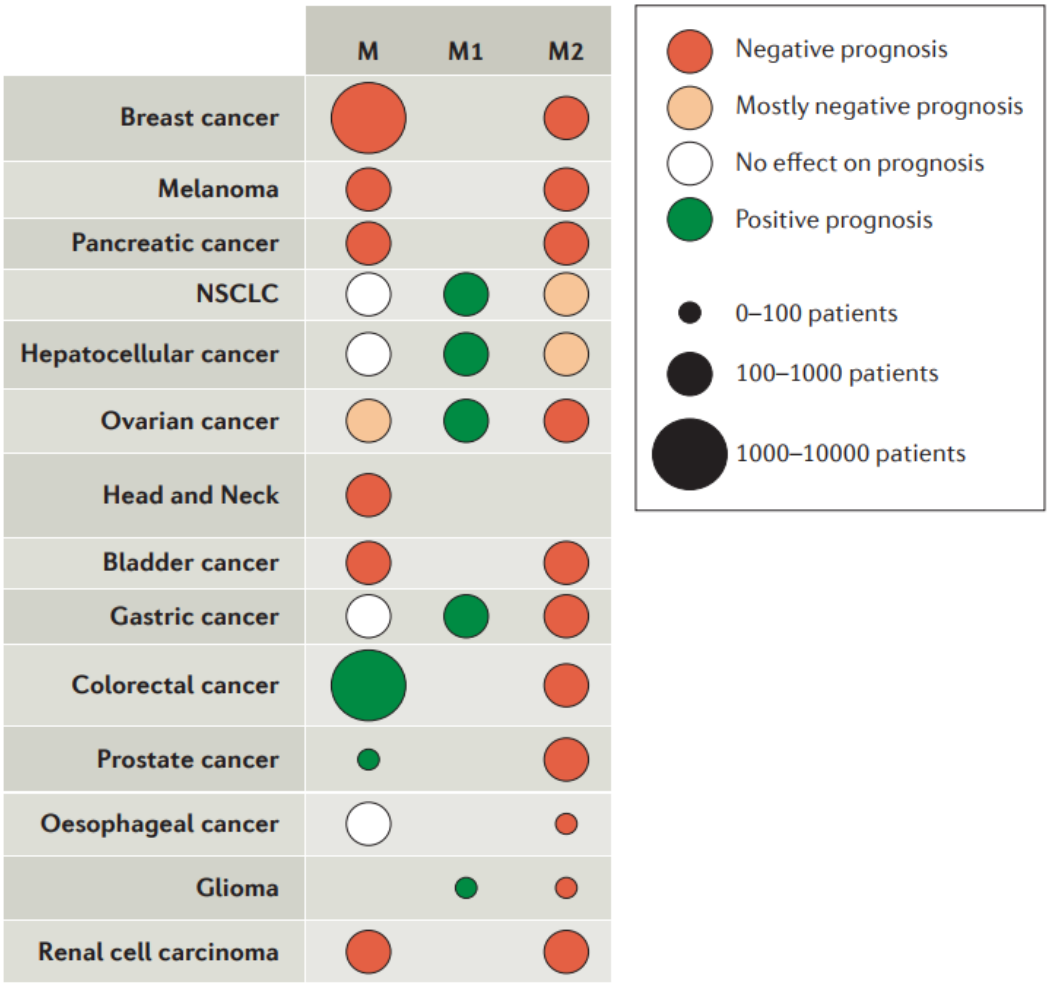

Figure 3. M1 or M2 macrophages (M) infiltration and clinical outcome in cancer patients. Bold colors indicate a positive (green) or a negative (red) prognostic association following analysis of all relevant studies; orange color indicates a predominantly negative prognostic association in the majority of studies analyzed. White circles indicate no statistically significant correlation, or that unclear prognostic associations were observed in a similar number of studies. The size of the circles relates to the size of the cohort in clinical studies. Small circles indicate 0–100 patients, Medium-sized circles indicate 100–1,000 patients and Large circles indicate 1,000–10,000 patients (adapted from Fridman et al. 2017).

Our current understanding regarding the impact of macrophages infiltration and clinical outcome of cancer patients is based on the M1/M2 differentiation model. In this respect, classically activated M1 macrophages were shown, in animal models and clinical studies, to possess a potent antitumor activity (Mills et al. 2016; Fridman

et al. 2017). In particular, the enhanced ability of M1 macrophages to inhibit tumor progression has been correlated mostly to their capacity to release high amount of TNF-α (Sica and Mantovani 2012; Fridman et al. 2017). Furthermore, CD14-derived M1 macrophages can selectively eliminate pre-malignant cells preventing tumor progression, at least in the animal model (Kang et al. 2011). In addition to these latter observations, the antitumor role of CD14-derived M1 macrophages is also based on their ability to orchestrate generation of innate and adaptive immune responses against cancer. Accordingly, it has been shown that IFN-γ production by NK cells, Th1 and CD8+ T lymphocytes might sustain M1 polarization of myeloid cells, which in turn release IL-12 and CXCL9, CXCL10 chemokines resulting in a sustained antitumor immune response (Biswas and Mantovani 2010). In vitro and in vivo studies have repeatedly shown a protective role for CD14-derived M1 macrophages (Grabstein et al. 1986; Mills et al. 2016).

However, a significant infiltration of pro-tumor M2 macrophages, particularly at the late stages of malignancy, has also been observed (Biswas et al. 2013; Noy and Pollard 2014).

In this regard, an increased attention has been focused, in the last years, regarding on the ability of chronically inflamed tissues, including many tumor types(Mantovani and Sica 2010), to promote preferential recruitment and polarization of TAMs toward an M2-like profile (Fridman et al. 2012; Sica and Mantovani 2012; Mills et al. 2016; Fridman et al. 2017). In this type of microenvironment, macrophages are exposed to cytokines and chemokines (such as M-CSF, IL-10, TGF-β, CCL2) produced by the malignant and tumor-associated stromal and inflammatory cells educating macrophages to become pro-tumoral cells with an M2-like phenotype (Biswas et al. 2013; Noy and Pollard 2014). These macrophages display a low production of IL-12 and high production of IL-10. Furthermore, they show low antigen presentation and tumoricidal activity, impaired production of RNI but large production of the tumor growth molecule ornithine as well as molecules involve in

tissue remodeling and angiogenesis (vascular endothelial growth factor [VEGF], epidermal growth factor [EGF], CXCL8) (Mills 2012; Biswas et al. 2013; Noy and Pollard 2014). Most importantly, when cancers become invasive, the tumor microenvironment appears to be dominated by cytokines that promote T cells skewing from a Th1 to a Th2 immune response. IL-4 and IL-13 cytokines, derived from Th2 cells, further sustain macrophages polarization to an M2 phenotype, which in turn express a set of chemokines (CCL17, CCL22, CCL24) resulting in the recruitment of other inflammatory cells (Treg, Th2, eosinophils, basophils) expressing cognate receptors CCR3 and CCR4. Once in the tumor microenvironment, these cells produce cytokines (IL-10, IL-4, IL-13) that further promote macrophage polarization through an M2/M2-like phenotype in a self-sustaining cycle promoting cancer progression (Biswas and Mantovani 2010; Noy and Pollard 2014) (Figure 4).

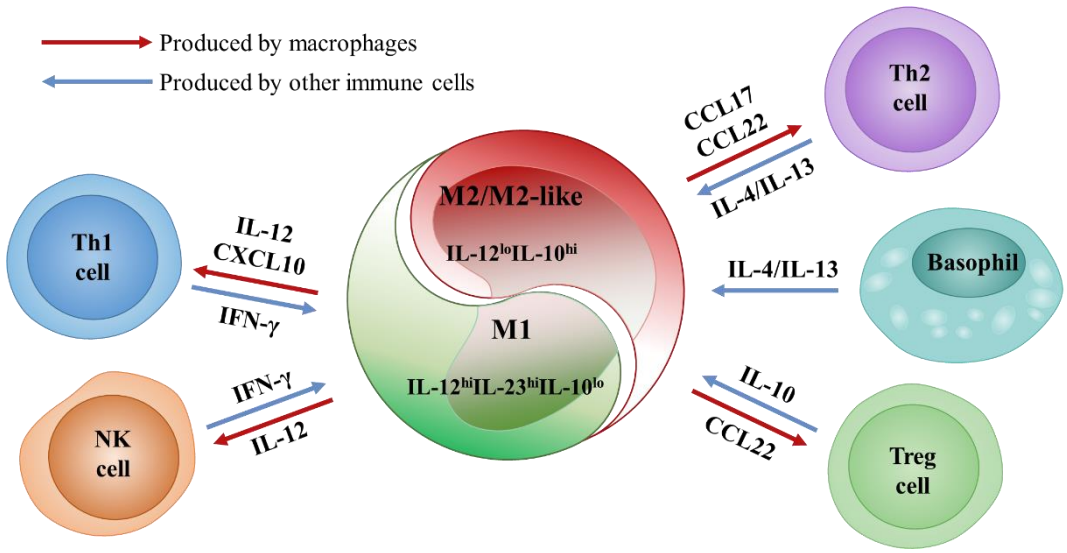

Figure 4. Macrophage's activation and polarization orchestrate by immune cells. M1-polarized macrophages and their crosstalk with Th1 and NK cells. M2 polarization of macrophages driven by Th2 cells and basophils, through their secretion of IL-4 or IL-13. M2-like macrophages polarized by interaction with Treg. In green M1 or in red M2/M2-like polarized macrophages (adapted from Biswas and Mantovani 2010).

Due to the pivotal role played by macrophages in either cancer progression or cancer eradication, a number of potential macrophage-centered therapeutic approaches are being explored. (Mantovani et al. 2017; Zheng et al. 2017). In particular, since it has been demonstrated that macrophage polarization can be reverted to some extent (Guiducci et al. 2005; Saccani et al. 2006) potentially leading to the elicitation of M1 direct anticancer activity, an appealing strategy appears to be the functional re-education of TAMs to an M1-like functional phenotype.

1.3.2 Targeting the CD40 pathway to enhance antitumor macrophage activity

Current understanding of successful cancer immunotherapeutic strategies largely focuses on the ability of tumor-reactive CD8+ T cells to mediate the elimination of transformed cells.

However, in the last years, the role of myeloid cells in tumor eradication has been revised. In particular, a potential protective role against cancer has been underlined for macrophages. Several studies have shown that these myeloid cells might mediate cancer cells clearance and tumor regression through mechanisms not requiring the activation of CD8+ T cells immunity (Beatty et al. 2011; Rakhmilevich et al. 2012; Mills et al. 2016). In particular, studies performed in animal models indicate that tumor infiltration by M1 macrophages results in decreased tumor progression as compared to M2-infiltrated tumors (Evans et al. 1977; Fidler and Poste 1982; Mills 2015; Mills et al. 2016). In addition, skewing M2 toward M1 phenotype might favor tumor regression (Mills et al. 1992; Beatty et al. 2011; O'Sullivan 2012). Notably, antitumor activity of myeloid cells of monocyte and macrophage lineage can be finely tuned by the CD40R : CD40L pathway (Rakhmilevich et al. 2012). Indeed, several *in vitro*, *ex vivo* and *in vivo* studies have demonstrated that myeloid cells once stimulated through CD40R develop an M1 pro-inflammatory phenotype that can rapidly lead to tumor cells elimination (Buhtoiarov et al. 2005; Lum et al. 2006).

The first evidence on the antitumor potential of myeloid cells was reported by Alderson and colleagues. This study showed that CD40L transfected cells were able to activate the tumoricidal activity of human monocytes against melanoma cells (Alderson et al. 1993). This initial observation was later confirmed by *in vitro*, *ex vivo* and *in vivo* studies aimed at clarifying the antitumor potential of macrophages with similar results. Taken together, these studies have revealed that following CD40R stimulation, murine macrophages are able to secrete effector cytokines and soluble cytotoxic molecules, most notably nitric oxide (NO), IL-12 and TNF-α, resulting in a significant cytostatic/cytotoxic activity against different types of transformed cells (Imaizumi et al. 1999; Buhtoiarov et al. 2005; Lum et al. 2006 [A]; Rakhmilevich et al. 2012).

