Activation of antitumor M1 macrophages for cancer immunotherapy

Doctoral thesis by

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To my family. Better together.

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Abbreviations

1400w	N-[[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride
Ab	Antibodies
ACT	Adoptive cell therapy
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
Ag	Antigen
AP1	Activator protein 1
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BCR	B cell receptor
BiTE	Bi-specific T cell engager
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
CAR	Chimeric antigen receptor
CCK-8	Cell-counting assay 8
CCL2	C-C motif ligand 2
CD	Cluster of differentiation
CM	Conditioned medium
cMoP	Common myeloid progenitor
CpG-ODN	CpG oligodeoxynucleotide
Cpm	Counts per minute
CREB	cAMP response element-binding protein
CRT	Calreticulin
CSF-1	Colony stimulating factor 1
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage/danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
Foxp3	Forkhead box transcription factor 3
GATA-3	GATA-binding protein 3
GM-CSF	Granulocyte-macrophage colony stimulating-factor
GSK	Glycogen synthase kinase
HDAC	Histone deacetylase
HLA-DR	Human Leukocyte Antigen – antigen D related
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
iNOS	Inducible nitric oxide synthase
irAE	Immune-related adverse events
IRAK-M	Interleukin receptor-associated kinase M
IRF	Interferon regulating factor
JAK	Janus kinase
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAF	Macrophage activating factor
MAL	MyD88 adaptor-like protein
MDM	Monocyte-derived macrophage
MDSC	Myeloide derived suppressor cell
MHC	Major histocompatibility complex
miRNA	MicroRNA
MMR	Macrophage mannose receptor
MOPC	Mineral oil induced plasmacytoma
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxygen species
NP	Nanoparticle
NSCLC	Non-small cell lung carcinoma
NSG	NOD-*Scid*-gamma or NOD-*scid* IL2Rgamma^{null}
PAMPs	Pathogen associated molecular patterns
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
pIC-NPs	Poly(I:C)-encapsulated nanoparticles
Poly(I:C)	polyriboinosinic acid-polyribocytidylic acid
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
Rag2	*Recombination activating gene 2*
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	ribosomal RNA
s.c	Subcutaneous

s.c.	Subcutaneous
SARM1	sterile-α- and armadillo-motif-containing protein 1
SFV	Semliki Forest Virus
SIRPα	Signal-regulatory protein α
SMT	S-methylisothiourea hemisulfate salt
STAT	Signal transducer and activator of transcription 1
T bet	transcription factor T box expressed in T cells
TAM	Tumor associated macrophage
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Tumor growth factor β
Th cell	T helper cell
TIL	Tumor infiltrating lymphocytes
TIR	Toll-IL-1-resistence
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
T-VEC	Talimogene laherparapvec
VEGF	Vascular endothelial growth factor
WT	Wild type

Acknowledgements

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Finally I would like to thank my husband Martin. He always kept faith and had my back, whether it was bringing takeaway dinner to lab, putting our boy to sleep on long working days or simply always believing in me. I could not have done this without his love and unconditional support. We are better together.

List of publications

Paper I

TLR ligands and IFN-γ synergize for induction of antitumor M1 macrophages.

Elisabeth Müller, Panagiotis F. Christopoulos, Sanjib Halder, Anna Lunde, Kahsai Beraki, Martin Speth, Inger Øynebråten and Alexandre Corthay

Frontiers in Immunology 8:1383 (2017)

Paper II

Generation and Functional *in vitro* analysis of Semliki Forest Virus vectors Encoding TNF-α and IFN-γ.

Baiba Kurena, Elisabeth Müller, Panagiotis F. Christopoulos, Ingvild Bjellmo Johnsen, Branislava Stankovic, Inger Øynebråten, Alexandre Corthay and Anna Zajakina

Frontiers in Immunology 8:1667 (2017)

Paper III

Role of type I and type II interferons for activation of antitumor M1 macrophages.

Elisabeth Müller, Martin Speth, Panagiotis F. Cristopoulos, Anna Lunde, Ajna Avdagic, Inger Øynebråten and Alexandre Corthay

Manuscript in preparation

Background

Cancer immunotherapy was declared "Breakthrough of the year 2013" by Science Magazine. This was triggered by the approval of several new breakthrough therapies aiming to strengthen or unleash the body's own immune response against cancers, and has given the research into cancer immunology a great push forward. Following this, the last five years have seen a massive surge in the interest in cancer immunotherapy research from both the public and the scientific communities. However, many clinical studies has been very narrow and focused mainly on cytotoxic T cells in one form or another. Other potential effector cells are antitumor macrophages. The collaboration of lymphocytes and macrophages in preventing the growth of lymphoma cells *in vivo* was described already in 1970 by Evans and Alexander, and Corthay *et al.* showed that primary immune responses against tumors can be mediated by antitumor macrophages in collaboration with tumor specific cluster of differentiation 4 $(CD4)^+$ T cells [1, 2]. Antitumor macrophages are able to block cancer cell proliferation *in vitro*, and they secrete factors that inhibit angiogenesis and stimulate antitumor immune responses *in vivo* [2, 3]. However, the current mainstream view is that the phenotype of macrophages infiltrating the tumor is being redirected by tumor-derived signals towards supporting tumor growth, angiogenesis and metastasis instead [4]. Interestingly, several proof-of-principle studies have shown the therapeutic potential of targeting macrophages as a strategy to induce antitumor immune responses against cancer [5-9].

This thesis aims at better understanding the signals controlling the antitumor functions of macrophages. Such knowledge might be used to develop new strategies for targeting tumor associated macrophages in cancer patients.

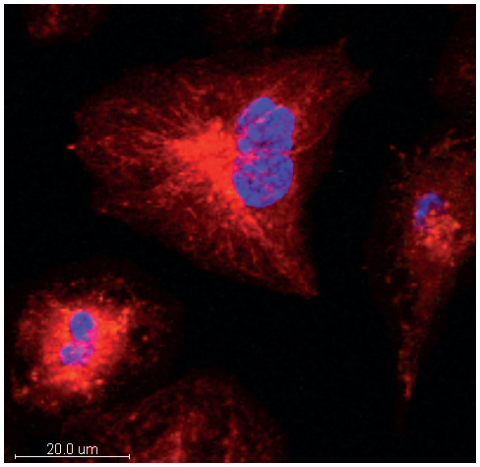

Figure 1 - Peritoneal macrophages.

Confocal microscopy picture of mouse peritoneal macrophages, stained with red lysosomal compartment tracker and Hoechst nuclear stain in blue. Created by Elisabeth Müller

Introduction

1. The immune system

The immune system is a highly complex system of cells and soluble agents responsible for protecting the body against pathogens, stress and damage, and for promoting healing and recovery to a homeostatic state. To accomplish this, the system must be able to respond to the correct stimuli and to regulate and control this response. Failure of the immune system in either of these aspects increases the risk of developing life threatening infections, severe autoimmunity or cancer.

The immune system is most commonly divided into two arms; the innate and the adaptive arm, based on germline encoded or somatically diversified immune receptors, respectively (Figure 2). The innate immune system has the advantage of being ready to act immediately as the first line of defense, while the adaptive immune system has the ability to create highly specific responses to almost any pathogen. This latter process requires time for recognition, somatic gene rearrangement and amplification of the specific response, but also confers memory upon re-challenge. Despite this dichotomy, the system is highly integrated, and crosstalk and interactions between innate and adaptive immune cells are taking place at multiple levels. New cell types and subtypes are still being discovered, and the intricate regulatory mechanisms controlling the immune system are still not sufficiently understood.

1.1. The innate immune system

The innate immune system is the first line of defense against any pathogen or damage, and appeared earlier in the evolutionary development of life. It consists of the epithelial barrier of the skin, gastrointestinal tract and the airways, which provides protection against entry of foreign pathogens into the underlying tissues. The innate immune system includes important humoral factors such as cytokines and chemokines, complement and pentraxins, as well as many different cell types including macrophages, dendritic cells (DCs), mast cells, basophils, eosinophils, neutrophils, innate lymphoid cells and natural killer cells (Figure 2).

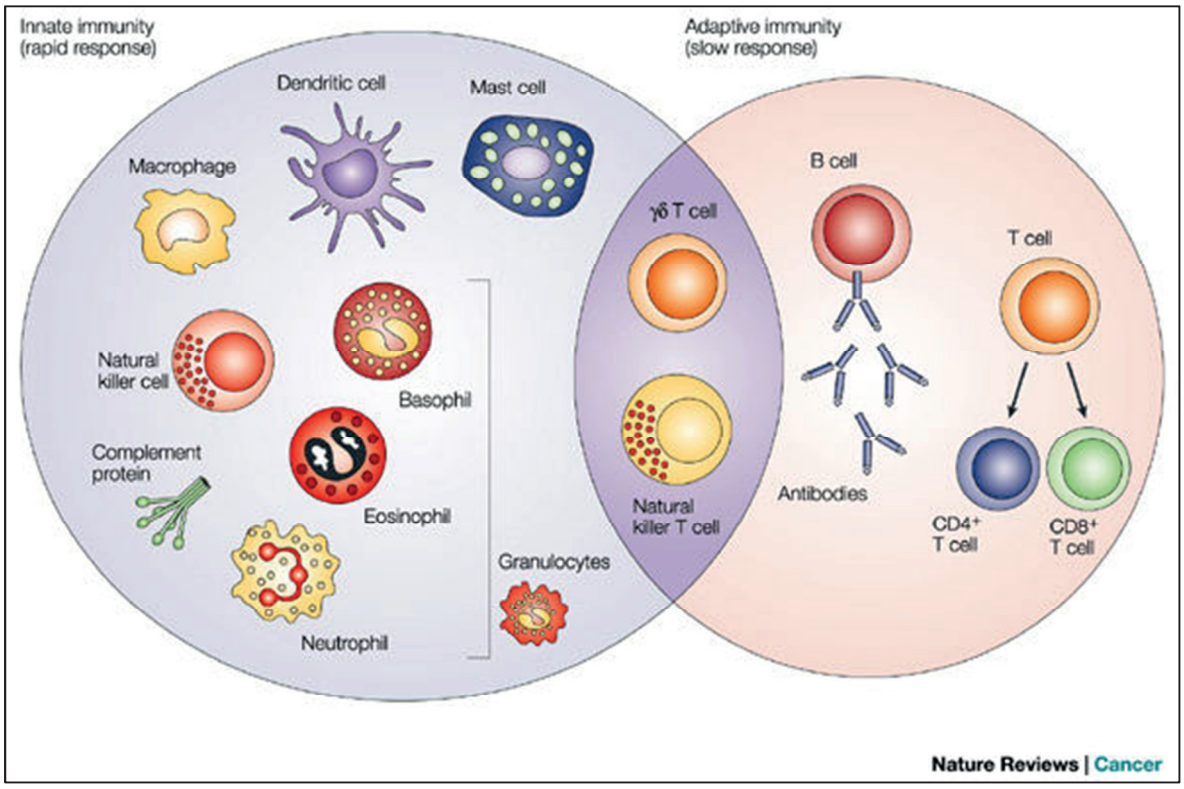

Figure 2 - The innate and adaptive immune response. The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granuloocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. Figure taken from [10] and reused with permission.

Macrophages –origin and functions

Macrophages play a dual role in the body, as they both contribute to maintaining healthy tissue homeostasis and protect the organism from infection, being a part of the innate immune system. It was previously believed that tissue-resident macrophages arose from blood-circulating monocytes, which originate from progenitors in the bone marrow (BM). This hierarchy originated from the "mononuclear phagocyte system" concept by Van Firth an colleagues in the 1970s [11]. However, recent studies have revealed that circulating monocytes contribute to the macrophage populations in only a few tissues, which include the gut, dermis and heart. Instead, most tissue-resident macrophage populations arise from embryonic precursors prior to birth, and maintain themselves locally throughout adulthood

(Figure 3) [12, 13]. Such tissue macrophages play critical roles during development and homeostasis, and are found in most tissues of the body.

Macrophages can also differentiate from bone-marrow derived monocytes. Monocytes originate from a common myeloid progenitor (cMoP) in the bone marrow (Figure 3). During steady state, monocytes may survey the tissues, pick up antigens and transport this back to lymph nodes, similar to conventional DCs [14]. Increased numbers of monocytes egress from the bone marrow into the blood in response to infection and TLR ligands [15] or upon tissue injury [16], and differentiate into macrophage-like or DC-like cells. There has been extensive confusion regarding the distinction between macrophages and DCs based on functions such as antigen-presentation [17], but it is now clear that conventional DCs and plasmacytoid DCs arise from precursors in the bone marrow distinct to monocytes [18]. To avoid misinterpretations, Guilliams *et al.* proposed the label "monocyte-derived cells" for all subsets of cells originating from blood monocytes (Figure 3) [19], but the debate continues [20].

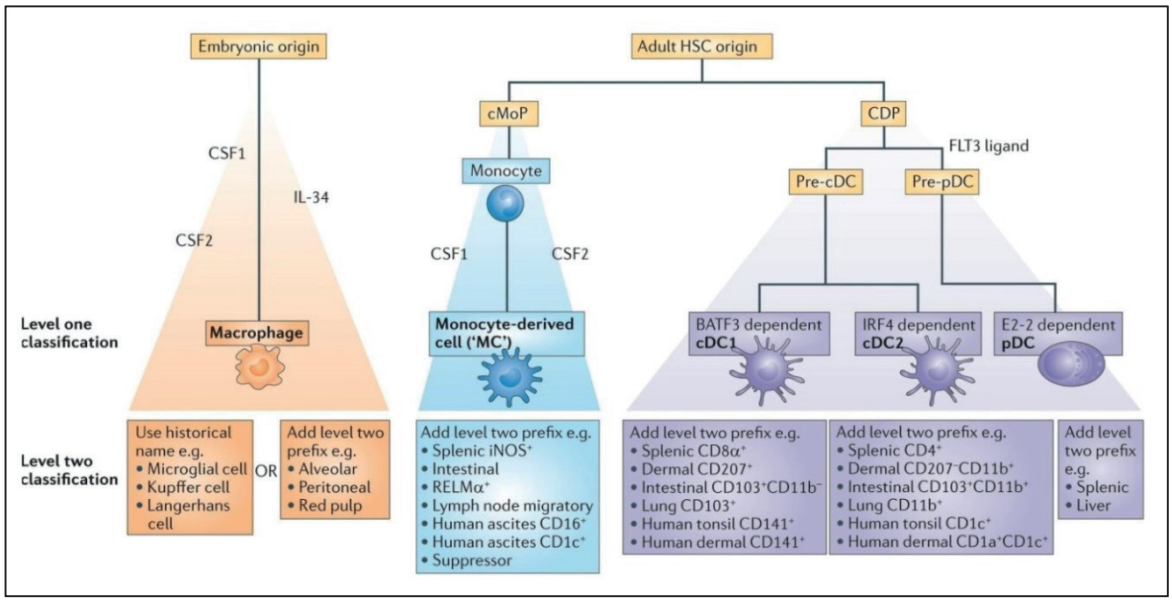

Figure 3 - Proposed categorization of mononuclear phagocytes. Guilliams *et al.* suggest that mononuclear phagocytes should be first defined on the basis of their ontogeny (level one), followed by their function, location and/or morphology (level two). This yields three main groups of cells – namely common dendritic cell (DC) precursor (CDP)-derived DCs, embryonic-derived macrophages and monocyte-derived cells. DCs could be further subdivided into "classical type I DCs (cDC1s)", "cDC2s" and plasmacytoid DCs (pDCs) due to their dependence on distinct sets of transcription factors. Level two could include surface markers used to indentify the cells, the functional specialization or cellular localization. Figure taken from [19] and reused with permission.

This thesis deals mainly with monocyte-derived cells of a macrophage phenotype which are induced upon inflammation, and the term macrophage is used for these cells. When appropriate, further annotation to the bone-marrow or blood monocyte origin of specific cells is given.

Macrophages were first described by Élie Metchnikoff as large cells capable of phagocytosing bacteria and other harmful objects [21]. Macrophages, along with DCs and B cells, are professional antigen presenting cells (APCs) and can take up foreign proteins and present it as peptides to T cells. Macrophages can further stimulate and guide immune responses though the secretion of cytokines and chemokines which affect other immune cells, and play important in roles host defense, immune regulation and wound healing [22]. These various functions of macrophages are regulated in response to signals from the environment.

Macrophage receptors

Macrophages express a wide variety of receptors on their surface which can bind endogenous and exogenous signals. Examples are Fc receptors, complement receptors, C-type lectin (CTL) receptors and Toll-like receptors (TLRs). Macrophages are important for the clearance of pathogens such as bacteria and viruses and can take up and kill pathogens that are opsonized by complement upon binding to complement receptors [23], or antibodies, upon binding to Fc receptors [24]. Furthermore, infected cells that have been coated with antibodies may be taken up and lysed, or killed by antibody-dependent cellular cytotoxicity (ADCC) [25].

In 1989, Janeway famously termed Freund's adjuvant "The Immunologist's Dirty Little Secret", aimed at the fact that activation of naïve T cells cannot be achieved by APCs and antigen alone, but requires additional stimuli in form of microbial agents [26]. Bretscher and Cohn were the first to propose that all lymphocyte activation requires two signals [27], with the first signal being delivered through the antigen receptor. Lymphocyte activation was found to also depend on the presence of co-stimulating factors on APCs presenting the antigens [28]. Janeway pointed out that the manner in which such co-stimulating factors were induced on APCs remained a mystery. He noted that exposure of APCs to bacteria or bacterial products (such as Freund's adjuvant) resulted in induction of costimulatory activity, and his conclusion was that APCs would be seen to express

receptors to detect such microbial signals, once appropriately studied. He further hypothesized that:

"...the immune system has evolved specifically to recognize and respond to infectious microorganisms, and that this involves recognition not only of specific antigenic determinants, but also of certain characteristics or patterns common on infectious agents but absent from the host." [26]

The receptors predicted by Janeway have become known as pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs), and the first type of PPRs described was the Toll-like receptors. The history of TLRs dates back to the cloning of the interleukin-1 receptor (IL-1R) in 1988 [29]. In 1991, homology between this receptor and the cytosolic domain of a developmental *D. melanogaster* protein called Toll was described [30]. Work by Jules Hoffmann and colleagues showed that Toll also regulates immune gene expression and that activation of Toll-pathways can induce the antifungal peptide Drosomycin [31]. A mammalian Toll gene homologue, termed *hToll*, was cloned and studied by Ruslan Medzhitov and Janeway in 1997, and hToll displayed ability to induce co-stimulatory genes [32]. The final bit of the puzzle came when Bruce Beutler's group identified *hToll*, now renamed *Tlr4*, as the gene encoding the LPS receptor [33], finally confirming that TLRs are indeed PRRs and recognize microbial products. LPS had been pictured as the ideal PAMP and PRR ligand, and the search for its receptor had been on for years [34]. Other receptors, such as CTL receptors, were also recognized as PRRs, as they recognize pathogens based on the presence of conserved oligosaccharides on their surface. Macrophages express several CTLs, such as dectin-1 and macrophage mannose receptor [35].

The discovery of TLRs, a molecular sensor system for the detection of microbes, shared by insects and mammals, was has greatly contributed to our understanding of and appreciation for the complexity of innate immune responses. Bruce A. Beutler and Jules A. Hoffmann received the 2011 Nobel Prize in Physiology or Medicine for "their discoveries concerning the activation of innate immunity", together with Ralph M. Steinman for "his discovery of the dendritic cell and its role in adaptive immunity" [36].

The TLR family is strongly conserved in both invertebrates and vertebrates, and comprises 12 member in mice (TLR1-13, TLR10 being a pseudogene) and 10 in human (TLR1-10) [37]. The TLRs are either located on the cell surface (TLR5, TLR1/2, TLR2/6

and TLR11), in endosomal vesicles (TLR3, TLR7/8, TLR9 and TLR13) or both (TLR4) (Figure 4). Binding of TLRs to their ligands result in either homo- or heterodimerization, which enable adaptor molecules to bind the Toll-IL-1-resistence (TIR) domain on the cytosolic side of TLRs. The first such adaptor to be described was Myeloid differentiation primary response 88 (MyD88), originally identified as an adaptor to the IL-1R [38]. Since then, TLR signaling pathways have been found to include four adaptor molecules in addition to MyD88: MyD88 adaptor-like protein (MAL), TIR domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM; also known as TICAM2) and sterile-α- and armadillo-motif-containing protein 1 (SARM1) [39].

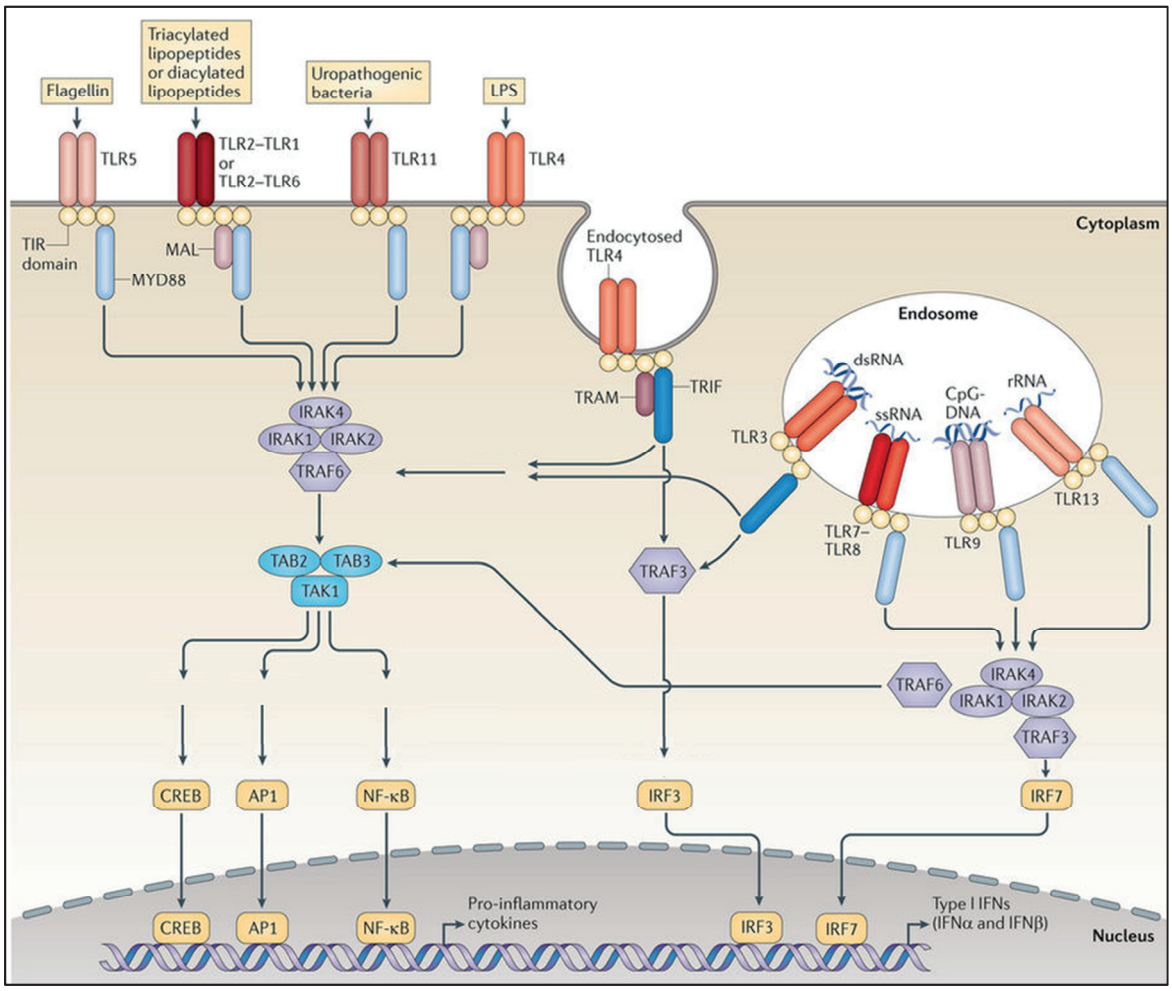

Figure 4 – Mammalian TLR signaling pathways. TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes. TLR4 localizes at both the plasma membrane and the endosomes. TLR signaling is initiated by ligand-induced dimerization of receptors, followed by engagement of TIR domains of TLRs with TIR domain-containing adaptor proteins (either MYD88 and MAL, or TRIF and TRAM). TLR4 moves from the plasma membrane

to the endosomes in order to switch signaling from MYD88 to TRIF. As a result, downstream signaling pathways that involve interactions between IRAKs and the adaptor molecules TRAFs are stimulated, which lead to the activation of transcription factors. Transcription factors activated by TLRs include Nuclear factor-κB (NF-κB) and Interferon responsive factors (IRFs), as well as cAMP response element-binding protein (CREB) and Activator protein 1 (AP1). A major consequence of TLR signaling is the induction of pro-inflammatory cytokines and the induction of type I interferon (IFN). Figure adapted from [34] and reused with permission.

Figure 4 describes in short the downstream signaling of mammalian TLRs, following binding of the different adaptor molecules to the various TLRs [34]. However, the full complexity of the TLR system has not yet been uncovered, although it is clear that activation of multiple TLRs, as would be the case during microbial infection, can have novel effects on gene transcription which needs to be explored [40-42]. Macrophages express most receptors of the TLR family [43], and the identity and sequence of receptors activated is regarded as a way for the innate immune system to tailor its response to the type of infectious agent it senses [44]. In addition, macrophages carry receptors for chemokines and cytokines produced by other cells, which can further tune macrophage function and phenotype [45].

1.2. The adaptive immune system

The adaptive immune system is found in humans and other vertebrate species, and evolved long after the appearance of multicellular organisms with complex innate immune systems [46]. The adaptive immune system consists of two types of specialized immune cells of the lymphocyte lineage, B cells and T cells, which develop from hematopoietic stem cells in the bone marrow. These cells carry antigen receptor gene segments which are somatically rearranged and then expressed to form a vast repertoire of diverse receptors. Each cell expresses receptor with a certain specificity and can, upon appropriate activation, expand to yield a clonal pool of highly specific immune cells ready to recognize and clear pathogens.

B cells develop and mature in the bone marrow, before entering the blood and traveling to secondary lymphoid structures. B cells express highly specialized recognition proteins called immunoglobulins (Ig), which can be membrane-bound and function as the B cell antigen receptor (BCR) or secreted as antibodies (Ab). Immunoglobulins contain a variable region responsible for binding of antigens and a constant region, which comes in five main isoforms. T cells carry receptors that are related to immunoglobulins, also

containing variable and constant regions, but the T cell receptor (TCR) cannot recognize antigens directly. The TCR require short peptide fragments of protein antigens to be presented by major histocompatibility complex (MHC) molecules on host cells for binding.

There are two main classes of MHC molecules, class I and class II. MHC class I molecules are expressed by all nucleated cells, and function by displaying peptide fragments of any protein present in the cell [47]. Upon viral infection, viral peptides are produced, and may be presented on the cell surface where they can be recognized by specific T cells. Similarly, cancerous mutations can create neoantigens which can be presented by the cell and recognized by TCR on T cells. The recognition depends on both a TCR with specificity for binding of that particular peptide-MHC complex, and expression of CD8, which binds to the invariant portion of the MHC class I molecule. $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs), can then directly kill infected or mutated cells, and mediate important effector functions of the adaptive immune system.

The other main type of MHC molecules is the class II, which is only expressed by professional APCs, *i.e.* macrophages, dendritic cells and B cells. APCs pick up extracellular pathogens, digest them and present such pathogenic peptides to the immune system on MHC class II molecules. T cells expressing CD4 and a specific T cell receptor can bind to APCs expressing peptides on MHC II molecules and initiate an immune response. $CD4^+$ T cells are also called T helper (Th) cells, and they play a key role in providing stimulatory help to $CD8^+$ T cells and B cells as well as orchestrating the response of other immune cells through secretion of cytokines. $CD4^+$ T cells are further separated into several subsets which differ in their functional properties and cytokine profile.

1.3. The integrated immune system at work – Types of immune responses

The immune system has developed to protect against a wide range of threats, spanning from viruses of a few nanometers to large parasitic worms. Furthermore, it is required to mediate tolerance to commensal bacteria and promote healing and return to homeostasis of damaged and inflamed tissues. These different functions are made possible by a differentiated immune response, tailored to the task. One of the best examples of such differentiation is the functionally distinct subsets of Th cells (Figure 5). Depending on the current cytokine microenvironment, the antigen and the T cell-APC interaction, an activated $CD4^+$ T cell will differentiate into Th1, Th2 or regulatory T cells (Treg) among

others [48]. There are additional important subsets, such as the Th17 and T follicular helper cells [49], but they will not be discussed further in this thesis.

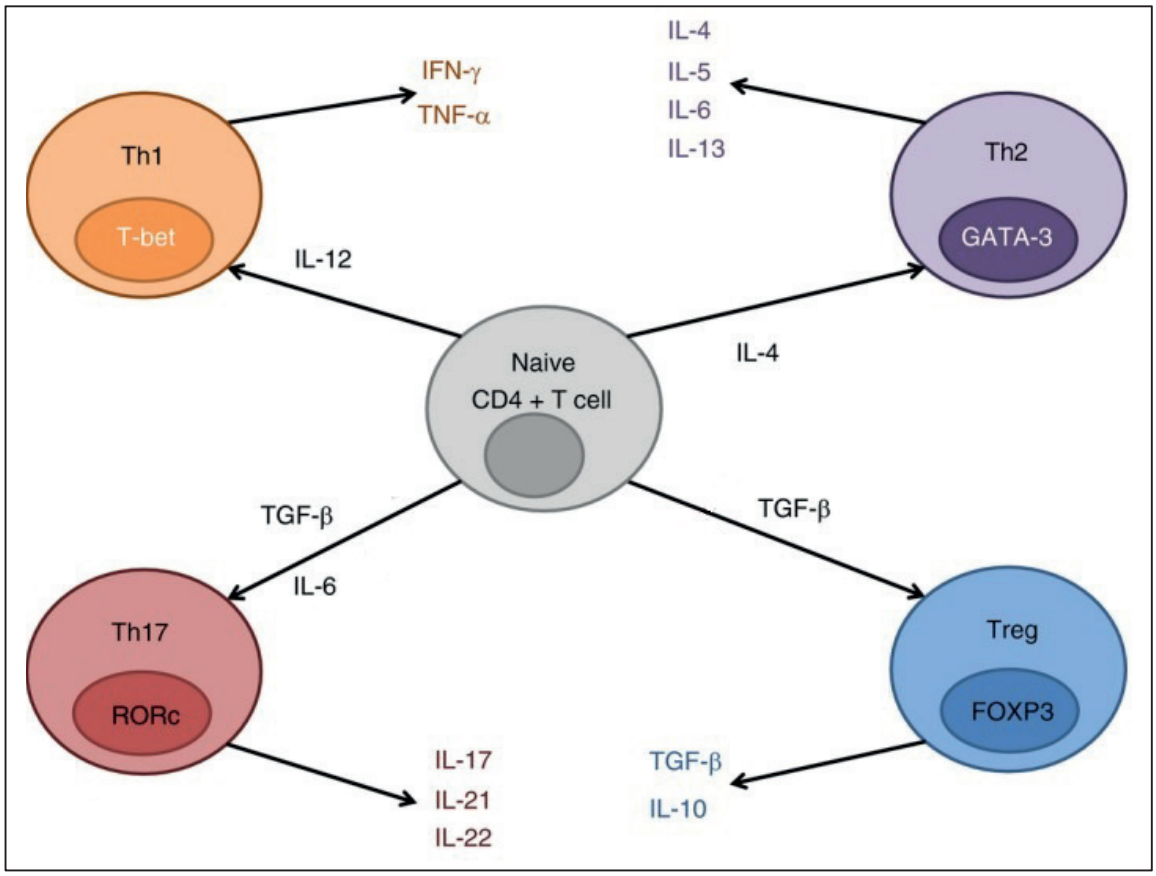

Figure 5 – Differentiation of naive CD4$^+$ T cells into T helper type 1 (Th1), Th2, Th17 or regulatory T (Treg) cells, depending on the cytokine profile. Interleukin-12 (IL-12) and interferon-γ (IFN-γ) stimulated naive T cells differentiate to Th1 cells. These cells express IFN-γ and tumour necrosis factor-α (TNF-α), and are responsible for intracellular parasite clearance and allergy conditions. IL-4 stimulated naive T cells induce a Th2 response. Th2 cells express IL-4, IL-5, IL-6 and IL-13, and are responsible for clearance of extracellular parasites. Transforming growth factor-β (TGF-β) stimulated naive T cells induce a Treg response. Treg cells express TGF-β and IL-10 and are responsible for tolerance and pregnancy success. These three cell types express one cytokine responsible for its induction, in a positive feedback mechanism. TGF-β and IL-6 stimulated naive T cells induce a Th17 response. Th17 cells express IL-17, IL-21 and IL-22 and are responsible for autoimmunity and pregnancy loss. Figure is adapted from [50] and reused with permission.

Th1 cells are typically elicited by viruses, microbes which persist in macrophage vesicles or extracellular bacteria, and depend on the presence of IL-12p70, which induce the expression of "transcription factor T box expressed in T cells" (T bet). T bet expression

leads to differentiation of naïve $CD4^+$ T cells into Th1 cells, which is characterized by production of IFN-γ [51]. The prototype inducer of Th2 cells is helminth parasites and the presence of IL-4. IL-4 induce the expression of the transcription factor GATA-binding protein 3 (Gata-3), which results in differentiation into Th2 cells that produce IL-4, IL-5 and IL-13 [51]. Contrary to Th1 and Th2 cells, which help activate other immune cells, Treg cells function by suppressing immune responses [52]. Treg cells are characterized by the dependence and expression of the transcription factor Forkhead box transcription factor 3 (Foxp3). Treg suppression can be mediated through soluble factors, such as IL-10, TGF-β and IL-35, or through cell-cell contact. Suppression may target T cell functions directly, inhibiting proliferation and activation, or indirectly through the modulation of APCs. [52]. The two-way communication between the Th cells and APCs, such as macrophages, represents an important mechanism of polarization the immune response towards appropriate effector functions, whether this is induction of tolerance or immune attack.

Macrophage activation

In the 1960s, G. B. Mackaness observed a phenomenon he called "acquired cellular resistance" in mice after infection with the intracellular bacteria *L. monocytogenes* [53, 54]. Macrophages from infected mice could kill a range of bacteria much more efficiently than macrophages from uninfected mice, and mice infected with *L. monocytogenes* became temporarily resistant to infections with such unrelated bacteria. The resistance did not last, but could be reaquired by rechallenge with the same *L. monocytogenes* bacteria, indicating the response was antigen-specific in its initiation, but not in its expression.

Carl Nathan picked up on the work of Mackaness and went on to show that a soluble product of antigen-stimulated lymphocytes could activate macrophages and enhance several antimicrobial functions [55]. He called this lymphocyte-derived agent macrophage-activating factor (MAF). Interestingly, Evans and Alexander had showed that co-culture of macrophages with lymphoid cells which had been immunized against lymphoma, resulted in macrophages being activated to kill such lymphoma cells [1]. Macrophages could also be activated to kill tumor cells by exposure to endotoxin (LPS) or double stranded RNA [56]. Hibbs *et al.* then showed that high concentrations of lymphocyte-derived MAF could mediate the effect of lymphocytes and activate macrophages for tumor cell killing [57]. Consecutive studies revealed that MAF could function as a priming factor, but a secondary stimuli, such as LPS, was required for induction of tumoricidal activity [58]. However, MAF acted synergistically and improved

the tumoricidal induction upon LPS activation [59]. This function was exploited for the development of a quantitative assay (Figure 6) for the measurement of MAF by Pace *et al.* [60].