IL-12 production by CD40R-stimulated macrophages plays a pivotal role in the elicitation of their antitumor activity. Indeed, it has been reported that IL-12 produced in response to CD40R triggering induces the production of IFN-γ, which in turn results in the activation and stimulation of macrophages antitumor effect (Buhtoiarov et al. 2005). Moreover, INF-γ was demonstrated to be responsible for CD40 upregulation on macrophages cell surface and, in combination with CD40R stimulation, to increase NO and TNF-α production by these myeloid cells (Imaizumi et al. 1999)

These initial findings were confirmed and further extended *in vivo*, by demonstrating that a CD40R specific agonist mAb stimulated murine macrophages to maturate into an M1 pro-inflammatory phenotype able to inhibit tumor growth in a T/B/NK independent manner (Lum et al. 2006; Rakhmilevich et al. 2012). In particular, CD40R stimulation of these cells led to their rapid activation, as indicated by upregulation of costimulatory (CD80, CD86, CD40, toll like receptor [TLR] 4) and MHC class II molecules. Most importantly, after CD40 stimulation, they produce pro-inflammatory Th1 cytokines (IFN-γ, IL-12; TNF-α) and display an increased

cytostatic activity against neuroblastoma and melanoma cancer cells (Lum et al. 2006).

In line with these findings underlining the critical role of macrophages in cancer eradication, important results were also obtained in pre-clinical and clinical studies performed on pancreatic ductal adenocarcinoma (PDA) (Beatty et al. 2011; Beatty et al. 2013).

PDA is a highly aggressive cancer, poorly responsive to standard therapies such as chemotherapy, which offer only minimal benefit in patients not eligible for surgery (Beatty et al. 2011; Vonderheide et al. 2013). New drugs approved for treatment of PDA (erlotinib) extend patients survival by only a couple of weeks compared to classical therapies. In contrast, the combination of standard care treatment (gemcitabine) with systemic administration of CD40R agonist (CP-870,893) for advanced/surgically incurable PDA, chemotherapy naïve patients gave encouraging results (Vonderheide et al. 2013). Although gemcitabine alone results only in approximately 5% partial response, the addition of a CD40R agonist resulted in 4/21 patients in partial response and 11/21 in disease stabilization with a median progression free survival of 5.6 months, while gemcitabine alone only achieves 2.3 months (Beatty et al. 2011; Beatty et al. 2013; Vonderheide et al. 2013). Histological analysis of surgically resected tumor lesions from patients with partial response revealed an immune infiltrate, which was enriched in macrophages, but not in T lymphocytes as expected. In particular, animal studies have contributed to clarify the antitumor activity of CP-870,893. Indeed, administration of a rat anti-mouse CD40R agonist mAb FGK45 (homolog of human CP-870,893) following gemcitabine treatment resulted in a similar antitumor activity as observed in cancer patients (Beatty et al. 2011). Most importantly, systemic administration of FGK45 resulted, similarly to CP-870,893, in a high infiltration of myeloid cells but not T lymphocytes into the neoplastic lesion (Beatty et al. 2011; Beatty 2013). Analysis of tumor-associated immune cells after FGK45 treatment provides evidence that macrophages

were activated as indicated by upregulation of MHC class II and CD86 molecule. These CD40R-activated macrophages acquired tumoricidal properties and the capacity to secrete high levels of IL-12 and TNF-α. Most importantly these cells shift their phenotype from stroma supporting to stroma degrading cells and mediate disruption of Type I collagen (a major component of extracellular matrix) (Beatty et al. 2011; Beatty et al. 2013).

These findings reinforce the idea that immunotherapeutic strategy aimed at targeting CD40R can drive both T cell dependent and independent mechanisms. In regard to the latter, triggering of the CD40 pathway has been demonstrated to efficiently restore tumor immune surveillance by targeting tumor-infiltrating macrophages (Vonderheide et al. 2013). Notably, these results also imply that the final antitumor activity may pass through a re-education of tumor promoting macrophages and stromal destruction (Vonderheide et al. 2013).

1.4 Vaccinia virus and cancer immunotherapy

Therapeutic efficacy of engineered CD40L-expressing recombinant viral vectors has been extensively evaluated in animal models and clinical studies (Ullenhag and Loskog 2012; Parviainen et al. 2014). Interestingly, several phase I/II clinical trials have addressed the clinical efficacy of CD40L-expressing recombinant adenovirus (AdCD40L) alone or in combination with IL-2 in patients with solid cancer and hematological malignancies (Wierda et al. 2000; Biagi et al. 2005; Rousseau et al. 2006; Malmström et al. 2010). Remarkably, following treatment, a significant increase in tumor-reactive T cells along with shrinkage of tumor masses was observed in cancer patients enrolled in clinical studies (Biagi et al. 2005; Rousseau et al. 2006) Most importantly, immune activation promoted by AdCD40L resulted in 5-year overall survival of 90% of treated patients with acute leukemia (Rousseau et al. 2006).

Among different viral vectors evaluated for cancer therapeutic purposes, recombinant vaccinia viruses (rVVs) have been extensively explored. Several studies in pre-clinical and clinical settings have addressed the antitumor potential of rVVs encoding together with tumor-associated antigens, costimulatory molecules of immunological relevance (Zajac et al. 1998; Adamina et al. 2010; Parviainen et al. 2014).

Vaccinia Virus (VV) belongs to the *Poxviridae* family. VV is a double-strand DNA virus (Shen and Nemunaitis 2005). The length of virus backbone is approximately of 190kbp and contains 250 genes (Larocca and Schlom 2011). The effectiveness of VV as preventive vaccine has been proven by the eradication of variola virus, officially achieved in 1980 (Moss 1991). Due to its historical role, VV biology has been extensively studied in laboratory resulting in its use as an effective vehicle for gene delivery in immunotherapy.

A peculiar feature of VV is represented by its ability to infect a broad range of mammalian cells. Notably, host cell infection by VV does not require any defined cell surface receptor, but it is due to its fusion with plasma membrane of mammalian cells. In this regard, it must be underlined that other viruses, including adenovirus, have a more restricted host cell range due to the requirement for specific surface receptors during the infection process.

VV genome can be easily engineered leading to the accommodation to up to 25 kb of foreign genetic material. Finally, VV displays an enhanced tumor tropism due to cancer cell dysregulation in cell cycle, immune evasion and uncontrolled replication. Importantly, the use of vaccinia virus is safe, since replication of the virus takes place in cytoplasmic structures defined “viral factories” and the virus does not integrate into the host genome (Shen and Nemunaitis 2005; Kirn and Thorne 2009; Kim and Gulley 2012).

The use of vaccinia virus (Copenhagen strain) in preclinical studies has been extensively explored at the University of Basel. In previous studies, the efficacy of rVV in the generation and the expansion of tumor-reactive CD8+ T cells was tested. Remarkably, the use of a rVV encoding tumor-associated antigens and costimulatory molecules has shown an enhanced ability to promote the expansion of CD8+ tumor-reactive T cells. This was observed with cells from peripheral blood of healthy donor and tumor-infiltrating lymphocytes, obtained from clinical specimens (Zajac et al. 1997; Zajac et al. 1998; Groeper et al. 2007).

Interestingly, rVV displayed also therapeutic efficacy in phase I/II clinical studies in stage III/IV melanoma patients. This viral vector was engineered to express three HLA-A0201 restricted epitopes from melanoma differentiation antigens ($gp100_{280-288}$; - Melan-A/$MART\text{-}1_{27-35}$; - $tyrosinase_{1-9}$) and CD80, CD86 costimulatory molecules.

The treatment administered to the patients enrolled in the study consisted in the intradermal administration or the recombinant virus, followed by re-call boosts with the antigenic soluble peptides together with GM-CSF injected intradermally.

Clinical observations indicate that 7/10 treated patients developed a CD8+ T cells response against at least one of the antigens and showed stable disease. Moreover, 3/7 responsive patients displayed single metastasis regression (Adamina et al. 2010). Thus, clinical benefit correlated with the generation of CD8+ T cells targeting at least one tumor-associated peptide, indicating that the administration of rVV is safe and induces the generation of tumor-specific T cells. In this regards Trella et al., more recently (2016), demonstrated that the use of a recombinant vaccinia virus encoding for CD40L (rVV40L) promotes the rapid "in vitro" differentiation of naïve CD8+ T cells into CD8+ T central memory cells reactive against human tumor-associated antigens. This work underlines once again the ability of such type of vector to fine tune CD8+ T cell-mediated immune response.

However, tumor regression is also associated to T cell independent mechanisms (see paragraph 1.3.2). In this respect, CD40 pathway targeting has been demonstrated to

efficiently restore tumor immune surveillance through re-education of tumor-infiltrating macrophages. Therefore, rVV40L could theoretically be able not only to promote the generation and expansion the CD8+ T cells responses, but also to generate antitumor activity through the modulation of functional profiles of tumor-infiltrating myeloid cells.

2. Outline of the thesis

In the present study, I have analyzed CD40 expression on malignant and tumor-infiltrating immune cells in a large (n>800) cohort of human cancers and identified malignancies potentially representing important clinical targets. This information was then used to perform tailored “in vitro” and “in vivo” experiments using CD40(-) and (+) cell lines of defined histological origin. In particular, I evaluated the ability of rVV40L to directly induce apoptosis on CD40(+) tumor cells. In addition to the direct effect on cancerous cells, I also investigated the ability of rVV40L to modulate the functional profile of differentially polarized macrophages potentially leading to in vivo inhibition of tumor progression.

3. Materials and methods

3.1 Immunohistochemistry

Tumor characteristics and peritumoral immune cell infiltration were evaluated by using the original hematoxylin and eosin (H&E) slides of resection specimens corresponding to microarray punches. Tissue microarray (TMA) slides were incubated with CD40 specific primary antibodies (Clone58612; Abcam). Percentages of CD40 positive cells were evaluated by experienced pathologists blinded to any prior information. In particular, percentages of positive malignant and tumor-infiltrating immune cells were evaluated in total tumor and peritumoral areas in each punch. Intensity of staining on malignant cells was also analyzed and samples were classified as negative (0), weakly positive (1), moderately positive (2) and strongly positive (3). Tumor cells were scored positive if they displayed at least intensity 2 staining. Positivity cut-off for tumor-infiltrating immune cells was 20% regardless of staining intensity.

3.2 CD40 ligand-expressing recombinant Vaccinia Virus (rVV40L)

rVV40L was generated as previously described (Feder-Mengus et al. 2015). In order to avoid the cytopathic/lytic effect of replicating virus and focus on the effect of the transgene expression, viral replication was inactivated by DNA cross-linking by using psoralen (1 μg/ml) and long-wave UV (365nm) irradiation (Feder-Mengus et al. 2015).

3.3 Established tumor cell lines

Established melanoma (A375), hepatocellular carcinoma (PLC, HepG2 and HuH-7), colorectal (HCT116, LS180 and HT29) and breast (MDA-231 and BT-474) cancer cell lines were purchased from European Collection of Cell Cultures (ECACC). Na-8 melanoma cell line was a gift from Dr.Jotereau (Nantes, France). Breast and

colorectal cancer cell lines were cultured in RPMI-1640 CM supplemented with 1% GlutaMAX-I, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, HEPES, 1% Kanamycin Sulfate (Gibco-Life Technologies), thereafter referred to as complete medium (CM) and 10% FBS. Melanoma and hepatocellular carcinoma, HepG2 and HuH-7 cells, were cultured in D-MEM CM 10% FBS. PLC cells were cultured in ALPHA-MEM supplemented with 10% FBS. When specific established tumor cell lines were required for indicated experiments, early passage cells were thawed and maintained in culture forless than 2 months.

3.4 In vitro generation of CD14+ derived macrophages

CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors to a > 95% purity by using anti-CD14 magnetic beads (Miltenyi Biotech). Magnetically isolated cells were cultured in RPMI-CM and 10% FBS in presence of 12,5 ng/ml GM-CSF (R&D System) to generate M1 macrophages or MCSF (R&D System) for M2 macrophages. On day 6, cells were collected and stained with anti-CD16, anti-CD163, anti-CD204 and anti-CD40R receptor specific, fluorochrome-conjugated mAbs.

3.5 CD40 triggering on tumor cells

Tumor cell lines were left untreated, infected with rVV40L or with vaccinia virus wild type (VV WT) at 10 multiplicity of infection (MOI). Furthermore, established tumor cell lines were also treated with soluble CD40L (s40L; 0,5 µg/ml; Enzo Lifescience) recombinant protein alone or following VV WT infection (WT+s40L). After 4 days, tumor cells from different culture conditions were collected and their viability was assessed by Annexin V apoptosis detection kit (Becton Dickson) according to the manufacturer's instructions. In brief, the cells were washed twice with cold PBS and then resuspended in the binding buffer at 1 x 10^6 cell/ml concentration. 100 µl of this solution (1 x 10^5 cells) were stained with 5 µl of Annexin V antibody plus 5 µl PI (ready-to-use solutions), then incubated for 15 min

at room temperature in the dark. Finally, the cells were diluted with 400 µl of the binding buffer and analyzed by flow cytometry.

3.6 Activation of CD14+ monocytes and macrophages differentiation

CD14+ monocytes obtained from peripheral blood of healthy donors were left untreated or infected in 500 µl RPMI-1640 CM 10% FBS for 1 hour at 37°C with rVV40L or with VV WT at MOI of 5. In addition, CD14+ monocytes were also treated with s40L (0,5 µg/ml) alone or following VV WT infection (WT+s40L). CD14+ monocytes were then cultured in RPMI-1640 CM 10% FBS in presence of either GM-CSF or M-CSF (see above). At indicated time points, supernatants from different culture conditions were collected and cytokine release was assessed by ELISA.

3.7 Co-culture of M1 and M2 macrophages with established tumor cell lines

LS180 and HuH-7 established tumor cell lines were left untreated or infected with rVV40L or with VV WT at 10 MOI. After 24 hours, 3000 untreated and VV WT or rVV40L-infected tumor cells were cultured alone or in presence of M1 or M2 macrophages at different effector: target ratios in 96 well-plates. On day 4, cytokines and chemokine released in supernatants from different culture conditions were evaluated by ELISA. Cytostatic activity of M1 and M2 macrophages on tumor cells was evaluated by adding 1µCi of ^{3}H-thymidine for the last 18 hours of culture. Cells were then harvested and tracer incorporation was measured by scintillation counting. TNF-α neutralization was achieved by adding to cultures anti-human TNF-α mAb (BioLegend) at 10 µg/ml final concentration.

3.8 Cross-presentation assays

Generation of MelanA/Mart-1_{27-35} specific HLA-A0201 restricted CD8+ T cell clones was described previously (Mariotti and Nisini 2009). 3000 cells from CD40 (-) HLA-A0201(-) HT29 cell line were left untreated or infected with VV WT or rVV40L and co-infected with a recombinant vaccinia virus encoding MART-1 full gene, inducing the production of the entire protein in infected cells at MOI of 10. After 24 hours, differentially infected HT29 cells were cultured alone or in presence of HLA-A0201 (+) M1 or M2 macrophages at 1:1 ratio. Two days later, 30.000 cells of a MelanA/Mart-1_{27-35} specific, HLA-A0201 restricted, CD8+ T cell clone were added to the different culture conditions. After 48 hours, the release of IFN-γ in the supernatants was assessed by ELISA.

3.9 Migration assay

Migration of CD8+ T cells towards supernatants from co-cultures of M1 macrophages and tumor cells was assessed in 96 well trans-well plates (5μm pore size; Corning Costars) upon 60 min culture at 37°C. When indicated, anti-CXCL10 antibody (10 μg/ml; R&D System) was also added to culture supernatants. Cell migration was quantified by flow-cytometry.

3. 10 In vivo experiments

In vivo experiments were approved by Basel Cantonal Veterinary Office (License Number 2266). NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) from Charles River Laboratories, were bred and maintained under specific pathogen-free conditions in the animal facility of Department of Biomedicine, University of Basel. Eight-ten week old mice were injected subcutaneously (s.c.) in the flank with tumor cells (10^5 cells/mouse), resuspended in 1:1 growth factor reduced Matrigel (BD Biosciences) / PBS solution. Tumor formation was monitored twice weekly by palpation and caliper measurements. Once tumor masses reached an approximate

diameter of 5 mm (1 x 10^6 tumor cells), 20μl of virus solution (10^7 eq. pfu of VV WT or rVV40L) or PBS were injected in the tumor tissues. After 48 hours PBS or 5 x 10^5 M1 or M2 macrophages were also injected intratumorally. Tumor size changes were followed every day by caliper measurements. One week after, all mice were sacrificed and tumors were harvested. Tumor volumes (in mm3) were determined according to the formula (length x width2)/2. Samples from all tissues were harvested for subsequent histological examination.

3.11 Gene expression analysis

Total cellular RNA was extracted by using the RNeasyVR Mini Kit (Qiagen) and reverse transcribed according to the manufacturer's instructions (Invitrogen-Life Technologies). Human TRAF-1 and NORE1A (RASSF5) gene expression were evaluated by quantitative RT-PCR (qRT-PCR) using specific primer sets (TaqMan® Assays, Applied Biosystems-Life Technologies) and normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression. Expression of genes displaying a delta to reference gene >13Ct were considered.

3.12 Flow cytometry

Fluorochrome-labeled monoclonal antibodies (mAbs) recognizing CD163, CD204, CD154 (CD40L), CD3, CD4, CD8, CD45RO, CD62L, CD183 (CXCR3) and HLA-A0201 were obtained from Becton Dickinson. A mAb recognizing CD16 was obtained from BioLegend whereas a mAb recognizing CD40 receptor (Clone 5C3) was obtained from eBioscience. Viability of tumor cells was measured by Annexin V apoptosis detection kit (Becton Dickson). Signals derived from mAbs and fluorochrome-conjugated annexin by exposure of phosphatidylserine and incorporation of propidium iodide (PI) were evaluated by flow cytometry (FACScalibur; Becton Dickinson). Data were analyzed by FlowJo software (Tree Star).

3.14 ELISA

Interleukin-10 (IL-10), interleukin-12 (IL-12p70) and interferon-γ (IFN-γ) release in culture supernatants was measured by using ELISA kits (Becton Dickinson). Tumor Necrosis Factor-α (TNF-α) release was measured by using an ELISA kit from BioLegend, whereas C-X-C motif chemokine 10 (CXCL10, IP-10) release was evaluated by using a kit from R&D System.

3.15 Histological evaluation

Cryo-sections embedded in OCT compound (Leica) were cut (10μm) from each tumor and fixed in formalin. Sections were either stained for Hematoxylin and Eosin using a Continuous Linear Stainer COT 20 (Medite) or incubated with rabbit anti-cleaved caspase 3 (Cell Signalling), followed by secondary species-specific Alexa Fluor 488-conjugated antibody (Invitrogen) and DAPI for nuclei counterstaining. Sections were examined with a Nikon TI fluorescence microscope (Nikon Switzerland) and images captured with 20x magnification using a digital camera and NIS-Elements software.

3.16 Statistical analysis

Statistical analysis software SPSS (Version 14.0, SPSS Inc) was used for statistical analyses. Skewness, Kurtosis distribution parameters and respective standard errors were used to test normality of the concerned populations. Mann-Whitney was used for non-parametric tests, with non-Gaussian distribution of the test population. All reported P-values were considered to be statistically significant at $P \leq 0.05$.

4. Results

4.1 CD40 receptor expression in clinical tumor specimens

CD40R has been reported to be expressed in nearly all B cell malignancies and approximately 70% of solid tumors (Vonderheide 2007). Triggering of CD40R expressed on surfaces of transformed cells has been associated to pro-survival effects or to inhibition of proliferation and/or apoptosis induction (Banchereau et al 1994; Elgueta et al. 2009). We initially evaluated CD40 expression in a TMA including 836 tumor specimens of 27 different histological origins, focusing on both malignant and tumor-infiltrating immune cells. Representative examples of CD40-specific staining are reported in Figure 5A.

CD40 expression was detectable in >15% of cases in breast and lung cancers subtypes, hepatocellular carcinoma (HCC) and esophageal cancers. Lower percentages of positive cases were observed in ovarian cancers, mesothelioma, gastrointestinal (GI) tract malignancies and head and neck cancers. In contrast, CD40 expression was virtually undetectable in prostate and pancreatic cancers (Figure 5B). Differential analysis in clinical specimens revealed that CD40 expression, in ≥ 5% malignant cells, was only observed in limited numbers of cancer types including medullary breast carcinoma (20%) and subtypes of ovarian and lung cancers (5-10%) (Figure 5C). In contrast, in a wide range of malignant tissues, CD40 expression was usually detectable in significantly higher percentages of infiltrating immune cells, except in endometroid ovarian carcinoma.

Taken together, TMA data underline that CD40R might serve as therapeutic target for different cell types present in the tumor microenvironment, and predominantly in tumor-infiltrating immune cells in sizeable percentages of cancers of high epidemiological relevance.

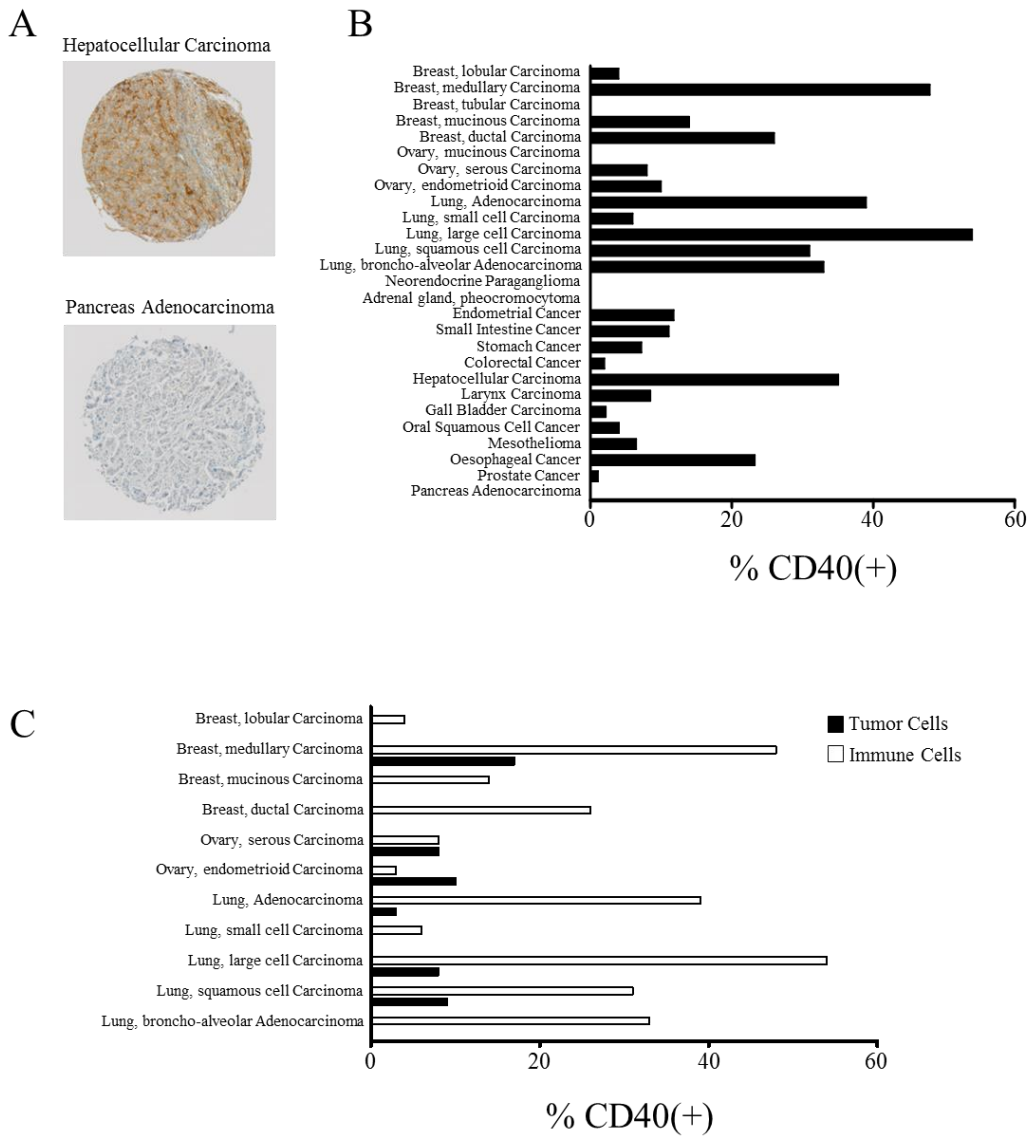

Figure 5. Evaluation of CD40R expression in clinical specimens. (A) Representative examples of CD40R-specific positive and negative staining. (B) Cumulative results of CD40R expression in tumor specimens included in the TMA. (C) CD40R expression on tumor and infiltrating immune cells was differentially analyzed in clinical samples derived from breast, ovarian and lung cancers.

4.2 rVV40L infection induces apoptosis of CD40(+) tumor cell lines

TMA data prompted us to first evaluate the biological responses induced by replication incompetent rVV40L infection in a panel of CD40(+) and CD40(-)

established tumor cell lines. In particular, CD40(+) Na8 melanoma, HCT116 colorectal cancer, MDA-231 breast cancer, PLC and HepG2 ($CD40^{DIM}$) hepatocellular carcinoma cell lines were tested. We also investigated cell lines of the same histological origin (A375, BT-474, LS180 and HuH-7) with undetectable expression of CD40R at mRNA and protein level (Figure 6A, B).