Then, in 1983, Pace and colleagues showed that T cell-derived lymphokines primed macrophages for tumoricidal activity in a manner that was attributed to IFN-γ [61], and they provided quantitative molecular evidence for the fact that MAF was identical to IFN-γ [62]. Only a few months later, Nathan *et al.* also showed that IFN-γ was the lymphokine responsible for macrophage activation leading to the production of hydrogen peroxide and the ability to kill intracellular microbial pathogens [63]. Since then, IFN-γ has become recognized as one of the most important endogenous mediators of immunity and inflammation, playing a key role in regulating the functions of macrophages, both in fighting infections and in cancer [64].

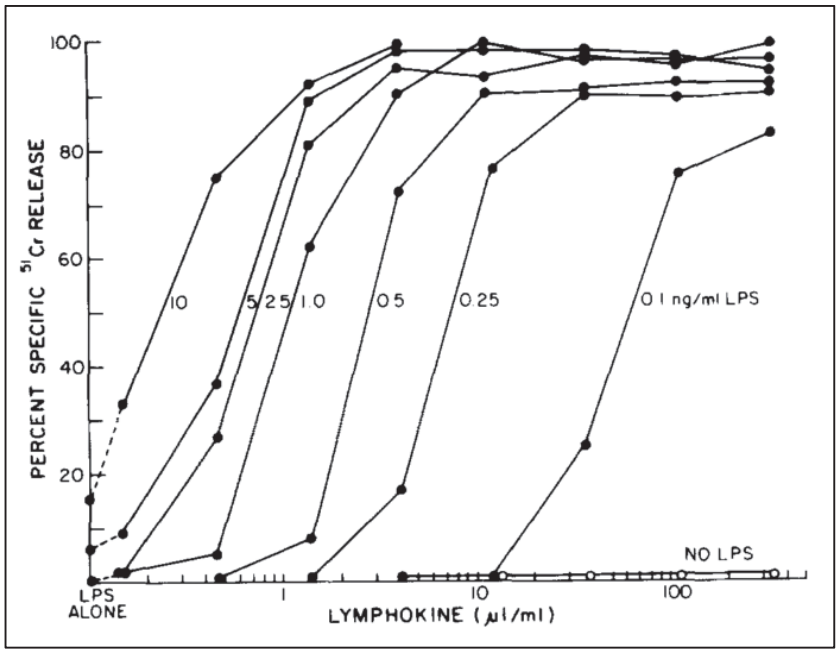

Figure 6 - The effect of LPS on the lymphokine dose response curve. Spleen cell culture supernatants were diluted in medium alone and in medium containing constant amounts of LPS ranging from 0.1 to 10 ng/ml. Inflammatory peritoneal macrophage were preincubated 4 hr with either lymphokine alone (○) or with the combination of lymphokine and LPS (●). Percent specific 51Cr-release was determined 16 hr after the addition of labeled P815 mastocytoma cells. Activating stimuli were present throughout the assay. Concentrations of activating stimuli present during the initial 4-hr preincubation period are indicated. Figure taken from [60] and reused with permision.

Nitric oxide – a product of activated macrophages

In the 1980s, a correlation between immune stimulation and elevated NO_3^- synthesis was observed in both humans and rats [65, 66]. This led Stuehr and Marletta to investigate the production of NO_2^- and NO_3^- in LPS-responsive and unresponsive mice strains, by LPS activation of cells *in vitro* or *in vivo*, upon injection of LPS or infection with Mycobacterium bovis [67]. They found that LPS activated macrophages, but not other lymphocytes, to produce NO_2^- and NO_3^-. The presence of T cells could enhance the resulting nitrate production, but LPS-induced nitrate synthesis was also observed in mice lacking T cells. They suggested that the synthesis of nitrate could directly participate in the endogenous formation of nitrosamines and be involved in some form of cytotoxicity [67].

Hibbs *et al.* first found that L-Arginine was required for the tumor cell cytotoxic effect of activated macrophages [68], and then went on to report that activated macrophages synthesize nitric oxide (NO) from a terminal guanidono nitrogen atom of L-arginine [69]. They also showed that exposure to NO caused the same pattern of cytotoxicity in hepatoma cells as activated macrophages, suggesting that NO was the effector molecule [69]. Stuehr and Nathan confirmed this, as they showed that the reactive radical NO is a mediator of macrophage-induced cytostasis and mitochondrial respiratory inhibition in lymphoma cells [70]. NO, formed from L-arginine by activated macrophages, was shown to be an important effector mechanism in macrophage anti-microbial activity [71-73], and the enzyme responsible for its production was identified as inducible nitric oxide synthase (iNOS) [74]. Other isoforms of the NOS enzyme were found to be expressed by endothelial cells and neurons, and NO was recognized as playing important roles in non-immune functions as well [75].

New and "alternative" functions of macrophages

The substrate for NO production by iNOS, L-arginine, can also serve as a substrate for another enzyme called arginase and yield ornithine and urea, in the urea cycle [76]. Arginase activity, through ornithine production, was found to promote cellular proliferation and tissue regeneration and to be increased in tumors [77, 78]. Interestingly, increased amounts of ornithine and reduced l-arginine was found at wounds and other sites of inflammation and shown to be a result of macrophage-derived arginase [79]. In parallel to the description of the anti-tumor and cytotoxic functions of activated macrophages, Alberto Mantovani had showed increased tumor cell proliferation *in vitro* upon co-culture with unstimulated peritoneal macrophages or tumor-infiltrating macrophages from certain

tumors [80]. Charles Mills and colleagues helped shed light on this new function of macrophages, as they used a model of sterile wound healing to investigate the arginine metabolism by macrophages over time. They showed that macrophages initially metabolize arginine to the toxic NO, in potential defense against microbes, before shifting to the production of ornithine, aiding tissue repair and healing [81]. Furthermore, Mills *et al.* showed that tumor rejection of P815 tumors in preimmunized mice was accompanied by an increase in NO production by intratumor macrophages, while tumor growth and progression in naïve mice was associated with increased production of ornithine and urea [82]. In the same year, Stein *et al.* described a new phenotype of macrophages, resulting from *in vitro* activation with IL-4, which contrasted the phenotype of IFN-γ-activated macrophages [83]. IL-4- or IL-13-activated macrophages displayed induction of the macrophage mannose receptor (MMR), inhibition of pro-inflammatory cytokines and restricted MHC class II antigen expression. Stein termed this alternative activation, to separate it from the previously well-described "classical" activation of macrophages with IFN-γ and/or LPS [83].

M1 and M2 macrophages

$CD4^+$ T cells had previously been described to separate into, among others, an IFN-γ-producing Th1 subset and an IL-4- and IL-13-producing Th2 subset, important for dealing with different types of pathogen infections [51]. Mills showed that macrophages from mice prone to elicit Th1 responses (C57BL/6) responded to LPS by making NO, while the response of macrophages from mice prone to elicit Th2 responses (Balb/c) to the same LPS was production of ornithine [84]. He termed these macrophage responses for M1 and M2, corresponding to the Th1 and Th2 T cell response respectively. However, he showed that this difference in macrophage response was also present in C57BL/6 Nude or SCID mice, indicating that Th1 or Th2 T cells were not the mediator of these macrophage phenotypes, but possibly the result [84]. This was going to form the basis for an ongoing discussion of the role and importance of macrophages guiding T cell responses or vice versa, a story of the chicken or the egg [85].

The interest into alternative or M2 activation of macrophages was further spurred on by a popular review by Mantovani *et al.* [86]. This review suggested that macrophages associated with tumors are activated towards a M2 phenotype, and that this underlies the immune-suppressive and tumor-promoting effects of such tumor-associated macrophages [86]. However, extensive research into activation of macrophages by various factors, and

characterization of macrophages infiltrating tumors have since drawn a much more complex and varied picture [45, 87, 88]. The phenotype and functions of macrophages in tumor will be discussed further in a later section.

1.4. Immune regulation and the importance of turning immune responses off

The immune system is immensely powerful in conferring protection, as proven by the successful vaccination against previously deadly diseases. However, severe autoimmune diseases, such as multiple sclerosis or rheumatoid arthritis, have made it equally clear that this effectiveness comes at the risk of serious tissue destruction. Immune responses must therefore be tightly controlled and the resulting inflammation must be resolved before tissue damage become too severe. Upon primary infection or tissue damage, the inflammatory response is initiated where plasma and leukocytes enter the affected tissues. Cells and soluble factors collaborate to remove the injurious stimuli, so that healing processes can take over and the acute inflammation is resolved. If this is not possible, such as in the case of a response to an auto-antigen which cannot be removed, the inflammation can progress to a chronic state with continued tissue destruction. Both chronic inflammation and an unhinged primary immune response can be detrimental.

The immune system has numerous safety breaks built in, and multiple steps are usually required to initiate any response. In order to be activated, T cells require co-stimulation through CD28 via binding to CD80 and CD86 molecules on APCs, in addition to specific binding of TCR to an antigen-MHC complex. T cells also express inhibitory receptors, such as CTLA-4 and PD-1, which help regulate immune responses by blocking the binding of CD80 and CD86 to co-stimulatory receptors or by binding to inhibitory ligands [89, 90]. Treg cells also play important roles in dampening or controlling immune responses through production of immune suppressive cytokines acting on T cell proliferation and on the capability of APCs to activate T cells. Treg cells may also sequester CD80, CD86 and IL-2 from naïve T cells, preventing their activation and proliferation [91]. Interestingly, activated antibody-secreting B cells can also contribute to regulation of immune responses through the production of IL-10 and IL-35 [92, 93].

IL-10 plays an important role in keeping macrophages and dendritic cells quiescent, and in preventing them from activating T cells and immune responses when this is not wanted, as in the case of commensal bacteria in the gut [94]. Furthermore, activation of

innate immune cells through pattern recognition receptors has been shown to simultaneously induce pro-inflammatory and anti-inflammatory cytokines through the activation of mitogen-activated protein kinases (MAPKs) and different downstream kinases. This contributes to regulatory feedback pathways which are important for controlling the innate immune responses [95].

Unfortunately, such regulatory switches can be exploited by pathogens which are under evolutionary pressure to evolve strategies for escaping immune detection and destruction. Such strategies include down-regulation of MHC class I or proteins required for antigen presentation to avoid detection or induction of immune suppression through Tregs or IL-10 homologs. Tumors are also under a similar evolutionary pressure by the immune system and develop analogous mechanisms for immune escape. It was demonstrated by Schreiber *et al.* in 2001 that tumors formed in the absence of an intact immune system are more immunogenic compared to tumors from an immunocompetent host [96]. The evasion from immune destruction has now been added to the hallmarks of cancer and this will be discussed further in the next section [97].

2. Tumor immunology

2.1. Hallmarks of cancer

Any multicellular organism depends on tightly regulated growth and death of individual cells to meet the needs of the tissue they belong to. Growth, death and any cellular functions are controlled by the expression of genetically encoded proteins in the cell's DNA, and damage to the DNA has the potential to unravel this control. Cancer is a group of diseases which is characterized by the uncontrolled growth and spread of cells which may result in life-threatening loss of tissue functions. Protective mechanisms have evolved to protect against individual cells going rogue and endangering the organism, and include DNA damage repair or induction of cell death if the damage cannot be fixed. In addition, there are several barriers preventing outgrowth of full blown cancer even when cells manage to escape apoptosis and start to proliferate in an uncontrolled manner. Interestingly, transformed cells which succeed in becoming cancerous, share common characteristic traits and functions, despite of being of different morphology and origin. Hanahan and Weinberg summarized these traits in their "Hallmarks of cancer" which was originally published in 2000 and updated with four new characteristic traits of cancers in 2011 [97] (Figure 7).

The hallmarks of cancer include the induction of angiogenesis and activation of invasion and metastasis, and among the new traits included in 2011 are tumor-promoting inflammation and evasion of immune destruction. These two last traits demonstrates the two-faced nature of the immune system, in that it may work as an effective barrier against tumor development (or infection), but at the same time holds the potential of contributing to host damage. Hanahan and Weinberg also conclude the following:

"Tumors are more than insular masses of proliferating cancer cells. Instead, they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. We depicted the recruited normal cells, which form tumor-associated stroma, as active participants in tumorigenesis rather than passive bystanders; as such, these stromal cells contribute to the development and expression of certain hallmark capabilities.[97]"

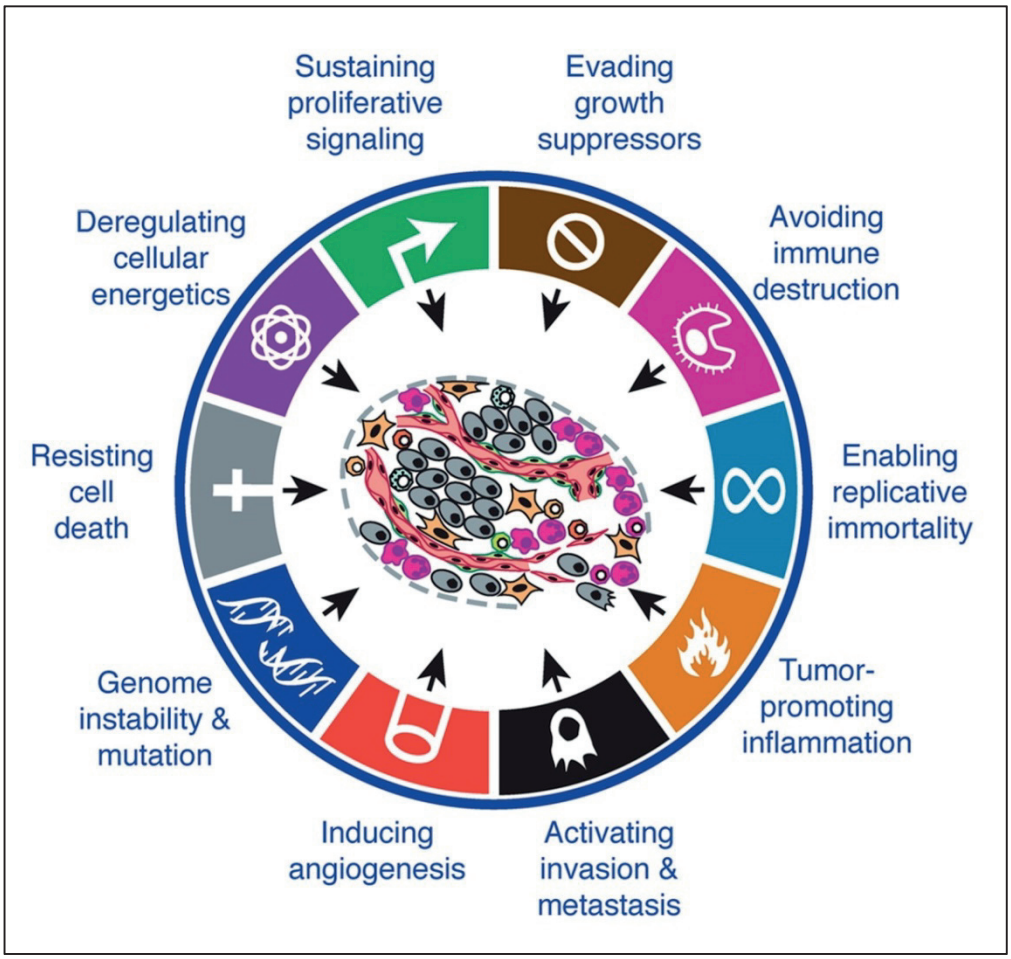

Figure 7 - The original "Hallmarks of cancer". Figure taken from [97] and adapted with permission.

This represents a general shift from a tumor cell-centered focus towards more interest in the non-cancerous cells in the microenvironment such as immune cells, fibroblasts and endothelial cells [98, 99]. Accumulating data are now pointing towards a major role of such supporting cells recruited from blood or the surrounding stroma by cancer cells in promoting angiogenesis, proliferation, invasion and metastasis. As a result, both immune cells and other stromal cells appears to be important treatment targets for cancer in the future [100].

2.2. The immune system protects against cancer

Tumor immunology and cancer immunotherapy recently received a lot of attention both in the scientific community and the public media. But the question of whether the immune system can respond to and protect against malignant tumors dates back more than a century. William Coley performed pioneering work in the end of the nineteenth century where he inoculated cancer patients with heat-killed bacteria [101-103]. In 1909, Paul Ehrlich

hypothesized that the immune system played a role in protecting against cancer: “First of all, with the exception of our Chondrosarcoma, I have yet to come across cancers against which individual animals are not (naturally) immune to” (translated by Elisabeth Müller) [104]. Ludwik Gross showed that, after intradermal inoculation of a small amount of cancer cells, mice were protected against re-challenge with a larger dose of the same cancer cell [105]. With the discovery of genetic mutations as a major driving force of cancer development, Frank Macfarlane Burnet picked up the possibility that the immune system could recognize mutated proteins in cancer cells. “It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hints of its existence” [106]. With input and suggestions from Lewis Thomas [107], Burnet further developed his ideas regarding a function of the immune system, specifically the adaptive arm, in preventing cancer and protecting the organisms against cancerous cells. This culminated in his definition of cancer immune surveillance as follows:

“In large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step towards malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character”[108]

However, the scientific community was not convinced that the immune system evolved to combat tumors. In properly matched donors and hosts and in the absence of alloreactivity, little or no resistance to tumor grafts was observed. It was proposed that antitumor immune responses were artifacts, or that these responses were possibly directed against endogenous viruses in tumors [109]. It was also found that athymic nude mice, which have major deficiencies in T cell development, have a normal incidence of tumors, and this was a major argument against a surveillance function of the adaptive immune system [110].

Since then, the tide has turned. Functional T cells were found in athymic mice [111], while a new model of immune deficiency, *Rag2*$^{-/-}$ mice, was found to completely lack mature B and T cells [112]. Shankaran *et al.* showed that *Rag2*$^{-/-}$ mice developed carcinogen-induced tumors earlier and with greater frequency than wild-type mice [96].

Mice deficient for IFN-γ or Signal transducer and activator of transcription 1 (STAT1) also showed increased susceptibility to carcinogen-induced tumors [96]. The presence of tumor-infiltrating lymphocytes (TILs) were shown to correlate positively with survival in large meta-analyses, and in particular $CD8^+$ TILs and a high $CD8^+/FoxP3^+$ ratio are reported to be advantageous [113]. Evidence have accumulated for a number of functions of the innate and adaptive immune system in recognition and killing of cancer cells, as well as in baseline protection against the development of cancerous lesions [114]. The buzz is on for new treatment options for cancer by targeting the immune system. The FDA approval of a monoclonal antibody targeting the T cell inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in 2010 created the foundation for a whole new field of immunotherapy called checkpoint inhibition [115]. Several other important steps in the field quickly followed, such as the approval of anti-Programmed death-1 (PD-1) and anti-PD-ligand 1 (PD-L1) antibodies (checkpoint inhibitor) and sipuleucel-T (dendritic cell vaccine) [116-118]. A strategy based on genetically modified T cells using chimeric antigen receptors also made major breakthroughs and improvements, putting adoptive T cell therapy on the map for cancer treatment in the future [119]. Cancer immunotherapy was announced as the breakthrough of the year by Science magazine in 2012, and the interest and progress has not slowed since [120].

2.3. Recognizing and responding to cancer

Since the early discovery that some animals can reject tumors that are successfully transplanted on to other individuals [104], the immunological and molecular basis for such recognition and rejection has been widely studied. There has been described an average of 40-90 mutations in protein-coding genes in cancers such as colorectal-, breast and pancreatic cancer and glioblastoma multiforme [121-123]. These mutated proteins can act as neoantigens, given that the proteins are expressed and that the mutated epitopes are presented by the cancer cells on MHC-I molecules. Similar to viral infections, which also result in the expression and presentation of non-self epitopes, such mutations renders the cells susceptible to recognition and elimination by cytotoxic $CD8^+$ T cells. In a study investigating the immunogenicity of mutations in expressed genes in B16F10 mouse melanoma cells, it was found that one-third of mutated peptides elicited immune responses. Furthermore, peptide immunization conferred tumor control *in vivo* in tumor transplant models [124]. However, early studies investigating the specificities of tumor-reactive T

cells from melanoma patients revealed that melanoma antigens may be tissue-specific, such as Mart-1 and gp100, rather than tumor-specific [125].

As cytotoxic $CD8^+$ T cells can only recognize and kill tumor cells upon binding of specific TCRs on the T cell to antigen-MHC I complex on the cancer cell, there is a selective pressure for cancer cells to acquire additional mutations which result in down-regulation or abrogation of the MHC-I peptide presentation pathway[126]. A similar effect is seen during viral infections and represents a problem for the immune system. One solution is the ability of natural killer (NK) cells to recognize and respond to "missing self", which was first formulated by Klas Kärre and Hans-Gustaf Ljunggren [127, 128]. NK cells carry both activating and inhibitory receptors, and killing mediated by NK cells depend on the presence of an activating signal and the absence of inhibitory signal. The activating signals can be various stress-induced ligands, such as MICA/MICB and ULBP1–6, not normally expressed by healthy cells. The inhibitory signal is provided by inhibitory receptors which recognize self-MHC-I. Therefore, cancer cells which have lost cell-surface expression of MHC-I may be a target of NK cells [129].

$CD4^+$ T helper cells usually cannot recognize cancer cells directly as most cancer cells do not express MHC-II. Exceptions to this include hematological cancers and some melanomas [130]. Contrary to cytotoxic $CD8^+$ T cells or NK cell, $CD4^+$ T cells require professional APCs to pick up and present neoantigens, in order to mediate an immune response [131]. Once activated, tumor-specific $CD4^+$ T cells can provide help for $CD8^+$ T cells for killing cancer cells [132]. Furthermore, $CD4^+$ T cells can mediate primary antitumor immune responses in the absence of $CD8^+$ T cells, as demonstrated by Corthay *et al.* in a mouse model of multiple myeloma [2]. Tumor growth inhibition was mediated by $CD4^+$ T cells which collaborated with tumor-infiltrating macrophages for antigen-presentation and reciprocal activation. T cell-derived IFN-γ was required for macrophage activation and inhibition of tumor growth [2].

The initiation of any T cell-mediated immune response, being $CD8^+$ or $CD4^+$ T cells, depends on antigen-recognition in the presence of co-stimulatory signals delivered by activated APCs, such as dendritic cells and macrophages [133]. Activation of APCs by bacteria or virus is achieved through the recognition of conserved PAMPs by pattern recognition receptors [134, 135]. However, the sterile inflammation of autoimmune diseases or the induction of antitumor immune responses in the absence of tumor-causing

viruses, poses a problem to the original self/non-self paradigm of Janeway. Polly Matzinger was the first to suggest that there might be an alternative way of inducing immune responses, which is not based on recognition of foreign vs. self, but rather based on the tissues of the body sensing damage or danger [136]. In short, Matzinger proposed that tissues that are distressed or damaged will release endogenous molecules, so-called damage/danger associated molecular patterns (DAMPs), which activate APCs and can initiate immune responses in the presence of antigen [137]. This mechanism of activation has been proven to be of great importance in cancer, as studies have demonstrated the release of DAMPs by apoptotic or necrotic cancer cells [138]. It has also shed light on how radiotherapy and chemotherapy of cancer might indirectly induce antitumor immune responses and how future therapies might be developed to exploit this [139-141].

As previously discussed, macrophages can contribute to direct cancer cell killing or growth inhibition when appropriately activated, as well as activate and guide adaptive immune responses to tumors, similar to bacterial infections, by production of proinflammatory and Th1-stimulating cytokines [3]. Another well-established function of macrophages is phagocytosis of apoptotic cells. Phagocytosis is induced upon recognition of a range of different ligands by receptors expressed by macrophages [142]. Examples of such phagocytosis-inducing ligands, or "eat-me" signals, are cell-surface phosphatidyl serine (PS) and calreticulin (CRT), and they may be counteracted by so-called "don't eat me" signals (

Figure **8**).

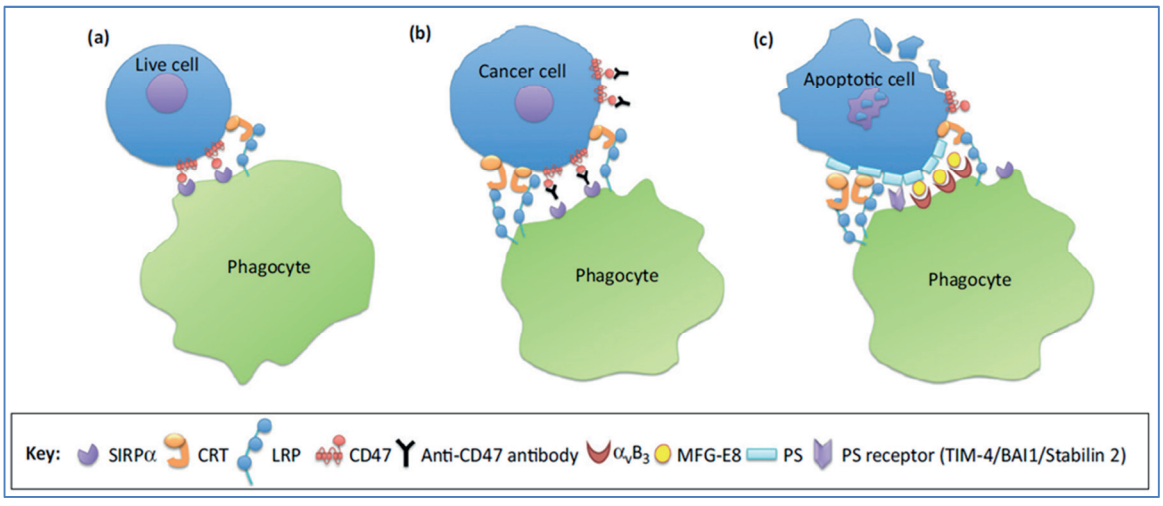

Figure 8 - "Eat-me" and "don't-eat-me" signals in cellular phagocytic uptake. A) Normal healthy cells B) Live cancer cells C) Apoptotic cells. SIRPα, signal-regulatory protein α; CRT, calreticulin; LRP, lipoprotein-related protein; αvβ3, PS receptor via MFG-E8; MFG-E8, milk fat globule EGF factor 8;PS, phosphatidyl serine. Figure adapted from [143] and reused with permission.

Whereas healthy cells do not express CRT on the cell surface, they express higher levels of the "don't eat me" signal CD47 and are protected from clearance by macrophages (Figure 8a) [144]. Cancer cells have been shown to express calreticulin on the cell surface, possibly as a result of ER stress, and to upregulate CD47 to avoid phagocytosis (Figure 8b) [145]. These findings have led to the development of anti-CD47 antibodies able to block the interaction of CD47 with its receptor signal-regulatory protein α (SIRPα) [146]. Anti-CD47 antibodies have been shown to induce cancer-cell phagocytosis by macrophages and priming of T cell immune responses [7]. Apoptotic cells lack such "don't eat me" signals, but display CRT on the cell surface, where it may co-localize with PS and induce phagocytosis and clearance [144, 147]. The phagocytosis and clearance (or absence of clearance) of cancer cells by macrophages is just one example of how the immune system is capable of protecting itself against stressed and damaged cells, and that tumors in turn can escape this protection by altering these same mechanisms. More such mechanisms will be discussed in the next section.

2.4. Tumor immune evasion

The model of cancer immune surveillance and immune protection against cancer triggers the question of how cancer can occur in immunocompetent individuals. Schreiber *et al.* formed a theory to explain this, based on his studies into how tumors that are formed in the absence of an intact immune system are more immunogenic than tumors formed in immunocompetent hosts [96]. His theory of immunoediting seeks to complete the immune surveillance theory with an answer to what happens when tumors outmaneuvers the host immune system and successfully escape this surveillance [148]. Schreiber and colleagues suggest that cancer immunoediting can be viewed as a process with two possible endpoints (Figure 9). Tumor cells which arise from normal cells upon acquiring multiple mutations are recognized and attacked by immune cells in an elimination process, corresponding to what was previously denoted as "immune surveillance". The tumor cells might be successfully eradicated and tissue integrity regained. Alternatively, some tumor cells might acquire additional mutations leaving them resistant to killing by immune cells or able to

inhibit or tune the immune responses to their advantage. This stage is called equilibrium, as the immune cells keep the tumor in check, but fail to remove all cells. Finally, during what is denoted as the escape stage, most remaining tumor cells have become resistant or the immune cells have become sufficiently suppressed to allow the tumor to start growing unchecked and fully transform into malignant cancer [149].

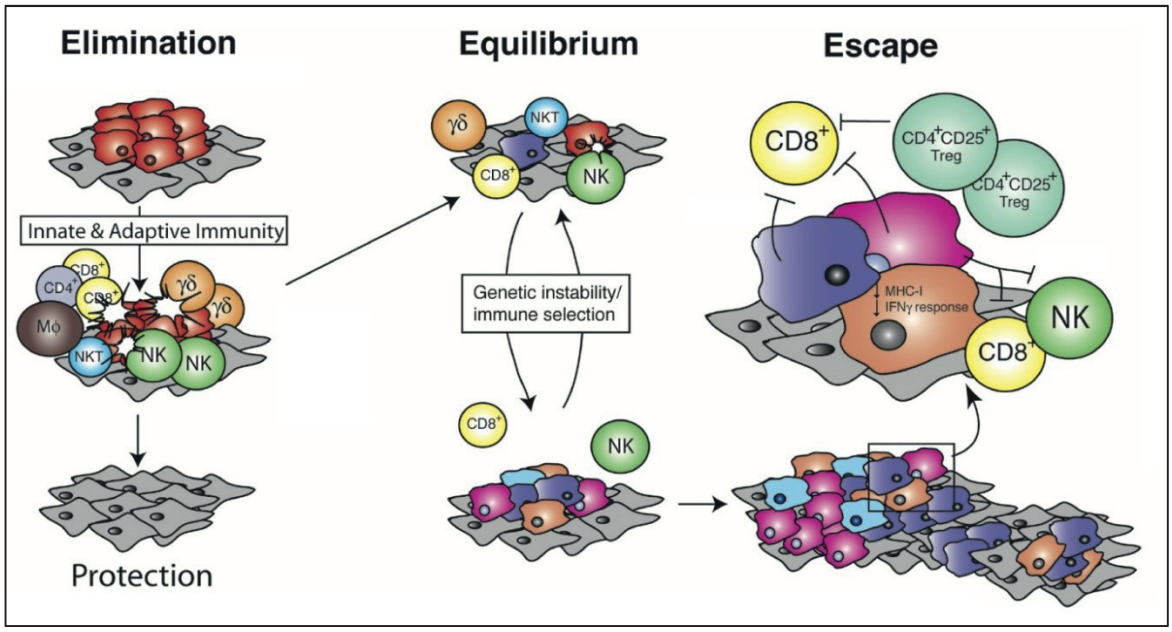

Figure 9 – The three Es of cancer immunoediting: elimination, equilibrium, and escape. Even at early stages of tumorigenesis, these cells may express distinct tumor-specific markers and generate proinflammatory "danger" signals that initiate the cancer immunoediting process. In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immunosurveillance network, may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculpted by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase. $CD4^+$, $CD8^+$, $CD4^+CD25^+$ Treg, γδ and NKT cells are all types of T cell; Mφ cells are macrophages and NK cells are natural killer cells. Figure is adapted from [150] and reused with permissions.

Several mechanisms underlying tumor immune escape have been described. Downregulation or abrogation of tumor antigen presentation has already been mentioned, and tumors may also limit antigen-presentation by interfering with the differentiation and function of APCs [151-153]. Tumor-derived vascular endothelial growth factor (VEGF)

was shown to play a role in this interference [154], possibly through the induction of PD-L1 on APCs [155] which may mediate an inhibitory signal via its receptor PD-1 on T cells [156]. Furthermore, other tumor-derived factors such as IL-10 [157] or tumor growth factor- β (TGF-β) [158] and environmental factors such as tumor hypoxia [159] or lactic acid build-up [160] can also influence the phenotype and function of APCs.

Upon appropriate priming and activation of tumor-specific T cells, there are still possible pitfalls which tumor cells might take advantage of, in order to prevent T cells from recognizing and eliminating them. T cells, as other immune cells, depend on chemokines for trafficking into cancer lesions, and aberrant modifications of expressed chemokines in tumors have been shown to reduce attraction of tumor-specific cytotoxic lymphocytes [161]. T cells which arrive in the tumor though the vasculature must then cross the endothelial barrier. The exact mechanism by which the tumor vasculature may prevent this, is not clear, but VEGF-mediated inhibition of adhesion molecules [162] or skewing of endothelial cells towards T cell inhibition [163] has been implicated. Once arrived in the tumor, effector T cells are subjected to several suppressive signals. Two cell types which may suppress the functions of effector T cells are tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs).

Tumor-associated macrophages and myeloid-derived suppressor cells

Tumors induce the accumulation of diverse cells of monocyte-origin through secretion of chemokines such as C-C motif ligand 2 (CCL2) [164]. These cells include fully differentiated macrophages, so-called TAMs [165, 166], and immature cells of myeloid origin, called MDSCs [167]. TAMs and MDSCs are regarded as separate entities, but they share many characteristics and boundaries between the cell types are not definite [168]. TAMs are commonly regarded in bulk as immune suppressive [86], however several studies have described subsets of differentially activated macrophages among TAMs in mice. Movahedi *et al.* showed that in a mouse model of subcutaneous mammary tumors, tumors contained high numbers of blood monocyte-derived macrophages with highly variable expression of surface markers such as MHC-II [169]. The different subsets of macrophages (MHC-II^{low} vs MHC-II^{high}) varied in their gene expression levels of important macrophage antitumor genes, such as iNOS, were localized to different tumor regions and displayed different kinetics, with accumulation of MHC-II^{low} macrophages as the tumors grew [169].

Cancer cells have been shown to tune the accumulating TAMs towards an immune-suppressive phenotype by multiple mechanisms. Production of TGF-β by Lewis lung carcinoma cells (LLCs) were shown to induce expression of Interleukin receptor-associated kinase (IRAK)-M in TAMs [170]. IRAK-M is a negative regulator of TLR signaling, and IRAK-M-deficient mice displayed reduced growth of LLCs and more TAMs of an M1 antitumor phenotype than wild type (WT) mice. This finding indicates that macrophages infiltrating tumors are not inherently of a protumor subtype, but is actively directed to this phenotype by tumor-derived factors.

Both TAMs and MDSCs may suppress T cell proliferation and cytokine production through several mechanisms, including production of anti-inflammatory cytokines or mediators such as arginase, nitric oxide (NO) and reactive oxygen species (ROS) [171]. Interestingly, MDSCs might account for the previously mentioned tolerogenic APCs, as MDSCs has been described to activate and expand tolerance-inducing Treg populations [172]. Tregs have been found to be increased at tumor sites [173] and carry antigen-receptors specific for tumor antigens [174]. However, the role of Tregs in cancer has been shown to be variable, exemplified by studies showing that $FoxP3^+$ Tregs has both a positive and a negative effect on overall survival, depending on tumor type, tumor site and stage [175].

In addition to the mechanisms already mentioned, both TAMs and tumor cells may express PD-L1 and PD-L2, which directly inhibit T cell proliferation, cytokine production and cytolytic function upon binding to PD-1 on T cells [156]. Inhibiting the interaction of PD-1 with its ligands and thereby releasing T cells from this inhibitory checkpoint has proven very beneficial in the treatment of several types of cancer [117]. However, macrophages have been shown to mediate escape from anti-PD-1 mAb therapies [176]. In the end, blocking one immune-suppressive mechanism is unlikely to be sufficient for most patients and targeting multiple pathways and cell types will be an important goal.

3. Cancer immunotherapy

Restoring or reactivating antitumor immune responses in advanced cancer has been regarded as a quite impossible task. Yet, with the improved understanding of basic immunology and the discovery of new regulatory pathways, this view is changing. The field of immunotherapy is in rapid development and expanding fast, covering a whole range of exciting treatment targets and effector mechanisms. This section aims to give an overview of the most important types of immunotherapy currently in use (summarized in Table 1) or being developed, and put the work of this thesis in the context of our ultimate goal: improved treatment options for cancer patients.

3.1. Cancer vaccines

The first therapy of cancer patients which resulted in an immunologically based effect is considered to have been performed by William B. Coley, who injected streptococcal organisms into patients with inoperable cancer [102]. He then optimized his treatments by using a mixture of bacterial products which became known as Coley's Toxins. Skepticism and critique resulted in the abandonment of his discovery [177], however, he is now recognized as a pioneer. The basic principle of his discovery has enjoyed a rebirth upon the discovery of pattern recognition receptors such as TLRs, which respond to bacterial products [178]. Activation of TLRs and other related receptors is crucial for the activation of innate immune responses, which in turn leads to the activation of adaptive and protective immune responses.

Similar to William Coley, the husband and wife team of John and Ruth Graham published a study ahead of their time in 1959, showing the first clinical effect in a cancer vaccine study [179]. They had treated a cohort of 114 patients with gynecologic cancer with autogenous tumor lysates mixed with Freund's adjuvant, which, similar to Coley's toxin, contain various bacterial products. The results were highly intriguing, with 22% remission or stable disease and even some complete remissions, but work went on un-noticed. At the time, no mechanism underlying these effects could not yet be postulated as both T cells, antigen-presenting dendritic cells and MHC restriction was yet to be discovered [180].

With a much improved understanding of the immune system and the immunogenicity of tumors, the generation of cancer vaccine antigens has moved from whole-tumor preparations towards molecularly-defined non-self antigens which can be

patient-specific. Approaches vary from peptide or mRNA-based to viral delivery, as well as the addition or not of adjuvants such as poly(I:C). Vaccines aim to enlarge the pool of tumor-specific T cells and induce T cell infiltration and tumor killing. Despite showing induction of tumor-specific T cells responses, most therapeutic vaccines have failed to treat established disease, and this is considered to be due to immune evasion mechanisms [181]. However, cancer vaccines have shown great promise in preventing disease recurrence [182, 183], and it is suggested that combining vaccines with other immunotherapies might improve clinical benefits [181]. Studies in mice have shown that combining cancer vaccines with T cell checkpoint inhibitors can succeed where vaccine alone failed to induce functional T cell responses [184, 185], indicating the importance of abrogating tumor-induced immune suppression.

In 2010, the FDA approved the first therapeutic cancer vaccine, sipuleucel-T, for use in metastatic prostate cancer [116]. This vaccine is based on isolation of APCs from each patient and culture of these cells with target proteins, before the APCs are infused back into the patient. Despite the challenge of an extensive manufacturing process, the approval of the vaccine marks an important step forward and is considered to be one of the two main events which lead to the revival of cancer immunotherapy [180]. The other event was the FDA approval of ipilimumab, which is a monoclonal antibody against CTLA-4, and the first drug in a new class of immunotherapies called checkpoint inhibitors [115].

3.2. Checkpoint inhibitors

In 1994 the group of Jeffrey Bluestone was the first to suggest that CTLA-4, a T cell surface protein first cloned by Burnet *et al.* in 1987 [186], inhibit T cell proliferation upon engagement [187]. This was confirmed by the group of Jim Allison, who showed that blockade of CTLA-4 with antibodies resulted in enhanced CD28-mediated stimulation and T cell proliferation [188]. CTLA-4 is closely related to the co-stimulatory receptor CD28, and binds the same ligands, co-stimulatory ligand B7-1 and B7-2 (now called CD80 and CD86 respectively), expressed on APCs [189]. In 1996, Allison and colleagues published results showing that *in vivo* blocking of CTLA-4 by antibodies resulted in rejection of pre-established tumors and immune protection against secondary tumor induction [190]. The authors hypothesized that the poor immunogenicity of many tumors is caused by their inability to overcome CTLA-4 inhibition and mediate CD28-costimulation [190]. Monoclonal anti-CTLA-4 antibody entered clinical trials, but challenges with interpreting and understanding the novel response kinetics of this immunotherapy first had to be solved.

Primary end points were changed from response-based end points (overall response rate or progression-free survival) to overall survival, which finally resulted in a positive clinical trial [115], and lead to the FDA approval of ipilimumab in 2011. The lessons learned from this very first checkpoint inhibitor resulted in the characterization of immune-related adverse events (irAEs) and the creation of corresponding treatment algorithms [191, 192].

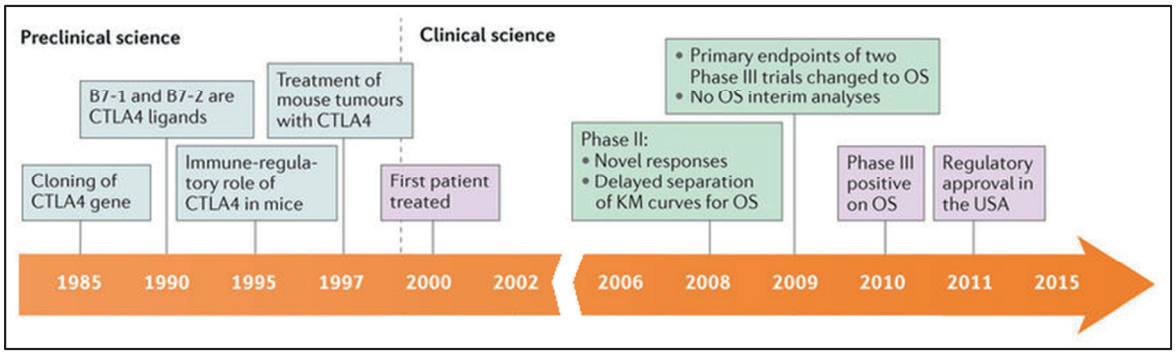

Figure 10 - Ipilimumab drug-development milestones. After basic and preclinical scientific advances, ranging from the cloning of the CTLA4 gene in 1987 to the elucidation of its role in tumor immunology by Allison and colleagues in the mid-1990s, the ipilimumab (anti-CLTA4) monoclonal antibody was introduced into clinical trials in melanoma in 2000. Lessons learned from extensive clinical trials, with regards to novel response kinetics and delayed separation of survival curves, led to the change of primary end points for the ipilimumab pivotal studies from response-based end points (overall response rate or progression-free survival) to overall survival (OS) and ultimately led to a positive Phase III study. Ipilimumab was approved for metastatic melanoma in 2011. B7-1, T lymphocyte activation antigen CD80; B7-2, T lymphocyte activation antigen CD86; KM, Kaplan–Meier. Figure adapted from [110] and reused with permission.

More than two decades spans the initial scientific discovery of CTLA-4 and its clinical application (Figure 10), but this one ground-breaking success was quickly followed by others. In 2014, a new checkpoint inhibitor called Pembrolizumab received special status as breakthrough therapy, priority review and orphan drug, and was granted an accelerated approval by the FDA for the use in treatment of advanced melanoma [193]. Pembrolizumab is a monoclonal antibody against PD-1, which blocks the interaction with its ligands PD-L1 or PD-L2 [193]. The PD-1 pathway was the second checkpoint pathway discovered and it functions similar to the CTLA-4 pathway in inhibiting T cell proliferation and stimulation, although the two pathways were discovered independently. PD-1 was first cloned in the lab of Tasuku Honjo in 1992 [194] and proven to be a negative regulator of lymphocyte activation [195]. Liebing Chen first described the major

ligand of PD-1, called PD-L1 (previously B7-H1), which was another member of the B7 family [196]. Furthermore, Chen showed that many human tumors up-regulate PD-L1 and that antibodies blocking the interaction between PD-1 and PD-L1 could lead to tumor regression in mice [197]. Thus, the PD-1 pathway emerged as another pathway of tumor-induced immune suppression that could be targeted by blocking antibodies with the goal of releasing anti-tumor immune responses [198]. Nivolumab, another anti-PD-1 antibody, was approved by the FDA in 2015, and the two anti-PD-L1 antibodies atezolizumab and durvalumab were approved in 2016 and 2017 respectively (www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs).

Since then, checkpoint inhibitors have been approved for multiple new cancers and there is intense investigation new potential theraputic targets involved in T cell activation control [199, 200]. T-cell activation has been shown to be regulated by the interplay of the stimulatory and inhibitory ligand–receptor interactions between T cells, dendritic cells, tumour cells, and macrophages in the tumour microenvironment (TME) (Figure 11). The combination of anti-CTLA-4 with anti-PD-1 antibodies has also been approved based on greatly improved clinical effect compared with single therapy, although at the cost of more severe side effects [201]. Despite great advances and encouraging improvements on 1-year or 3-year survival, the rate of patients responding to checkpoint inhibitors is in the range of ~20% (monotherapy with anti-CTLA-4) to ~60 % (combination of anti-CTLA-4 with anti-PD-1) [202]. Response rates have been found to correlate with tumor immunogenicity in the form of overall mutational load and active immune evasion by tumor cell expression of PD-L1, as well as other factors [200, 203].The search for biomarkers able to predict response is currently ongoing and the validation of markers, such as PD-L1 expression, immune cell infiltration or mutational load, will be an important next step for improving the clinical use of these checkpoint inhibitors [200].

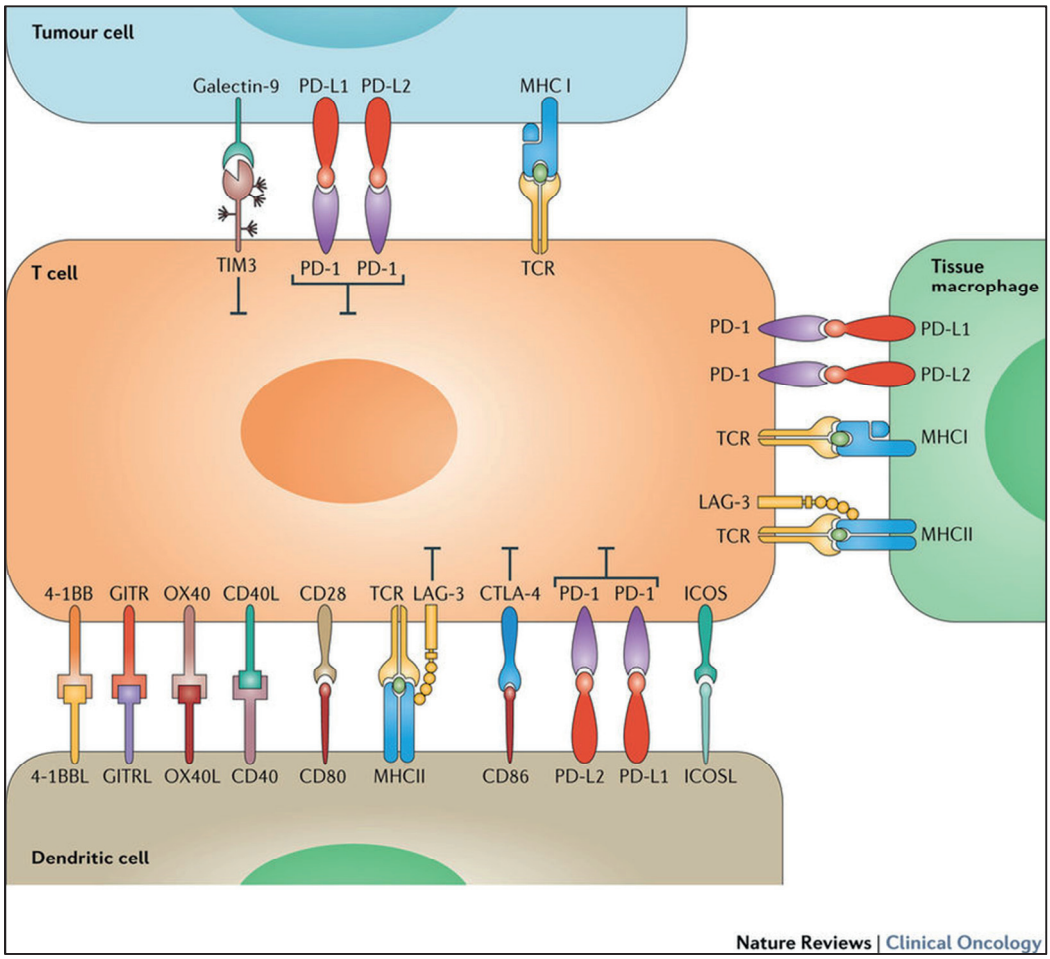

Figure 11 – Suppressive and stimulatory receptor-ligand pairs controlling T cell activation. Figure taken from [204] and reused with permission.

3.3. Adoptive transfer of immune cells

Checkpoint inhibitors aiming to release tumor-specific T cells from tumor-mediated suppression would be predicted to fail in the absence of or insufficiency in previous infiltration of anti-tumor T cells. T cell infiltration has been shown to vary greatly between tumor types as well as individual patients, but the reasons for this have not yet be properly elucidated [205]. One solution to this problem is adoptive cell transfer (ACT) of tumor-infiltrating T cells (TILs) in high numbers. The concept, originally consisting of isolation of patient-derived TILs followed by expansion of these cells *ex vivo* and the subsequent re-infusion into the same patient was pioneered by Steve Rosenberg [206, 207]. Adoptive transfer of TILs administered in conjunction with interleukin-2 (IL-2) in patients with metastatic melanoma resulted in overall response rates and complete response rates of around 50% and 20%, respectively [208]. Most interestingly, a subset of patients receiving

a single infusion of T cells became long-lasting complete responders, demonstrating the great potential of antitumor T cell responses.

The application of TIL therapy to other types of cancer was limited though, due to poor antigenicity, tumor growth at less accessible sites and problems to obtain sufficient number of cells [209]. Current strategies to improve ACT are based on utilizing genetic engineering of T cells to express tumor-specific antigen receptors, and the use of allogenic cells from healthy donors. The strategies can be divided into two main categories depending on the receptors used: traditional αβ TCRs, which recognize epitopes of intracellular antigens presented by MHC molecules, or chimeric antigen receptors (CARs). CARs are antibody single-chain variable fragments joined with T-cell receptor (TCR) and T-cell costimulatory receptor signaling domains. CAR T cells can recognize cell-surface antigens in the absence of MHC molecules, as binding of the CAR receptor alone provides sufficient stimulatory signaling to induce T cell proliferation and killing of the target cell [209] (Figure 12). B cell lymphomas are especially well suited for treatment with CARs. These cells can be specifically recognized and targeted by their cell-surface expression of CD19, as CD19 is only expressed by B cells, both normal and malignant. CD19 CAR T cell therapy has displayed an impressive efficacy and long lasting responses in clinical trials treating B cell acute lymphoblastic leukemia (B-ALL), with reported complete response rates in the range of 70-90% [210-212]. This resulted in FDA approval of two different CD19 CAR-T cell therapies in 2017, and such treatment has completely changed the outlook for B-ALL patients, where the previous median survival was 24 weeks and the 5-year survival was 7% in the pre-CAR-T-cell therapy era [213].

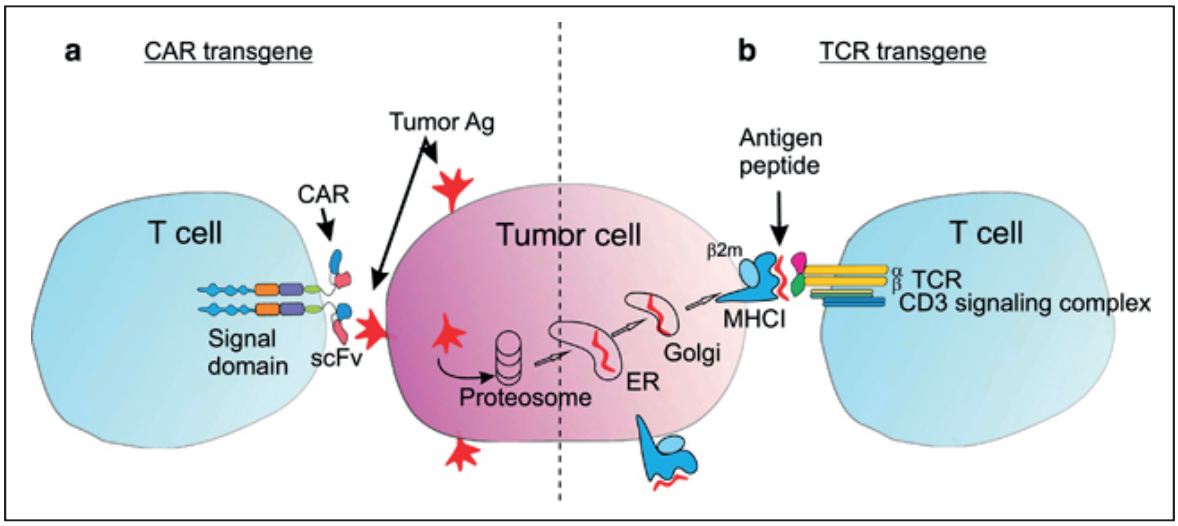

Figure 12 – Two strategies for genetically engineering antitumor T cells. (a) Cell surface antigen can be recognized by a CAR expressed by T cells. The CAR is composed of an extracellular single-chain antibody domain (scFv) linked by a hinge and transmembrane domains to several intracellular signaling domains, here represented by different colors. CARs are often expressed as dimers, as shown here. **(b)** Intracellularly processed antigen can be recognized by a transgene-encoded TCR expressed by T cells. The TCR associates with endogenous signaling molecules derived from the CD3 signaling complex. Figure taken from [214] and reused with permission.

Other CD markers on hematological tumors are being investigated as targets for CAR T cell therapies, including CD38, CD22, CD123 and others [215]. However, the use of CAR T cell therapies in solid tumors is limited by several factors. These include the presence of an immune suppressive microenvironment, antigen heterogeneity across the same malignancy and toxic "on-target, off-tumor" effects if target antigens are expressed in essential organs [216]. Current investigations into solid tumor targets include VEGFR2, EGFRvIII for glioblastoma and mesothelin for mesothelioma, pancreatic and ovarian cancer [209, 217].

In an alternative strategy, traditional MHC-restricted αβ TCRs are genetically engineered to recognize the target gene of interest (Figure 12). Such genetically engineered TCRs have the great advantage of being theoretically able to recognize any expressed protein in the cell, and can specifically target mutated proteins while sparing the non-mutated form [218].The choice of target antigen remains the big question, and several approaches have been tried, including tumor-associated antigens, such as gp100 and MART-1, or germline antigens only expressed on tumors or during fetal development,

such as NY-ESO-1 and MAGE [219]. Recently, efforts have concentrated on identifying cancer-specific mutations and the corresponding TCRs able to recognize them [220, 221]. Ideally, mutations in cancer-driving genes would be targeted, in order to limit antigen-loss and development of therapy resistance. Whole-exome sequencing of tumor samples have enabled easier identification of mutated peptides expressed by tumor cells, and TILs which specifically recognizes such antigens have been identified [222]. Additional issues which need to be addressed includes determining the composition and characteristics of the final T-cell product to be made ($CD8^+$ vs. $CD4^+$, effector vs memory features), coping with T cell exhaustion and immune suppression, and including sufficient safety switches such as suicide genes [219]. Combining TCR/CAR T cell therapies with recombinant cytokines, such as IL-2, has been proven to improve outcomes [223], and further cytokines, including IL-7 and IL-15 are being tested [224].

3.4. Cytokine therapy

Currently, IFN-α and IL-2 are the only cytokines to have received FDA approval for cancer treatment. IFN-α belong to the type I IFN family and comprise a group of at least twelve distinct isoforms, where IFN-α-2a, IFN-α-2b and IFN-α-2c are most commonly used in the clinic [225]. IFN-α along with IFN-β bind to the interferon-α/β receptor (IFNAR), activate Janus kinase (JAK) and STAT signaling pathways, and affect immune cells and tumor vasculature[226], as well as cancer cells directly [227]. Recombinant IFN-α has been widely studied, but is currently only approved a limited number of malignancies [228]. It is thought that the clinical benefit has been limited due to dose-limiting side effects and lack of understanding of the mechanisms of actions involved [228, 229].

In 1992, IL-2 received FDA approval as the second recombinant cytokine. IL-2 drives the proliferation of effector lymphocytes and recombinant IL-2 has been crucial for the development of adoptive T cell therapy [230]. IL-2 is used for the *in vitro* expansion of patient derived T cells as well as given alongside cellular therapies to improve the survival and expansion of T cells *in vivo.*

3.5. Oncolytic viruses

Cancer cells accumulate damage in cell signaling and responses to stress or infection, which enable certain viruses to selectively enter and replicate within such cancer cells [97, 231, 232]. The ability of viruses to infect and kill cancer cells was described many decades ago [233, 234], but only in 2015 did the first oncolytic virus, Talimogene laherparapvec

(T-VEC), receive FDA approval. T-VEC is a genetically modified herpes simplex virus, type 1, which is attenuated by the deletion of several virulence genes and is expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) to help promote priming of immune responses [235]. The approval was based on improved durable responses in patients with melanoma upon T-VEC therapy in a phase III study [236].

T-VEC has been shown to mediate clinical benefit through two major mechanisms: direct killing of cancer cells and induction of an immune response against the cancer. The lysis of cancer cells is beneficial in itself, but also results in the release of new viral particles, tumor-associated antigens and danger-associated molecular factors. Oncolytic viruses may thus work as an in situ cancer vaccine, yielding both cancer antigens and immune stimulating agents. The complex interactions between virus, cancer cells and the immune system are still incompletely understood, but a summary of the current knowledge of T-VEC mechanism of action is depicted in Figure 13.

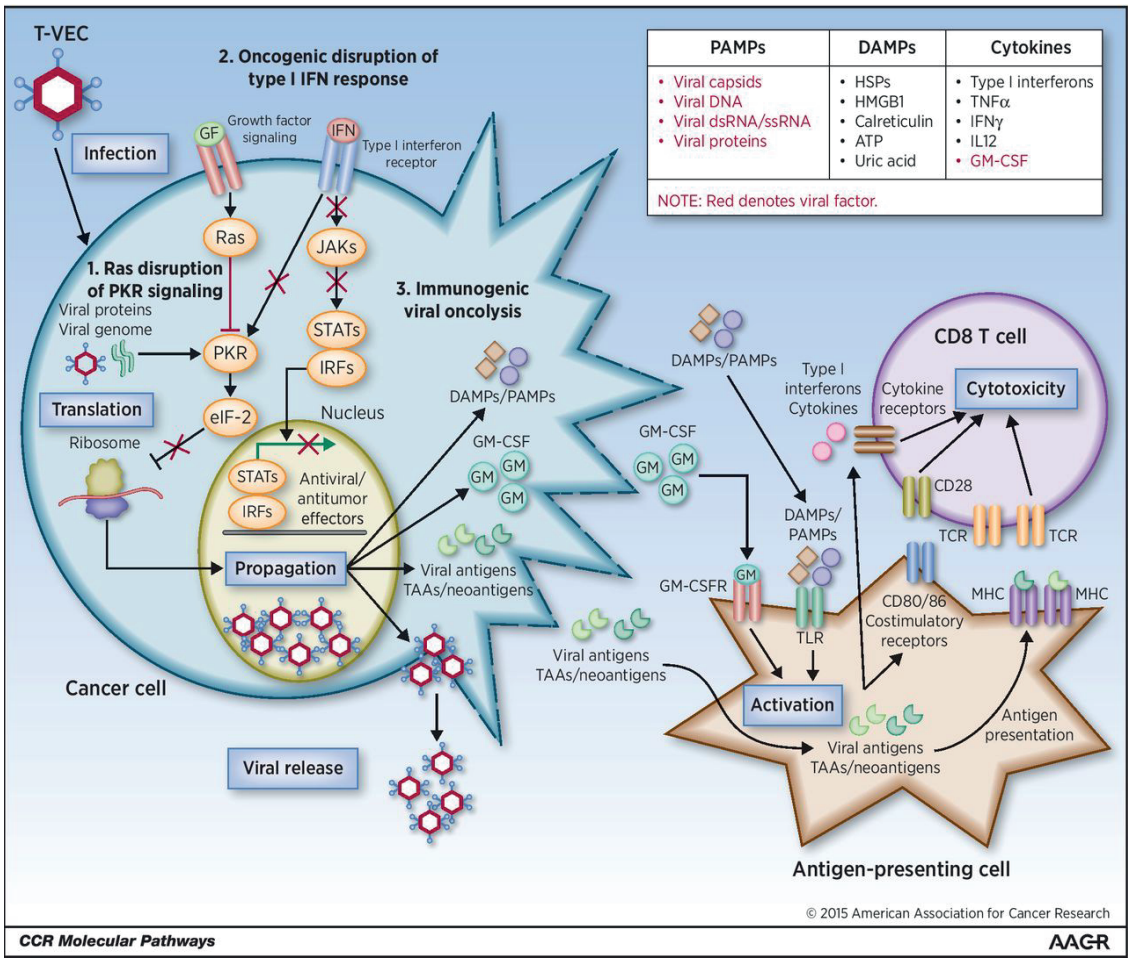

Figure 13 - Mechanism of action for T-VEC. T-VEC preferentially replicates in cancer cells because of disrupted PKR activity, which may be lower due to overactive Ras signaling. PKR

signaling regulates cell proliferation by phosphorylating eIF-2, which prevents delivery of Met-tRNAi to ribosomes blocking protein translation. T-VEC also replicates in cancer cells because of disrupted type I IFN signaling. Type I IFN signaling activates PKR to block protein translation and induces transcription of IFN-stimulated genes that promote antitumor and antiviral responses. Following viral replication and propagation in the nucleus, mature virions assemble in the cytoplasm and induce cell lysis releasing new progeny virions that can infect other tumor cells and various proimmunogenic factors, such as viral-based pathogen-associated molecular patterns (PAMP), cell-derived DAMPs, and cytokines. These factors recruit antigen-presenting cells, such as dendritic cells that can present tumor-associated antigens to cytotoxic CD8+ T cells. The T cells, in turn, can mediate direct rejection of other tumor antigen-expressing cells. Depicted in red are factors that are directly produced by T-VEC infection and in black are factors produced as a result of an antiviral response. eIF-2, eukaryotic initiation factor 2; GM, GM-CSF; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; HMGB1, high mobility group box 1; IRF, IFN regulatory factor; PKR, protein kinase R; TAA, tumor-associated antigen; TCR, T-cell receptor; TLR, Toll-like receptor. Figure taken from [232] and reused with permission.

The development and approval of T-VEC as a first-in-class agent have demonstrated that oncolytic viruses may be safe, and able to enhance immune responses and promote tumor rejection [237]. This has caused significant interest in the development of classes of oncolytic viruses, including herpesvirus, poxvirus, picornavirus, adenovirus, paramyxovirus, parvovirus, reovirus, Newcastle Disease virus and rhabdovirus [232]. The ability of each virus to infect cancer cells depends on the presence of specific viral entry receptors on the cancer cells, and clinical indications for oncolytic viruses are therefore likely to vary [232]. The presence of pre-existing neutralizing antibodies to viruses that are common in the general population cause a barrier to infection of cancer cells which needs to be overcome, along with physical barriers to infection caused by the tumor microenvironment such as decreased tissue vascularization or dense extracellular matrix composition [232].

So far, therapeutic use and benefit of T-VEC monotherapy in melanoma has been limited, due to the fast development of other potent immunotherapy drugs and lack of insight into optimal patient selection [238]. This might soon change, as several innovative strategies to improve the potency of oncolytic viruses are being developed. Examples include the use of oncolytic viruses as viral expression vectors for intratumoral cytokine delivery or the combination of oncolytic viruses with immunotherapies that improve the T cell immune responses induced by cancer cell infection [231].

3.6. Monoclonal antibodies

Since the creation of the hybridoma technique in 1975 [239], production of monoclonal antibodies (mAbs) have been critical for medical sciences and the first therapeutic mAb was approved by the FDA in 1992 (Orthoclone OKT3 against CD3 in organ transplant rejection). In 1997 the approval of rituximab, a mAb against CD20, followed, and marked the first use of mAb in cancer therapy [240]. MAbs can be targeted towards most any target on the surface of cancer cells, and be designed to function in multiple different ways. MAbs can be designed to block the interaction of a receptor-ligand pair and ameliorate signaling, or to tag cancer cells and direct them for antibody-dependent cellular cytotoxicity (ADCC). Macrophages express the receptors required for ADCC, namely Fcγ receptors which recognize the constant region of antibodies, and are important effector cells for ADCC alongside NK cells [241]. Monocytes and macrophages mediate antibody-dependent cellular phagocytosis (ADCP) in addition to ADCC [242], and studies have shown that macrophage depletion with liposomal clodronate eliminated the ability of anti-CD20 antibodies to deplete normal and malignant B cells [243]. Optimization of mAb-design for augmentation of the macrophage response is currently being investigated, and one promising strategy is targeting of the macrophage inhibitory CD47/SIRPα axis [241].

Development of novel mAb-based therapies are also ongoing, exemplified by the creation of Bi-specific T cell engagers (BiTE). BiTEs are a specialized form of bispecific monoclonal antibodies aimed to direct cytotoxic T cells to cancer cells by simultaneous binding of CD3 and a cancer marker. The BiTE Blinatumomab is a CD19-/CD3-bispecific antibody which allows T cells to directly recognize CD19+ lymphoma cells and has received FDA approval [244, 245].

3.7. Strategies targeting monocytes and macrophages

As previously described, macrophages are important effector cells for cancer immunotherapies such as vaccines or monoclonal antibodies. Macrophages can also be targeted directly by therapeutic strategies in order to improve the antitumor functions or limit the tumor-promoting functions of TAMs [246]. Four main strategies aimed to shift this balance have been described, as summarized in Figure 14.

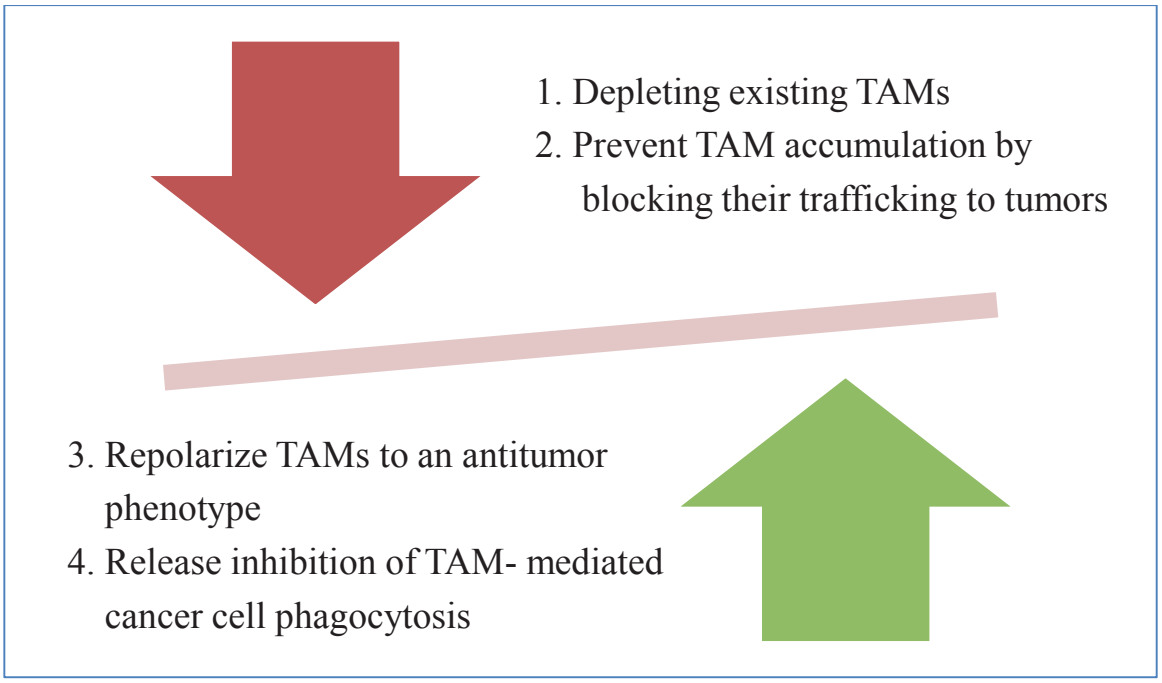

Figure 14 – Four strategies for therapeutic targeting of tumor-associated macrophages (TAMs).

The chemotherapeutic drug trabectedin is an example of the first strategy, as it was shown that its anticancer effect was due to selective induction of cell cycle arrest and death in monocytes and macrophages [247]. Secondly, TAM accumulation in tumors have been shown to depend on production of CCL2 by cancer cells [164] and the expression of CCR2 on monocytes and macrophages [248], and targeting this axis has shown preliminary treatment responses in mouse models and patients with advanced solid tumors [249-251]. A monoclonal antibody against CCL2, Carlumab, was tested in several phase I trials and showed both a promising safety profile and clinical responses [6, 252].

Colony-stimulating factor-1 (CSF-1 or M-CSF) has been shown to be over-expressed in many tumors and to be involved in TAM accumulation, differentiation and survival [253]. Thus, inhibitors of the CSF-1 receptor (CSF-1R) have been investigated for therapeutic effect in several types of cancer [254-257]. Interestingly, one study in glioblastoma multiforme revealed that CSF-1R inhibition affected the TAM phenotype without depletion, reducing markers of tumor-promoting functions [258]. This indicates that blocking the CSF-1/CSF-1R axis may be beneficial in all three strategies targeting TAMs, and different agents including mAbs have been tested in clinical trials [259, 260].

Reprogramming of TAMs from tumor-promoting to tumor-suppressive phenotypes has been achieved both *in vitro* and *in vivo* by a range of agents and such reprogramming have been shown to be of clinical significance. One striking example is a study by Beatty *et al.*, in which gemcitabine chemotherapy was combined with agonistic CD40 mAb in order to reverse immune suppression and drive antitumor T cell responses [5]. The combination treatment successfully resulted in tumor regression in patients with pancreatic ductal adenocarcinoma (PDA), however the tumor infiltrating immune cells were dominated by macrophages with an absence of lymphocytes [5]. The combination of gemcitabine and anti-CD40 was further studied in a mouse model of PDA, and the therapeutic benefit was shown to be mediated by macrophages, independent of T cells.

CD40 signaling was shown to result in activation of TAMs to a tumoricidal phenotype [5], similar to what had previously been shown in mouse peritoneal macrophages by Buhtoiarov *et al.* [261]. Macrophages activation by CD40 stimulation depended on endogenous IFN-γ production [261], and combined treatment with anti-CD40 and LPS or the TLR9 agonist CpG oligodeoxynucleotide 1826 (CpG-ODN) resulted in a synergistic effect with increased killing of cancer cells *in vitro* [262]. Treatment of B16 melanoma-bearing mice with CpG-ODN alone also induced macrophage-dependent antitumor effects, which involved NO, TNF-α and IFN-γ [263]. Finally, Buhtoiarov *et al.* showed that a combination of multidrug chemotherapy with anti-CD40 + CpG-ODN immunotherapy synergized for the induction of antitumor effects in mouse models of melanoma and neuroblastoma [264]. Combination treatment with chemotherapy and anti-CD40 + CpG-ODN immunotherapy resulted in the phenotypic repolarization of TAMs towards a pro-inflammatory and antitumor phenotype [264]. The findings of Buhtoiarov and Beatty demonstrate that modulating an immune suppressive tumor microenvironment by targeting the phenotype of TAMs is possible, and that agonistic CD40 mAbs are strong therapeutic candidates for cancer immunotherapy [265].