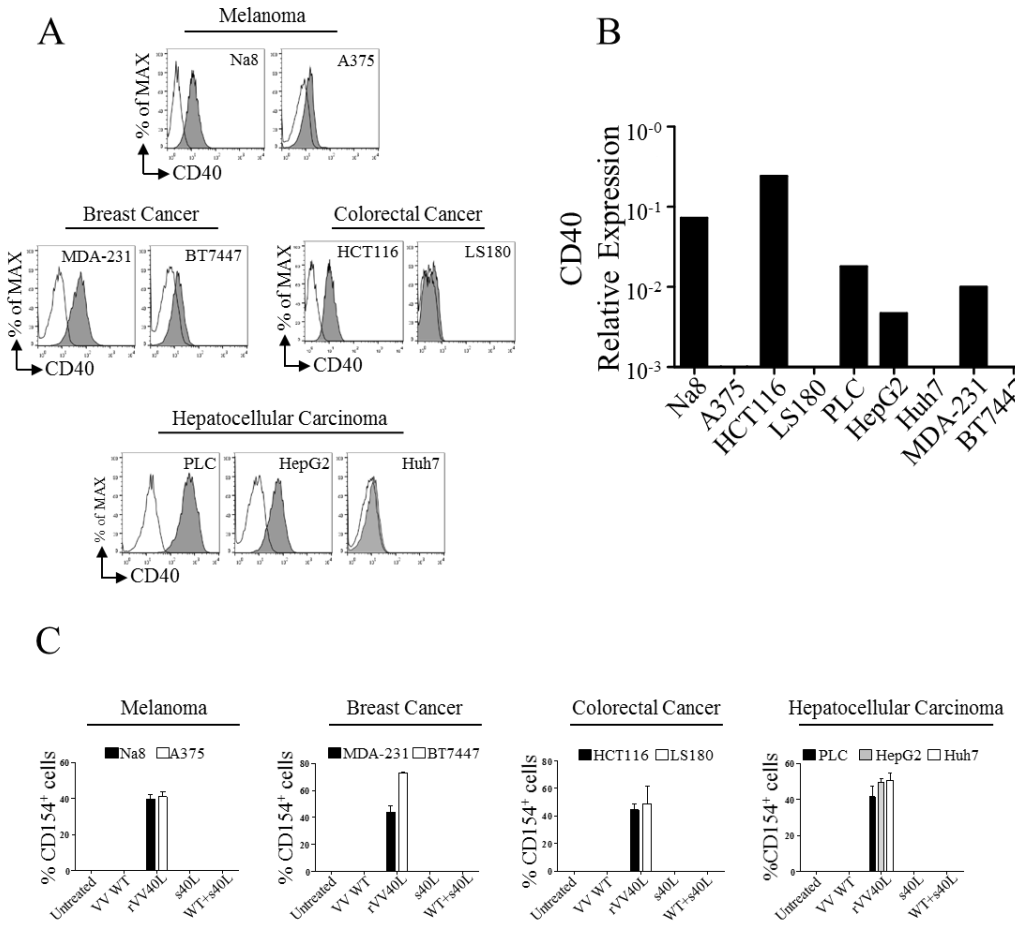

Figure 6. Characterization of established tumor cell lines. (A) Expression of CD40R (shaded histograms), as compared to isotype control reagents (open histograms) was evaluated on indicated cell lines by flow cytometry analysis. (B) CD40R expression was also evaluated by qRT-PCR and normalized in comparison to GAPDH in all established tumor cell lines. (C) Tumor cells from different cell lines were left untreated or infected with rVV40L or VV WT at MOI 10. Different cell lines were also treated with soluble CD40L recombinant protein (s40L; 0.5 μg/ml) alone or following VV WT infection (WT+s40L). After 24h of culture, untreated and differentially treated cells were collected and stained with

anti-CD154 (CD40L) fluorochrome-labeled mAbs. Data are reported as mean + SEM (n:5). *p<0.05, **p<0.01; Mann-Whitney nonparametric test.

Expectably, rVV40L infection of tumor cells, regardless of their histological origin and CD40R status, resulted in significant expression of CD40L on cell surfaces (Figure 6C).

Importantly, rVV40L infection of CD40(+) Na8 melanoma (Figure 7A), MDA-231 breast (Figure 7B) and PLC hepatocellular carcinoma cell line (Figure 7D) resulted in significant increases of percentages of apoptotic cells, as compared to control cultures. In contrast, s40L-stimulation of the same tumor cell lines was totally ineffective (Fig. 7A, B, D). Surprisingly however, ligation of CD40R expressed on cellular surfaces of HCT116 colorectal cancer cells did not impact on their survival (Fig. 7C). As expected, both rVV40L infection and s40L-treatment failed to induce apoptosis in CD40(-) cell lines.

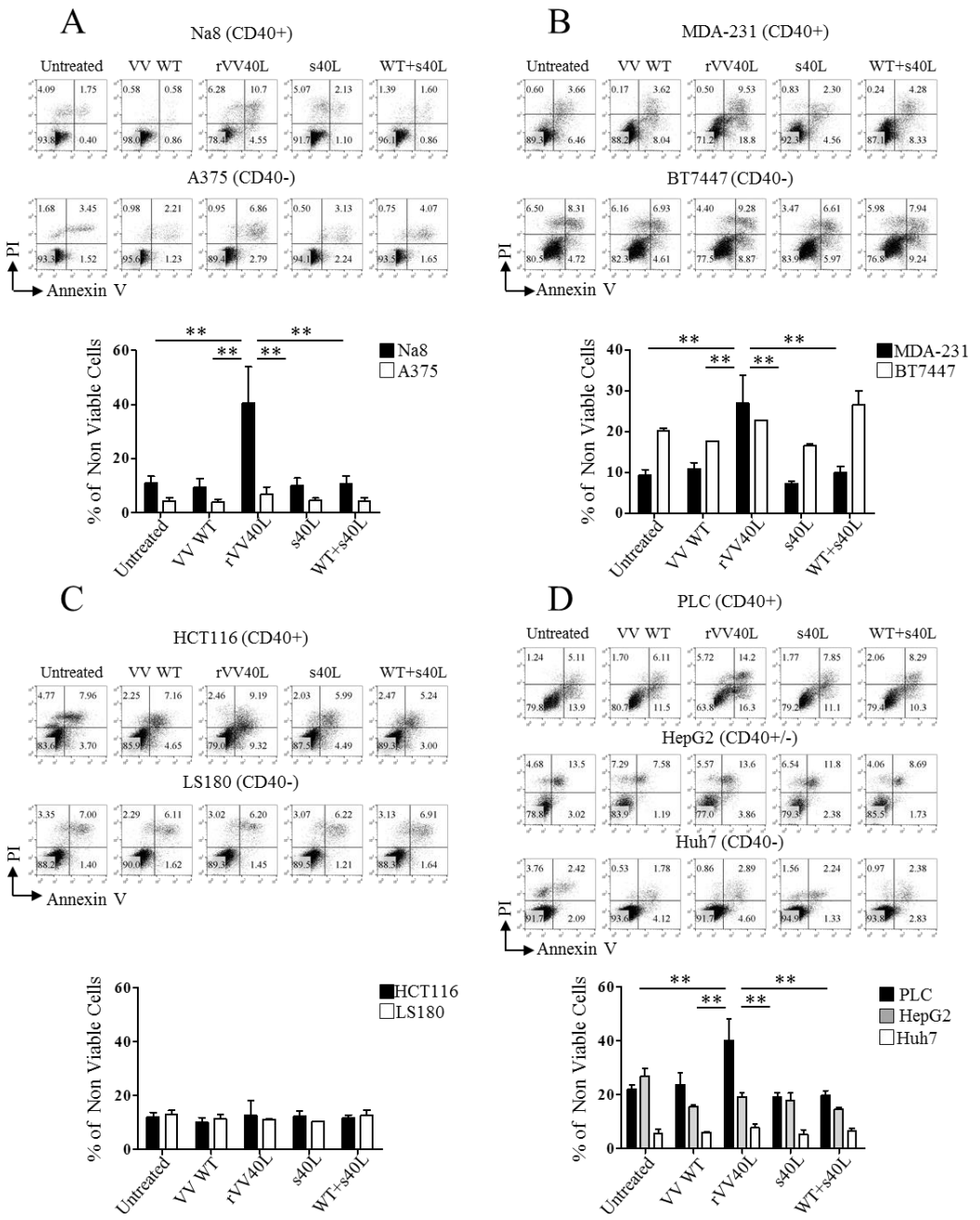

Figure 7. CD40L-expressing recombinant vaccinia virus but not s40L treatment induces cytotoxic effects on CD40(+) tumor cells in 'vitro'. Established tumor cell lines from (A) melanoma (Na8 and A375), (B) breast cancer (MDA-231 and BT-474), (C) colorectal cancer (HCT116 and LS180) and (D) hepatocellular carcinoma (PLC, HepG2 and HuH-7) were left untreated or infected with CD40L-expressing recombinant vaccinia virus (rVV40L) or vaccinia virus wild type (VV WT) at MOI of 10. Tumor cells were also treated with soluble CD40L recombinant protein (s40L; 0.5 μg/ml) alone or following VV WT

infection (VV WT). At day 4, cells were collected and stained with annexin V and propidium iodide (PI). (A, B, C and D) Data are referred to a representative experiment for each established tumor cell line (upper panels) and report collective results (mean +/- SEM) from five different experiments (lower panels). *p<0.05, **p<0.01; Mann-Whitney nonparametric test.

4.3 Impaired CD40 signaling pathway is associated with tumor cell resistance to rVV40L induced apoptosis

CD40R ligation has been shown to result in receptor clustering, inducing in turn recruitment to its cytoplasmic domain of TNF receptor-associated factors (TRAFs), mediating intracellular signaling (Banchereau et al 1994). However, only TRAF-1 is regulated at transcription level in response to CD40R ligation and initiates signaling cascades leading to cell death (Elmetwali et al. 2010). Furthermore, cytotoxic effects on tumor cells elicited by CD40R ligation have been recently reported to result in upregulation of NORE1A protein mediating proapoptotic JNK pathway and caspase activation inducing apoptosis of target cells (Elmetwali et al. 2016).

Thus, we investigated CD40R signaling in HCT116 cells using TRAF-1 and NORE1A expression as downstream markers.

As expected, in apoptosis-responsive CD40(+) Na8 and MDA-231 cells, a significant upregulation of TRAF-1 gene expression was observed upon rVV40L infection whereas s40L, alone or in combination with VV WT, was ineffective (Figure 8A, B, D). A similar trend (p=0.0671) was also observed in PLC CD40(+) hepatocellular carcinoma cells. In sharp contrast, triggering of CD40R expressed on cellular surfaces of HCT116 did not result in upregulation of TRAF-1 gene expression levels (Figure 8C).

Similarly, gene expression level of NORE1A was clearly increased following rVV40L infection but not s40L or WT+s40L treatment, only in ‘sensitive’ CD40(+) PLC but not in ‘insensitive’ CD40(+) HCT116 cells (Figure 8E).

These data underline that rVV40L, but not s40L treatment, might induce apoptosis upon infection. However, our results indicate that CD40 expression is not ‘per se’

predictive of sensitivity to rVV40L cytotoxic effects and induction of functional adapter proteins is required for apoptosis induction (Elmetwali et al. 2010; Elmetwali et al. 2016).

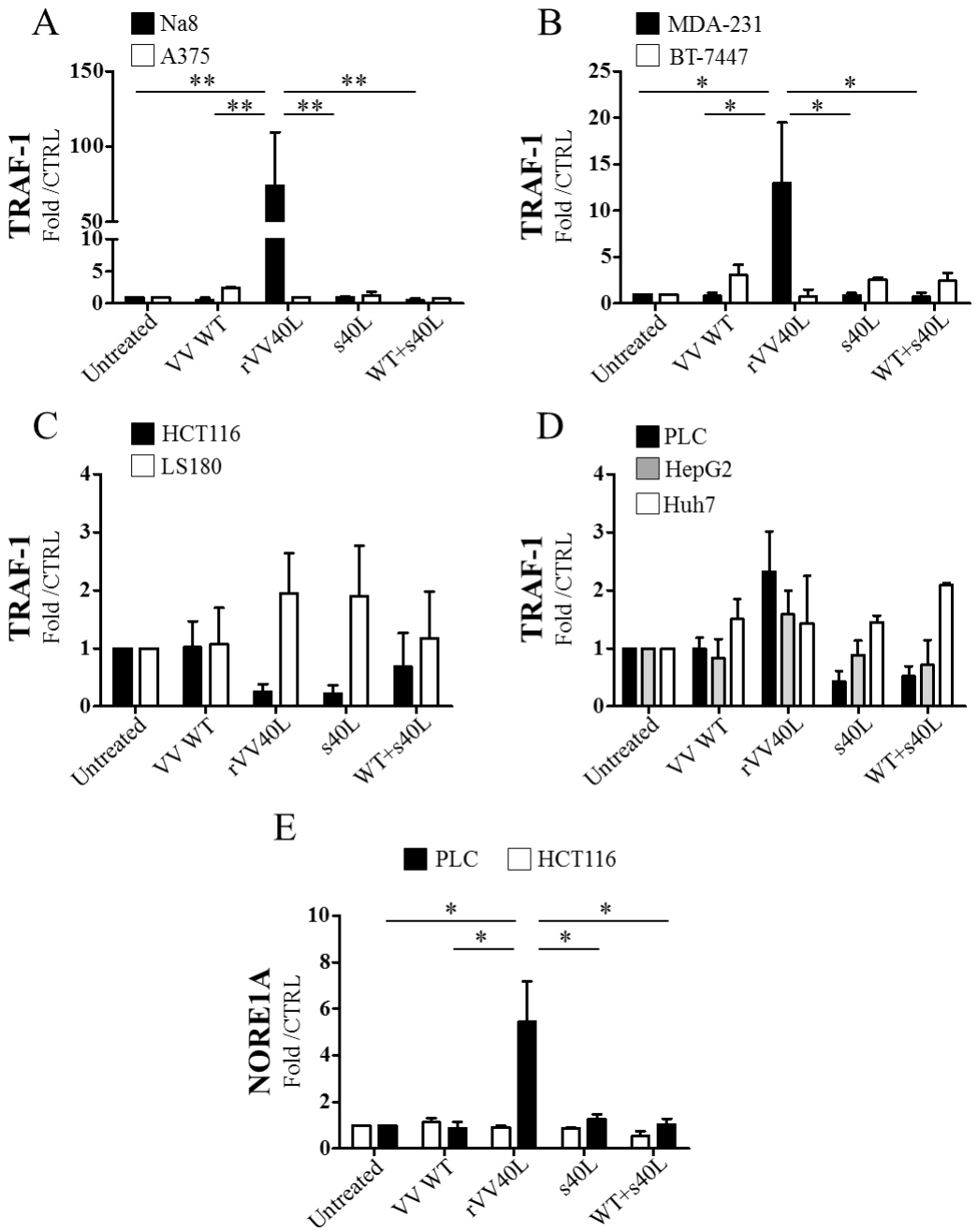

Figure 8. Impaired CD40 signaling pathway is associated with a reduced tumor cell cytotoxicity following rVV40L infection. Established melanoma (Na8 and A375) (A), breast cancer (MDA-231 and BT-474) (B), colorectal cancer (HCT116 and LS180) (C) and hepatocellular carcinoma (PLC, HepG2 and HuH-7) (D) cell lines were treated as indicated. After 4 days, TRAF-1 gene expression was evaluated by qRT-PCR. HCT116 (CD40+) colorectal cancer and PLC (CD40+) hepatocellular carcinoma cell lines were treated as described and NORE1A gene expression was assessed by qRT-PCR (E). Data are expressed as fold increase, as compared to untreated tumor cells. (n=5 A, B, C, D and n=3 E). *p<0.05, **p<0.01; Mann-Whitney nonparametric test.

4.4 Modulation of M1/M2 functional profiles by rVV40L infection

TMA data underline the relevant expression of CD40 in tumor-infiltrating immune cells. A potential role of tumor-associated macrophages in the control of tumor progression has been repeatedly reported. A key feature of macrophages is represented by their high plasticity. Indeed, M1/M2 macrophages may represent extremes of a continuum of differentiation states, characterized by specific functional attributes (Mantovani et al. 2002; Sica and Mantovani 2012). Therefore, we addressed the possibility that targeting CD40 might condition in vitro differentiation of CD14+ monocytes towards M1/M2 functional profiles.

We generated M1 and M2 CD14-derived macrophages by culturing CD14+ monocytes in presence of GM-CSF (M1) or M-CSF (M2) (Nebiker et al. 2014). Phenotypic characterization of M1 and M2 CD14-derived macrophages confirmed a significantly higher expression of CD16 and reduced level of CD163 and CD204 on M1 as compared to M2 macrophages (Nebiker et al. 2014, Ruffell and Coussens 2015) (Figure 9A, B).

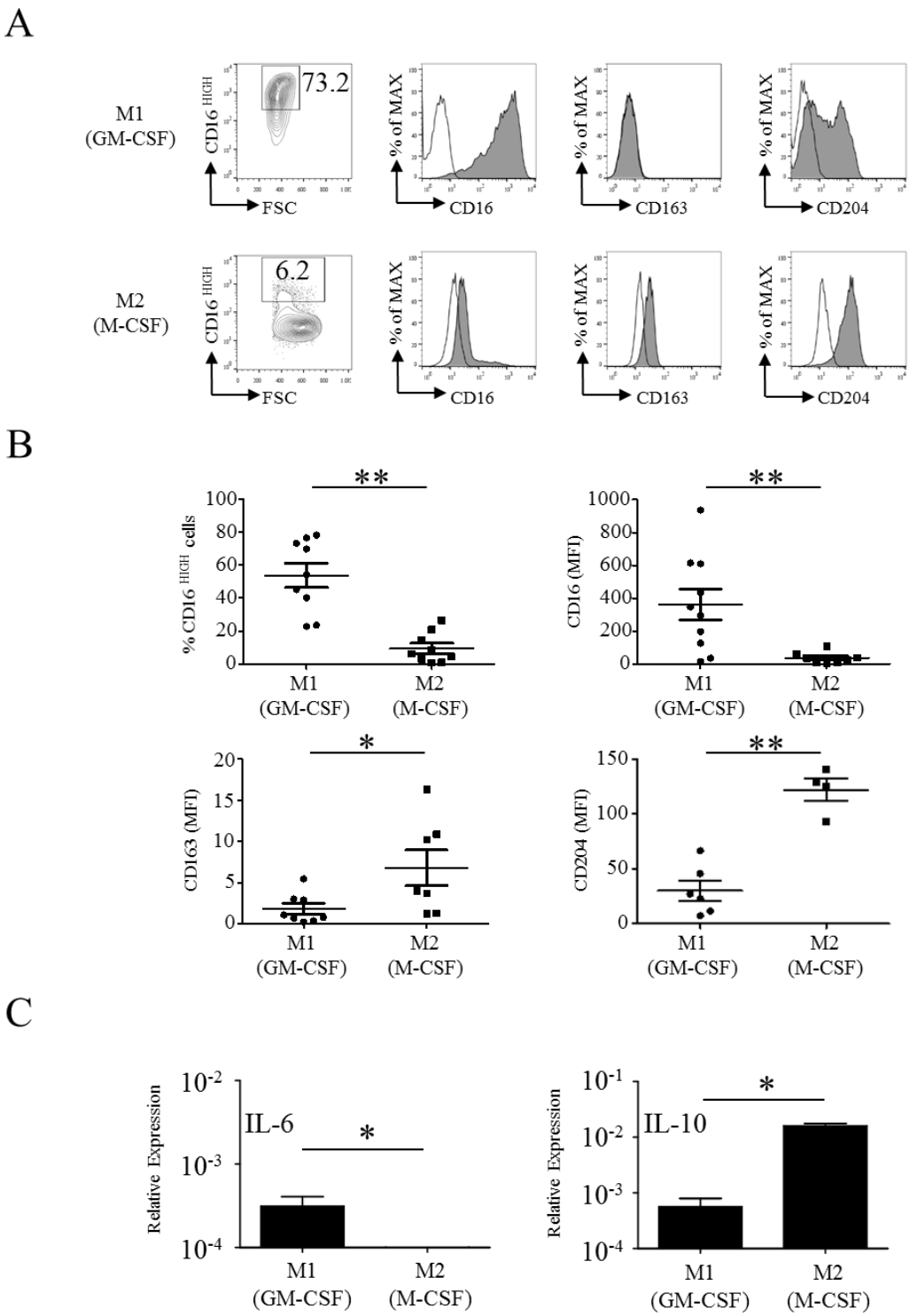

Figure 9. Phenotypic profile of in vitro generated GM-CSF (M1) and M-CSF (M2), CD14+ monocyte-derived macrophages. CD14+ monocytes magnetically isolated from

peripheral blood of healthy donors were cultured in presence of GM-CSF (12,5 ng/ml) or M-CSF (12,5 ng/ml). At day 6, cells were harvested and phenotypic profiles and cytokine gene expression patterns were evaluated by flow cytometry analysis and qRT-PCR, respectively. (A+B) Expression of CD16 (% and MFI), CD163 (MFI) and CD204 (MFI) surfaces antigens on GM-CSF (M1) and M-CSF (M2) CD14-derived macrophages. (C) In addition, expression level of IL-6 and IL-10 genes was also evaluated on GM-CSF (M1) and M-CSF (M2) conditioned macrophages Data are referred to representative (A) experiments and display cumulative results from eight independent experiments (B+C). *$p<0.05$, **$p<0.01$; Mann-Whitney nonparametric test.

Accordingly, analysis of cytokine gene pattern profiles revealed a significant IL-6 gene expression in M1 macrophages whereas IL-10 gene expression was higher in M2 macrophages (Figure 9C). Notably, we also observed a significantly higher expression of CD40R in M1, as compared to M2, CD14-derived macrophages (Figure 10A).

At difference with sCD40L treatment alone or in combination with WT infection, rVV40L infection promoted a significant IL-12 production by CD14+ monocytes cultured in presence of GM-CSF. Moreover, unexpectedly, rVV40L infection also induced detectable IL-12p70 release by M2 macrophages. On the other hand, IL-10 production was induced in M2 by sCD40L treatment alone or in combination with WT infection, but not by rVV40L (Figure 10B).

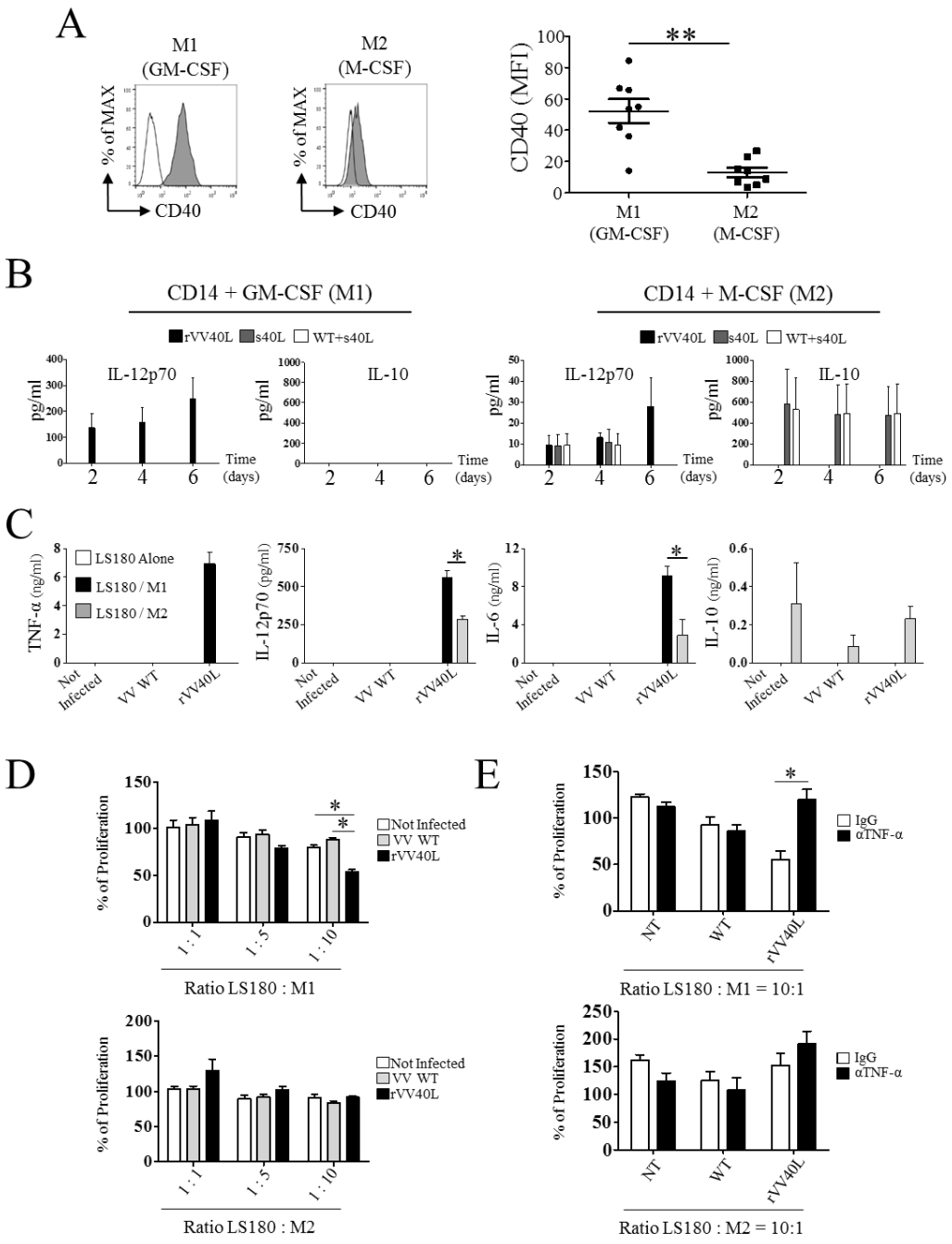

Figure 10. rVV40L modulates functional profile of M1/M2 CD14-derived macrophages. (A) Expression of CD40, on surfaces of CD14+ cell-derived M1 or M2 macrophages, was evaluated by flow cytometry. (B) M1/M2 cells were infected with VV WT or rVV40L (MOI 5) or cultured in presence s40L alone or following VV WT infection (WT+s40L). On day 2, 4 and 6 of culture supernatants were collected and IL-12p70 and IL-10 release was evaluated by ELISA. (C) M1 and M2 macrophages were co-cultured (1:1

ratio) with LS180 CRC cells infected with VV WT or rVV40L (MOI 10). After 4 days, supernatants were collected and TNF-α, IL-12p70, IL-6 and IL-10 release was evaluated by ELISA. (D) M1 (GM-CSF) and M2 (M-CSF) macrophages were co-cultured with LS180 CRC cells, treated as described above, at the indicated ratios. On day 4, proliferation of LS180 CRC cells was evaluated by ^{3}H-thymidine incorporation and expressed as percentage of proliferation as compared to untreated tumor cells. (E) Proliferative capacity of LS180 CRC cells, treated as described in C+D, co-cultured with M1 and M2 macrophages, as evaluated by ^{3}H-thymidine incorporation in presence of anti-TNF-α at 10 μg/ml concentration. Data are referred to cumulative results from eight (A) or four (B, C, D, E) independent experiments. *$p<0.05$, **$p<0.01$; Mann-Whitney nonparametric test.