TLR agonists, such as CpG, have been investigated for antitumor effects through multiple mechanisms, as reviewed by Dajon et al [266], including direct stimulation of tumor cells leading to apoptosis, reduced immune suppression by Tregs and increased cytotoxicity of NK cells and T cells. However, many of the studies referred to, failed to test the effect of TLR agonists on the phenotype and function of TAMs. Repolarization of TAMs towards an antitumor phenotype have been demonstrated upon treatment with TLR3 agonist poly(I:C) [267], a combination of TLR7 agonist and TGF-β inhibitor [268],

and TLR2 stimulation through infection with attenuated Listeria monocytogenes [269]. Furthermore, attenuated TLR signaling through IRAK-M activation and increased IL-10 production has been shown to be one of the pathways whereby cancer cells polarize TAMs towards a protumor phenotype [170, 270]. This indicates that repolarizing TAMs through stimulation of TLRs, likely in combination with additional signals, is a potent strategy for macrophage-targeted immunotherapies.

Interestingly, several microRNAs (miRNAs) have been shown to be important in macrophage activation through their regulation of TLR signaling pathways, although the exact mechanisms remain incompletely understood [271-273]. MiRNA-155 was shown to be upregulated upon TLR activation and downregulated in TAMs from a mouse sarcoma model [274]. The overexpression or depletion of miR-155 in TAMs shifted the activational status towards an antitumor or protumor phenotype respectively [274]. Depletion of miRNA-21 was shown to promote an antitumor phenotype of TAMs, resulting in therapeutic benefit *in vivo* [275]. Furthermore, several miRNAs have been shown to mediate the cancer cell-induced polarization of TAMs towards a protumor phenotype [276-278]. Targeting miRNAs is emerging as a novel strategy for macrophage-directed cancer immunotherapies [273, 279].

As a fourth strategy targeting TAMs, several studies have investigated the therapeutic potential of removing cancer cell-induced inhibition of macrophage phagocytosis [7, 146]. The main molecule targeted is CD47, which is expressed at high levels by many types of cancer cells and works by binding SIRPα, an inhibitory receptor on macrophages [145], as previously discussed in introduction section 2.3. The anti-CD47 antibody Hu5F9-G4 is currently being tested in safety and efficacy against both solid tumors and hematological malignancies (NCT02216409, NCT02678338, NCT02953509, NCT03248479 and NCT02953782), and additional antibodies have also made it to clinical trials [280]. There are some controversies and problems with this strategy [281], but also great hope stemming from the recent advances in T cell check point inhibitors.

Finally, a study by Guerriero *et al.* demonstrated a highly novel strategy for targeting TAMs by modulating the epigenetic regulation these cells [9]. The class IIa histone deacetylase (HDAC) inhibitor TMP195 was shown to repolarize TAMs from mouse mammary carcinoma towards an antitumor phenotype and induce macrophage-dependent reduction in tumor burden [9]. TMP195 treatment induced the recruitment of

highly phagocytic and immune stimulatory macrophages into the tumors and synergized with chemotherapy and checkpoint blockade [9]. To summarize, there are multiple different ways of targeting TAMs with the aim of limiting their protumor effect and stimulate their antitumor potential. Such strategies may successfully be combined with other immunotherapies for improved therapeutic benefit, but to date, none have been approved for clinical use.

Class	Target	Drug	Clinical indication	Approved
Therapeutic vaccine	GM-CSF + PAP fusion	Sipuleucel-T (Provenge)	• Met. castrate-resistant prostate cancer	2010
Checkpoint inhibitor	CTLA-4	Ipilimumab (Yervoy ®)	• Met. melanoma	2011
	PD-1	Pembrolizumab (Keytruda®)	• Met. urothelial carcinoma, melanoma, NSCLC, HNSCC • Classical Hodgkin lymphoma • Met. gastric adenocarcinoma • Met. MSI-H or dMMR tumors	2014
	PD-1	Nivolumab (Opdivo ®)	• Met. melanoma and HNSCC • Squamous NSCLC • Advanced renal cell carcinoma • Classical Hodgkin lymphoma • Met. urothelial carcinoma • MSI-H or dMMR metastatic colorectal cancer	2014
	PD-L1	Atezolizumab (Tecentriq™)	• Met. bladder cancer and NSCLC	2016
	PD-L1	Durvalumab (Imfinzi™)	• Met. urothelial carcinoma and bladder cancer	2017
	PD-L1	Avelumab (Bavencio)	• Met. bladder cancer • Merkel cell carcinoma	2017
CAR T cell therapy	CD19	Tisagenlecleucel (Kymriah)	• Refractory or recurrent B-cell ALL	2017
	CD19	Axicabtagene (Yescarta™)	• Refractory large B-cell lymphomas	2017
Cytokine therapy	IFN-α 2b	Intron® or Roferon ®	• Hairy-cell leukaemia • CML and follicular NHL • Melanoma • AIDS-related Kaposi's sarcoma	1986
	IL-2	Aldesleukin (Proleukin)	• Malignant melanoma • Renal cell carcinoma	1992
Monoclonal antibodies	CD20	Rituximab (Rituxan)	• NHL	1997

	HER2	Trastuzumab (Heceptin)	• $HER2^+$ breast cancer	1998
	CD52	Alemtuzumab (Campath)	• B-cell CLL	2001
	VEGFR	Cetuximab (Erbitux)	• Metastatic colon cancer	2004
	VEGR	Bevacizumab (Avastin)	• Colon cancer	2004
	RANKL	Denosumab (Xgeva)	• Cancers with bone metastases	2010
	CD38	Daratumumab (Darzalex)	• Multiple myeloma	2015
	GD2	Dinutuximab (Unituxin)	• Neuroblastoma	2015
BiTE	CD19	Blinatumomab (Blincyto)	• Refractory or recurrent B-cell ALL	2014
		IL-2 (Proleukin ®)	• Metastatic renal cell carcinoma • Metastatic melanoma	1992
TLR agonists	TLR2 (+)	BCG	• Non-invasive bladder cancer	1990
	TLR7	Imiquimod	• Actinic keratosis • Superficial basal cell carcinoma • External genital warts	1997
	TLR4	MPL in Cervarix®	• Prophylactic vaccine against HPV-16 and 18-induced cervical cancers	2009
Virus	Oncolytic, GM-CSF	T-VEC (Imlygic)	• Advanced melanoma	2015

Table 1 – List of important, currently approved cancer immunotherapy drugs. Granulocyte macrophage colony-stimulating factor; GM-CSF, prostatic acid phosphatase; PAP, cytotoxic T-lymphocyte-associated protein 4; CTLA-4, programmed death-1; PD-1, programmed death ligand 1; PD-L1, Met; metastatic, HNSCC; head and neck squamous cell carcinoma, Non-small cell lung cancer; NSCLC, microsatellite instability-high; MSI-H, mismatch repair deficient; dMMR, Chimeric antigen receptor; CAR, Acute lymphoblastic leukemia; ALL, Bispecific T cell engager; BiTE, Acute lymphoblastic leukemia; ALL, chronic lymphocytic leukemia; CLL, Chronic myeloid leukemia; CML, Non-Hodgkin lymphoma; NHL, interleukin-2; IL-2, toll-like receptors; TLRs, Bacillus Calmette-Guérin; BCG, monophosphoryl lipid A; MPL.

Aims of the study

The main aims of this thesis were to study the signaling requirements for induction of antitumor macrophages and characterize effector mechanisms involved in the antitumor function of these cells. Alexandre Corthay had previously demonstrated that IFN-γ is critical for primary antitumor immune responses mediated by $CD4^+$ T cells in collaboration with tumor-associated macrophages [2]. However, exogenous IFN-γ was not sufficient to induce an antitumor phenotype *in vitro* in tumor-associated macrophages harvested from SCID mice that are unable to mount an antitumor immune response [282]. The additional activation of the macrophages with LPS or IL-1β in combination with IFN-γ however, was highly effective. The finding that both LPS and IL-1β can synergize with IFN-γ for induction of antitumor macrophages was intriguing. The receptors for LPS and IL-1β, TLR4 and IL-1R respectively, signal through similar pathways that dependent on the adaptor molecule MyD88 and result in activation of the transcription factor NFκB. This finding opened up the search for other agents able to replace LPS, which is highly toxic, with the purpose of developing new strategies for macrophage-based cancer immunotherapies. The first scientific question we asked was:

1. Can TLR agonists other that LPS induce antitumor macrophages *in vitro*?

The following studies focused on IFN-γ and the role of interferons for antitumor macrophage activation:

2. As IFN-γ is critical for macrophage activation, can we find novel ways of therapeutically deliver this protein while aiming at reducing systemic toxicity?

3. Can we find alternatives to IFN-γ for synergizing with potent TLR agonists for induction of antitumor macrophages, with the prospect of application in cancer immunotherapies?

Summary of Papers

Paper I - TLR ligands and IFN-γ synergize for induction of antitumor M1 macrophages. Elisabeth Müller, Panagiotis F. Christopoulos, Sanjib Halder, Anna Lunde, Kahsai Beraki, Martin Speth, Inger Øynebråten and Alexandre Corthay. Frontiers in Immunology 8:1383 (2017)

In this study, we investigated the induction of antitumor M1 macrophages *in vitro* by the use of TLR agonists alone or in combination with IFN-γ. The antitumor macrophage phenotype was characterized by high NO production and inhibition of tumor cell growth. We found that the classical macrophage activator LPS was the only TLR agonist capable of inducing these functions in macrophages in the absence of IFN-γ. However, a range of TLR agonists, binding both intracellular and extracellular TLRs, could synergize with IFN-γ for induction of antitumor M1 macrophages, while IFN-γ alone had no effect. Two different mouse tumor cell lines were used, and both primary mouse bone marrow-derived macrophages and a mouse macrophage cell line showed similar effects. As the level of NO production was closely correlated with induction of tumor cell growth inhibition, we tested blockade of the inducible nitric oxide synthase enzyme responsible for NO production in macrophages. We found that the inhibitor s-methylisothiourea hemisulfate salt (SMT) could effectively block NO production, and also blocked induction of macrophage-mediated growth inhibition of tumor cells. Finally we showed that the combination of TLR agonists and IFN-γ increased the production of TNF-α, IL-12p40 and IL-12p70, and reduced the production of IL-10 when compared with TLR agonists alone, indicating that these two signals synergize for the induction of a pro-inflammatory, pro-Th1 macrophage phenotype. We conclude that two signals from the microenvironment are required for optimal induction of antitumor M1 phenotype.

Paper II - Generation and Functional in vitro analysis of Semliki Forest Virus vectors Encoding TNF-α and IFN-γ. Baiba Kurena, Elisabeth Müller, Panagiotis F. Christopoulos, Ingvild Bjellmo Johnsen, Branislava Stankovic, Inger Øynebråten, Alexandre Corthay and Anna Zajakina. Frontiers in Immunology 8:1667 (2017)

In our first paper, we describe a key function of IFN-γ on activating macrophages to an antitumor phenotype in combination with TLR agonists and indicate a potential for IFN-γ

in combination with TLR agonists for macrophage-based cancer immunotherapy. In this study, we developed two new recombinant Semliki Forest virus (SFV) vectors, encoding either IFN-γ or TNF-α, for therapeutic protein delivery. While the SFV vector showed high infection rate in mouse and human lung carcinoma cells *in vitro*, mouse and human macrophages were resistant. The rSFV vector inhibited cancer cell growth, induced cancer cell death and simultaneously exploited cancer cells for production of SFV-encoded TNF-α and IFN-γ. By *in vitro* assays, we could show that the cytokines were produced at functional levels. Furthermore, the rSFV-encoded IFN-γ activated macrophages towards an antitumor phenotype *in vitro* when combined with a TLR agonist. We believe that these new SFV vectors could be useful in cancer immunotherapy, as they carry the potential of combining direct anti-tumor effects with immune-stimulatory effects on tumor associated macrophages. Experiments aimed at testing these vectors *in vivo* are planned for the near future.

Paper III - Role of type I and type II interferons for activation of antitumor M1 macrophages. Elisabeth Müller, Martin Speth, Panagiotis F. Cristopoulos, Anna Lunde, Ajna Avdagic, Inger Øynebråten and Alexandre Corthay. *Manuscript*

In the last paper, we sought to better understand why LPS, a TLR4 agonist, but none of the other TLR agonists tested, was able to induce an antitumor macrophage phenotype in the absence of IFN-γ. Martin Speth had previously shown that nanoparticle-encapsulated poly(I:C), a TLR3 ligand, could synergize with BCG infection or TLR2 activation through the induction of autocrine type I IFN signaling, and result in induction of NO production by mouse macrophages [283]. We found that LPS induced production of type I IFNs, which activated BMDMs in an autocrine fashion to produce NO and inhibit cancer cell growth macrophages. Furthermore, we could show that exogenous type I IFNs synergized with TLR agonists in inducing macrophage-mediated cancer cell growth inhibition, which was dependent on NO production. We were also able to replace exogenous IFNs altogether by using Pam3 in combination with poly(I:C), which induced type I IFNs, to induce activation of antitumor macrophages. Our findings establish a novel role of type I IFNs for antitumor activation of macrophages, paralleling those of IFN-γ. This opens up for new strategies to induce antitumor functions of macrophages, including combinations of TLR agonists.

Methodological considerations

1. Effector and target cells used

Our study of antitumor macrophages is based on *in vitro* experiments involving differently stimulated macrophages as effector cells and cancer cell lines as target cells, as described in the methods section of each paper. We utilized both primary human and mouse macrophages, and the mouse macrophage cell line J774A.1. However, most of the experiments were done using bone marrow-derived macrophages (BMDMs) from C57BL/6 mice, and the rational for using these cells is described in the following section. The target cells used were the mouse multiple myeloma cell line MOPC315, the mouse Lewis lung carcinoma (LLC) cell line and the human lung carcinoma cell line A549. The LLC cell line will be discussed further in section 1.2.

1.1. Mouse BMDMs as a model for human tumor-associated macrophages (Paper I, II and III)

At the beginning of our study, we sought to reproduce experiments from some of the original papers describing tumoricidal macrophages, and most papers had utilized primary mouse peritoneal macrophages [56, 59]. We harvested peritoneal macrophages from C57BL/6 mice, without any macrophage eliciting agents, and tested the cells in our functional assays. We found that the macrophages responded well and could inhibit cancer cell growth and produce NO upon appropriate activation, however there were several drawbacks. The macrophage yield was limited, requiring a lot of mice, and it was very time consuming to obtain a sufficient number of macrophages for each experiment. In addition, we found that the method of selecting macrophages by attachment only to culture dishes resulted in high numbers of contaminating B cells. Positive selection by CD11b-beads resulted in loss of cells, thus further reducing the yield of macrophages per mouse.

We then switched to using bone marrow-derived macrophages (BMDMs), which were differentiated from bone marrow precursors by *in vitro* culturing for 6 days in the presence of L929 cell-conditioned medium (CM) by use of an established protocol [284]. The yield was typically 10^8 BMDMs per mouse and the BMDMs were shown to be ≥99% pure $CD11b^+F4/80^+$ cells, which responded efficiently to stimuli and survived well in culture for the duration of our assays. Large batches of fully differentiated BMDMs can be frozen and stored for later experiments, reducing time consumption and allowing

experimental repeats to be performed on the same batch of BMDMs to check for inter-experimental variation. The main differentiation factor found in CM is macrophage-colony stimulating factor (M-CSF), also called colony stimulating factor 1 (CSF-1) [285]. One drawback of using CM instead of recombinant M-CSF is potential batch-to-batch variation, and the presence of other additional factors which could affect the activation of macrophages. However, we have compared the function of BMDMs differentiated with CM and M-CSF, and the cells function equally well in our antitumor assays.

Tumor associated macrophages (TAMs) in mice have been shown to originate from monocyte precursors [169]. Furthermore, the infiltration of TAMs into tumors is guided, at least in part, by M-CSF [286]. M-CSF has been shown to be important for differentiation of monocytes into a MHC-II^{low} TAM phenotype [8] and to control pro-tumor effects of TAMs, such as progression of metastasis [286]. Therefore, macrophages of myeloid origin that are differentiated using M-CSF and other factors may be a better model for TAMs than peritoneal macrophages, which are considered to comprise mainly macrophages of embryonic origin at steady state [287].

The difference as well as similarity between mouse and human macrophages when it comes to arginine metabolism into NO or ornithine, have been a topic of debate for several decades [288]. The topic was recently reviewed by Thomas and Mattila [289], and they noted that the two main enzymes involved in the differential metabolism of arginine into NO or ornithine, iNOS and arginase respectively, are highly conserved in mammals. However, studies of macrophage activation in mouse cells often failed to be reproduced in human cells, with lower or absent levels of NO and ornithine [288]. iNOS and arginase were detected in human macrophages at mRNA and protein level by some, but were found to be absent by others. Furthermore, studies have demonstrated differences in the epigenetic regulation of *Nos2* between human and mouse macrophages, with human *Nos2* being in a constitutively more closed confirmation [290]. However, Thomas and Mattila pointed out that there were significant differences in the origin of the studied macrophages, originating from healthy controls or diseased patients, and varying from tissue resident macrophages to macrophages produced *in vitro* from peripheral monocytes, referred to as monocyte-derived macrophages (MDMs). Thomas and Mattila suggested that the current system of differentiating macrophages into such MDMs requires further refinement, and that mouse macrophages remain a useful model for investigating aspects of macrophage activation.

Toll-like receptors, which are a main point of interest in this thesis, are evolutionary old and the genes encoding TLRs are highly conserved between mouse and human [37]. Nevertheless, not all TLRs are shared between mouse and human [37], and there are differences in the regulation of TLR expression and in which cell types express the various TLRs [291]. Furthermore, mice and humans have been shown to respond differently to LPS [292]. An extensive study of TLR4-regulated gene expression found that 24% of orthologues genes were divergently expressed in mouse and human macrophages upon LPS activation [293]. The authors also showed that mouse macrophages displayed enhanced feedback regulation which may dampen the primary LPS response.

Human monocyte-derived macrophages has been shown to be able to kill human cancer cell lines after activation with LPS or LPS in combination with IFN-γ [294]. Human non-small cell lung carcinoma (NSCLC) samples were shown to contain macrophages expressing markers of an antitumor phenotype (iNOS, HLA-DR and TNF-α), and patient survival correlated with increased numbers of antitumor macrophages in tumor islets [295]. Such studies indicates that both mouse and human macrophages can be activated to an antitumor phenotype by combination of IFN-γ and TLR agonists, and that TAMs of an antitumor phenotype might be clinically important in antitumor immune responses. However, our results on induction of antitumor macrophages must be confirmed in human monocyte-derived cells and ultimately in human TAMs isolated from tumor samples.

1.2. LLC cells as target cells for antitumor macrophages (Paper I, II and III)

The Lewis lung carcinoma line 1 (LLC1), was established from mice harboring metastasis of Lewis lung carcinoma as described in [296]. The cells are simply referred to as LLC cells in this thesis. LLC cells have been extensively studied and used as a syngeneic mouse model for lung cancer. We observed that LLC cells are extremely potent in developing subcutanous (s.c.) tumors, both in immune deficient NOD *Scid* gamma (NSG) mice and immune competent C57BL/6 mice, as s.c. injection of cell numbers as low as 1000 cells per mouse resulted in tumor development in 6/8 mice (Figure 15). Furthermore, differentially activated subsets of TAMs have been described in LLC tumors, including antitumor-like MHC II^{high} and protumor-like MHC II^{low} subsets [169]. TAMs in LLC tumors have also been shown to be able to change phenotype and mediate cytotoxic and antitumor effects upon activation with the TLR agonist poly(I:C) [267]. With the increased

interest into cancer immunotherapy in NSCLC, the LLC cell line is an interesting target for testing the effect of antitumor macrophages both *in vitro* and *in vivo*.

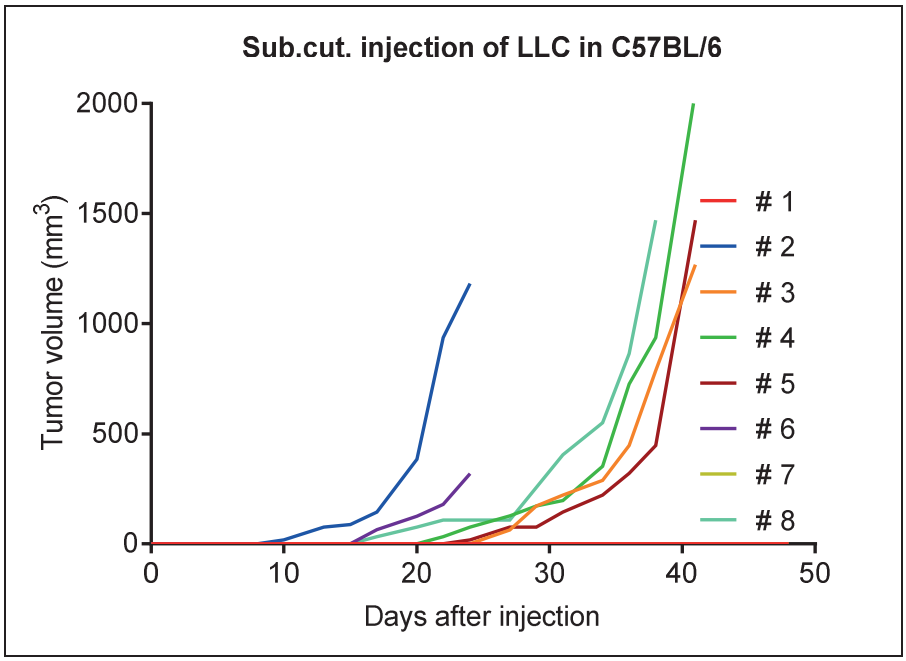

Figure 15 - Growth if tumor following s.c. injection of the Lung carcinoma cell line LLC. LLC cells were injected s.c. in C57BL/6 mice with 1000 cells/mouse. Tumor growth was measured every 2-3 days using calipers, and tumor volume was calculated using the following formula: tumor width^2 x tumor length x 0.5. The graph shows the tumor volume of 8 individual mice at the indicated time point after injection. 6/8 mice developed tumors, while 2/8 (#1 and 7) remained without palpable tumors throughout the experiment.

2. *In vitro* assays

2.1. Indirect quantification of NO production by the Griess assay (Paper I, II and III)

Activation of macrophages by appropriate stimuli may induce expression of *Nos2,* the gene encoding iNOS, and result in production of the gaseous free radical NO, as previously discussed (introduction section 1.3). Induction of this pathway has long been used as a marker of pro-inflammatory and antitumor macrophages, but the detection and measurement of NO is problematic, due to its extremely short half-life [297]. Quantification of the NO metabolite NO_2^- (nitrite) however, can be performed in simple colorimetric assays such as the Griess assay. NO is rapidly oxidized to NO_2^- in the presence of oxygen, and NO_2^- may be further oxidized to NO_3^- (nitrate) in the presence of certain oxyhemoproteins [298]. Treatment of samples with nitrate reductase would convert NO_3^- back to NO_2^-, allowing for analysis of both metabolites. However, in analysis of cell culture supernatants such as in our studies, this step is not commonly used as the main metabolite of NO was considered to be NO_2^-. The Griess assay takes advantage of a two-step diazotization reaction where NO_2^- forms a colored azo compound that can be quantified on a spectrophotometer (Figure 16).

The Griess assay was performed on supernatants from BMDMs activated for 24h, in some cases removed just prior to the addition of target cells in the growth inhibition assay. This experimental setup provided an indirect measurement of how much NO had been produced up to the start of co-culture of BMDMs with cancer cells, but could not give any information about the actual presence of radical species or the continued secretion of NO. There are other, more sensitive assays for measuring NO metabolites, including High Performance Liquid Chromatography (HPLC) [298]. However, the Griess assay can be performed with standard lab equipment and affordable reagents, and worked well to distinguish between agents that induce high, low or no apparent NO production by macrophages.

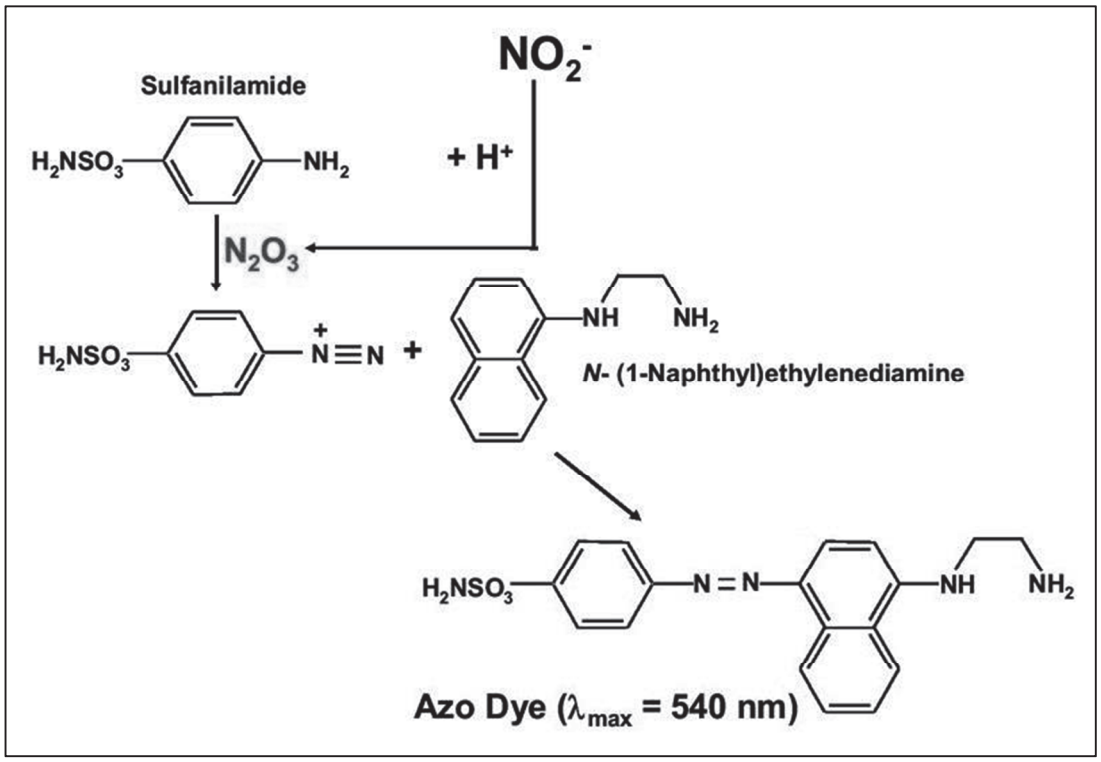

Figure 16 – The Griess reaction. The nitrosating agent dintrogen trioxide (N_2O_3) generated from acidified nitrite reacts with sulfanilamide to yield a diazonium derivative. This reactive intermediate will interact with N-1-naphthylethelene diamine to yield a colored diazo product that absorbs strongly at 540 nm. Figure adapted from [298] and reused with permission.

In order to study the importance of NO production for the function of antitumor macrophages, we utilized two inhibitors of the iNOS enzyme. BMDMs were incubated with inhibitors both during the activation phase and during co-culture with LLC cells. In paper I, we used S-Methylisothiourea hemisulfate salt (SMT, #M84445, Sigma-Aldrich), which has previously been shown to be a potent inhibitor of NO production with selectivity towards the iNOS isoform [299]. This inhibitor successfully inhibited both NO production by BMDMs and the macrophage-mediated growth inhibition of LLC cells induced by TLR agonists in combination with IFN-γ. However, in experiments for the third manuscript, we faced some problems with LLC cell toxicity upon incubation with SMT at high concentrations. An alternative iNOS inhibitor called 1400w (or N-[[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride, #1415, Tocris/Bio-Techne) had been tested in parallel in our lab with promising results, and had also showed the ability to inhibit iNOS activity *in vivo* [300]. We changed to using 1400w in Paper III for blocking NO production upon activation with Pam3 and type I or type II IFNs, and this iNOS inhibitor also blocked cancer cell growth inhibition by macrophages. In conclusion,

the results from both paper I and III, using two different iNOS inhibitors, indicated that NO production is an important effector mechanism of antitumor macrophages.

2.2. Cancer cell growth inhibition assay (Paper I, II and III)

Most of the early studies of antitumor macrophages were functional, quantifying the induction of a macrophages-mediated cytostatic or cytotoxic effect on tumor cells in vitro [1, 56]. Contrary to this, most current studies are based on the measurement of surface markers, gene expression profiles or cytokine production. There is not yet clear evidence that antitumor macrophages constitute a defined subset rather than a functional phenotype, and there is no consensus as to any markers absolutely identifying antitumor versus protumor macrophages. We sought to find which signals are required for induction of a functional antitumor phenotype *in vitro*, as this might be a better predictor of antitumor effects of macrophages *in vivo.*

Activated macrophages have been shown to induce both cytostatic and cytotoxic effects on cancer cells *in vitro*, as described previously in the introduction. The most widely used assay for measurement of macrophage-mediated cytotoxicity is based on release of chromium-51 (^{51}Cr) from cancer cells pre-labeled with this radioactive isotope. However, ^{51}Cr is an external exposure hazard requiring particular working conditions and shielding to limit exposure to gamma-radiation. Another commonly used assay is based on incorporation of tritium(^{3}H)- radiolabeled thymidine, which is incorporated in dividing cells and can be used to measure cell growth. ^{3}H-thymidine only emits low energy beta radiation, and requires much less safety precautions.

We established a ^{3}H-thymidine-based growth inhibition assay in which cancer cells were cultured alone or in the presence of various numbers of BMDMs, resulting in macrophage to target cell ratios ranging from 20:1 to 1:1. One drawback of this method is that thymidine will be incorporated in all dividing cells, which required us to block the growth of BMDMs. We did this by incubating BMDMs with Mitomycin C, a DNA crosslinking agent, prior to seeding the cells. Wells with BMDMs alone were included as a control to ensure that the macrophages did not contribute to the measured thymidine incorporation. The BMDMs were treated with TLR agonists and/or cytokines for 24h prior to addition of target cells. Half of the supernatant was then removed and used for analysis of secreted factors, before target cells were added. As a result, a decreased concentration of the stimulatory signals remained for the duration of the assay. In order to control for any

direct effects of TLR agonists and/or cytokines on the growth of target cells, we included wells with target cells alone and added the same concentrations of stimulatory signals as remained in the treated co-culture wells. Table 2 shows an example of what a typical 96 well plate layout for this assay would look like:

Ratio:	**20:1**			**10:1**			**1:1**			**Target cells alone**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	BMDM alone			BMDM alone			BMDM alone			Empty		
B	Untreated			Untreated			Untreated			Untreated		
C	IFN-γ			IFN-γ			IFN-γ			IFN-γ		
D	Pam3			Pam3			Pam3			Pam3		
E	Pam3 + IFN-γ			Pam3 + IFN-γ			Pam3 + IFN-γ			Pam3 + IFN-γ		
F	SMT			SMT			SMT			SMT		
G	Pam3 + IFN-γ + SMT			Pam3 + IFN-γ + SMT			Pam3 + IFN-γ + SMT			Pam3 + IFN-γ + SMT		
H	-			-			-			-		

Table 2 – Example of plate layout for the growth inhibition assay.

We found that an excess of macrophage was required for inhibition of cancer cell growth. Effective ratios ranged from 6:1 to 20:1 (macrophage:target cell), depending on stimulus and the type of target cell. In a previous study by Haabeth *et al.*, it was found that active antitumor macrophages were present at an approximate 1:1 ratio relative to target cancer cells [3]. This indicates that the *in vitro* requirement for a higher ratio of macrophages to target cells might be an artifact. This could be caused a diluting effect of culture medium, artificial positioning of the cells in a monolayer or the limited contact between cells at the 1:1 ratio, as the total number of cells here was very low (6000 or 10 000 cells in total).

The readout from this growth inhibition assay is the levels of incorporated ^{3}H-thymidine, measured as counts per minute (cpm). We found that the absolute values of cpm varied greatly from one experiment to the next, ranging from 10 000 to 40 000 cpm in untreated controls. As a result, we were limited to showing one representative experiment instead of pooled data. However, the relative growth inhibition mediated by BMDMs upon activation, for instance with Pam3 and IFN-γ, was reproducible and consistent between experiments. Activation with 100 ng/ml Pam3 in combination with 40 ng/ml IFN-γ was

used as a positive control, and was in the range of 1 and 5% of untreated BMDMs for 10 independent experiments.

A major drawback of the growth inhibition assay is implicated in the name. The assay is unable to quantify the number of apoptotic or necrotic cancer cells after co-culture with activated macrophages, as it measures only the growth (or lack of growth) of viable cancer cells. It has also proved challenging to establish alternative assays to quantify the macrophage-mediated killing of cancer cells by microscopy or flow cytometry. Dead cancer cells may be cleared and phagocytosed by macrophages and escape detection, and the distinction between effector cells, macrophages, from cancer target cells is made difficult by the loss of specific markers or unspecific binding of antibodies in dead or dying cells. During the final stage of this thesis, we successfully established an assay measuring the induction of caspase-3-dependent apoptosis. We prepared whole cell extracts from cancer cells in co-culture with stimulated or untreated macrophages, and analyzed the enzyme activity of caspase-3 by measuring fluorescence resulting from hydrolytic cleavage of the Ac-DEVD-AMC substrate ((#14459, Cayman Chemical, Ann Arbor, MI, USA). It was not possible in this assay to determine whether the caspase-activity induced originated from BMDMs or LLC cells in the co-culture, but similarly treated wells containing each cell type separately were analyzed in parallel as controls.

The preliminary data from such caspase-3 experiments confirmed our previous assumption based on microscopic observations and literature reports. Macrophages activated with TLR agonists in combination with IFN-γ, but not by either factor alone, induce apoptotic cell death in co-cultures with cancer cells *in vitro* (Figure 17A). No apoptosis induction was observed in single cultures with BMDMs or LLC cells treated with the same factors (Figure 17A). Furthermore, induction of cancer cell apoptosis depended on NO production by macrophages, as blocking NO production using the iNOS inhibitor SMT was shown to inhibit the induction of apoptosis in co-cultures of activated BMDMs and LLC cells (Figure 17B and C). These findings are in agreement with the findings in the growth inhibition assay reported in Paper I, and support the deduction that cancer cell growth inhibition, as observed upon co-culture with TLR agonist and IFN-γ-stimulated macrophages, represents a useful measurement for the activation of functional antitumor macrophages. The caspace-3 assay, as well as additional flow cytometry assays for quantification of apoptosis, necrosis and phagocytosis are currently being developed in our lab, and may contribute to better understanding of antitumor macrophages in the future.