These data prompted us to further evaluate the relevance of CD40R ligation in the modulation of effector functions of fully in vitro differentiated M1 or M2 macrophages. We analyzed cytokine production pattern of polarized macrophages upon co-culture with untreated, VV WT or rVV40L-infected CD40(-) HuH-7 HCC and LS180 CRC cells. In these conditions, TNF-α production was only detectable following CD40R ligation in M1 differentiated macrophages. CD40R ligation also induced IL-12p70 and IL-6 production in M1 cells. Interestingly, IL-12p70 and IL-6 release by M2 cells, albeit to lower extents, was detected following CD40R ligation. Induction of IL-10 production was observed in M2 macrophages only upon co-culture with rVV40L-infected HuH-7 (Figure 11A), but not LS180 cells (Figure 10C). These data indicate that CD40R is functional in both M1 and M2 macrophages and suggest that its ligation might partially steer M2 cells towards M1-like functional profile.

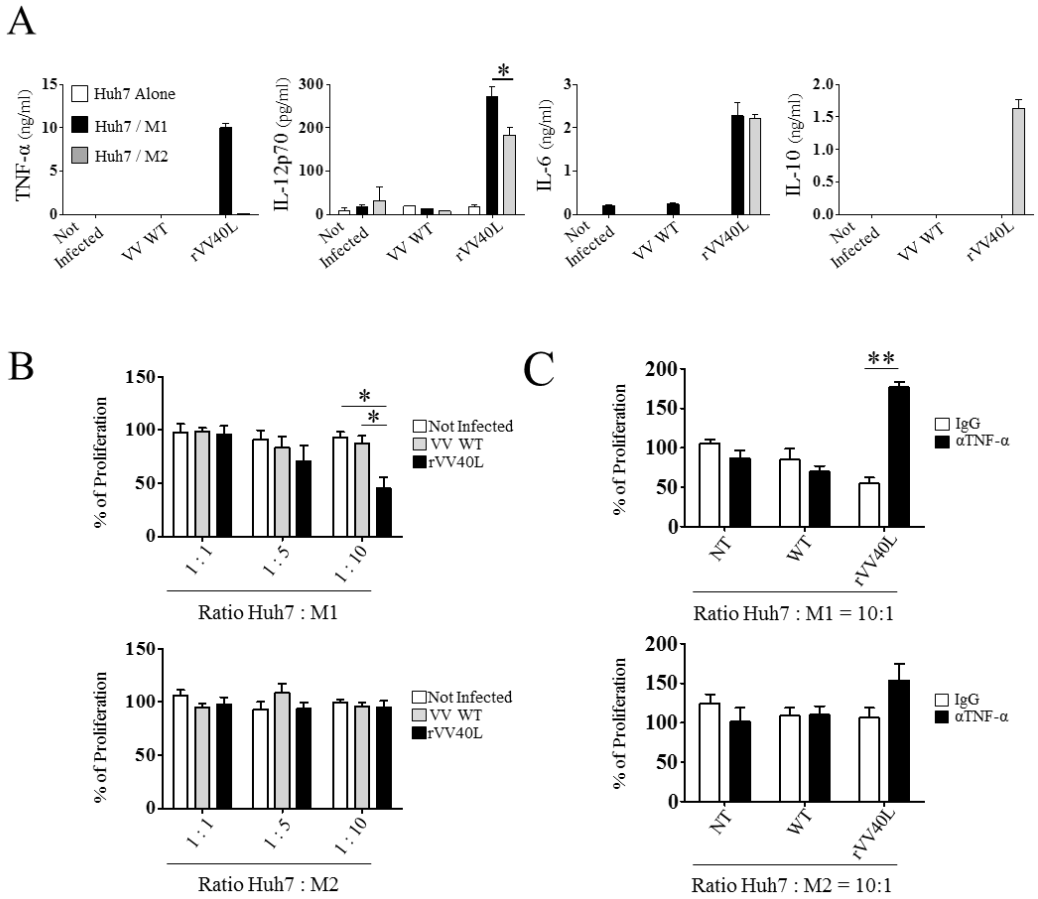

Figure 11. rVV40L-infected HuH7 HCC promote the activation of M1/M2 macrophages. (A) M1 and M2 CD14+ monocyte-derived macrophages were co-cultured at 1:1 ratio with Huh7 hepatocellular carcinoma (HCC) cells left untreated or infected with VV WT or rVV40L at MOI of 10. After 4 days, supernatants from different culture conditions were collected and TNF-α, IL-12p70, IL-6 and IL-10 release was evaluated by ELISA (B) M1 (GM-CSF) and M2 (M-CSF) CD14+ monocyte-derived macrophages were co-cultured with LS180 CRC cells, treated as described above, at indicated ratios in flat bottom 96 well plates in quadruplicates. On day 4, Huh7 HCC cell proliferation was evaluated by ^{3}H-thymidine incorporation and expressed as percentage of proliferation, as compared to tumor cells cultured alone. (C) Proliferative capacity of Huh7 cells, treated as described in C+D, and co-cultured with M1 and M2 macrophages was also evaluated by ^{3}H-thymidine incorporation in the presence of anti-TNF-α antibodies at 10 μg/ml concentration. Data refer to cumulative results from four independent experiments. *p<0.05, **p<0.01; Mann-Whitney nonparametric test.

Consistently with their TNF-α production, M1, but not M2, macrophages displayed significant cytostatic activity on rVV40L-infected LS180 CRC (Figure 10D) and

HuH-7 HCC established tumor cell lines (Figure 11B). Although macrophages could generate a variety of cytotoxic/cytostatic mediators, the pivotal role of TNF-α was confirmed by its neutralization resulting in significant inhibition of antiproliferative effects of CD40R-stimulated M1 cells on rVV40L-infected tumor cells (Figure 10E and Figure 11C).

4.5 CD40 receptor-stimulated M1 and M2 macrophages promote the recruitment of CD8+ T cells

In a variety of solid tumors, high infiltration by CD8+ T cells is associated with favorable prognosis. Thus, their recruitment may represent a critical step in the elicitation of antitumor immune responses (Fridman et al. 2011; Fridman et al. 2012; Fridman et al. 2013; Fridman et al. 2017).

CXCR3 chemokine receptor is expressed in CD8+ lymphocytes, and, preferentially in "central memory" T cells, characterized by strong antitumor potential (Klebanoff et al. 2006; Trella et al. 2016) (Figure 12A). CXCR3 ligands include CXCL9, CXCL10 and CXCL11. A main role in CD8+ T cells recruitment has been attributed to CXCL10 (Yue et al. 2015).

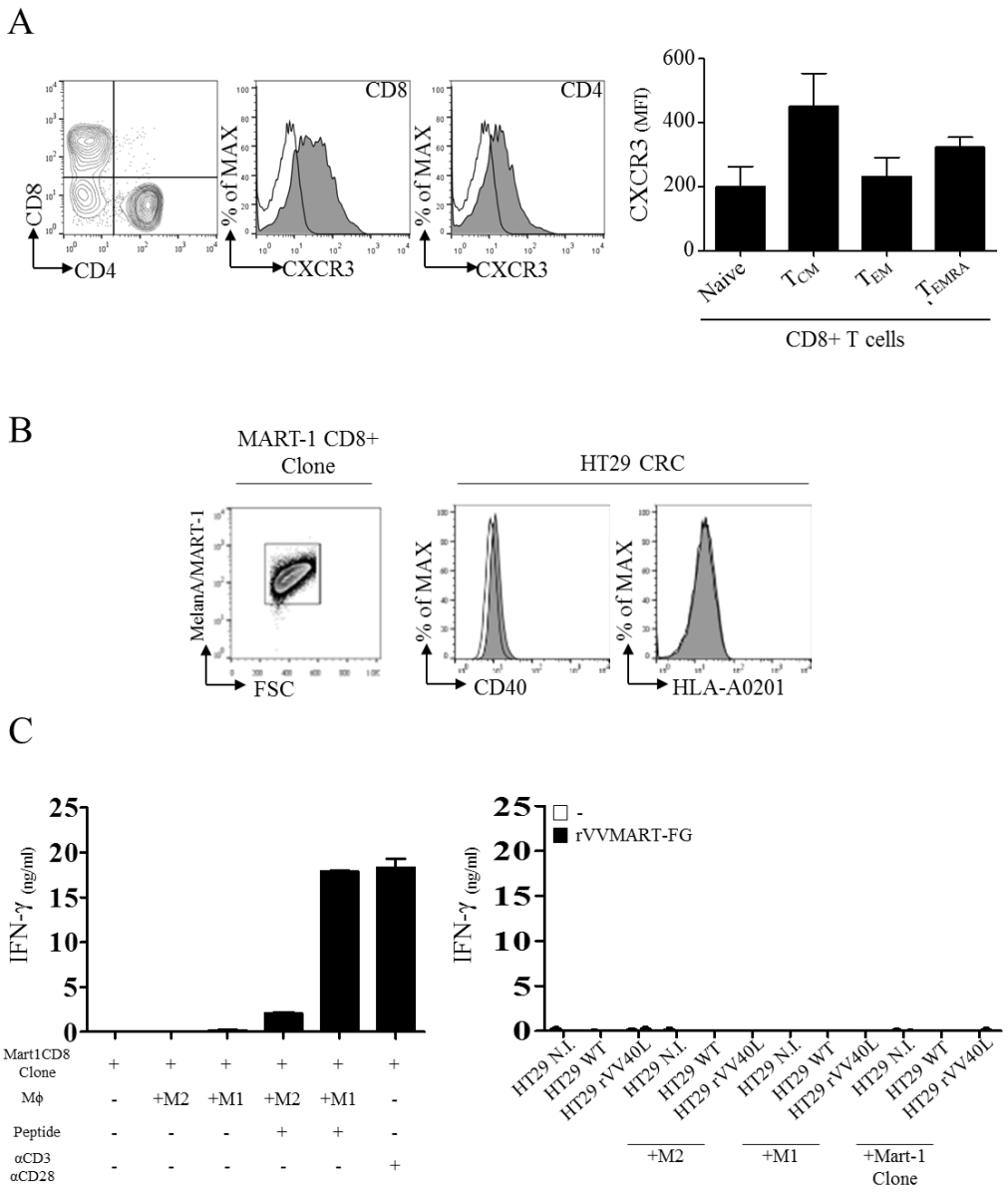

Figure 12. CXCR3 expression on immune cells and M1/M2 cross-presentation of cellular antigens. (A) CXCR3 expression on naïve and memory CD8+ T lymphocytes subsets, as according to CD45RO and CD62L surface marker expression. (B) Phenotypes characterization of the HLA-A0201 restricted, MelanA/MART-1_{27-35}-specific CD8+ T cell clone (left) and HLA-A0201- CD40(-) HT29 CRC cell lines were used (right) used in cross-presentation assay. (C) IFN-γ release in the supernatant from the indicated control conditions of the cross-presentation assay.

We tested CXCL10 release in supernatants of co-cultures of M1 or M2 macrophages and LS180 CRC and HuH-7 HCC cell lines. As depicted in Figure 13A, rVV40L but not VV WT infection of tumor cells, resulted in significant increases of CXCL10 production by both M1 and M2 macrophages.

Migration assays were performed to formally prove the ability of CD40R-stimulated M1 and M2 macrophages to promote CXCL10-mediated CD8+ T cell recruitment. Supernatants from macrophages, CD40L-stimulated as described above, induced the migration of CD8+ T cells to significantly higher extents, as compared to control supernatants. Importantly, antibody-mediated CXCL10 depletion from these supernatants, abrogates CD8+ T cells migration (Figure 13B), confirming its crucial significance in lymphocyte recruitment.

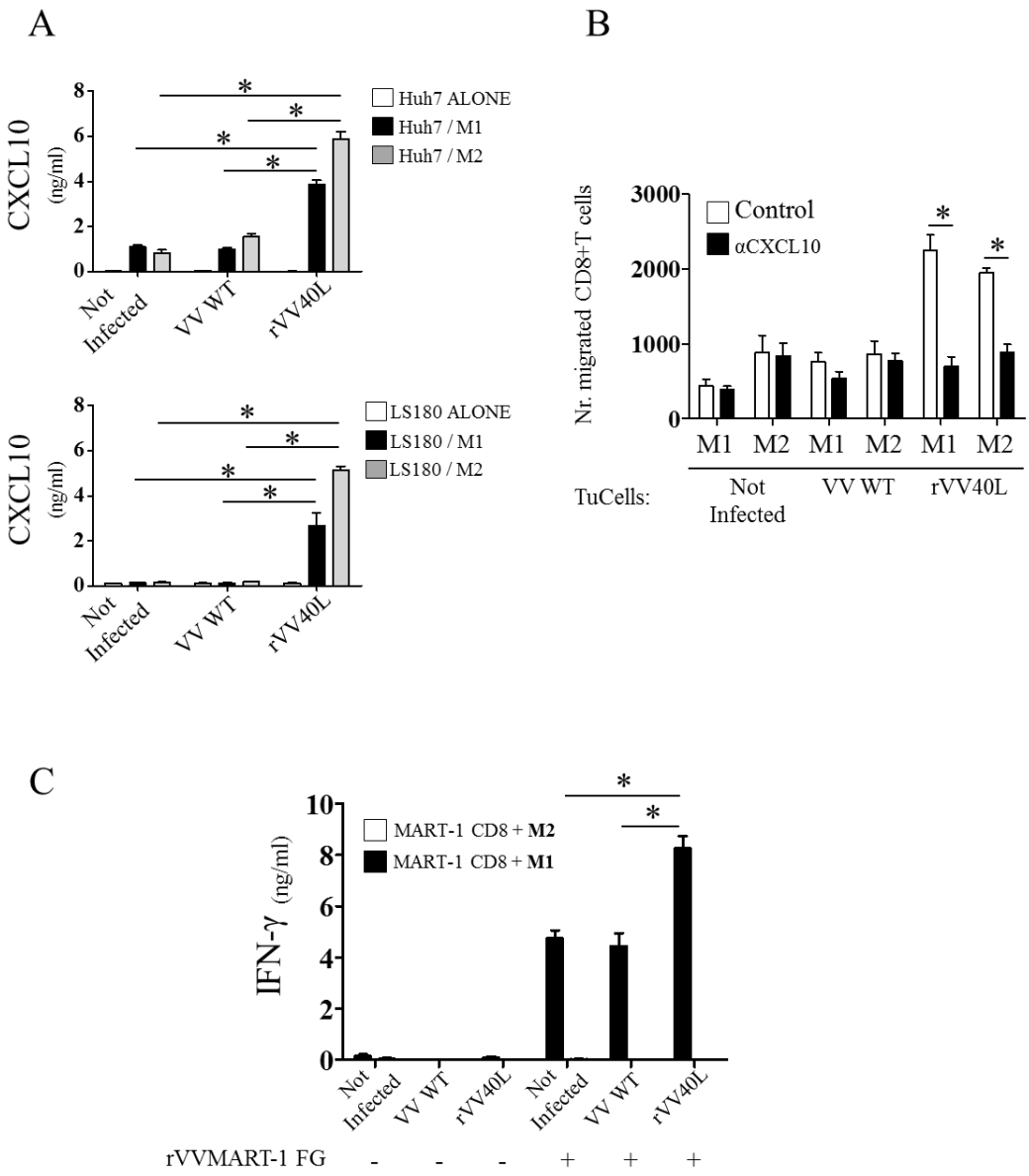

Figure 13. rVV40L induces CXCL10-mediated recruitment of CD8+ T cells and promotes cross-presentation of tumor-associated antigens by M1 macrophages. (A) M1 or M2 macrophages were co-cultured at 1:1 ratio with LS180 CRC (upper panel) or HuH-7 HCC (lower panel) cell lines untreated or infected with VV WT or rVV40L at MOI 10. After 4 days, supernatants were collected and CXCL10 release was evaluated by ELISA. (B) CD8+ T cell migration, induced by supernatants from co-cultures of M1/M2 macrophages with differentially infected tumor cells, as evaluated by flow cytometry. (C) Cross-presentation of MelanA/Mart-$1_{27\text{-}35}$ epitope from HLAA0201(-) CD40(-) HT29 CRC cell lines co-infected with VV WT or

rVV40L and with a rVV encoding MelanA/MART-1 full gene (rVVMART1 FG). After 24h, HT29 were co-cultured, at 1:1 ratio, with HLA-A0201+ M1 or M2 macrophages. After 48 additional hours, 20.000 HLA-A0201 restricted CD8+ T cells were added to the cultures and concentration of IFN-γ release was evaluated by ELISA two days later. *p<0.05; Mann-Whitney nonparametric test.

4.6 rVV40L promotes M1-mediated cross-presentation of tumor-associated antigens (TAAs) to CD8+ T cells

Macrophages have been shown to be able to cross-present antigens derived from apoptotic cells to specific T cells (Heath and Carbone 2001). Cross-presentation-based vaccination strategies have shown promising results for cancer treatment (Zwaveling et al. 2002; Melief 2008). Therefore, we evaluated the capacity of rVV40L-stimulated macrophages to promote the activation of tumor-reactive CD8+ T cells through cross-presentation of cellular antigens.

HLA-A0201(-) CD40(-) HT29 CRC cells (Figure 12B) were co-infected with rVV40L and a recombinant vaccinia virus expressing MelanA/MART-1 full gene (rVVMART-1 FG). HT29 CRC cells were then co-cultured with HLA-0201(+) M1 or M2 macrophages and, after 4 days, the ability of macrophages to cross-present MART-1_{27-35} HLA-A0201 restricted epitope was evaluated by measuring IFN-γ release by a specific CD8+ T cell clone (Figure 12B). The clone efficiently responded to presentation of the target peptide by M1, but not M2 antigen presenting cells, or to antibody-mediated CD3 triggering (Figure 12C left panel). Most importantly, our data clearly indicate that priming of HLA-A0201(+) M1 macrophages by rVV40L-infected HLA-A0201- tumor cells resulted in significantly enhanced activation of MART-1_{27-35}-reactive CD8+ T cells, as compared to control culture conditions (Figure 13C). On the other hand, as expectable, HLA-A0201(-) tumor cells infected by the reagents under investigation, were unable “per se” to induce IFN-γ production in macrophages alone or in CTL alone (Figure 13C right panel).

4.7 rVV40L promotes in vivo tumor regression through macrophage activation

To address antitumor efficacy of rVV40L in vivo, NSG immunodeficient mice were subcutaneously inoculated with LS180 CRC and HuH-7 HCC cells. Upon development, tumors were first injected with replication incompetent VV WT or rVV40L followed, after 48 hours, by human M1 or M2 macrophages.

Control and VV WT-infected tumors rapidly and similarly progressed in the presence or absence of M1 or M2 cells (Figure 14A-F). In sharp contrast, consistently with in vitro observations, rVV40L infection of HuH-7 HCC and LS180 CRC resulted in a complete inhibition of tumor progression upon intratumor injection of M1 cells (Figure 14B and E). Similar effects were also observed upon injection of the positive control LPS-stimulated M1 cells (data not shown). Injection of M2 also resulted in a modest nonsignificant inhibition of LS180 CRC tumor progression (Figure 14F).

Remarkably, histological analysis of excised tumors clearly revealed that rVV40L-mediated activation of M1 macrophages resulted in a significant disruption of tumor tissues, comparably to LPS-stimulated M1 macrophages (Figure 14G-H).

Consistently, immunofluorescence studies provided evidence of significant caspase 3 cleavages in these tumors (Figure 14G-H).

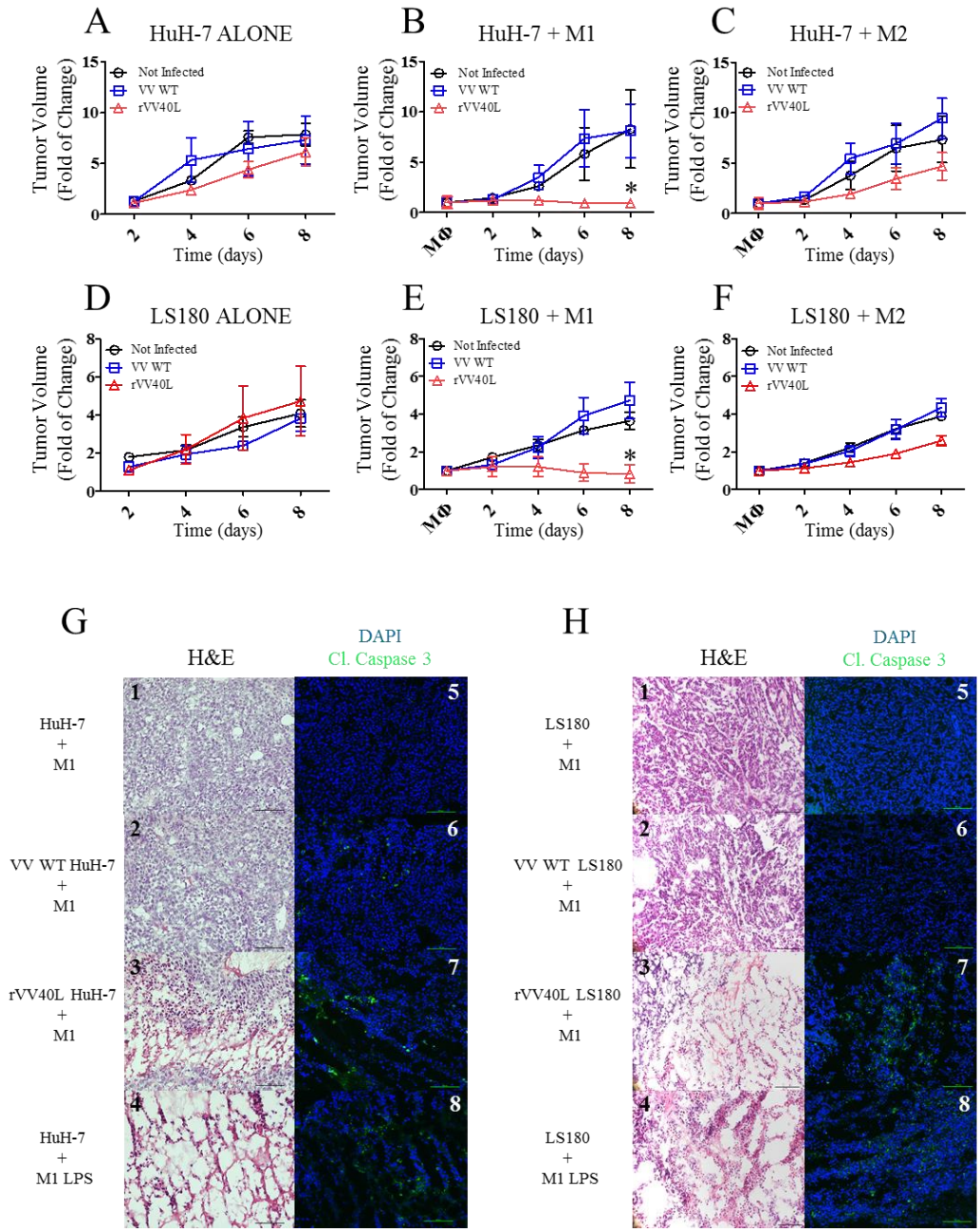

Figure 14. rVV40L promotes antitumor activity of M1 macrophages in vivo. 10^5 LS180 CRC or HuH-7 HCC cells were inoculated subcutaneously (s.c.) into the flanks of NSG mice. Established tumors were then injected with PBS or 10^7 pfu of replication incompetent VV WT or rVV40L. 48 hours later, $5*10^5$ M1 or M2 macrophages were injected in the tumor masses. Tumor volume of LS180 (A-C) and Huh7 (B-F) is expressed as fold increases, as compared to tumor volumes measured at the time of macrophage inoculation. Data refer to two independent experiments with 3 mice in each condition. On day 8, mice were sacrificed

and histology (H&E) and cleaved Caspase 3 expression (green) were evaluated in LS180 (G) and HuH-7 (H) tumors. Data are referred to representative stainings out of two tumors per condition displaying similar results (Magnification 20x; scale bar 100μm).