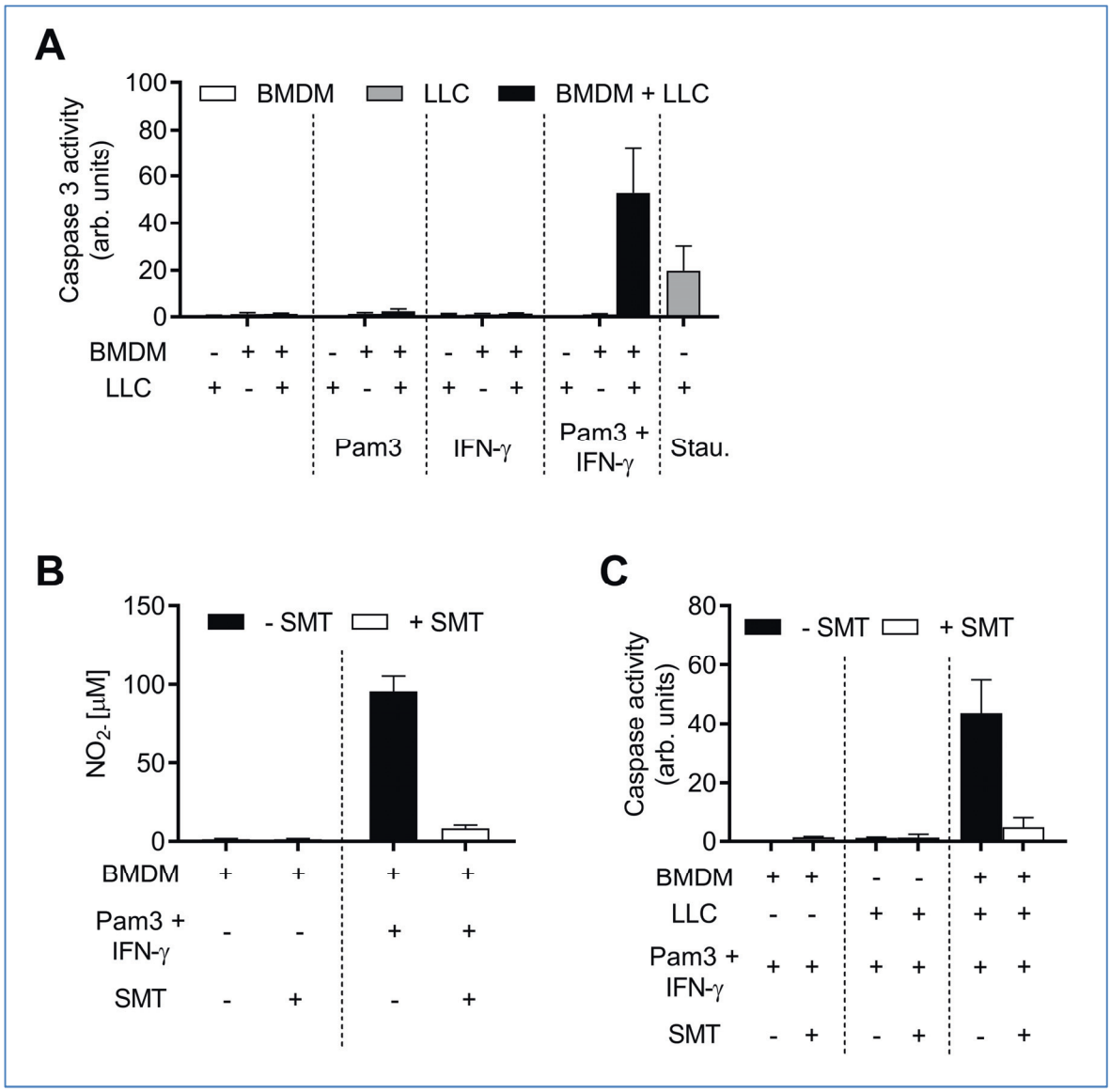

Figure 17 – Stimulation of BMDMs with TLR1/2 agonist Pam3 in combination with IFN-γ induces BMDM-mediated and NO-dependent apoptosis in LLC-BMDM co-cultures.

(A) BMDMs (1.75 x10^6 cells in 4 ml) were left untreated or stimulated with Pam3 (100 ng/ml), IFN-γ (40 ng/ml) or a combination of both for 24 h before LLC cells (1.75 x10^6 cells/well) were added, resulting in a LLC: BMDM ratio of 1:1. Control wells containing only BMDMs or LLC cells were treated similarly. Activity of the apoptosis indicator caspase 3 was measured in whole cell extracts after another 12 h of incubation. Treatment of LLC cell cultures with the apoptosis inducer staurosporine (0.5 µM) for 12 h was used as a positive control. **(B)** Effect of iNOS inhibition by SMT (1 mM) on NO-production in BMDMs left untreated or stimulated with Pam3 in combination with IFN-γ for 24 h. **(C)** Caspase 3 activity in BMDM-LLC co-cultures or in cultures of LLC cells or BMDMs alone after stimulation with Pam3 and IFN-γ, in the presence or absence of the iNOS inhibitor SMT (1 mM). Data are presented as means ± SD of three independent experiments

2.3. Cytokine quantification by Luminex technology (Paper I, II and III)

The amount of cytokines secreted was measured using multiplex bead assays from Bio-Rad laboratories and ThermoFischer Scientific on the Bio-Plex MAGPIX Multiplex Reader. The assay is based on immunodetection of cytokines by monoclonal antibodies attached to dyed beads, where one bead population is coupled to one target specificity. The sensitivity is very high, typically down to ~10 pg/ml. Multiple cytokines can be detected simultaneously, as the bead populations are classified based on their dye intensity. This is a great advantage when working with high number of samples and yields a lot of information from small sample volumes. Reporter antibodies are biotinylated and subsequently labelled with a fluorescent reporter. Using a standard curve of known cytokine concentrations, the detection of specific beads and the associated recorded reporter intensity is used to calculate the amount of the given cytokine.

This analysis could be applied to various cell supernatants, including the supernatants from the growth inhibition assay, and be performed directly or on frozen samples. To limit cytokine degradation, samples were kept at -80 and repeated freezing and thawing was avoided. Cell supernatants were centrifuged at 400G for 5 min in order to remove non-adherent cells. Variation in absolute levels of cytokines between experiments could potentially be improved by adding protease inhibitors to the supernatants before storage to further avoid degradation, but was not tested in our studies. Some cytokines, such as IL-1β, are produced in an inactive form and are only secreted and active upon appropriate stimulation [301]. As we were interested in the effect of different TLR agonists and IFN-γ on macrophage functional phenotype, we measured the cytokines released into the supernatant rather than cytokines present in complete cell lysates.

2.4. Determination of *Nos2* mRNA levels by Real-time qPCR (Paper III)

Increased transcription of *Nos2*, the gene encoding inducible nitric oxide synthase (iNOS), is used by many as a marker of macrophage activation. This enzyme enables macrophages to produce NO in response to stimuli, such as LPS, and to mediate various antitumor functions [302, 303]. We chose to measure iNOS at the mRNA level rather than the protein level as both LPS- and IFN-γ-induced activation of the iNOS enzyme have been described to be mediated mainly by transcriptional activation [304]. We analyzed the levels of *Nos2* transcripts relative to the 18S rRNA housekeeping gene in extracts containing total RNA of activated or untreated BMDMs. For our study, we only analyzed samples after 24 h of

activation, and this poses the possibility that we underestimate the gene expression if the maximum is reached at an earlier or later time point. A second drawback is that by studying only one time point, we could potentially miss differences in the temporal dynamic of *Nos2* transcriptional induction by the different activation stimuli. However, this is not critical to our main conclusion in paper III that both IFN-γ and IFN-β synergize with Pam3 for the induction of NO production by inducing *Nos2* gene expression.

2.5. Proliferation assay using Cell counting kit-8 (CCK-8) (Paper III)

We used the Cell counting kit-8 (CCK-8) from Sigma Aldrich to confirm our data regarding the direct inhibitory effect of IFN-β on the growth of LLC cells. CCK-8 is based on the reduction of colorless WST-8 agent to an orange formazan product by cellular dehydrogenases, which allows for quantification of cell proliferation by measuring the increase in number of viable cells over a certain time period. This assay, similar to the ^{3}H-thymidine-based growth inhibition assay, uses 96 well plates and allows for the simultaneous measurement of multiple treatment conditions. Our main reason for implementing a second, independent measure of LLC cell growth was the variability in the cpm counts of the thymidine-based assay, which made detection of smaller effects on LLC proliferation harder to measure with confidence. The same problem was not observed in the CCK-8 assay, and we could confirm that IFN-β directly inhibited the growth on LLC cells to about half of untreated controls.

3. Strategies for the development of novel macrophage-targeted immunotherapies

3.1. Oncolytic virus for intratumoral delivery of cytokines (Paper II)

In Paper I, we had shown how multiple TLR agonists can activate macrophages to an antitumor phenotype, but these TLR agonists, with the exception of LPS, require co-stimulation with IFN-γ. As systemic IFN-γ therapy has several drawbacks related to side effects and targeting of cancer cells, we sought to develop alternative strategies for delivery of IFN-γ to tumors for the induction of antitumor macrophages. Intratumoral cytokine delivery by viral expression vectors is an interesting approach for cancer immunotherapy, especially when used with an oncolytic virus strain. As previously described, oncolytic viruses selectively infect and kill cancer cells, and have the potential to induce cancer cell-antigen presentation and anti-tumor immune responses [305]. T-VEC, the so far only oncolytic virus to be approved by the FDA, encodes additional GM-CSF for induction of antigen-presentation cells [235]. Oncolytic viruses themselves may stimulate immune responses through activation of anti-viral immune response including production of type I IFN [232]. Such an immune response may be further enhanced by virally encoded proinflammatory cytokines [306, 307].

Semliki Forest Virus (SFV) is an enveloped positive-sense single-stranded RNA virus that belongs to the genus *Alphavirus* of the *Togaviridae* family [308]. It is found in central, eastern and southern Africa, and causes mild disease in humans [309]. Unlike more globally prevalent viruses such as Herpes Simplex Virus, high titers of anti-SFV antibodies are unlikely to cause problems for therapeutic infection of patients from areas outside Africa. In 1991, Liljeström and Garoff developed a recombinant SFV vector system which yields replication deficient virus particles that are able to infect cancer cells and express the encoded transgene in a transient fashion, but do not result in production of new virus particles [310]. These SFV vectors have become widely used, as they provide high level of expression of the inserted transgene without the risk of disseminated infection. Recombinant SFV has been effectively used in a number of preclinical studies to express genes of interest and has been shown to induce effective tumor immune responses [307, 311-317]. SFV have also been shown to induce p53-independent apoptosis, which is a great advantage as many cancer cells carry mutations in this tumor suppressor gene [318]. Recombinant SFV has also showed great effect in mouse models of hard-to-treat cancers

such as glioblastoma multiforme and neuroblastoma [319], and emerges as an excellent candidate for novel immunotherapies.

In Paper II, we generated replication-deficient SFV vectors encoding the cytokines TNF-α and IFN-γ, which specifically infected LLC cancer cells and resulted in functional protein production *in vitro*. Virus-derived IFN-γ successfully activated BMDMs towards an antitumor phenotype when combined with the TLR1/2 agonist Pam3, demonstrating that this strategy is potentially useful for macrophage-targeted cancer immunotherapy when combined with appropriate co-stimulatory factors. So far, testing of these recombinant SFVs in mouse cancer models is lacking, and important questions regarding optimal route of delivery of both virus and additional TLR agonists need to be answered. A future goal would be to study the effect of IFN-γ-encoding SFV and Pam3 therapy on tumor associated macrophages *in vivo*, as well as the direct and indirect effect on antitumor T cell responses. This could help determine the therapeutic potential of such therapies, and whether combining such macrophage-targeted therapy with checkpoint inhibitors could provide additional therapeutic benefit, as previously shown for other recombinant SFVs [307].

3.2. Poly(I:C)-encapsulating nanoparticles (paper III)

In Paper III, we further developed a strategy by Martin Speth where simultaneous activation with MyD88-dependent TLR agonist BCG or lipomannan and TRIF-dependent TLR agonists poly(I:C) resulted in induction of macrophage NO production. We could show that similar activation also induced cancer cell growth inhibition and represents a novel strategy for activation of antitumor macrophages. However, limiting factors for using poly(I:C) both *in vitro* and *in vivo* are the stability of this synthetic double stranded RNA molecule and the uptake into the endocytic compartment where TLR3 is present. This had previously been addressed by incorporating poly(I:C) into chitosan-based nanoparticles (poly(I:C)-NP) [283].

Nanoparticles are man-made or modified natural structures which have specific advantages due to their limited size (below 1000 nm in pharmacology), but can range widely in their composition, shape and properties [320]. Nanoparticles are taken up by cells through the endocytic pathway, and APC, such as macrophages, are particularly efficient in endocytosis and phagocytosis, making nanoparticles a promising delivery system for macrophage-targeted therapies [321]. Targets that are vulnerable to enzymatic

degradation or systemic elimination can be protected by incorporation into appropriate nanoparticles and achieve longer half-life and stability.

Speth et al. had previously developed nanoparticles that were able to greatly improve both the stability (Figure 18C) and uptake (Figure 19) of poly(I:C) into BMDMs, when compared with soluble poly(I:C) [283]. Poly(I:C)-NP were made by mixing poly(I:C) with chitosan, a natural polysaccharide polymer derived from chitin, which has previously been shown to be a promising candidate in biomaterials, being both biocompatible and biodegradable [322]. Being positively charged, chitosan spontaneously forms particles upon appropriate mixing with negatively charged poly(I:C) (Figure 18A-B), and the NPs were collected by centrifugation on a glycerol bed [283]. In order to fully resuspend and dissociate the NPs, they were sonicated in a water bath sonication before use, and prepared fresh for each experiment. The amount of poly(I:C) and chitosan in the poly(I:C)-NPs were measured indirectly by analyzing the supernatant after NP collection. Soluble poly(I:C) was measured by absorbance at 260 nm using a Nanodrop, and free chitosan was measured using a fluorescamine assay[323].

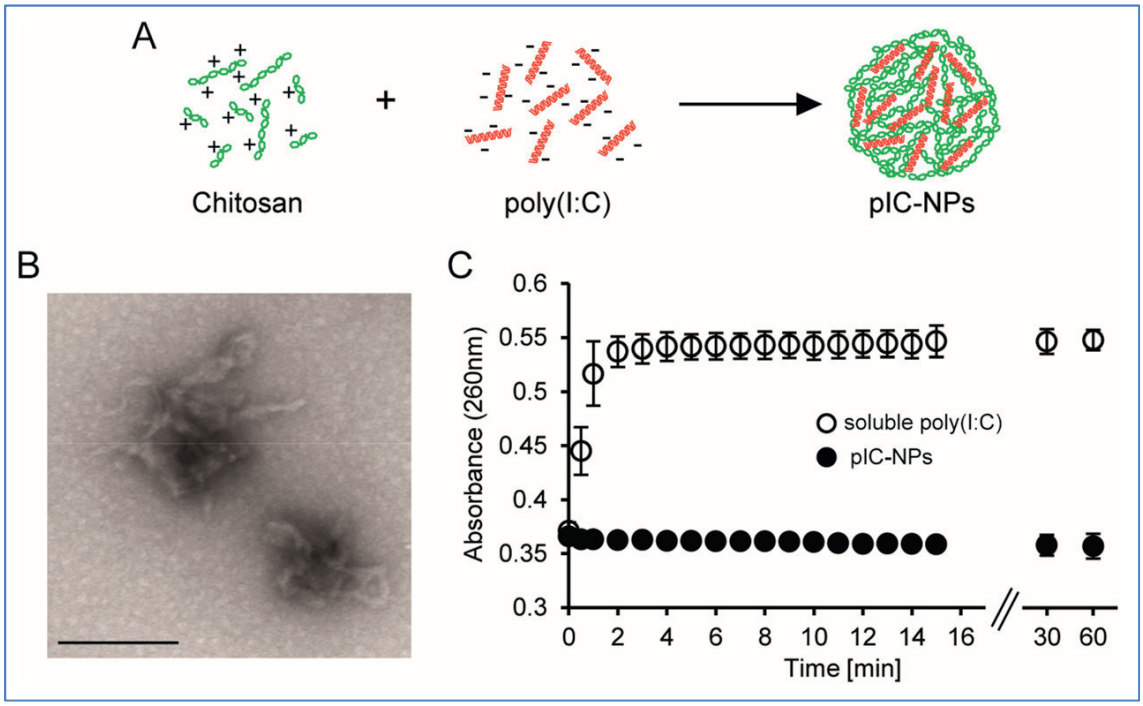

Figure 18 - Characterization of poly(I:C) encapsulating chitosan nanoparticles (pIC-NPs).
(A) Schematic illustration of the preparation process of pIC-NPs. (B) Transmission electron microscopy (TEM) of pIC-NPs; scale bar 200 nm. (C) Nuclease protection assay with soluble poly(I:C) and pIC-NPs. Degradation of poly(I:C) was measured as increase in absorbance at 260

nm (hyperchromatic effect). Adapted with permission from [324]. Copyright (2017) American Chemical Society.

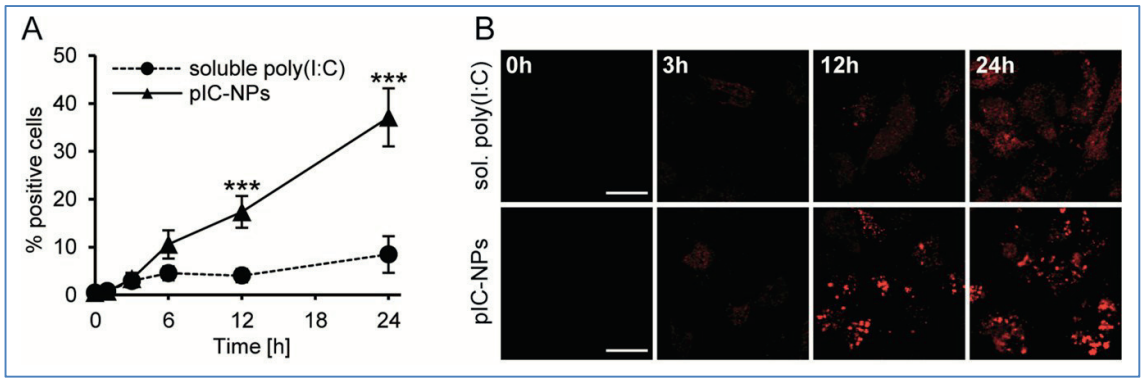

Figure 19 - Uptake of soluble poly(I:C) or pIC-NPs and intracellular stability of pIC-NPs in BMDM. (A) Flow cytometry analysis of the uptake of red-fluorescent pIC-NPs in comparison to soluble red-fluorescent poly(I:C) (both 2 μg/mL) by BMDM at different time points. Uptake was measured as percentage of cells positive for red fluorescence. (B) Confocal microscopy images of the uptake of red-fluorescent pIC-NPs compared to soluble red-fluorescent poly(I:C) by BMDM after different time periods; scale bars: 20 μm. Adapted with permission from [324]. Copyright (2017) American Chemical Society.

In paper III, we compared the effect of poly(I:C)-NP and soluble poly(I:C) and found that nanoparticle-encapsulation greatly improved the ability of poly(I:C) to induce endogenous IFN-β production by BMDMs. Consequently, we found that poly(I:C)-NPs were effective at up to 100-fold lower concentrations than soluble poly(I:C) for the induction of antitumor macrophages, when combined with MyD88-dependent TLR agonist Pam3. The technologically simple production of poly(I:C)-NPs, combined with the biocompatible and biodegradable properties of chitosan polymer used, is a great advantage for the further development of this strategy to improve the potency and effect of poly(I:C). Poly(I:C)-NP will be an interesting agent for future studies into the induction of antitumor TAMs *in vivo* in combination with MyD88-dependent TLR agonists.

Future perspectives

Mechanism underlining the direct antitumor effects of macrophages

In paper I and III, we could confirm previous reports that appropriately activated macrophages mediate growth inhibition of cancer cells *in vitro* through a NO-dependent mechanism. This does not however, exclude a possible contribution of additional factors. Preliminary experiments indicated that macrophage-mediated induction of caspase-3 dependent apoptosis of LLC cells could explain at least part of the observed inhibition of LLC cells. However, further studies of apoptosis, as well as any involvement of the macrophage phagocytic pathway, are required, and such findings need to be confirmed in repolarized TAMs and *in vivo*. In addition, activated macrophages have been shown to contribute to antitumor immune responses through the secretion of proinflammatory and Th1-stimulating cytokines such as TNF-α, IL-6, IL-12p40 and IL-12p70, and to secrete antiangiogenic factors [3]. The effects of inducing antitumor macrophages *in vivo* are likely to involve improved antigen presentation to T cells and release of TAM-mediated immune suppression, and will need to be investigated in appropriate cancer models.

Could our strategies for inducing antitumor M1 macrophages be effective in human primary macrophages and even human TAMs?

As the final goal is the treatment of human cancers, not mice, the most uncertain factor for the interpretation of our results is how they translate to the human system. This is especially true regarding the importance of NO production for human antitumor macrophages, as there is substantial evidence that human macrophages produce very little NO upon activation [288, 289]. Testing of macrophages from different protocols and in different assays will be required to determine if human primary macrophages can be activated to an antitumor phenotype by the same factors as mouse primary macrophages, and whether human TAMs can be repolarized similarly.

Switching TAM phenotype: a potent strategy for stimulation of long-lasting antitumor immune responses or a fight against the tide of immune suppression?

With the increasing understanding how cancer cells direct TAM phenotype towards mediating immune suppression and promotion of tumor growth and metastasis [277, 278, 325-330], a reasonable concern is that any therapeutic repolarization of TAMs towards an antitumor phenotype would quickly be overcome by tumor-derived signals. Therefore, the

recent report by Van den Bossche *et al.* that mitochondrial dysfunction prevents repolarization of inflammatory macrophages was very welcome [331]. It demonstrates that metabolic changes induced by combined activation with IFN-γ and the TLR agonist LPS may arrest macrophages in a proinflammatory phenotype, which would be a beneficial outcome upon therapeutic repolarization of TAMs in cancer.

In this study, the authors show that stimulation with LPS and IFN-γ *in vitro* of both human and mouse macrophages resulted in a M1-like phenotype [331]. Subsequent stimulation with IL-4 *in vitro* failed to repolarize such macrophages to an M2-like phenotype normally induced by IL-4. Furthermore, IL-4 challenge *in vivo* also failed to convert proinflammatory M1-like macrophages to an M2-like phenotype. Analysis of macrophage metabolism upon stimulation with LPS and IFN-γ revealed blunted oxidative phosphorylation, which prevented IL-4-induced polarization. Treatment of LPS+IFN-γ-stimulated mouse macrophages with the iNOS inhibitor 1400w was shown to improve mitochondrial function and partially restore IL-4-induced M2-like repolarization. As human macrophages did not produce detectable levels of NO upon LPS and IFN-γ stimulation, additional mechanisms may underline the observed abruption in oxidative phosphorylation and contribute to the block in macrophage plasticity [331]. This intriguing study has spurred further investigations into the metabolic modulation of macrophage effector functions [332], and we will curiously await the results.

Combination therapies: a place for macrophage-targeted immunotherapies in the future?

The therapeutic potential of various macrophage-targeted immunotherapies are still being explored, but several studies have indicated a clear benefit of targeting TAMs in combination with other immune cell subsets. Combination of anti-CD47 Abs, which target cancer-induced suppression of macrophage antitumor functions, or TLR agonists with anti-PD1 Abs greatly improved the response in several mouse models of cancer [333, 334]. Therapeutic benefit was also observed upon combining the induction of antitumor TAMs with therapeutic cancer vaccines in several tumor models [335]. Furthermore, combining the induction of antitumor macrophages with inhibition of further accumulation of suppressive TAM populations has demonstrated increased survival compared with monotherapy in mouse tumor models [336]. It seems very likely that adding macrophage-targeted strategies to the fast-expanding arsenal of immunotherapies will be of clinical benefit in many types of cancer.

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Scientific papers (I-III)

PAPER I

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Toll-Like Receptor Ligands and Interferon-γ Synergize for Induction of Antitumor M1 Macrophages

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Tumor-associated macrophages may either promote or suppress tumor growth depending on their activation status. Interferon-γ (IFN-γ) has been identified as a key factor for inducing tumoricidal M1 phenotype in macrophages. However, it remains unclear whether IFN-γ is sufficient or if additional stimuli are required. Here, we tested IFN-γ and a panel of toll-like receptor (TLR) agonists for the ability to activate murine macrophages toward a tumoricidal M1 phenotype. The following TLR ligands were used: TLR1/TLR2 agonist Pam3CSK4, TLR2/TLR6 agonist lipotechoic acid, TLR3 agonist poly(I:C), TLR4 agonist lipopolysaccharide (LPS), TLR5 agonist flagellin, TLR7 agonist CL264, and TLR9 agonist CpG. We used an *in vitro* growth inhibition assay to measure both cytotoxic and cytostatic activity of mouse macrophages against Lewis lung carcinoma (LLC) and MOPC315 plasmacytoma tumor cells. Production of nitric oxide (NO) and cytokines by activated macrophages was quantified. We found that IFN-γ alone was not able to render macrophages tumoricidal. Similarly, macrophage activation with single TLR agonists was inefficient. In sharp contrast, IFN-γ was shown to synergize with TLR agonists for induction of macrophage tumoricidal activity and production of both NO and pro-inflammatory cytokines (TNF-α, IL-12p40, and IL-12p70). Furthermore, IFN-γ was shown to suppress macrophage IL-10 secretion induced by TLR agonists. NO production was necessary for macrophage tumoricidal activity. We conclude that two signals from the microenvironment are required for optimal induction of antitumor M1 macrophage phenotype. Combination treatment with IFN-γ and TLR agonists may offer new avenues for macrophage-based cancer immunotherapy.

Keywords: macrophages, tumoricidal, toll-like receptors, interferon-γ, cancer, nitric oxide, immunotherapy

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INTRODUCTION

Macrophages are multifunctional cells whose activities are triggered in response to stimuli from the microenvironment. The stroma of solid tumors contains tumor-associated macrophages (TAMs) which may either suppress or promote tumor development depending on their activation phenotype (1, 2). According to a widely used nomenclature, macrophages with antitumor or

Abbreviations: BMDM, bone marrow derived macrophage; cpm, counts per minute; DETA/NO, diethylenetriamine/NO adduct; FBS, fetal bovine serum; FLA, flagellin; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LLC, Lewis lung carcinoma; LPS, lipopolysaccharide; LTA, lipotechoic acid; MAF, macrophage-activating factor; MIG, monokine-induced by IFN-γ; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Pam3, Pam3CSK4; Poly(I:C), polyinosinic:polycytidylic acid; SMT, s-methylisothiourea hemisulfate salt; TLR, toll-like receptor; TAM, tumor-associated macrophage.

killing activity are called M1 while tumor-promoting or healing macrophages are named M2 or M2-like (3, 4). Because TAMs are commonly assumed to have a tumor-promoting phenotype, research in the field has mainly focused on detrimental aspects of macrophages in tumors (5) and therapeutic strategies were designed accordingly for the depletion of TAMs (6). However, it has also been reported that TAMs may be rendered tumoricidal upon activation by tumor-specific Th1 cells (7). Furthermore, several recent reports revealed the potential of re-programming TAMs toward a tumoricidal M1 phenotype rather than depleting them (8–10). Therefore, it is of therapeutic importance to clarify the molecular requirements for activation of macrophages toward an antitumor M1 phenotype.

Antitumor M1-polarized macrophages are characterized by their direct cytostatic and cytotoxic effect on tumor cells, secretion of pro-inflammatory cytokines, and stimulation of T cell immunity (7, 11, 12). The ability of macrophages to kill tumor cells *in vitro* was reported already in 1970 (13), and it was shown that supernatant of spleen cells from tumor-immunized mice contained a factor that could render macrophages tumoricidal *in vitro* (14). Investigations into the cooperation of lymphoid cells and macrophages led to the identification of interferon-γ (IFN-γ), previously known as macrophage-activating factor (MAF), as a major agent responsible for regulating macrophage tumoricidal activity (15, 16). Bacterial endotoxin [lipopolysaccharide (LPS)] and viral RNA were also reported to render macrophages cytotoxic to tumor cells (17). Later studies suggested that IFN-γ may not be sufficient to render macrophages tumoricidal and that a second signal from the microenvironment was required (18, 19). Dead bacteria or purified LPS were shown to provide such a second signal to render macrophages tumoricidal in combination with IFN-γ (20–22). Still, many current reviews refer to IFN-γ as the major inducer of tumoricidal M1 macrophages or do not make a distinction between the phenotype resulting from activation with IFN-γ alone, LPS alone or both factors (23, 24). A popular view is that activation of M1/M2 macrophage phenotypes depend on cytokines from adaptive immune cells (such as IFN-γ from Th1 cells or IL-4 from Th2 cells), rather than signals from innate receptors such as toll-like receptors (TLRs) (25). There is confusion regarding the current annotation of macrophage phenotype and the M1/M2 classification has been criticized (24, 26). Recent studies investigating macrophage activation do not describe the direct tumoricidal activity of macrophages, but rather focus on production of cytokines, nitric oxide (NO) and reactive oxygen species, and changes in gene expression or surface markers (27, 28). As a result, it remains unclear whether IFN-γ is sufficient or if additional stimuli such as LPS are required for induction of tumoricidal M1 macrophages.

Lipopolysaccharide binds to TLR4, a member of the TLR family of receptors which recognize pathogen- and damage-associated molecular pattern molecules. These receptors signal through adaptor molecules and downstream mediators to modulate gene transcription and induce a pro-inflammatory response. The great potency of LPS in stimulating immune responses has led to clinical trials investigating the use of LPS against cancer. Unfortunately, severe side effects have been reported and therapeutic use of LPS against cancer has so far not been approved (29). However, TLR4 agonists different from LPS as well as agonists of other TLRs have been investigated for their potential use in cancer therapy, either as vaccine adjuvants or immune modulators (30). Several TLR agonists have been shown to activate macrophages similarly to LPS, inducing cytokine production, upregulation of antigen-presentation and co-stimulatory molecules, and induction of the enzyme inducible NO synthase (iNOS) with resulting NO production (31, 32). Viral double stranded RNA, an agonist of TLR3, was shown to induce tumoricidal activity in macrophages already in the 1970s (17), and a synthetic analog, poly(I:C), was also efficient (33). Other TLR agonists that have shown potency for induction of antitumor M1 macrophages includes lipotechoic acid (LTA) (34), gardiquimod (35), R848 (36), and CpG (37). However, none of these studies investigated the role of IFN-γ in potentiating the effect of the TLR agonists despite accumulating evidence for the strong synergistic effect of this cytokine on TLR signaling. Furthermore, the experimental setup, including the source of macrophages, the functional assay used, and the activation regimen with single or combination treatment varied greatly between these studies. This makes it difficult to conclude on the background of the current literature as to which TLRs are effective in inducing tumoricidal M1 macrophages and whether IFN-γ is important for such activation.

This prompted us to systematically test a range of TLR agonists in functional assays for M1 polarization of mouse macrophages, including tumoricidal activity, and to test the synergistic effect of IFN-γ when combined with LPS or other TLR agonists. We found that several TLR agonists could induce macrophage-mediated tumor cell growth inhibition, but only when combined with IFN-γ. IFN-γ synergized with all TLR agonists for NO production and secretion of pro-inflammatory and Th1-polarizing (IL-12p70) cytokines. We conclude that optimal activation of antitumor M1 macrophages require two signals, which can be provided by a combination of IFN-γ and TLR agonists. Seven TLR agonists were shown to be effective and thereby emerge as potential therapeutic agents for cancer immunotherapy based on targeting of TAMs.

MATERIALS AND METHODS

Mice

C57BL/6NRj mice from Janvier Labs (Le Genest-Saint-Isle, France) were bred at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet (Oslo, Norway) in specific pathogen free conditions. The study was approved by the Norwegian National Committee for Animal Experiments. All experiments were performed in accordance with the institutional guidelines and regulations, including EU directive 2010/63/EU.

Cell Lines

MOPC315 is a mineral-oil induced plasmacytoma cell line which was derived from a BALB/c mouse and was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) (38). Lewis lung carcinoma (LLC, also called LLC1) is a cell line originating from a spontaneous lung carcinoma in a C57BL/6 mouse and was obtained from CLS Cell Lines Service (Eppelheim, Germany) (39). L929 is a fibroblast-like cell line

originating from connective tissue of a C3H/An mouse and was obtained from ATCC (40). J774.A1 is a macrophage-like cell line originating from the ascites of a BALB/c mouse with reticulum cell sarcoma and was kindly given by Anders Ø. Gammelsrud at the Norwegian Veterinary Institute (Oslo, Norway) (41). All of the above described cell lines were negative for mycoplasma infection as tested by use of MycoSensor PCR Assay kit (#302109, Agilent, Santa Clara, CA, USA).

Generation of Bone Marrow Derived Macrophages

Confluent L929 cells were cultured in RPMI 1640 medium (#61870044, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, #BCHRS0405, Biochrom GmbH, Berlin, Germany) for 10 days before the medium was centrifuged, filtered and stored at −20°C. Such L929 cell-conditioned medium contains macrophage colony-stimulating factor and was used for induction of macrophage differentiation according to standard protocols (42). Femurs and tibiae of the hind legs from 8- to 12-week-old male and female C57BL/6NRj mice were harvested and flushed with RPMI 1640 medium containing 10% FBS under sterile conditions. The isolated bone marrow was passed through a cell strainer with 70-µm pores (#CLS431751-50EA, Sigma-Aldrich, St. Louis, MO, USA) and cultured in 15 mm non-tissue culture treated dishes (#734-2359, VWR, Radnos, PA, USA) in RPMI 1640 medium containing 30% L929-derived conditioned medium. The bone marrow cells were cultured for 5 days, after which non-adherent, cells were washed off using phosphate buffered saline (PBS, # D8662, Sigma-Aldrich) and the adherent macrophages were cultured for 1 more day. Macrophages were harvested by incubation for 30 min at 4°C with cold PBS without $CaCl_2$ and $MgCl_2$ (#D8537, Sigma-Aldrich). Macrophages were then flushed off the plate, collected and kept frozen in aliquots at −150°C in FBS with 10% dimethyl sulfoxide (#0231-500 ml, VWR) for future experiments. The purity of the cells was 99% when analyzed by flow cytometry using the macrophage markers CD11b (#101219, BioLegend, San Diego, CA, USA) and F4/80 (#123116, BioLegend), and the cells were then referred to as bone marrow derived macrophages (BMDMs).

Tumor Cell Growth Inhibition Assay

Bone marrow derived macrophages were thawed and cultured for 3 days in non-tissue culture treated dishes (VWR) in RPMI 1640 medium containing 10% FBS and 10% L929-derived conditioned medium. The BMDMs were harvested by scraping, incubated for 2 h at 37°C with 10 mg/ml mitomycin C (#M4287, Sigma-Aldrich) to inhibit proliferation, and then washed thoroughly. Next, the BMDMs were resuspended in medium and seeded out in triplicates in flat bottom 96 well plates (#734-1793, Costar, Washington, DC, USA) at 3 cell densities: 6×10^4, 3×10^4, and 3×10^3 cells/well in a final volume of 200 µl/well. After 24 h of incubation, the medium was replaced with medium containing various stimuli, see section below, and incubated for another 24 h. Then, half of the cell supernatants (100 µl) were removed and used for quantification of NO_2^-. Target cell suspensions, consisting of 5,000 cells/well of MOPC315 or 3,000 cells/well of LLC cells were added, resulting in varying ratios of effector to target cells. After 24 h of co-culture, cells were pulsed with [^{3}H]thymidine (#MT6032, Hartmann Analytic, Braunschweig, Germany) and harvested 18 h later by a freeze and thaw cycle. The amount of radiolabeled DNA was measured on a 1450 MicroBeta Trilux Microplate Scintillation counter (Perkin Elmer, Waltham, MA, USA). The same assay was performed using the cell line J774.A1 as effector cells instead of BMDMs. Mitomycin C (10 mg/ml) was also used to block proliferation of J774.A1 cells before they were seeded out in 96 well plates in triplicates at a density of 1×10^5 cells/well in a final volume of 200 µl/well.

For the purpose of statistical analysis of pooled data from several experiments, the percentage of remaining cancer cell growth was calculated by dividing counts per minute (cpm) values from the macrophage-LLC co-cultures at the 20:1 ratio with the cpm values of the corresponding wells with LLCs alone using the following equation:

$$\frac{\text{cpm}_{20:1}}{\text{cpm}_{\text{LLC alone}}} \times 100 = \%\ \text{growth remaining.}$$

TLR Agonists and Cytokines

The following TLR agonists were used: TLR1/TLR2 agonist Pam3CSK4 (Pam3, #tlrl-pms, InvivoGen, San Diego, CA, USA); TLR3 agonist poly(I:C) of high molecular weight type (#tlrl-pic, InvivoGen); TLR4 agonist LPS from *E. coli* (#L4391, Sigma-Aldrich); TLR5 agonist flagellin (FLA) from *S. typhimurium*, ultrapure type (#tlrl-epstfla-5, InvivoGen); TLR2/TLR6 agonist LTA from *S. aureus* (#L2515, Sigma-Aldrich); TLR7 agonist CL264 (#tlrl-c264e-5, InvivoGen); and TLR9 agonist CpG, class C ODN 2395 (#tlrl-2395-1, InvivoGen). The TLR agonists were used alone or in combination with mouse recombinant IFN-γ (#315-05, Peprotech, Rocky Hill, NJ, USA) at a 40 ng/ml concentration.

Quantification of Nitrite by the Griess Test

Supernatants were centrifuged at 410 g to remove cellular debris and immediately assayed for nitrite as a measure for the amount of NO that was produced. 50 µl of macrophage supernatant was added to 50 µl of Griess reagent A consisting of distilled water with 1% sulphanilamide (#S9251, Sigma-Aldrich) and 5% phosphoric acid (#W290017, Sigma-Aldrich). The mixture was incubated in the dark for 10 min. Next, 50 µl of Griess reagent B consisting of 0.1% *N*-(1-napthyl) ethylenediamine (#N9125, Sigma-Aldrich) in distilled water was added and the absorbance at 540 nm was measured with a microplate reader (BioTek Instruments, Winooski, VT, USA). Serial dilution of $NaNO_2$ served to create a standard curve of nitrite in the range of 1.56–100 µM.

iNOS Inhibition and NO Donor

S-Methylisothiourea hemisulfate salt (SMT, #M84445, Sigma-Aldrich) is a potent inhibitor of iNOS (43) which was used to block the production of NO by activated macrophages. Diethylenetriamine/NO adduct (DETA/NO) (#D185, Sigma-Aldrich) was used to produce controlled release of NO in solution.

Cytokine Quantification by Luminex Technology

Supernatants harvested from macrophages that had been stimulated with TLR agonists and/or IFN-γ for 24 h were centrifuged to remove cellular debris and stored at −80°C for maximum 1 week and assayed for cytokines. The cytokine concentrations were determined by multiplex bead assays, Bio-Plex Pro Mouse cytokine singleplex sets for TNF-α (#171-G5023M), IL-12p40 (#171-G5010M), IL-12p70 (#171-G5011M), monokine-induced by IFN-γ (MIG) (#171-G6005M), and IL-10 (#171-G5009M) from Bio-Rad Laboratories (Hercules, CA, USA) according to the manufacturer's instructions. Samples in duplicates were analyzed, using a Bio-Plex MAGPIX Multiplex Reader and Bio-Plex Manager 6.1 software (Bio-Rad Laboratories).

Statistical Analysis

Multiple groups were compared by using one-way ANOVA followed by a *post hoc* Tukey test for multiple comparisons and p values of less than 0.05 were considered statistically significant ($^*p$-value < 0.05, $^{**}p$-value < 0.01, $^{***}p$-value < 0.001). Statistical analysis, including column statistics, was performed using GraphPad Prism 7.02 software.

RESULTS

LPS and IFN-γ Synergize to Activate Macrophages to Inhibit Tumor Cell Growth

Table 1 shows an overview of the literature on induction of tumoricidal activity of macrophages by various TLR agonists. The most widely used agonist, LPS, has shown effect in a number of studies that utilized different functional assays, macrophages and target cells. LPS has been used alone, in combination with MAF/IFN-γ, other TLR agonists, agonistic anti-CD40 antibodies, or TGF-β inhibition. However, basic questions regarding the induction of tumoricidal activity in normal macrophages remain to be answered. Many of the studies from the 1970s and 1980s lacked reliably pure (LPS free) reagents or macrophages, and more recent articles typically lack functional assays for tumoricidal activity.

We used an *in vitro* growth inhibition assay (7, 13) in order to measure both the cytotoxic and cytostatic activity of macrophages toward tumor cells (**Figure 1A**). Macrophages were first treated with the DNA crosslinker mitomycin C to block cell division and thereby avoid that macrophage growth could interfere with the detection of tumor cell growth inhibition. Thereafter, macrophages

TABLE 1 | Literature on induction of macrophage tumoricidal activity by TLR agonists.

TLR agonist	Functional assay used	Effector cells	Target cells	Conclusion	Ref
Lipopolysaccharide (LPS), lipid A (TLR4), RNA, and poly(I:C) (TLR3)	GI by manual counting of cells	PM from DBA/2 or CBA mice	L5178Y	All agents induced tumoricidal activity in macrophages	(17)
LPS	GI by cell number, cell death by release of thymidine	PM from C3H/HeN, C3H/HeJ mice	3T12	LPS induced tumoricidal activity, and MAF acted synergistically	(20)
LPS	Cell death by thymidine labeling	Human macrophages from PBMCs	SK-BR-3 and HT-29	LPS induce tumoricidal activity, and MAF acted synergistically	(44)
Lipotechoic acid (LTA) (TLR2/6), lipomannan (TLR2)	Cell death by release of thymidine	Bone marrow derived macrophages from DA rats	P-815 and DA tumor cells	Some LTAs induced strong tumoricidal activity, other LTAs and lipomannan less	(34)
LPS, CpG (TLR9)	GI by thymidine labeling and cell death by flow cytometry	PM from C3h/HeJ, CB17 SCID/beige or C57BL/6 mice	L5178Y, B16, RENCA, M21, NXS2, OVCAR	CpG and LPS combined or either factor combined with *in vivo* anti-CD40 ligation induced tumoricidal activity	(45)
LPS, BCG	Cell death by chrome release assay	PM from C3H/HeN mice	MBT-2	Both LPS and BCG induced tumoricidal activity	(46)
LPS, CpG	GI by thymidine labeling and cell death by flow cytometry	PM from C57BL/6 mice	B16, L5178Y and NXS2	*In vivo* stimulation with CpG induced tumoricidal activity *in vitro*, which was improved by adding LPS	(47)
Poly(I:C)	Cell death by chrome release assay	Tumor-associated macrophages (TAMs) from C57BL/6 mice	3LL Lewis	*In vivo* poly(I:C) induced *in vitro* tumoricidal activity	(33)
LPS, CpG	GI by thymidine labeling	PM from C57BL/6 mice	B16	LPS synergized with *in vivo* stimulation with anti-CD40 and CpG to induce *in vitro* tumoricidal activity	(48)
LPS, gardiquimod (TLR7)	Cell death by assay for luciferase	$CD11b^+$ $CD11c^-$ TAMs from C57/BL6 mice	MN/MCA1	TGF-β inhibition combined with TLR4 or TLR7 ligation induced tumoricidal activity	(35)
R848 (TLR7/8), Pam3 TLR(1/2)	Cell death by flow cytometry	mMDCs from human PBMCs	A549	R848 induced tumoricidal activity, but not Pam3	(36)

GI, growth inhibition; PM, peritoneal macrophages; ds, double stranded; MAF, macrophage-activating factor (interferon-γ); PBMCs, peripheral blood mononuclear cells; BCG, Bacillus Calmette–Guérin; TGF-β, tumor necrosis factor beta; iNOS, inducible nitric oxide synthase; mMDCs, monocytic myeloid-derived suppressor cells.

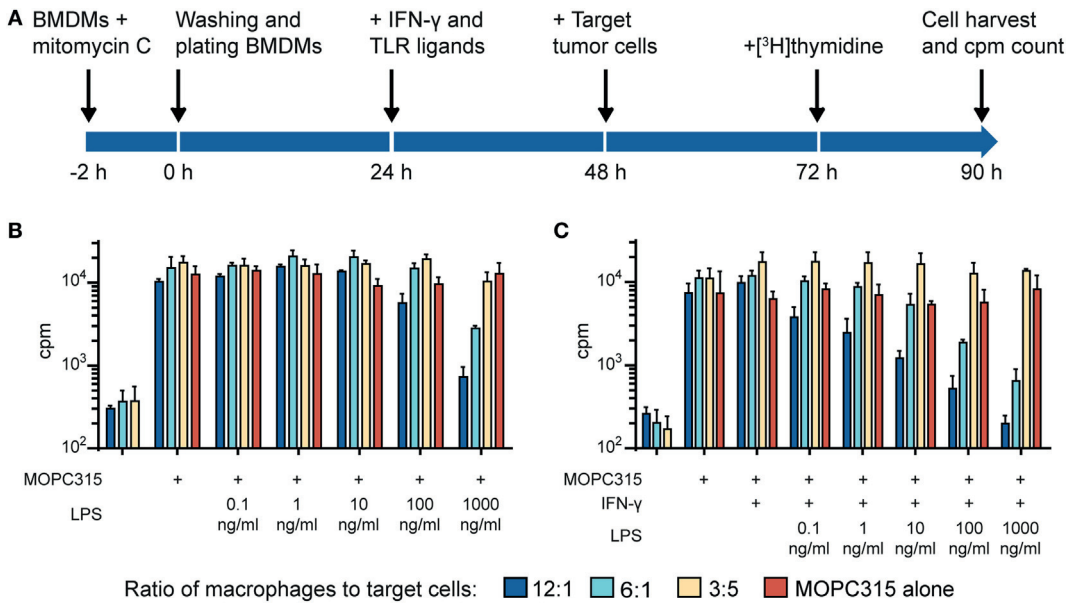

FIGURE 1 | Lipopolysaccharide (LPS) and IFN-γ synergize to induce tumoricidal activity in macrophages. **(A)** Time-line for the growth inhibition assay used for measuring macrophage cytotoxic and cytostatic activity toward tumor cells. **(B)** Mitomycin C-treated bone marrow derived macrophages (BMDMs) were stimulated for 24 h with various LPS concentrations before addition of 5,000 MOPC315 tumor cells/well, resulting in the indicated macrophage to target cell ratios. The growth of the tumor cells was quantified by measuring incorporation of radiolabeled thymidine and is shown on the *y*-axis as mean counts per minute (cpm) values of triplicates ± SD. The three columns to the left show proliferation of BMDMs alone (tumor cells were not added). **(C)** BMDMs were stimulated with IFN-γ (40 ng/ml) in combination with different concentrations of LPS for 24 h before target cells were added. Radiolabeled thymidine incorporation in growing cells is shown on the *y*-axis as mean cpm values of triplicates ± SD. The three columns on the left show proliferation of BMDMs alone. **(B,C)** All experiments were performed three times and representative experiments are shown.

were cultivated in the presence or absence of IFN-γ and TLR agonists for 24 h, before tumor cells were added and co-cultured for 42 h. Radiolabeled thymidine was used to detect tumor cell growth and was added to the co-cultures 18 h before cell harvest. As inhibition of tumor cell growth is known to depend on the number and density of macrophages, we seeded out macrophages in three different cell concentrations while the number of tumor cells remained constant within an experiment. The resulting ratio of macrophages to tumor cells, i.e., ratio of effector to target cells varied from 20:1 to 1:1 in various experiments.

In the first set of experiments, we investigated the effect of the classical macrophage activators IFN-γ and LPS. We used C57BL/6-derived BMDM as a source of normal mouse macrophages and the MOPC315 plasmacytoma as target tumor cells. When used alone, a high concentration (1,000 ng/ml) of LPS was required to activate BMDMs for inhibition of MOPC315 cell growth (**Figure 1B**). The potency of LPS was greatly improved when the macrophages were stimulated with LPS in combination with IFN-γ (**Figure 1C**) in accordance with previous reports (20, 22). Notably, macrophage stimulation with IFN-γ alone had no inhibitory effect on tumor cell growth (**Figure 1C**). Taken together, the experiments showed that activation with both LPS and IFN-γ was required for optimal induction of tumoricidal activity in macrophages. LPS alone could induce tumoricidal M1 macrophages, but only at high concentrations, while stimulation with IFN-γ alone had no effect.

Several TLR Agonists Other than LPS Synergize with IFN-γ for Rendering Macrophages Tumoricidal

To explore the potential of other natural or synthetic TLR agonists for inducing tumoricidal M1 macrophage phenotype, we tested a panel of agonists targeting different TLRs. In these experiments, the Lewis lung carcinoma (LLC) cell line was used as target cell line anticipating that macrophage-mediated tumor cell growth inhibition was not restricted to a single cell line. The target tumor cells were added to BMDMs activated by the following TLR agonists; TLR1/2 agonist Pam3, TLR2/6 agonist LTA, TLR3 agonist poly(I:C), TLR5 agonist flagellin, TLR7 agonist CL264, and TLR9 agonist CpG (**Figures 2A–F**). Pam3 was very potent at stimulating the BMDMs and it resulted in strong growth inhibition of LLC cells, even at concentrations as low as 1 ng/ml, but only when it was used together with IFN-γ (**Figure 2A**). Similarly, IFN-γ in combination with LTA (**Figure 2B**), CL264 (**Figure 2E**), and CpG (**Figure 2F**) induced tumor cell growth inhibition by BMDMs. Stimulation of BMDMs with poly(I:C) resulted in growth inhibition both alone and together with IFN-γ. Similar to LPS, the effect of poly(I:C) was potentiated by IFN-γ (**Figure 2C**). Stimulation of BMDM with flagellin yielded no growth inhibition (**Figure 2D**). Statistical analysis was performed for two TLR agonists (Pam3 and CpG) by pooling data from experimental repeats. Because baseline cpm values varied between experiments, the percentage of growth remaining was

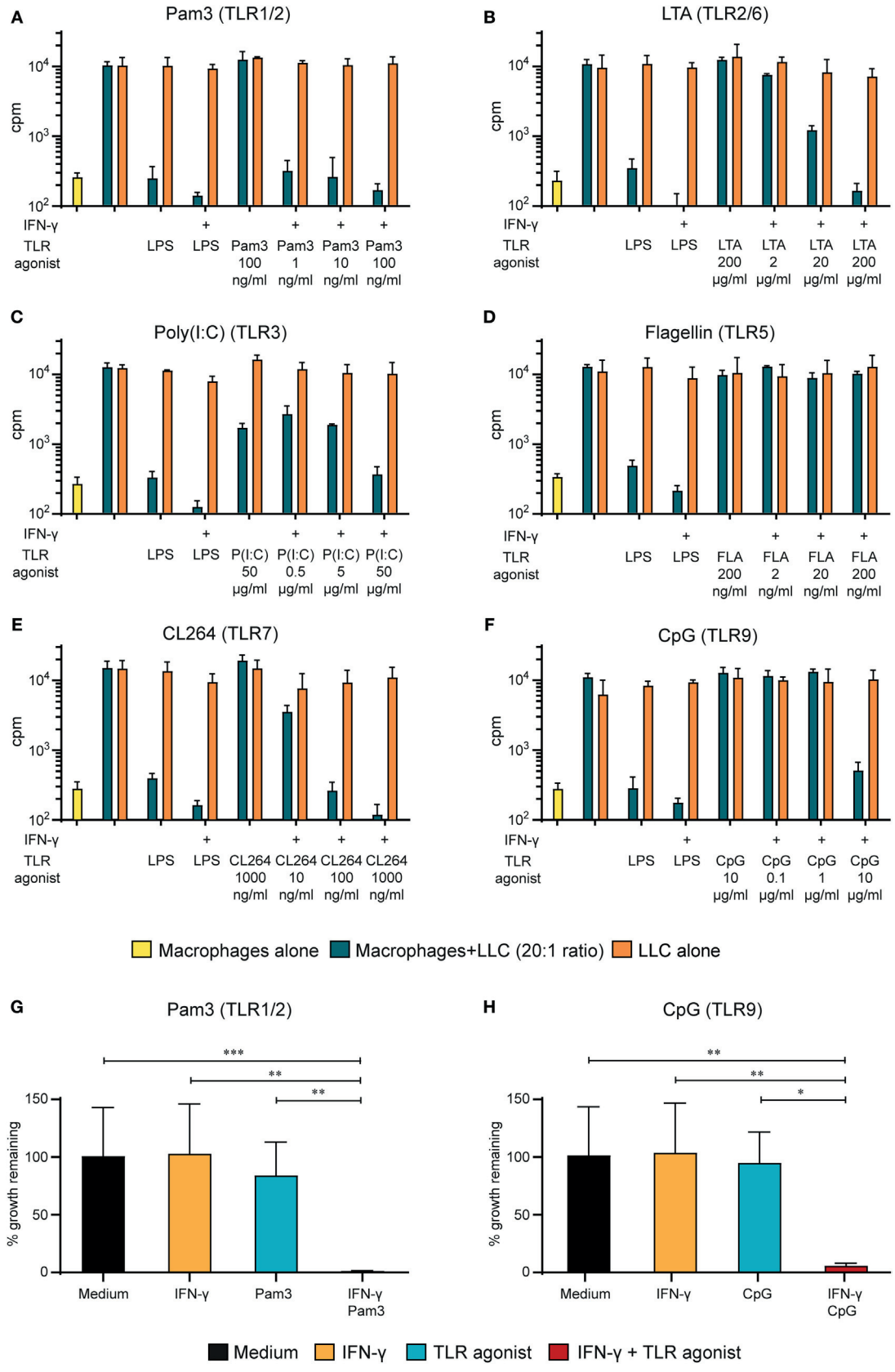

FIGURE 2 | Continued

FIGURE 2 | Continued
Synergy between IFN-γ and several TLR agonists for M1 macrophage activation. **(A–F)** Mitomycin C-treated bone marrow derived macrophages (BMDMs) (6×10^4 cells/well) were stimulated for 24 h with several TLR agonists at various concentrations in the presence or absence of IFN-γ (40 ng/ml) before addition of 3,000 LLC tumor cells/well, resulting in a 20:1 macrophage to target cell ratio. lipopolysaccharide (LPS) (1 μg/ml) + IFN-γ (40 ng/ml) was used as a positive control for macrophage activation. Radiolabeled thymidine incorporation in growing cells is shown on the *y*-axis as mean cpm values of triplicates ± SD. The first column on the left show proliferation of BMDMs alone. The following TLR agonists were tested at the indicated concentrations: **(A)** TLR1/2 agonist Pam3CSK4; **(B)** TLR2/6 agonist lipotechoic acid (LTA); **(C)** TLR3 agonist Poly(I:C); **(D)** TLR5 agonist Flagellin; **(E)** TLR7 agonist CL264; and **(F)** TLR9 agonist CpG. All experiments were performed three times and representative experiments are shown. **(G,H)** Statistical analysis of the pooled results from 5 **(G)** and 4 **(H)** growth inhibition assays performed as described above with the indicated TLR agonists. *y*-axis show % remaining growth calculated by dividing $cpm_{20:1}$ by cpm_{LLC} alone and multiplying with 100. *p*-values from multiple comparison test using one-way ANOVA is displayed as follows: **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001.

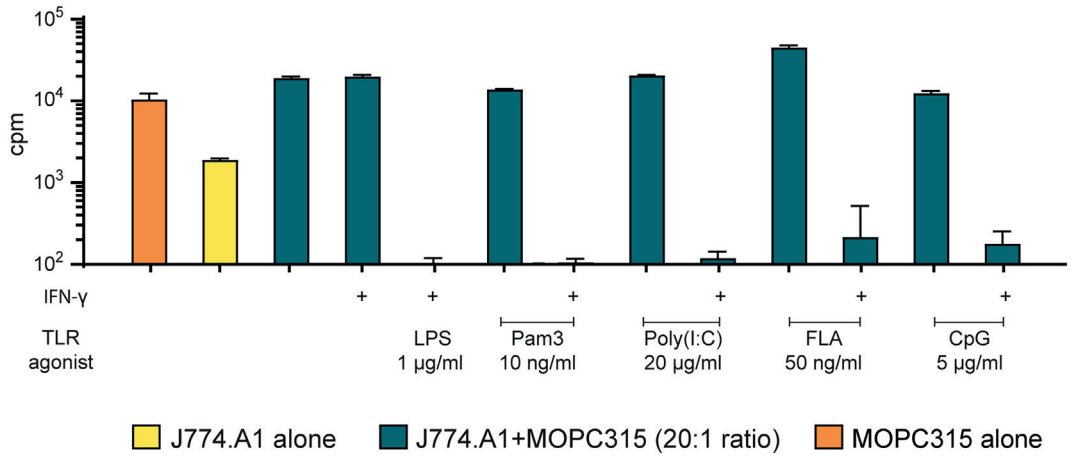

FIGURE 3 | The macrophage cell line J774.A1 inhibits tumor cell growth in a similar manner as bone marrow derived macrophages after two-signal activation. Growth inhibition assays. Mitomycin C-treated J774.A1 cells (1×10^5 cells/well) were stimulated with TLR agonists as indicated in the presence or absence of IFN-γ (40 ng/ml) for 18 h before addition of 5,000 MOPC315 tumor cells/well, resulting in a 20:1 effector to target cell ratio. Radiolabeled thymidine incorporation in growing cells is shown on the *y*-axis as mean cpm values of triplicates ± SD. The first column on the left shows proliferation of target cells alone and the second column shows proliferation of effector cells alone. This experiment was performed three times and a representative experiment is shown.

used in the comparisons. The analysis revealed a statistically significant stronger growth inhibition when macrophages were activated by two signals (TLR agonist and IFN-γ) compared to one signal only (**Figures 2G,H**). Thus, induction of tumoricidal M1 macrophages can be achieved through stimulation of the TLRs 1/2, 2/6, 3, 4, 7, or 9 when combined with IFN-γ. Stimulation of TLR 3 and 4 has some effect alone at high ligand concentrations. The only TLR agonist tested that did not activate BMDMs was flagellin (TLR5).

A Macrophage Cell Line also Inhibits Tumor Cell Growth Following Stimulation with TLR Agonists and IFN-γ

To investigate whether our findings were of a more general value rather than being specific to BMDMs, we tested an immortalized murine macrophage cell line, J774A.1, in the growth inhibition assay. Mitomycin C was used to block J774A.1 cell growth, before stimulation with TLR agonists alone or in combination with IFN-γ. When activated by LPS and IFN-γ, the macrophage cell line induced very strong growth inhibition of MOPC315 cells (**Figure 3**). These results were consistent with the growth inhibition mediated by BMDMs (**Figure 2**). We observed similar effect of co-stimulation with IFN-γ and the agonists Pam3 and CpG for the J774.A1 cell line and the BMDMs, whereas the effect of poly(I:C) combined with IFN-γ was stronger for the cell line. IFN-γ and flagellin also successfully stimulated J774A.1 to inhibit growth. Thus, murine macrophages, either primary cells or a cell line, could be activated toward a tumoricidal M1 phenotype by stimulation with IFN-γ and a second signal. Several TLR agonists could provide this second signal.

Tumor Cell Growth Inhibition by Activated Macrophages Is Mediated by NO

Production of the cytotoxic free radical NO is considered a hallmark of M1-polarized pro-inflammatory macrophages (49). NO was shown to be crucial for macrophage-mediated defense against bacteria during normal immune responses (50) and has been reported to be important for the killing of tumor cells *in vitro* (51, 52). Due to the extremely short half-life of NO, we quantified it indirectly using the Griess assay. This assay is based on the Griess diazotization reaction of the NO metabolite nitrite (NO_2^-) which forms a colored azo compound that can be quantified with a spectrophotometer. We analyzed the supernatant of BMDMs during the growth inhibition assay just before tumor cells were added. Macrophage activation with LPS alone for 24 h resulted in a concentration-dependent NO production

(**Figure 4A**). Stimulation with LPS in combination with IFN-γ greatly potentiated the effect and yielded more than 10 μM NO_2^- already at the lowest concentration of LPS that was tested (0.1 ng/ml) (**Figure 4A**). At 1,000 ng/ml of LPS, there was no clear additive effect of co-stimulation with IFN-γ, and the NO_2^- production seemed to reach a maximum level around 15 μM. These results, where stimulation with IFN-γ greatly improved the effect of LPS, are in accordance with previous studies on NO induction (53). These data also support our finding in the growth inhibition assay, showing that stimulation with two signals is required for optimal induction of M1 macrophage phenotype, defined either by tumoricidal activity or NO production.

To investigate the importance of NO in macrophage-mediated tumor cell growth inhibition, we used the iNOS-specific inhibitor SMT to block NO production (43). SMT completely blocked NO production by activated BMDMs when used at 10 mM concentration, whereas 1 mM only partly hindered NO production (**Figure 4B**). When tested in the growth inhibition assay, 1 mM SMT was sufficient to abolish the growth inhibition induced both by LPS alone (**Figure 4C**) and by LPS in combination with IFN-γ (**Figure 4D**). These data strongly suggest that macrophages mediate growth inhibition of tumor cells through a NO-dependent mechanism.

Cell-free NO Is Cytotoxic at a High Concentration

To test whether we could recreate the growth inhibitory effect of NO without the presence of macrophages, we used the chemical compound diethylenetriamine/NO adduct (DETA/NO), which functions as an NO donor and releases NO to the medium. We set up a modified growth inhibition assay where tumor cells were exposed to DETA/NO in the absence of macrophages (**Figure 5A**). DETA/NO was dissolved in cell culture medium and used immediately. Just before adding the DETA/NO solution to the MOPC315 target cells, the amount of NO released

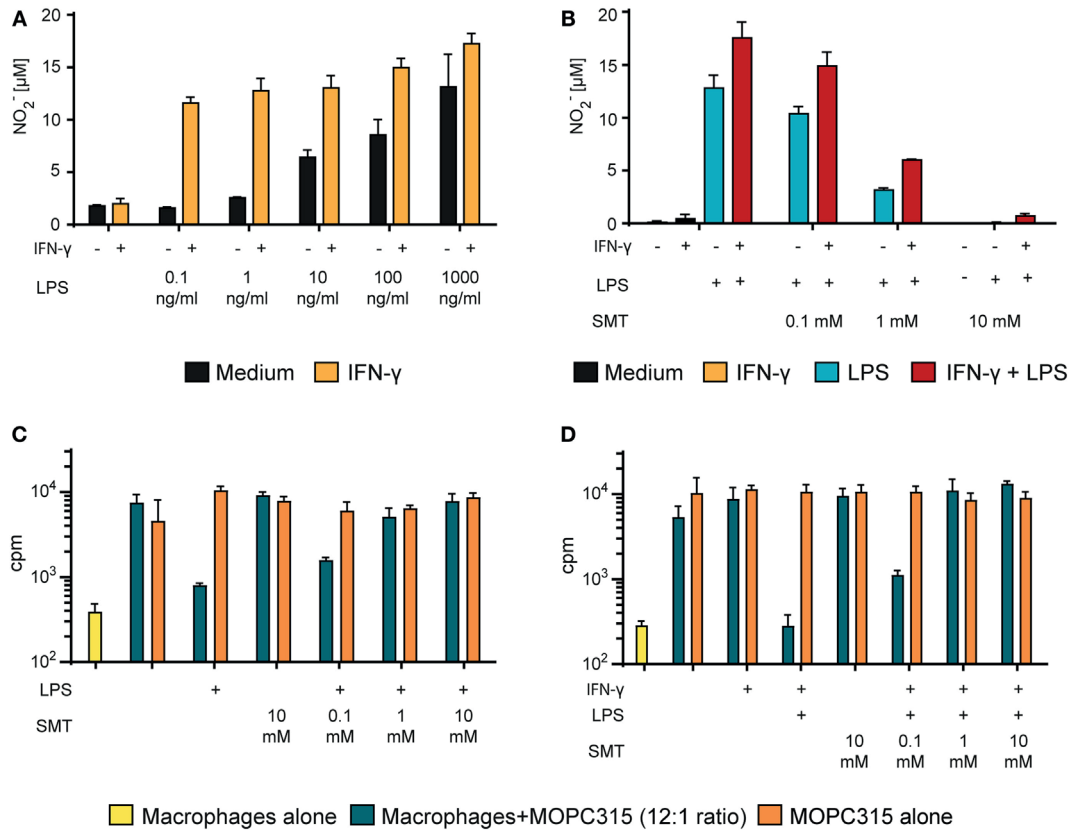

FIGURE 4 | Tumor cell growth inhibition by activated macrophages is mediated by NO. **(A)** Bone marrow derived macrophages (BMDMs) (6×10^4 cells/well) were stimulated with different concentrations of lipopolysaccharide (LPS) alone or in combination with IFN-γ (40 ng/ml) for 24 h. The Griess assay was used to measure NO in the supernatants indirectly as nitrite (NO_2^-). NO_2^- levels (μM) are presented as mean values of triplicates ± SD. **(B)** BMDMs (6×10^4 cells/well) were incubated with various concentrations of the inducible NO synthase inhibitor SMT (S-Methylisothiourea hemisulfate salt) and stimulated with LPS (1 μg/ml) alone or in combination with IFN-γ (40 ng/ml) for 24 h. NO_2^- concentration (μM) in the supernatants was measured using the Griess assay and presented as mean values of triplicates ± SD. **(C,D)** Growth inhibition assay. Mitomycin C-treated BMDMs (6×10^4 cells/well) were stimulated for 24 h with LPS alone (1 μg/ml) **(C)** or with LPS (1 μg/ml) + IFN-γ (40 ng/ml) **(D)** and treated with various concentrations of SMT before addition of 5,000 MOPC315 tumor cells/well, resulting in a 12:1 macrophage to target cell ratio. Radiolabeled thymidine incorporation in growing cells is shown on the *y*-axis as mean cpm values of triplicates ± SD. The first column on the left show proliferation of BMDMs alone. **(A–D)** All experiments were performed three times and representative experiments are shown.

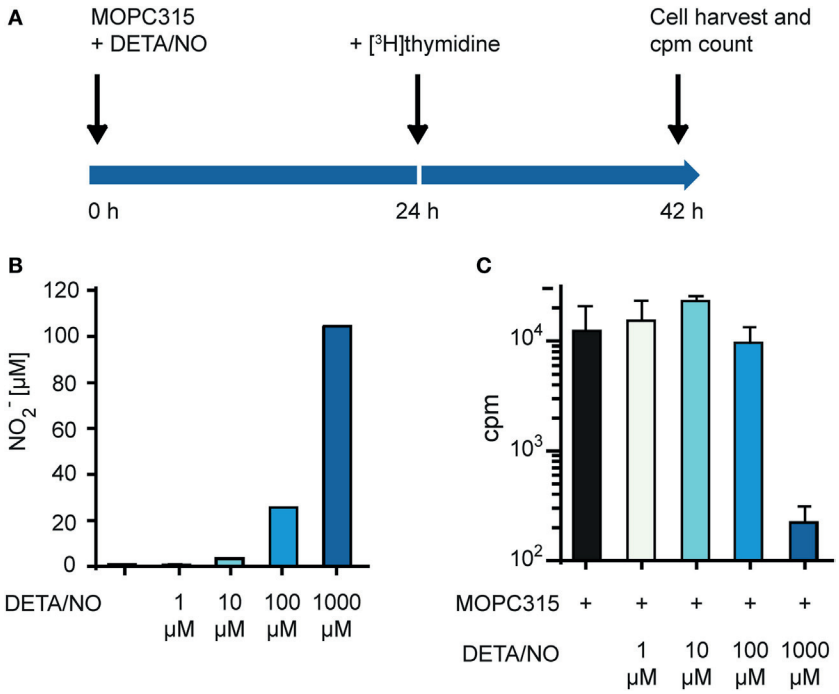

FIGURE 5 | Tumor cell proliferation is inhibited by high concentrations of NO. **(A)** Time-line for the growth inhibition assay used for measuring direct cytotoxic and cytostatic activity of NO released from diethylenetriamine/nitric oxide adduct (DETA/NO) toward tumor cells. **(B)** Varying concentrations of DETA/NO in media was analyzed for NO_2^- using the Griess assay. The *y*-axis shows the µM concentration of NO_2^- measured. **(C)** Growth inhibition assay. MOPC315 cells were cultured in varying concentrations of DETA/NO for 42 h before analysis. Radiolabeled thymidine incorporation in growing cells is shown on the *y*-axis as mean cpm values of triplicates ± SD. All experiments were performed three times and representative experiments are shown.

in the medium was quantified indirectly by measuring NO_2^- for each concentration of DETA/NO used (**Figure 5B**). MOPC315 growth was then quantified by measuring incorporation of radiolabeled thymidine as for the standard growth inhibition assay (**Figure 5C**). We found that inhibition of growth was obtained only at the highest tested DETA/NO concentration, i.e., 1 mM, which corresponds to a NO_2^- concentration of around 100 µM (**Figures 5A,B**). These data confirm that our target tumor cells are sensitive to NO in a concentration-dependent way and are consistent with a key role of NO secretion for macrophage tumoricidal activity.

TLR Agonists Mediate Tumor Cell Growth Inhibition *via* Production of NO

To examine whether TLR agonists other than LPS also induce NO production, we stimulated BMDMs with TLR agonists both alone and in combination with IFN-γ and measured the levels of NO_2^- in the supernatants (**Figure 6A**). For each TLR agonist, three concentrations were chosen and arbitrary defined as low, intermediate, and high. We found that all TLR agonists synergized with IFN-γ for induction of NO production, as the NO_2^- levels were 2- to 10-fold higher when BMDMs were activated with TLR agonists in combination with IFN-γ compared with TLR agonists alone. IFN-γ together with a low concentration of the TLR agonists still yielded more NO than single activation with TLR agonist at a 100-fold higher concentration (**Figure 6A**). Thus, several TLR agonists can replace LPS as an activating signal for macrophage NO production.

Next, we wanted to verify that the inhibitor SMT could inhibit the NO production induced by any TLR agonist. Measurements by the Griess assay revealed that SMT reduced the levels of NO_2^- in the BMDM cultures stimulated with TLR agonists in combination with IFN-γ (**Figure 6B**). Furthermore, we observed that the growth inhibition induced after co-stimulation with IFN-γ and LPS or any other tested TLR agonist was abolished when the iNOS inhibitor was present (**Figure 6C**). Therefore, *in vitro* tumor cell growth inhibition after macrophage activation with any TLR agonist appears to depend on NO production. **Table 2** shows a summary of the effect of all tested TLR agonists in combination with IFN-γ. There was a strong, but incomplete correlation between induction of NO production and tumor cell growth inhibition.