5. Discussion

Due to a peculiar expression pattern and multifunctional potential, CD40 represents an important target for the development of innovative cancer immunotherapy protocols (Vonderheide 2007; Loskog et al. 2016).

Administration of agonistic anti-CD40R mAbs and CD154 (CD40L)-expressing recombinant adenovirus has been shown to result in tumor regression in experimental models (Ullenhag and Loskog 2012; Vonderheide et al. 2013). These effects have initially been largely attributed to CD40-mediated-APC licensing and activation of T cell-mediated antitumor immune responses (Ridge et al. 1998).

However, data from clinical studies in human pancreatic cancers, typically lacking T cell infiltration, suggest that elicitation of antitumor effects of therapeutic anti-CD40R agonistic mAbs might rather rely on macrophage activation (Beatty et al. 2011; Beatty et al. 2013; Vonderheide et al. 2013).

Notably however, effectiveness of CD40R-targeting strategies developed so far appears to be limited. In particular, CD40 expression by tumor-infiltrating immune cells does not effectively predict responsiveness to agonistic anti-CD40R mAbs (Beatty et al. 2011; Beatty et al. 2013).

Furthermore, epitope specificity and isotype might critically affect the activity of therapeutic reagents (Vonderheide and Glennie 2013; Richman and Vonderheide 2014).

Regarding CD40L recombinant adenovirus, while safety has been reported, convincing evidence of clinical efficacy is still missing (Malmström et al. 2010; Hangalapura et al. 2012; Loskog et al. 2016). Potential limitations of adenovirus-mediated gene transfer approach might be associated to the requirement of specific cell entry pathways and of the administration of high viral titers (Malmström et al. 2010; Hangalapura et al. 2012; Loskog et al. 2016).

In order to develop a multipotent anticancer biological, we generated a replication incompetent CD40L-expressing recombinant vaccinia virus (rVV40L) targeting multiple functional aspects of tumor-immune system interaction and tested its antitumor effects in vitro and in vivo. In particular, we addressed the ability of rVV40L to promote direct CD40(+) tumor cell apoptosis, to modulate functional profiles of differentially polarized macrophages, to recruit immune cell populations of proven anticancer relevance, and to inhibit tumor progression in vivo.

Evaluation of CD40 expression in a large cohort of clinical specimens (n: 836) derived from different tumor types (n: 27) indicates that, with some exceptions, CD40 is expressed on cellular surface of transformed cells in limited percentages of tumors included in a TMA. Instead, CD40 expression was often detectable in infiltrating immune cells in a majority of malignant tissues analyzed. This observation, further underlines that the effectiveness of CD40-based therapeutic strategies should focus on immune infiltrating cells in addition to direct cytostatic/cytotoxic effects on CD40(+) malignant cells.

rVV40L infection but not soluble CD40L recombinant protein treatment (s40L), resulted in cytotoxic effects on CD40(+) melanoma, hepatocellular carcinoma and breast cancer tumor cell lines. In line with previous reports (Elmetwali et al. 2010; Elmetwali et al. 2016), our data underline the different biological properties of membrane-bound CD40L as compared to its soluble counterparts. Additional limitations of therapeutic strategies targeting CD40R on cancer cell surfaces should also be considered (Elmetwali et al. 2010; Ullenhag and Loskog 2012; Vonderheide and Glennie 2013; Elmetwali et al. 2016). Indeed, CD40 expression cannot be considered a sufficient signature predicting the effectiveness of "direct" strategies, since lack of cytotoxic effects, following receptor ligation on malignant cells, may correlate with impaired intracellular signaling. Thus, TRAFs adapter molecules alterations and defective activation of effector proteins may result in defective triggering of apoptosis induced by CD40 binding (Elmetwali et al. 2010; Elmetwali et al. 2016).

Tumor microenvironment (TME) includes, in addition to malignant cells, a variety of different non-transformed cell types exerting a major impact on clinical course (Fridman et al. 2011; Fridman et al. 2012; Fridman et al. 2013).

Initial evidence suggested antitumor activity of macrophages based on their capacity to mediate elimination of transformed cells and to promote in situ adaptive tumor-reactive immune responses (Fidler and Poste 1982). This interpretation has subsequently been largely challenged since different pro-tumorigenic functions, such as supporting tumor-associated angiogenesis, proliferation of malignant cells and suppression of antitumor T cell responses have been convincingly demonstrated in experimental models (Bingle et al. 2002; Pollard 2004; Biswas et al. 2006; Mantovani and Sica 2010; Qian and Pollard 2010; Biswas et al. 2013; Noy and Pollard 2014).

Furthermore, clinical evidence underlines that in a majority of human tumors, high macrophage infiltration is frequently associated with severe prognosis (Bingle et al. 2002; Pollard 2004; Biswas et al. 2006; Mantovani and Sica 2010; Qian and Pollard 2010; Biswas et al. 2013; Noy and Pollard 2014).

Thus, macrophage infiltrate emerges as a critical target of antitumor therapeutic strategies and the clinical potential of mAbs neutralizing CCL2 (Sandhu et al. 2013) and M-CSF (Cassier et al. 2015) receptor is currently being evaluated in specific clinical trials. These strategies point to the prevention of recruitment and differentiation of macrophages (Qian and Pollard 2010; Sandhu et al. 2013; Cassier et al. 2015). Alternative approaches focus on re-conditioning of tumor-infiltrating macrophages through the modulation of their functional activity (Beatty et al. 2011; Beatty et al. 2013; Vonderheide et al. 2013) by exogenous signals enhancing their antitumor activity (Beatty 2013; Ruffell and Coussens 2015; Mills et al. 2016).

Therefore, we evaluated the ability of rVV40L to steer, upon infection of tumor cells, functional profiles of M1 and M2 polarized macrophages into antitumor properties.

In vitro, co-culture with rVV40L-infected tumor cells resulted in a significant production of TNF-α by M1 but not M2 macrophages. More importantly, TNF-α production appears to represent a critical mediator of cytostatic/cytotoxic activity exerted by CD40-activated M1 macrophages on malignant cells.

Macrophages have been also shown to be able to orchestrate anticancer adaptive immune responses "in situ" (Mills et al. 2016). Several studies have underlined their capacity to mediate, through chemokines production, selective recruitment of defined immune cell subsets, such as CD8+ T cells, with a critical antitumor activity (Fridman et al. 2011; Fridman et al. 2012; Fridman et al. 2013) in malignant tissues (Qian and Pollard 2010; Panni et al. 2013). CXCL10 is a main mediator of CD8+ T lymphocyte recruitment (Yue et al. 2015) and, in our study, rVV40L driven CD40R-stimulation resulted in a significant release of CXCL10 by both M1 and M2 polarized macrophages leading to effective migration of CD8+ T cells.

The elicitation of CD8+ T cell immune responses is conditioned by cytokines produced by APC (Ridge et al. 1998; Mescher et al. 2006; Trella et al. 2016). Production of IL-12 or IL-10 has been associated to the ability of tumor-associated macrophages to promote or, respectively, inhibit adaptive anticancer T cell-mediated immune response (Mantovani and Sica 2010; Mills et al. 2016). Ligation of CD40R expressed on cellular surface of M1 macrophages significantly modulated their cytokine pattern profile. Interestingly, similar functional effects were also elicited in M2 macrophages, despite a significantly lower CD40 expression.

In particular, triggering of CD40R resulted in robust production of IL-12 by M1 macrophages and, more surprisingly, although to a lower extent, also by M2 macrophages. Notably, CD40R-stimulated M2 macrophages did not show increased production of immunosuppressive IL-10 (Panni et al. 2013; Mills et al. 2016). Most importantly, our data underline that infection of tumor cells by rVV40L enhances M1-mediated cross-presentation of tumor-associated antigens to CD8+ T cells.

In vivo studies nicely reinforce our in vitro results. Indeed, we observed a significant inhibition in the progression of CD40(-) tumors upon rVV40L infection. In particular, exogenous administration of human M1 macrophages, induced massive destruction of tumor tissue mediated by cleaved-caspase 3 activation. Intriguingly, detectable, albeit not significant, effects were elicited by M2 macrophages.
Considering the absence of overt toxic effects of macrophage administration in animals bearing rVV40L-infected tumors, and the replication incompetent nature of our reagent, these data may also suggest innovative adoptive cancer immunotherapy protocols, based on re-infusion of differentiated, patient-derived macrophages.

Taken together, our data underline the major antitumor potential of a CD40L-expressing recombinant vaccinia virus. Indeed, rVV40L infection might lead to direct inhibitory effects on CD40(+) malignant cells. In addition, inhibition of tumor progression could also result from the marked ability of rVV40L to promote M1 activation and possibly from a partial re-education of M2 macrophages. Most importantly, CD40-activated macrophages are able to induce direct TNF-α-mediated antitumor cytotoxic activity, lymphocyte recruitment and cross-presentation of cellular antigens to CD8+ T cells.

6. Conclusion and outlook

Collectively, the data presented in this study pave the way for additional research.

1) First, we have demonstrated that CD40R stimulation of monocytes, cultured in presence of GM-CSF, efficiently promotes their activation. Most importantly, ligation of CD40R on monocyte cultured in the presence of M-CSF, e.g. M2 induces functional features which resemble the M1-like, as indicated by a detailed analysis of their cytokine expression pattern. This latter observation is particularly important and of potential clinical relevance. In particular, it is tempting to speculate that through the use of this CD40L-expressing vaccinia virus, monocytes recruited within the tumor mass during cancer progression, might partially overcome their polarization to an M2-like phenotype and educate to exert M1-like functions, even in an immunosuppressive environment.

2) Second, our data clearly indicate that stimulation of CD40(+) tumor might result in the elimination of cancerous cells. Nevertheless, CD40 expression on transformed cells appears to be limited to a minor fraction of tumor cells and, most importantly, CD40 expression per se is not predictive of apoptosis induction following rVV40L infection or CD40R stimulation. However, in this scenario, expression of CD40L on surfaces of infected tumor cells might result in the elicitation of cytotoxic activity by tumor-infiltrating M1 macrophages resulting in disruption of malignant tissues. Of note, partial effects of M2 (M-CSF) macrophages on tumor growth are also detectable. Therefore, the use of such type of viral vector in the context of clinical trials could potentially have a major antitumor impact.

3) Most importantly, the data here presented extended beyond the direct effect of rVV40L/macrophages on tumor cells. Tissue disruption observed after CD40R stimulation of M1 (GM-CSF) macrophages and their ability to cross-present antigen to CD8+ T cells suggest another intriguing possibility to be verified in the context of clinical studies. In particular, tumor-associated antigens released during cancer cells

lysis could be potentially presented to CD8+ T cells, actively recalled in the tumor microenvironment by CD40R-stimulated macrophages, therefore promoting a specific CD8+ T cells-mediated antitumor response.

A potential limitation preventing the use of vaccinia virus as viral vector for gene delivery is represented by its high immunogenicity. On one hand, administered recombinant vaccinia virus might be rapidly eliminated particularly vaccinated cancer patients. On the other hand, this potential caveat restraining the use of rVV40L as a potential tool for cancer immunotherapy might also support its clinical validation. Indeed, intratumoral injection of the virus, which displays preferential tropism for cancer cells, could induce the activation of CD8+ T cells toward tumor-associated antigens, but also the re-activation of memory CD8+ T cells specific for viral antigens expressed on cellular surfaces of rVV40L-infected tumor cells.

Last but not least, our study demonstrates that rVV40L presents many promising features that make this type of approach highly promising specially in the context of tumor highly infiltrated with macrophages. In conclusion, such type of research might also contribute to a better understanding of the pivotal role played by macrophages in the tumor microenvironment and in the orchestration of innate and adaptive immune responses.

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- Fallarini S, Brittoli A, Fiore M, Lombardi G, Renaudet O, Richichi B, Nativi C. Immunological characterization of a rigid α-Tn mimetic on murine iNKT and human NK cells. *Glycoconj J*. 2017; 34(4):553-562.

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- Trella E, Brittoli A, Mele V, Governa V, Pinamonti M, Terracciano L, Muenst S, Iezzi G, Spagnoli G, Zajac P. 2017. CD154/40L recombinant vaccinia virus induces direct and macrophage-mediated in vitro and in vivo anti-tumor effects. Submitted to Cancer Research

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