IFN-γ and TLR Agonists Synergize for Production of Pro-inflammatory and Th1-Polarizing Cytokines by BMDMs

In the next set of experiments, we wanted to examine whether release of particular cytokines was affected by single versus two signal activation of macrophages. We measured the levels of the pro-inflammatory cytokines TNF-α and IL-12p40, the Th1-polarizing cytokine IL-12p70, the anti-inflammatory cytokine IL-10, and the chemokine MIG/CXCL9 in the supernatant of

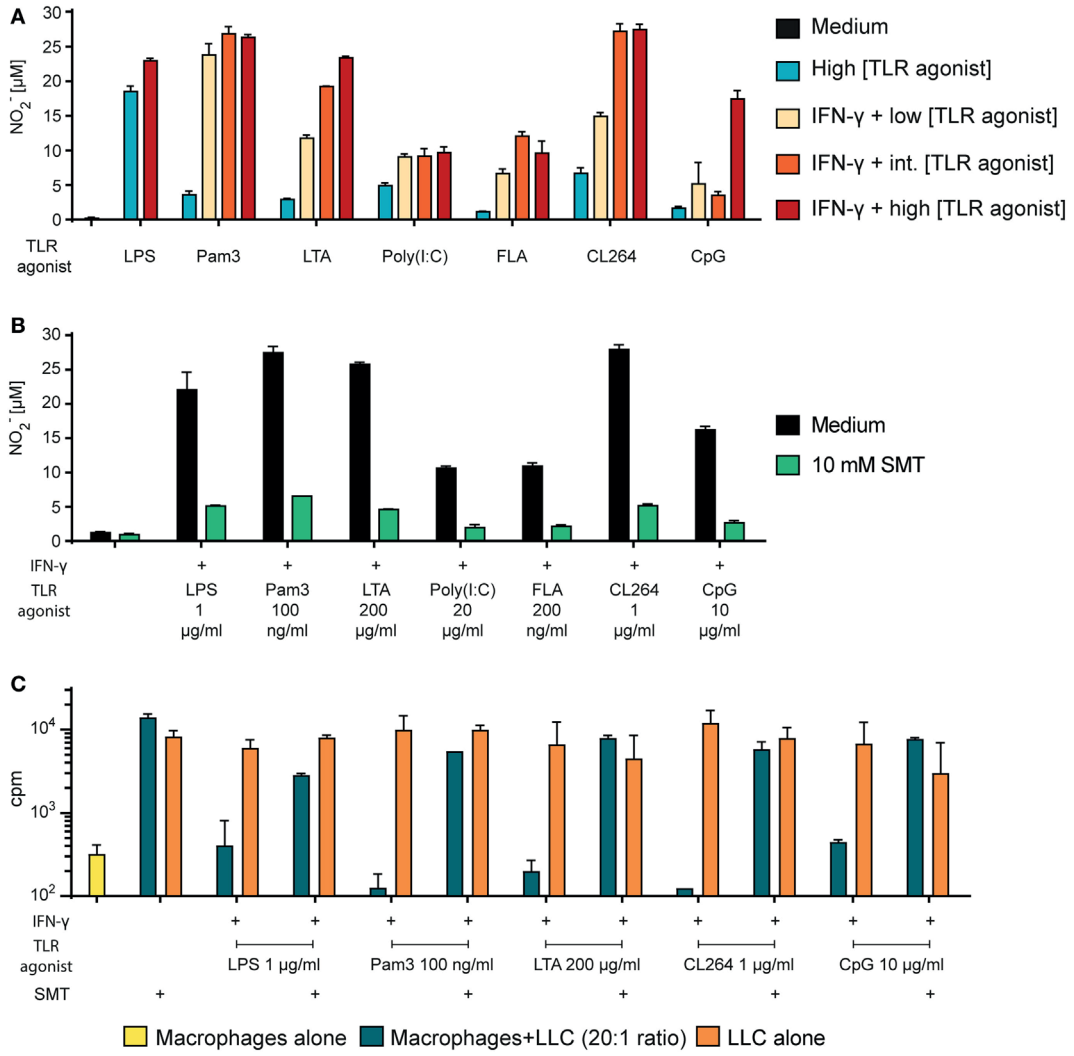

FIGURE 6 | Tumor cell growth inhibition by macrophages activated by any TLR agonist requires NO. **(A)** Bone marrow derived macrophages (BMDMs) (6×10^4 cells/well) were stimulated with TLR agonists alone or in combination with IFN-γ (40 ng/ml) as indicated for 24 h. NO_2^- concentration (µM) in the supernatants was measured using the Griess assay and presented as mean values of triplicates ± SD. Each of the TLR agonists were tested at three concentrations (low/intermediate (int)/high): Pam3 (1/10/100 ng/ml); lipotechoic acid (LTA) (2/20/200 µg/ml); poly(I:C) (0.5/5/50 µg/ml); flagellin (FLA) (2/20/200 ng/ml); CL264 (10/100/1,000 ng/ml); CpG (0.1/1/10 µg/ml). **(B)** BMDMs (6×10^4 cells/well) were incubated in the absence or presence of the inducible NO synthase inhibitor s-methylisothiourea hemisulfate salt (SMT) (10 mM) and stimulated with TLR agonists as indicated and IFN-γ (40 ng/ml) for 24 h. NO_2^- concentration (µM) in the supernatants was measured using the Griess assay and presented as mean values of triplicates ± SD. **(C)** Growth inhibition assay. Mitomycin C-treated BMDMs (6×10^4 cells/well) were incubated in the absence or presence of SMT (10 mM) and IFN-γ (40 ng/ml) in combination with TLR agonists as indicated for 24 h before addition of 3,000 LLC tumor cells/well, resulting in a 20:1 macrophage to target cell ratio. The growth of the tumor cells was quantified by measuring incorporation of radiolabeled thymidine and is shown on the *y*-axis as mean cpm values of triplicates ± SD. The first column to the left show control wells with BMDMs alone (no tumor cells). **(A–C)** All experiments were performed three times and representative experiments are shown.

BMDMs stimulated for 24 h (**Figure 7**). There was a clear synergistic effect of IFN-γ and most TLR agonists on the secretion of TNF-α, IL-12p40, and IL-12p70. Activation with TLR agonists alone resulted in relatively low to medium cytokine levels which increased in response to LPS, Pam3, LTA, and CL264 when IFN-γ was added (**Figures 7A–C**). IL-10 production was induced by TLR agonists alone with LPS giving the strongest response. Strikingly, IL-10 production induced by TLR triggering was reduced in the presence of IFN-γ (**Figure 7D**). Exceptions were poly(I:C) and flagellin, which resulted in no or very low secretion of any cytokine both when used alone and in combination with IFN-γ. Untreated BMDMs produced no cytokines. BMDMs activated with IFN-γ alone secreted no cytokines except for MIG/CXCL9 as expected for this IFN-γ-inducable chemokine. The chemokine MIG/CXCL9 was strongly induced by IFN-γ alone and the levels were further increased upon combined activation with all TLR agonists, except LPS (**Figure 7E**). Thus, IFN-γ and TLR agonists synergize to make macrophages produce high levels of pro-inflammatory and Th1-polarizing cytokines (TNF-α, IL-12p40, and IL-12p70) and low levels of IL-10.

TABLE 2 | Summary of TLR-mediated activation of macrophages in synergy with IFN-γ.

Activation signal[a]		Bone marrow derived macrophages		J774.A1	
Agonist	**TLR**	**GIA[b]**	**NO[c]**	**GIA**	**NO**
Lipopolysaccharide	TLR4	++	++	++	++
Pam3	TLR1/2	++	++	++	+
Lipotechoic acid	TLR2/6	++	++	ND	ND
Poly(I:C)	TLR3	+	+	++	+
Flagellin	TLR5	–	+	++	++
CL264	TLR7	++	++	ND	ND
CpG	TLR9	++	++	++	+

[a]*Given in combination with IFN-γ.*
[b]*Tumor cell growth inhibition assay (GIA): +, some inhibition; ++, strong inhibition; –, none; ND, not determined.*
[c]*Nitric oxide (NO) production. +, some; ++, strong; –, none; ND, not determined.*

DISCUSSION

In this paper, we show that activation with two molecular signals from the microenvironment is required for efficient induction of M1 phenotype in murine macrophages as defined by tumoricidal activity, NO production, and secretion of pro-inflammatory and Th1-polarizing cytokines. We evaluated first two classical macrophage stimulators, namely LPS and IFN-γ. We found that IFN-γ greatly potentiates the effect of LPS, resulting in strong tumoricidal activity at low LPS concentrations, whereas no tumoricidal activity was induced by IFN-γ alone. A similar synergistic effect of LPS and IFN-γ on induction of tumoricidal macrophages was shown previously by several investigators in the 1970s and 1980s (16, 18, 19, 21, 22). However, the interpretation of many of these early studies is problematic due to variability in the source of macrophages and potentially impure or LPS-contaminated IFN-γ (previously called MAF)

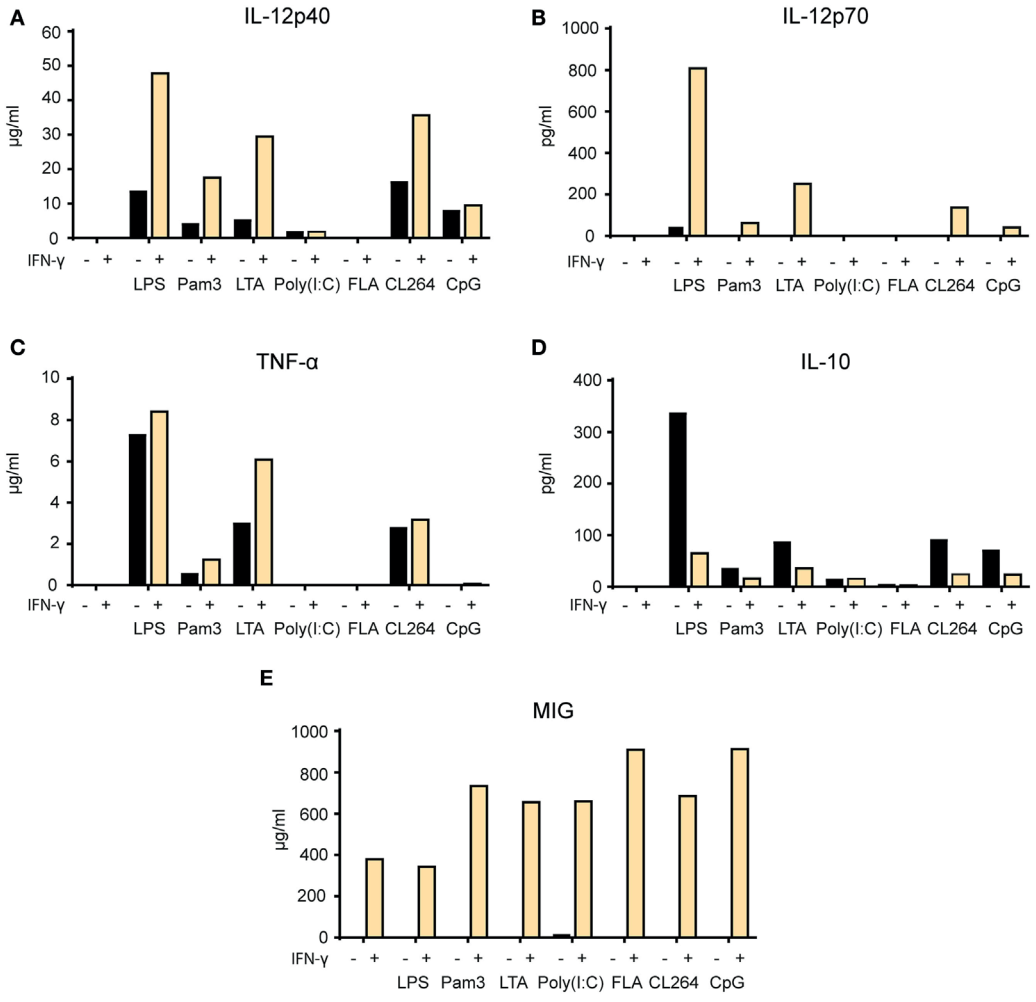

FIGURE 7 | Synergy between IFN-γ and TLR agonists for induction of pro-inflammatory cytokine secretion by macrophages. **(A–E)** Mitomycin C-treated bone marrow derived macrophages (2.4×10^4 cells/well) were stimulated for 24 h with the following TLR agonists in the presence or absence of IFN-γ (40 ng/ml): lipopolysaccharide (LPS) (1 µg/ml), Pam3 (100 ng/ml), lipotechoic acid (LTA) (200 µg/ml), poly(I:C) (50 µg/ml), flagellin (200 ng/ml), CL264 (1 µg/ml), and CpG (10 µg/ml). Cell supernatants were analyzed by Luminex technology and the cytokine content is shown on the *y*-axis as mean pg/ml or ng/ml values of duplicates. The following cytokines were measured: **(A)** IL-12p40, **(B)** IL-12p70, **(C)** TNF-α, **(D)** IL-10, and **(E)** monokine-induced by IFN-γ (MIG). All experiments were performed three times and representative experiments are shown.

preparations. Peritoneal macrophages were used in most of the studies, and often peptone or thioglycollate was injected into the peritoneum to increase the yield of macrophages. These compounds may themselves give an inflammatory stimulus to the macrophages (54). Moreover, peritoneal macrophages may be contaminated by other cell types (55), and this is not accounted for in all studies. The literature also contains reports on induction of tumoricidal M1 macrophages by single activation with IFN-γ or LPS (56, 57), and most recent reviews make no distinction between the macrophage phenotypes resulting from activation with IFN-γ, LPS, or both. Due to the potential of M1 macrophages for immunotherapy for cancer, a clarification of which signals are required for an optimal induction of these cells was needed. In pilot studies, we used peritoneal macrophages, but considerable variability between experiments was observed (data not shown). Therefore, we decided to use BMDM generated by standard protocols as source of normal mouse macrophages. Using BMDMs as effector cells, we could clearly show that IFN-γ alone is ineffective at activating macrophages to a tumoricidal M1 phenotype. LPS had some effect alone, but only when it was used in high concentrations, indicating that M1 activation by LPS alone is sub-optimal. When macrophages were activated with IFN-γ in combination with LPS, a potent tumoricidal phenotype was obtained even with the use of very low LPS concentrations. Thus, our data confirm earlier *in vitro* studies with LPS and IFN-γ that revealed that two signals are required for inducing a tumoricidal M1 macrophage phenotype. This is also in line with our previous findings from an *in vivo* model of myeloma, where IFN-γ was required, but not sufficient to explain the cytotoxic effect of TAMs, indicating the involvement of another signal (7, 11).

Based on our findings of the synergistic effect of IFN-γ and the TLR4 agonist LPS, we wanted to investigate whether stimulation with LPS could be replaced by triggering any other TLR. Some TLR agonists have previously been reported to be able to induce tumoricidal M1 macrophages, but the TLR ligands were mostly used in combination with other agents such as TGF-β inhibitors or CD40 agonists rather than IFN-γ (see **Table 1**). Synergistic effects of several TLR agonists and IFN-γ on macrophage expression of cytokines and NO production has been described (58, 59), but to the best of our knowledge the only TLR ligands that have been shown to synergize with IFN-γ for induction of tumoricidal functions of macrophages are LPS and poly(I:C) (60). We therefore set up a panel of agonists covering most of the well-described TLRs in mice. We found that all TLR agonists synergized with IFN-γ to induce a tumoricidal M1 macrophage phenotype. Flagellin, a TLR5 agonist, combined with IFN-γ did not induce any tumor cell growth inhibition by BMDMs, but it activated the macrophage cell line J774.A1. This could be explained by various factors such as lower TLR5 receptor expression by BMDMs compared to J774.A1. Importantly, all TLR agonists, with the exception of LPS and poly(I:C), had no effect when used alone, but induced potent macrophage-mediated tumor cell growth inhibition when combined with IFN-γ. This may explain the lack of reports on induction of tumoricidal M1 macrophages by other TLR agonists, as previous studies have not included IFN-γ in the activation protocol (**Table 1**). Several recent studies revealed the therapeutic potential of activating TAMs toward an antitumor M1 phenotype, resulting in macrophage-mediated tumor immune surveillance with tumor regression *in vivo* (8–10). These proof-of-principle reports support the potential application of our findings in the development of novel macrophage-targeted cancer therapies by combining IFN-γ with TLR agonists.

Our experiments demonstrated that the presence of NO was necessary for cancer cell growth inhibition by macrophages, which is consistent with recent studies reporting the importance of NO in macrophage-mediated antitumor effects (61, 62). NO was found to be the main mediator of the tumoricidal effect of activated macrophages in a number of studies from the 1980s (51, 52, 63), but some reports also indicate the existence of iNOS-independent mechanisms (64). Inhibition of iNOS in activated macrophages resulted in a concentration-dependent abrogation of both NO production and tumor cell growth inhibition. Production of NO by BMDMs correlated with tumor cell growth inhibition, but could not be used as a predictive surrogate marker for tumoricidal activity (**Table 2**). This finding has consequences for the interpretation of previous studies as well as the planning of future studies aimed at inducing tumoricidal M1 macrophages. M1 macrophages were originally defined as having a killer phenotype with a characteristic shift in L-arginine metabolism into NO production, as opposed to healing M2 macrophages which use L-arginine to generate L-ornithine and urea (3, 65). Consequently, induction of iNOS, the enzyme responsible for production of NO by activated macrophages, has been established as a hallmark of tumoricidal M1 macrophages (66). We would argue that the widespread use of iNOS-expression or NO production by macrophages as a surrogate marker of tumoricidal M1 macrophages should be replaced or accompanied by functional assays that directly measure the tumoricidal activity of macrophages.

We observed a synergistic effect of IFN-γ and TLR agonists on the induction of the pro-inflammatory cytokines TNF-α and IL-12p40, and the Th1-polarizing cytokine IL-12p70 (also called IL-12p75), while the angiostatic chemokine MIG (or CXCL9) was induced by IFN-γ alone. We have previously reported in mouse models for myeloma and lymphoma that the secretion of these cytokines was associated with successful immunity against cancer (11). Furthermore, production of TNF-α and IL-12 cytokines plays important roles in macrophage-mediated immune responses to pathogens and cancer (67), and the observation that TLR agonists and IFN-γ synergize for this function fits well with previous studies (68, 69). Interestingly, the results for the anti-inflammatory cytokine IL-10 were different, as IFN-γ reduced the induction of IL-10 seen by TLR activation alone. The ability of IFN-γ to inhibit IL-10 production has been previously described and suggested as a potential mechanism underlying the synergistic effect of IFN-γ on TLR-mediated macrophage activation (70). IL-10 is induced at a low level upon TLR activation and mediates a negative feedback loop involving induction of STAT3 (71, 72). IFN-γ was shown to inhibit IL-10 production by increasing the activity glycogen synthase kinase 3β (GSK-3β), a negative regulator of AP-1 and CREB signaling

(70). GSK-3β mediated the synergistic activity of IFN-γ on increasing NF-κB activity, NO production and IL-6 secretion in TLR-activated macrophages (73, 74). So far, GSK-3β has been shown to be a key regulator of TLR2 and TLR4 signaling, and potentially also TLR3 (75). However, more studies are required to clarify the role of GSK-3β in the synergistic effect of IFN-γ and other TLRs, as well as whether this regulatory pathway can explain why a combination of IFN-γ and TLR agonists are required for optimal induction of tumoricidal M1 activity in macrophages.

Our data confirm previous findings showing that LPS and poly(I:C) may induce some macrophage-mediated tumor cell growth inhibition in the absence of IFN-γ (17). At first glance, this contradicts our conclusion that M1 macrophage polarization requires two signals. However, it has been reported that LPS and poly(I:C) might in fact act by combining TLR signaling with autocrine type I interferon signaling (76). Torres and Johnson demonstrated that both LPS and poly(I:C) induced secretion of IFN-α/β and that the tumoricidal activity induced by LPS or poly(I:C) could be abrogated by neutralizing antibodies against IFN-α/β, but not against IFN-γ (76). Adding IFN-γ to poly(I:C)-activated macrophages after IFN-α/β-blocking could rescue the tumoricidal activity. Furthermore, Pace et al. observed that both IFN-α and β could synergize with LPS or heat killed *Listeria monocytogenes* for the induction of tumoricidal activity, however less potently than IFN-γ (77). After the discovery of the receptors that recognize LPS and poly(I:C), TLR4 and TLR3 respectively, and the signaling pathways involved, it has become clear that these two TLRs share the ability to signal *via* a TRIF-dependent pathway, resulting in activation of IRF3 and induction of type I interferons (78, 79). The other main signaling pathway used by TLRs depends on MyD88 and results in activation of NFκB rather than IRF3 (80). TLR4 is the only TLR that is able to activate both pathways, and this has been suggested to explain the powerful effect of LPS on macrophage activation. Synergistic effects on cytokine production and T cell stimulation from combined activation of macrophages with MyD88-dependent and TRIF-dependent TLR agonists have previously been described (81), and may provide a novel way of inducing tumoricidal M1 macrophages. Thus, the two-signal model for induction of tumoricidal M1 macrophages might be extended to encompass interferon-α/β/γ-signaling and signaling through a large range of TLRs. Such insight on 2-signal requirement should be valuable for the development of future macrophage-targeted cancer therapies.

Our data suggest a general mechanism of TLR and IFN-γ-mediated signaling that synergizes for induction of antitumor M1 macrophage phenotype. The striking functional similarities between different TLR agonists suggest that differential TLR expression between mouse and human macrophages might not represent a major problem for therapy development, since multiple TLR agonists may potentially be used. It has been shown that monocyte-derived human macrophages could inhibit tumor cell growth *in vitro* upon combined activation with LPS and IFN-γ (44), suggesting that the rules for induction of M1 macrophage phenotype may be conserved across these two species. Another important issue that will need clarification is whether TLR activation in combination with IFN-γ will be sufficient to induce M1 phenotype in TAMs which are considered to be polarized differently in M2 or M2-like modus. Such repolarization has been reported using several activation protocols, including miRNA (82). Interestingly, a TLR7 agonist was shown to be effective at reversing the pro-tumor phenotype of murine TAMs *in vitro,* but only in combination with TGF-β blockade (35). We propose that exploiting the synergistic effect of combined macrophage activation with IFN-γ and TLR agonist may have a great potential for development of novel tumor immunotherapies.

ETHICS STATEMENT

The study was approved by the Norwegian National Committee for Animal Experiments. All experiments were performed in accordance with the institutional guidelines and regulations, including EU directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

EM performed most experimental work, analyzed results, and wrote the manuscript. PC performed experimental work, analyzed results, and contributed to writing the final version of the manuscript. SH performed experimental work on the cell line and analyzed results. AL performed early, preliminary experimental work. KB provided help with cell culture work and materials. MS provided BMDM material and protocol and contributed to writing the final version of the manuscript. IØ provided supervision and experimental help, discussed the results, and contributed to writing the final version of the manuscript. AC designed, supervised, and evaluated the experiments and contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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PAPER II

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Generation and Functional *In Vitro* Analysis of Semliki Forest Virus Vectors Encoding TNF-α and IFN-γ

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Cytokine gene delivery by viral vectors is a promising novel strategy for cancer immunotherapy. Semliki Forest virus (SFV) has many advantages as a delivery vector, including the ability to (i) induce p53-independent killing of tumor cells *via* apoptosis, (ii) elicit a type-I interferon (IFN) response, and (iii) express high levels of the transgene. SFV vectors encoding cytokines such as interleukin (IL)-12 have shown promising therapeutic responses in experimental tumor models. Here, we developed two new recombinant SFV vectors encoding either murine tumor necrosis factor-α (TNF-α) or murine interferon-γ (IFN-γ), two cytokines with documented immunostimulatory and antitumor activity. The SFV vector showed high infection rate and cytotoxicity in mouse and human lung carcinoma cells *in vitro*. By contrast, mouse and human macrophages were resistant to infection with SFV. The recombinant SFV vectors directly inhibited mouse lung carcinoma cell growth *in vitro*, while exploiting the cancer cells for production of SFV vector-encoded cytokines. The functionality of SFV vector-derived TNF-α was confirmed through successful induction of cell death in TNF-α-sensitive fibroblasts in a concentration-dependent manner. SFV vector-derived IFN-γ activated macrophages toward a tumoricidal phenotype leading to suppressed Lewis lung carcinoma cell growth *in vitro* in a concentration-dependent manner. The ability of SFV to provide functional cytokines and infect tumor cells but not macrophages suggests that SFV may be very useful for cancer immunotherapy employing tumor-infiltrating macrophages.

Keywords: Semliki Forest virus, cancer immunotherapy, gene delivery, cytokines, macrophage activation, Lewis lung carcinoma

Abbreviations: 3H-TdR, [methyl-3H]-thymidine; BHK-21, baby hamster kidney fibroblasts; BMDM, bone marrow-derived macrophage; CM, conditioned medium; cpm, counts per minute; DsRed, *Discosoma* sp. red fluorescent protein; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HMDM, human monocyte-derived macrophage; IFU, infectious units; LLC, Lewis lung carcinoma; MOI, multiplicity of infection; NO, nitric oxide; NO2-, nitrite; Pam3, Pam3CSK4; PBS−/−, phosphate-buffered saline without magnesium and calcium; PBS+/+, phosphate-buffered saline with magnesium and calcium; PI, propidium iodide; rIFN-γ, recombinant interferon-γ; rSFV, recombinant Semliki Forest virus; rTNF-α, recombinant tumor necrosis factor-α; SeV, Sendai virus; SFV, Semliki Forest virus; TLR, toll-like receptor; vdIFN-γ, vector-derived interferon-γ; vdTNF-α, vector-derived tumor necrosis factor-α.

INTRODUCTION

Viral vectors have been used for cancer immunotherapy to intratumorally deliver and express transgenes and to thereby increase therapeutic protein delivery while simultaneously reducing systemic toxicity (1). Recombinant Semliki Forest virus (rSFV) has been successfully used as a cancer immunotherapy in preclinical tumor models for cytokine gene delivery (2–15) and for immunization with tumor-associated antigens against mastocytoma (2, 16) and human papilloma virus (HPV)-associated cervical cancer (17, 18). Semliki Forest virus (SFV) belongs to the *Alphavirus* genus of the *Togaviridae* family and possesses an enveloped nucleocapsid that contains a positive-sense single-stranded (+ss) RNA genome (19). The replication-deficient SFV vector system delivers genes of interest by infecting the cells with viral particles and thereby providing a transiently high level of transgene expression without further virus replication (20). The SFV-based vector is an attractive tool for cancer immunotherapy because of its oncolytic nature and ability to induce *p53*-independent apoptosis of tumor cells (21), which may facilitate uptake and presentation of tumor antigens from the apoptotic bodies by antigen-presenting cells, thus enhancing cancer immunogenicity (22, 23). In addition, SFV vector elicits endogenous type-I interferon (IFN) responses, which may be required for the therapeutic effect of a vector-encoded cytokine (3).

Cytokines can contribute to antitumor immune responses in the tumor microenvironment (24, 25) and are, therefore, promising tools for cancer immunotherapy. rSFV has been used in preclinical studies to modulate the tumor microenvironment by locally delivering cytokines, such as interleukin (IL)-12 (2–11), IL-18 (12), IFN-α (13), granulocyte-macrophage colony-stimulating factor (14), and endostatin (15). An SFV vector encoding the heterodimeric murine IL-12 (SFV-IL12) was shown to have a strong therapeutic antitumor effect when locally injected in tumor models of melanoma (4), glioma (5), colon adenocarcinoma (3, 6, 7), hepatic colon adenocarcinoma (8), hepatocellular carcinoma (9), mastocytoma (2), fibrosarcoma (7), breast carcinoma (7), and lung carcinoma (3) in mice, orthotopic hepatocellular carcinoma in rats (10), and spontaneous hepatocellular carcinoma in woodchucks (11). The therapeutic activity of SFV-IL12 involved immune responses that were mediated by IL-12 in combination with the virus-induced endogenous type-I IFN (3, 9, 11), which lead to an angiostatic effect (4) and enhanced CD8+ T-cell responses (3, 5–8, 11).

Two well-known cytokines that may be potentially useful for cancer immunotherapy are IFN-γ and tumor necrosis factor-α (TNF-α). The pleiotropic cytokine IFN-γ is a tumor-suppressive factor that protects against carcinogen-induced and spontaneous tumor development (26, 27), inhibits tumor angiogenesis (28–31) and may improve tumor immunogenicity by upregulating major histocompatibility complex class I molecules (32, 33). Furthermore, it has been shown that IFN-γ enhances macrophage activation toward a tumoricidal phenotype *in vitro* (34–36) and *in vivo* (31, 37–39). TNF-α was discovered in 1975 as a serum factor inducing haemorrhagic necrosis in tumors (40) and, therefore, this cytokine was proposed as a potential anti-cancer agent. TNF-α has been shown not only to selectively destroy tumor vasculature (41, 42), but also increase tumor vessel permeability, thus, improving drug penetration into tumors (43–45). Moreover, low doses of TNF-α have been shown to promote antitumor immune responses by enhancing T-cell infiltration and by activating macrophages toward a tumor-suppressive phenotype (46). Notably, a synergistic action of IFN-γ and TNF-α was reported in early studies showing tumor growth inhibition in mice (47, 48) and tumor disappearance in patients after local limb perfusions with IFN-γ and TNF-α in combination with a chemotherapeutic agent (49). The antitumor effects were likely due to decreased endothelial cell adhesion and survival in response to TNF-α and IFN-γ leading to destruction of tumor vasculature (50). The synergism may also be explained by the fact that IFN-γ enhances TNF-α receptor expression in malignant cells (51, 52), thus improving TNF-α treatment. Another synergistic action of IFN-γ and TNF-α has been shown on macrophage activation toward a tumoricidal phenotype *in vitro* (53). However, the clinical usefulness of TNF-α and IFN-γ is limited by their systemic toxicity (54, 55) and short *in vivo* half-lives (56, 57).

To the best of our knowledge, no previous studies have reported using rSFV vectors that encode the cytokine IFN-γ or TNF-α. To provide new tools for cancer immunotherapy, we developed two rSFV vectors that encoded either murine TNF-α or IFN-γ and tested the functionality of the resulting rSFV-encoded cytokines *in vitro*. Here, we show that the rSFV vectors were capable of infecting Lewis lung carcinoma (LLC) cells and exploiting them to produce rSFV-encoded TNF-α and IFN-γ. Moreover, in this study, we show that rSFV-encoded IFN-γ effectively enhanced macrophage activation toward a cancer-suppressive phenotype *in vitro*.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

Baby hamster kidney (BHK-21) fibroblasts were obtained from American Type Culture Collection (Cat. No. CCL-10™; ATCC/LGC Prochem, Boras, Sweden) and cultured in Glasgow's MEM (Cat. No. 11710; Thermo Fisher Scientific, Boston, MA, USA) supplemented with 10% fetal bovine serum (FBS; Cat. No. F7524; Sigma-Aldrich, St. Louis, MO, USA), 10% Tryptose Phosphate Broth solution (Cat. No. T4049; Sigma-Aldrich), 20 mM HEPES (Cat. No. H0887; Sigma-Aldrich), 2 mM L-Glutamine (Cat. No. 25030; Thermo Fisher Scientific), 100 U/mL penicillin, and 100 mg/mL streptomycin (Cat. No. P0781, Sigma-Aldrich). The LLC cell line (also called LLC1; Cat. No. CLS 400263) and mouse macrophage cell line J774A.1 (Cat. No. CLS 400220) were obtained from Cell Lines Service (CLS GmbH, Eppelheim, Germany). The LLC cells were grown in RPMI-1640 medium (Cat. No. 61870; Thermo Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich). The J774A.1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Cat. No. S0415; Biochrom GmbH, Berlin, Germany). The human lung carcinoma cell line A549 (Cat. No. CCL-185™) and murine fibrosarcoma cell line L929 (Cat. No. CCL-1™) were obtained from American Type Culture Collection (ATCC/LGC Standards GmbH, Wesel, Germany). The A549 cells were propagated in Advanced Dulbecco's MEM

(Cat. No. 12491; Thermo Fisher Scientific) supplemented with 5% FBS (Sigma-Aldrich) and 2 mM L-glutamine. The conditioned medium (CM) of L929 cells was used as a source of macrophage colony-stimulating factor (M-CSF) (58) for the maintenance and differentiation of bone marrow-derived progenitors into macrophages. Briefly, L929-CM was produced by cultivating L929 cells in RPMI-1640 with 10% FBS (Sigma-Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin in T75 flasks until confluency. The medium was then removed, 30 mL of RPMI-1640 supplemented with 10% FBS (Biochrom) without antibiotics was added, and cells were incubated for 10 days. The L929-CM was harvested and spun at 300 *g* for 10 min. The collected supernatant was filtered through a 0.22-µm strainer and stored at −20°C until used. All cells were cultured at 37°C in a humidified incubator in an atmosphere containing 5% CO_2 and 95% air.

Mice

C57BL/6NRj mice (Janvier Labs, Le Genest-Saint-Isle, France) were bred at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet (Oslo, Norway). All animal experiments were approved by and performed in accordance with the regulations and guidelines of the Norwegian Food Safety Authority.

Isolation and Culturing of Bone Marrow-Derived Macrophages (BMDMs)

Murine BMDMs were differentiated from bone marrow progenitors obtained from C57BL/6NRj mice as previously described (59, 60) with a few modifications. Femur and tibia were aseptically dissected from 8- to 10-week-old C57BL/6NRj mice, and bone marrow cells were collected by flushing the femurs and tibias with RPMI-1640 supplemented with 10% FBS (Biochrom) using a 25 G needle. After the cells were centrifuged for 5 min at 400 *g*, the erythrocytes were lysed in lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA-Na_2, pH 7.2–7.4, filtered through a 0.2-µm strainer), and the remaining cells were then filtered through a 70-µm cell strainer (Cat. No. CLS431751; Sigma-Aldrich) and centrifuged. The cells were seeded in 90-mm untreated cell culture dishes (Cat. No. 734-2359; VWR, Radnor, PA, USA) at a concentration of 8×10^6/dish and then differentiated *via* cultivation for 7 days in medium referred to hereafter as complete BMDM differentiation medium (consisting of RPMI-1640 with 10% FBS and 30% L929-CM containing M-CSF). The adherent cells were considered CD11b+F4/80+ macrophages since flow cytometry revealed that these cells were more than 99% pure (data not shown). After 7 days, the cells were detached by incubating them in cold Dulbecco's phosphate-buffered saline without Mg^{2+}/Ca^{2+} (referred to as PBS−/−; Cat. No. D8537; Sigma-Aldrich) for 15–20 min at 4°C. The harvested cells were centrifuged and frozen in FBS containing 10% DMSO (Cat. No. 0231; VWR). The BMDMs were cultivated in RPMI-1640 supplemented with 10% FBS and 10% L929-CM.

Generation of Human Monocyte-Derived Macrophages (HMDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats *via* centrifugation in Lymphoprep™ density gradient medium (Cat. No. 1114547; Alere Technologies AS, Oslo, Norway) according to the manufacturer's protocol. Buffy coats were obtained from the blood bank of St. Olav's Hospital (Trondheim, Norway). Monocytes were enriched from total PBMCs *via* plastic adherence in 8-well TC Lab-Tek Chamberslides (Cat. No. 80826; ibidi GmbH, Martinsried, Germany) and maintained in RPMI 1640 medium supplemented with 10% human serum (obtained from the blood bank of St. Olav's Hospital, Trondheim, Norway). Monocyte-derived macrophages were differentiated from monocytes by incubating the cells in RPMI 1640 containing 10% human serum and 10 ng/mL human M-CSF (Cat. No. SRP6165; Sigma-Aldrich) for 7 days.

Plasmids and Construction of Expression Vectors

The pSFV1-DsRed vector, which carries a gene for *Discosoma* sp. red fluorescent protein (*DsRed*), was generated in our lab as previously described (61) using the pSFV1 vector (20). The pSFV1 and pSFV-Helper1 plasmids (20) were generously provided by Garoff (Karolinska Institute, Stockholm, Sweden).

To generate the pSFV1-Tnfa-Flag plasmid, we used the pSFV1-NruI vector, which is a derivate of pSFV1 that was generated in our lab by deleting a 527 bp *Stu*I-*Hind*III fragment from pSFV1 (bp 7603–8130) and changing a unique *Spe*I site to a *Nru*I site (62). The murine TNF-α-coding gene (*Tnfa*) (GenBank No. NM_013693.2) fused to a Flag-tag sequence was inserted into the pSFV1-NruI vector under the control of the SFV 26S subgenomic promoter. The *Tnfa-Flag* fragment was amplified from the template plasmid pCMV3-mTNF-Flag (Cat. No. MG50349-CF; Sino Biological Inc., Beijing, China) using PCR with the following primers (synthesized by Microsynth AG, Balgach, Switzerland): 5′-GC**GGATCC**ATGAGCACAGAAAGCATGATC-3′ (forward) and 5′-TG**CCCGGG**TTTACTTATCGTCGTCATCCTTG-3′ (reverse). *BamH*I and *Sma*I restriction sites (shown here in bold) were introduced to facilitate the ligation of the PCR fragment into the pSFV1-NruI vector.

The pSFV1/Enh-Luc plasmid was generously provided by A. Merits (Institute of Technology, University of Tartu, Estonia) and has been previously described (63). The pSFV1/Enh-Ifng vector was generated by replacing the *Xma*I-*Apa*I fragment (bp 7572-9268), which contained the firefly luciferase gene (*Luc*) of pSFV1/Enh-Luc, with the murine IFN-γ-coding gene (*Ifng*) (GenBank No. NM_008337.3). The *Ifng* gene was amplified from the template plasmid pMD19-mIFNG (Cat. No. MG50709-M; Sino Biological Inc.) using PCR with the following primers (synthesized by Microsynth AG): 5′-TCCG**CCCGGG**ATGAACGCTACACACTGC-3′ (forward) and 5′-TCC**GGGCCC**TCAGCAGCGACTCCTTTTCC-3′ (reverse). *Xma*I and *Apa*I (shown here in bold) restriction sites were incorporated to facilitate cloning into the pSFV1/Enh-Luc vector. The sequences *Tnfa-Flag* and *Enh-2A-Ifng* of the constructs were verified by GATC Biotech AG (Constance, Germany) and found to share 100% identity with the reference sequences *Tnfa* (GenBank No. NM_013693.2), *Flag* (shown in the product description of pCMV3-mTNF-Flag (Cat. No. MG50349-CF; Sino Biological)), *Enh-2A* [shown in Ref. (7)] and *Ifng* (NM_008337.3).

RNA Synthesis and Production of Viral Particles

RNAs were synthesized and transfected into BHK-21 cells using electroporation as previously described (63). The plasmids pSFV/Enh-Ifng, pSFV-DsRed, and pSFV-Helper1 were linearized using the restriction enzyme *SpeI* (Cat. No. ER1251; Thermo Fisher Scientific), and pSFV/Tnfa-Flag was linearized using *NruI* (Cat. No. ER0111; Thermo Fisher Scientific). The linearized plasmids (1 µg of each) were used as templates for the *in vitro* transcription (SP6 RNA polymerase; Cat. No. AM2071; Thermo Fisher Scientific) of SFV-Helper1 RNA and recombinant RNAs (rRNAs) carrying *Ifng*, *Tnfa*, or *DsRed*. The transcribed rRNAs were capped by adding 1 mM 3′-*O*-Me-m^{7}G(5′)ppp(5′)G cap-structure analog (Cat. No. S1411S; New England Biolabs, Hitchin, UK) during the transcription reaction. To package the rRNAs into viral particles, each of the rRNAs was co-electroporated with the SFV-Helper1 RNA in 1×10^7 BHK-21 cells by pulsing the mixture twice at 850 V, 25 mF using a Gene Pulser-II apparatus (Bio-Rad, Hercules, CA, USA). The Helper1 RNA provided *in trans* the SFV structural proteins (20) for encapsidation of the rRNA. The electroporated BHK-21 cells were resuspended in 15 mL of BHK-21 cultivation medium containing 1% FBS (Sigma-Aldrich) and then incubated at 33°C for 48 h. The cell growth medium containing the infectious rSFV particles was then harvested, rapidly frozen in liquid nitrogen, and subsequently used as a source of rSFV particles. The titers of the rSFV particles were determined based on the "one virus particle—one infected cell" hypothesis regarding the replication-deficient virus particles. BHK-21 cells were infected with serial dilutions of the rSFV particles. Infected cells expressing DsRed were counted in 10 viewfields using fluorescence microscopy at 24 h post-infection. The cells that were infected with either SFV-Tnfa or SFV-Ifng were stained with rabbit polyclonal antibodies that were specific for the nsp1 subunit of SFV replicase (generously provided by A. Merits, Institute of Technology, University of Tartu, Estonia) and with a fluorochrome-conjugated secondary antibody (Cat. No. A11034; Thermo Fisher Scientific). The stained cell monolayers were observed using EVOSfl digital inverted fluorescence microscope (Thermo Fisher Scientific), and infection-positive cells were counted in 10 viewfields using fluorescence microscopy. The rSFV particle titers were expressed as infectious units (IFU)/mL relative to virus infectivity in BHK-21 cells and were used later in this study to calculate multiplicity of infection (MOI), which indicated how many infectious viral particles were added per target cell during infection. Cells infected with the rSFV stocks did not secrete infectious virus particles, as shown in cell reinfection experiments, confirming that the virus stocks contained replication-deficient SFV.

Cancer Cell and Macrophage Susceptibility to SFV and Cell Viability after Infection

Baby hamster kidney, LLC, A549, and J774A.1 cells and murine BMDMs were seeded in 12-well plates (Cat. No. 150628; Thermo Fisher Scientific) or 24-well plates (Cat. No. 142475; Thermo Fisher Scientific) and cultivated until 50–80% confluency. The cells were washed once with PBS containing Mg^{2+} and Ca^{2+} (referred to as PBS+/+; Cat. No. 14040; Thermo Fisher Scientific), and 140—600 µL of an infectious suspension containing SFV-DsRed at a MOI = 10 or MOI = 15 (defined as 10 or 15 IFU in BHK-21 cells) in PBS+/+ was added to duplicate sets of wells. PBS+/+ was used as a negative control. After the cells were incubated at 37°C for 80 min, the infectious suspension was replaced with cell-specific growth medium, and the expression of DsRed in the infected cells was observed 24–48 h later using an EVOSfl digital inverted fluorescence microscope (AMG). The LLC and J774A.1 cells were detached *via* scraping, the BHK-21 cells were detached using a 0.25% trypsin-EDTA solution (Cat. No. T4049; Sigma-Aldrich), and the BMDMs and A549 cells were detached by treatment with accutase (Cat. No. A6964; Sigma-Aldrich). The cells were then centrifuged and DsRed-positive cells were quantified 24 h post-infection using BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). To evaluate cell viability BHK-21 and LLC cells were labeled 48 h post-infection with 1 µg/mL of fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V-FITC from Apoptosis Detection Kit; Cat. No. 556547; BD Biosciences) for 15 min followed by staining with 3 µM of DAPI (Cat. No. 422801; BioLegend, San Diego, CA, USA) for 5 min. Cells were immediately quantified using BD LSRFortessa flow cytometer (BD Biosciences). The data were analyzed using FlowJo V10 software (FlowJo LLC., Ashland, OR, USA).

After differentiation in 8-well TC Lab-Tek Chamberslides (Cat. No. 80826; ibidi GmbH), HMDMs were washed once with PBS+/+ and incubated for 1 h with 200 µL of an infectious suspension containing SFV-DsRed at a MOI = 15 (defined as 15 IFU in BHK-21 cells) diluted in PBS+/+. A recombinant paramyxoviral vector Sendai virus (SeV)-GFP that was based on a wild-type infectious SeV clone that carried a supplemental GFP transgene (64) was generously provided by D. Kolakofsky (Department of Microbiology and Molecular Medicine, University of Geneva School of Medicine, Geneva, Switzerland) and used at a MOI = 15 as a positive control for HMDM infection. The infectious suspension was replaced with normal cell growth medium, and the cells were observed at 24 h post-infection using phase-contrast and confocal fluorescence microscopy with a Zeiss Axiovert 100-M inverted microscope equipped with a LSM 510 laser-scanning unit and a 1.4 NA × 63 Plan-Apochromat oil-immersion objective. To calculate the percentage of fluorescent protein-expressing cells, the total number of cells was manually counted in at least 10 viewfields using phase-contrast microscopy. Fluorescent protein-expressing cells were simultaneously counted within the same viewfields using fluorescence microscopy.

Cancer Cell Growth Inhibition after Infection with SFV Vectors

Baby hamster kidney cells (1.97×10^4 cells/well) and LLC cells (7.03×10^4 cells/well) were plated in 96-well plates (Cat. No. 3595; Corning, Corning, NY, USA) and incubated for 22 h. The cells were then washed once with PBS+/+ and incubated at 37°C for 80 min with 50 µL of an infectious suspension containing rSFV particles at MOI = 15 (defined as 15 infectous units in BHK-21 cells) diluted in PBS+/+. The infectious suspension was then replaced

with normal BHK-21 or LLC growth medium. Cells incubated in PBS+/+ without viral particles were used as a negative control. Cell morphology was observed 47 h after infection using bright-field microscopy, and 1 μCi of [methyl-^{3}H]-thymidine (^{3}H-TdR; Cat. No. MT6032; Hartmann Analytic GmbH, Braunschweig, Germany) per 1 mL of cell growth medium was then added. After a further 24 h of incubation, the cells were subjected to two freeze/thaw cycles and harvested using a Tomtec-96 cell harvester (Tomtec, Hamden, CT, USA) onto fiberglass filters (Cat. No. 1450-421; PerkinElmer Inc., Waltham, MA, USA). Cell growth was determined by measuring the incorporation of ^{3}H-TdR into proliferating cells as counts per minute (cpm) using a microplate scintillation counter (MicroBeta Trilux 1450, PerkinElmer Inc.).

Production of SFV Vector-Encoded Cytokines

To produce rSFV-encoded TNF-α and IFN-γ, BHK-21 cells were cultivated in 75 cm^2 cell culture flasks (Cat. No. CLS430641; Sigma-Aldrich) until 80% confluency, washed once with PBS+/+ and then incubated at 37°C for 80 min with 5 mL of a suspension containing 1.92×10^8 IFU of SFV-Tnfa or 1.21×10^8 IFU of SFV-Ifng in PBS+/+. The virus-containing suspensions were then replaced with BHK-21 cultivation medium containing 1% FBS. After 36 h, the cell growth medium was harvested and centrifuged at 300 *g* for 5 min to remove the cells before the supernatants were transferred to a new tube, which was then centrifuged at 11,000 *g* for 8 min. The remaining supernatants were stored at −80°C and used as a source of vector-derived (vd) TNF-α or vdIFN-γ. The concentrations within the supernatants of the cytokines vdTNF-α or vdIFN-γ were 332.28 and 795.92 ng/mL, respectively. The levels of cytokines within the supernatants were quantified using Luminex bead-based assay with Bio-Plex Mouse Cytokine Group I IFN-γ or TNF-α sets (Cat. No. 171G5017M and 171G5023M; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Secretion of SFV Vector-Encoded Cytokines from Cancer Cells

Baby hamster kidney and LLC cells were plated in 24-well plates (Cat. No. 142475; Thermo Fisher Scientific) and cultivated until the next day when the cells had reached 100 and 50% confluency, respectively. The cells were washed once with PBS+/+, and 600 μL of infectious suspension containing either SFV-Tnfa or SFV-Ifng particles at a MOI = 1, MOI = 10 or MOI = 40 (calculated according to the titration in BHK-21 cells) in PBS+/+ was added per well. After cells were incubated with the infectious suspension for 80 min at 37°C, the infectious suspension was discarded, and 500 μL of fresh cell growth medium was added per well. The cells were cultured for 24 h before the growth medium was collected and centrifuged for 6 min at 300 *g*. The supernatant was then transferred to a new tube, which was centrifuged at 11,000 *g* for 10 min. The supernatants were stored at −80°C, and the levels of secreted vdTNF-α and vdIFN-γ were determined using Luminex bead-based assay with Bio-Plex Mouse Cytokine Group I IFN-γ and TNF-α sets (Cat. No. 171G5017M and 171G5023M; Bio-Rad) according to manufacturer's recommended protocols.

Induction of Cell Death in L929 Fibroblasts and LLC Cancer Cells by TNF-α and IFN-γ

L929 fibroblasts or LLC cells were plated in 24-well plates (Cat. No. 142475; Thermo Fisher Scientific) and cultivated in RPMI 1640 containing 10% FBS (Sigma-Aldrich) for approximately 24 h until the cells reached 50–90% confluency. To verify the functional activity of vdTNF-α, it was added to L929 cells in parallel with recombinant (r)TNF-α (Cat. No. 315-01A; PeproTech, Rocky Hill, NJ, USA) to reach a final concentration of 6.6, 20, or 60 ng/mL, and the cells were then cultivated for 24 h. L929 cells that were treated with 1 μM staurosporine (Cat. No. S4400; Sigma-Aldrich) for 24 h were used as the positive control for apoptosis induction (65), and untreated cells were used as the negative control. LLC cells were treated with either 50 ng/mL rTNF-α or 100 ng/mL rIFN-γ for 48 h to test cytotoxicity of cytokines to LLC cancer cells. LLC cells were also treated with 50 ng/mL rTNF-α in combination with 100 ng/mL rIFN-γ for 24 h or 48 h. LLC cells were pretreated with 100 ng/mL rIFN-γ for 24 h followed by 24 h treatment with 50 ng/mL rTNF-α. Untreated cells were used as the negative control, whereas L929 cells that were treated with 50 ng/mL of rTNF-α were used as the positive control for apoptosis induction. After treatments L929 or LLC cells were detached using 0.25% trypsin-EDTA and centrifuged before they were labeled with annexin V-FITC and PI using an Apoptosis Detection Kit (Cat. No. 556547; BD Biosciences) according to the manufacturer's protocol. Briefly, the cell pellet was resuspended in 100 μL 1 x annexin V binding buffer and stained using 1 μg/mL annexin V-FITC and 5 μg/mL PI by incubating the cells at RT for 15–20 min. Then, 400 μL of additional annexin V binding buffer was added. The cell suspension was immediately analyzed using flow cytometry in a BD LSRFortessa instrument (BD Biosciences). The data were analyzed using FlowJo V10 software.

Verification of the Functional Activity of vdIFN-γ Using a Cancer Cell Growth Inhibition Assay

Murine BMDMs (4×10^6 cells/dish) were seeded in 90-mm nontreated cell culture dishes (Cat. No. 734-2359; VWR) and cultivated in complete BMDM growth medium (RPMI-1640 with 10% FBS (Biochrom) and 10% L929-CM) for 2–3 days. The BMDMs were detached by incubating the cells in cold PBS−/− at +4°C for 15–20 min and then harvested *via* centrifugation for 6 min at 300 *g*. To block cell proliferation, the BMDMs were incubated with 10 μg/mL mitomycin C (Cat. No. M4287; Sigma-Aldrich) at 37°C for 2 h. The cells were then washed twice with PBS−/− and plated in triplicate at one of three different densities (i.e., 6×10^4, 3×10^4, and 3×10^3 cells/well) in 96-well plates in 200 μL of macrophage growth medium. The cells were then cultivated for 24 h. To stimulate the macrophages, we treated them with 100 ng/mL of the toll-like receptor (TLR) 2/1 heterodimer agonist Pam3CSK4 (referred to as Pam3; Cat. No. tlrl-pms; InvivoGen, San Diego, CA, USA), 100 ng/mL of recombinant (r)IFN-γ (Cat. No. 315-05; PeproTech) or 100 ng/mL of vdIFN-γ for 24 h. Untreated BMDMs served as the negative control, and BMDMs that were treated with 100 ng/mL of Pam3 in combination with 100 ng/mL of rIFN-γ were used as

the positive control. Some BMDMs were treated for 24 h at 37°C with 100 ng/mL of Pam3 in combination with fourfold dilutions of rIFN-γ at concentrations ranging from 0.0015 to 100 ng/mL. Alternatively, other BMDMs were treated with 100 ng/mL of Pam3 in combination with 16-fold dilutions of vdIFN-γ at concentrations ranging from 0.0015 to 100 ng/mL. After 24 h of treatment, 100 µL of the cell culturing medium was replaced with 100 µL of a LLC cell suspension containing 3×10^4 cells/mL to form co-cultures of macrophages and cancer target cells. The resulting macrophage:cancer cell ratios were 20:1, 10:1, and 1:1. BMDMs that were incubated alone served as the negative control for macrophage growth, and LLC cells that were incubated alone served as the positive control for normal LLC cell growth. After 20 h, ^{3}H-TdR was added to all the wells at a concentration of 1 µCi per 1 mL of growth medium, and the cultures were incubated for an additional 24 h. Next, the plates were submitted to two freeze/thaw cycles and then harvested using a Tomtec-96 cell harvester onto fiberglass filters. Cell growth was analyzed by measuring the incorporation of ^{3}H-TdR into proliferating cells as cpm using a microplate scintillation counter (MicroBeta Trilux 1450).

Determination of Nitric Oxide (NO) Production by Activated Macrophages

Nitrite (NO_2^-) levels were measured using the Griess test in cultivation medium harvested from BMDMs at 24 h post-treatment. Cultivation medium (100 µL) was obtained from the wells with the highest densities of BMDMs and transferred to new 96-well round-bottom plates (Cat. No. 3799; Sigma-Aldrich). These were centrifuged for 6 min at 400 *g*. Then, 50 µL of the supernatant was pipetted into new 96-well flat-bottom plates (Cat. No. 3595; Corning), and an equal volume of a solution containing 1% sulfanilamide (Cat. No. S9251; Sigma-Aldrich) and 5% phosphoric acid (Cat. No. 345245; Sigma-Aldrich) in H_2O was added. The plates were incubated in the dark for 10 min at RT to allow the sulfanilic acid to convert to diazonium salt. After the incubation period, 50 µL of 0.1% *N*-(1-naphthyl)ethylenediamine (NED; Cat. No. N9125; Sigma-Aldrich) in H_2O was added to the samples, standards, and blank to induce the immediate formation of azo dye. Solutions of sodium nitrite ($NaNO_2$) in twofold dilutions at concentrations ranging from 3.13 to 100 µM were used as the standard and set up in triplicate. Water was used as the blank. Absorbance was measured at 540 nm using a microplate spectrophotometer (Epoch; BioTek Instruments, Winooski, VT, USA).

RESULTS

Construction of SFV-Based Vectors and Production of Recombinant Viral Particles

We constructed two new replication-deficient SFV-based vectors that encoded either mTNF-α or mIFN-γ (**Figure 1A**). To express mTNF-α in the SFV vector, a sequence containing the *Tnfa* gene followed by a Flag-tag-encoding sequence at the 3-prime end was subcloned into the pSFV1-NruI vector (**Figure 1A**). The pSFV-Ifng vector was made by inserting the mIFN-γ gene (*Ifng*)

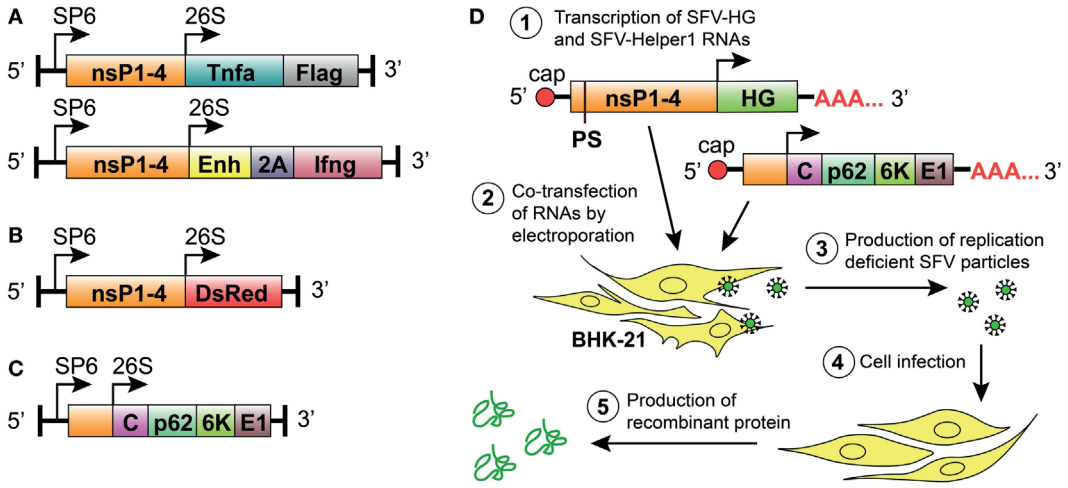

FIGURE 1 | Semliki Forest virus (SFV) vectors used in this study for replication-deficient expression system. All vectors comprised the prokaryotic SP6 RNA polymerase promoter for *in vitro* transcription of the recombinant SFV replicons. **(A)** Replication-deficient vectors encoding SFV non-structural protein genes (nsP1–4) and the heterologous genes (HGs) *Tnfa* or *Ifng* downstream of the SFV 26S subgenomic promoter. The mouse TNF-α gene (*Tnfa*) was followed by a FLAG-tag-encoding sequence. The mouse IFN-γ gene (*Ifng*) was inserted in frame with the minimal capsid translation enhancer sequence (*Enh*) using the 2A autoprotease sequence (*2A*) from foot and mouth disease virus as a linker. **(B)** Replication-deficient reporter construct consisting of SFV nsP1–4 and the fluorescent protein-encoding gene *Ds-Red* downstream of an SFV 26S subgenomic promoter. **(C)** The SFV helper vector SFV-Helper1, which encodes the full length of the structural protein ORF (i.e., C, capsid protein; p62, 6K and E1, and envelope proteins) downstream of an SFV 26S subgenomic promoter. **(D)** Production of recombinant particles and proteins by the replication-deficient SFV vector system: (1) *in vitro* transcription of RNA encoding the HG and the nsP1–4, and the transcription of helper RNA carrying SFV structural genes. The packaging signal (PS) for the selective encapsidation of the RNA encoding nsP1-4 and the HG is present in the nsP2 region of SFV-HG RNA. Both RNAs are capped at the 5-prime end and polyadenylated at the 3-prime end; (2) BHK-21 cells are transfected with SFV-HG and SFV-Helper RNAs by electroporation; (3) BHK-21 cells produce replication-deficient SFV particles carrying HGs; (4) target cells are infected with replication-deficient SFV particles; and (5) the heterologous protein is produced at high levels without production of new viral particles.

into the pSFV1/Enh vector downstream and in-frame with the SFV capsid translation enhancer sequence (*Enh*) that was fused to the 2A auto-protease sequence of foot and mouth disease virus (FMDV) (**Figure 1A**). The 2A-encoding sequence was included to induce the cleavage of the vdIFN-γ protein from the 34 amino acid-long peptide that was translated from the *Enh* sequence. It has been shown that by incorporating an enhancer element within SFV vector, the expression of the heterologous protein can be increased by up to 10-fold due to enhanced translation (7, 66). In contrast to the SFV-Ifng construct, we did not incorporate the enhancer element into the SFV-Tnfa vector aiming to retain low levels of translated TNF-α if necessary since lower levels of TNF-α might be important for normalization of tumor vasculature (46). The previously constructed vector pSFV-DsRed, which encode a fluorescent protein (**Figure 1B**), was used as a control to detect cell infection. All vectors comprised the prokaryotic SP6 RNA polymerase promoter for *in vitro* transcription of recombinant SFV replicons, as well as the vectors encoded SFV replicase complex, which consists of non-structural proteins (nsP) 1–4 (**Figures 1A–C**). The SFV structural proteins C, p62, 6 K, and E1, which are necessary for the formation of infectious virus particles, were encoded by the SFV-Helper1 vector (**Figures 1C,D**). The heterologous genes (HGs) and the genes encoding the structural SFV proteins were inserted downstream of the SFV 26S promoter (**Figures 1A–C**). In this study, replication-deficient rSFV particles were produced by BHK-21 cells that were co-transfected with RNA transcribed from the SFV-Helper1 vector and RNA transcribed from a vector encoding a HG (*Tnfa*, *Ifng*, or *DsRed*) (**Figure 1D**). The rSFV viral particles were quantified in BHK-21 cells and revealed titers ranging from 10^7 to 10^8 IFU/mL. More specifically, the mean viral titers were 1.15×10^8 IFU/mL of SFV-Tnfa, 7.23×10^7 IFU/mL of SFV-Ifng, and 1.46×10^8 IFU/mL of SFV-DsRed. The SE of the mean viral titers between decuplicates did not exceed 10%.

Mouse and Human Lung Carcinoma Cells Are Susceptible to rSFV Infection, While Macrophages Are Resistant

We wanted to use rSFV to deliver cytokine genes into cancer cells for subsequent production of the tumor-suppressive factors. Cell susceptibility to viral infection is a prerequisite for successful gene delivery into cells using a viral vector. We investigated the ability of rSFV particles to infect both cancer cells and macrophages using the reporter vector SFV-DsRed. We infected cells with SFV-DsRed at a concentration 15 virus particles per cell (i.e., MOI = 15). We observed the expression of the DsRed fluorescent protein using fluorescence microscopy and quantified the DsRed-expressing cells using flow cytometry at 24-h post-infection (**Figures 2A–E**). In all the tested cell types, non-infected cells were used as the negative control for DsRed expression. The BHK-21 cell line was used as a positive control due to being highly susceptible to SFV infection, as demonstrated by flow cytometry analysis that showed that 88.2% of the BKH-21 cells were infected under the experimental conditions used in this study (**Figure 2A**). We verified that both the murine (LLC) and the human (A549) lung carcinoma cell lines were infected by the rSFV particles because 41% of both cell types were DsRed-positive (**Figures 2B,C**). However, we also found that neither the mouse BMDMs nor the murine macrophage cell line J774A.1 was susceptible to SFV because less than 1.5% of the cells were DsRed-positive (**Figures 2D,E**). Macrophages remained resistant to SFV infection also when we increased the concentration of SFV particles during infection (data not shown).

To confirm these findings in human macrophages, we differentiated macrophages from blood-derived monocytes and infected the cells with SFV-DsRed virus particles at a MOI = 15 (calculated according to the titer that was determined in BHK-21 cells). The SeV is known to infect human macrophages (67), and we therefore included a SeV that encoded GFP (SeV-Gfp) at a MOI = 15 as a positive control. In parallel, we infected the human lung carcinoma cell line A549 with both types of recombinant viruses at a MOI = 15, and used the infected A549 cells as a positive control for SFV infection. To calculate the percentages of infected cells, we counted the total number of cells as well as the number of fluorescent protein-expressing cells within the same viewfields (multiple viewfields per sample) using phase-contrast and fluorescence microscopy at 24 h post-infection. In these experiments, 30% of the positive control sample (the human cancer cell line A549) was infected with SFV-DsRed, whereas the HMDMs remained uninfected (**Figure 3**, middle panel). Contrary to our findings for SFV, SeV-Gfp infected both HMDMs and A549 very efficiently, with 82 and 95% of the HMDMs and A549 cells, respectively, being positive for GFP (**Figure 3**, lower panel). Taken together, these data show that lung carcinoma cells are efficiently infected by rSFV, whereas primary mouse and human macrophages are resistant to SFV infection, suggesting that rSFV may be used to deliver genes for cancer therapy without killing tumor-associated macrophages.

Macrophages Remain Viable after Challenge with SFV Particles

We wanted to test macrophage viability after challenge with rSFV particles. Both J774A.1 macrophages and LLC cancer cells were subjected to rSFV-DsRed particles at MOI = 10 during 80-min infection protocol. Cell infection rate was determined 48 h post-infection by quantifying DsRed-expressing cells using flow cytometry. Cells were also stained with annexin V-FITC and DAPI followed by flow cytometry analysis to determine cell viability.

As expected, we observed that 98% of non-infected macrophages stayed viable after 48 h as determined using flow cytometry and retained round morphology, which is characteristic to J774A.1 cells in normal culture conditions (**Figure 4A**). Also 48 h after challenge with rSFV-DsRed particles 90% of macrophages remained viable, whereas 6% of macrophages were single-positive for Annexin V (**Figure 4B**). Interestingly, light microscopy revealed that macrophages changed morphology 48 h after challenge with rSFV by gaining spindle-like or polygonal shape (**Figure 4B**). LLC cells were used in parallel as a positive control for cell infection. As expected, the non-infected LLC cells showed 92% viability and were characterized

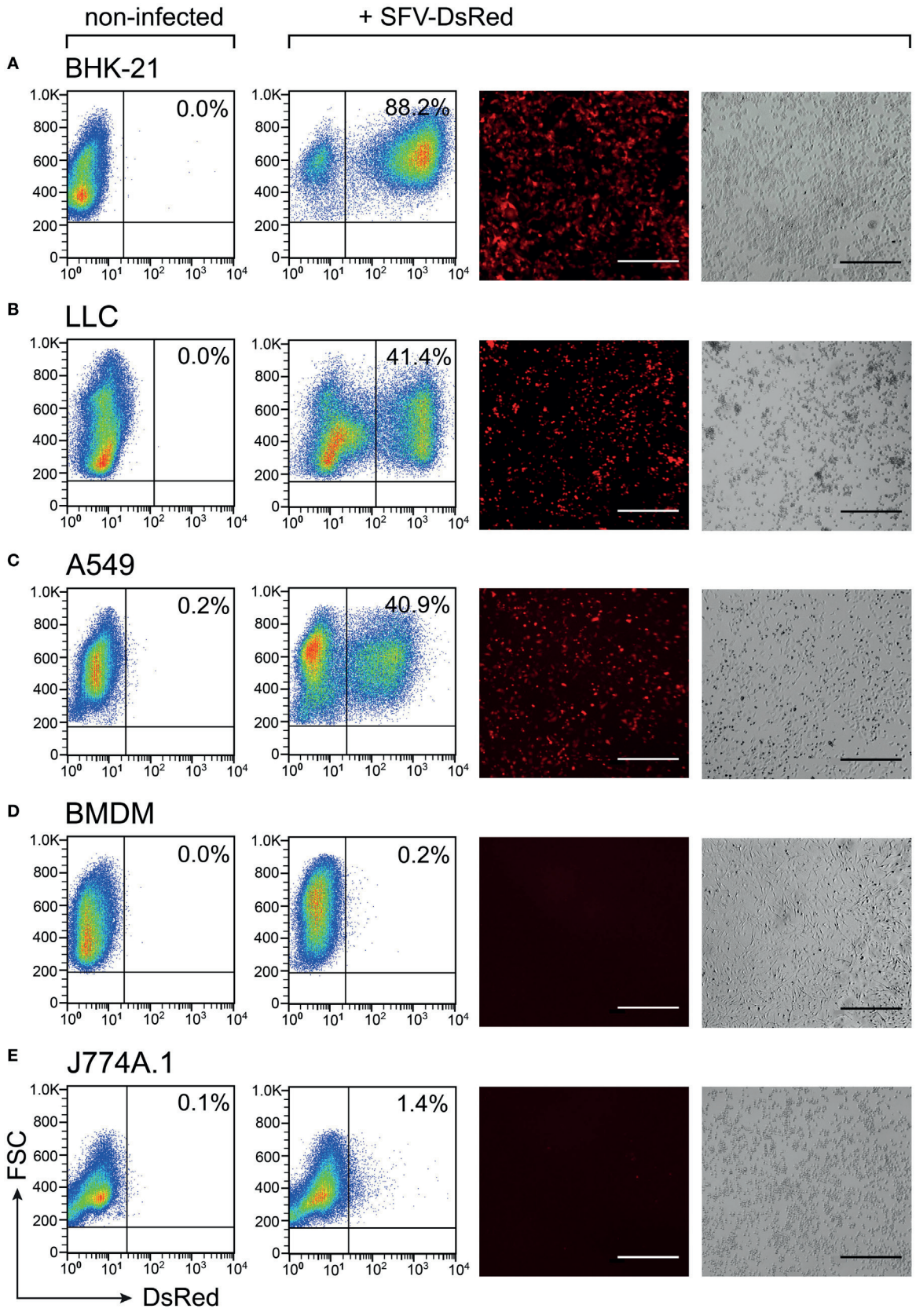

FIGURE 2 | Continued

FIGURE 2 | Continued
Susceptibility to Semliki Forest virus (SFV) infection varies between cell types. Cells were plated and infected with SFV-DsRed particles at MOI = 15 [determined using baby hamster kidney (BHK-21) cells] the next day. DsRed expression was evaluated at 24 h post-infection using flow cytometry and fluorescence microscopy. Non-infected cells were used as a negative control. From left to right: flow cytometry data for DsRed expression in non-infected and infected samples with the numbers representing the percentage of DsRed-positive cells as the mean of duplicates; and fluorescence microscopy images are shown with the corresponding bright-field microscopy images (scale bars, 50 μm). **(A)** The hamster fibroblast cell line BHK-21 was used as a positive control; **(B)** mouse lung carcinoma cell line Lewis lung carcinoma (LLC), **(C)** human lung carcinoma cell line A549, **(D)** mouse bone marrow-derived macrophages (BMDMs), and **(E)** mouse macrophage cell line J774A.1. The experiment was repeated three or more times with the SE not exceeding 10% between independent experiments. The average of duplicates with SEM <10% from one representative experiment is shown.

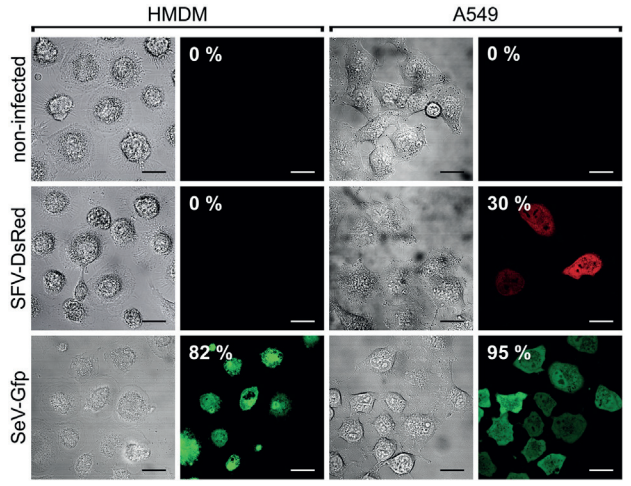

FIGURE 3 | Human macrophages are resistant to Semliki Forest virus (SFV) infection. Human monocyte-derived macrophages (HMDMs), and the human lung carcinoma cell line A549 were infected with either SFV-DsRed or SeV-Gfp virus particles. The resulting cell monolayers were analyzed using phase-contrast microscopy, and the expression of DsRed and GFP was evaluated using fluorescence microscopy 24 h post-infection (scale bars, 20 μm). The numbers in the images indicate the percentages of infected cells. Non-infected cells were used as negative controls. HMDMs were not susceptible to infection with SFV-DsRed, whereas A549 cells were infected with SFV, as expected (shown in the second row). Sendai virus (SeV)-Gfp virus particles were used as a positive control because they are capable of infecting both HMDMs and A549 cells, as shown in the third row. Total HMDM resistance to SFV infection was confirmed in three independent experiments using HMDMs from different donors. Data from one representative experiment are shown where the percentages represent average fluorescent protein-expressing cell population with SEM <5%.

by mixed population of adherent and floating cells with round or spindle-like morphology (**Figure 4C**). However, the infected LLC cells underwent cell death 48 h after infection with rSFV, and only 35% of LLC cells were viable (**Figure 4D**). We observed that 33% of rSFV-challenged LLC cells were double-positive for Annexin V-FITC and DAPI suggesting apoptotic/necrotic cell death. A LLC population of 30% was single-positive for DAPI 48 h after infection suggesting affected cell membrane permeability (**Figure 4D**). Light microscopy revealed that LLC cells possess apoptotic morphology characterized by round swollen cells with translucent cytoplasm after infection with rSFV (**Figure 4D**). We observed microscopically that both DsRed-positive (infected) and DsRed-negative (non-infected) cells undergo apoptosis after challenge with rSFV particles (**Figure 4D**, red and white arrows), whereas part of infected DsRed-expressing cells remain viable 48 h post-infection (**Figure 4D**, yellow arrows). Flow cytometry analysis revealed that 69% of all DsRed-expressing cells undergo cell death characterized mostly by being double-positive for Annexin V-FITC and DAPI (**Figure 4E**). Our data show that macrophages are not only resistant to SFV infection but also remain viable after challenge with rSFV particles.

SFV Infection Inhibits Growth of Murine Lung Carcinoma Cells

It has been shown that SFV infection induces cellular death *via* p53-independent apoptosis (21). To determine whether rSFV has a direct effect on cancer cell growth, we used *in vitro* assay to evaluate proliferation of infected LLC cells. The cells were cultivated until approximately 70–80% confluency, and they were then infected with SFV-Ifng or SFV-Tnfa viral particles at MOI = 15. To determine cell growth, a radioactive thymidine, which incorporates into new DNA strands during cell division, was added to the cell cultures at 47 h post-infection. The experimental setup is illustrated in **Figure 5A**.

BHK-21 cells were used in parallel as a positive control for efficient cell infection by SFV and showed nearly complete growth inhibition (**Figure 5B**). Growth inhibition was similar independent on whether SFV carried a gene encoding TNF-α or IFN-γ (**Figure 5B**). It was observed using light microscopy that BHK-21 cells lost confluency and gained apoptotic cell morphology characterized by translucent cytoplasm, granularity, or round shape 47 h post-infection (**Figure 5C**). LLC cell growth was inhibited by half 47 h after infection with SFV-Ifng and by 80% after infection with SFV-Tnfa (**Figure 5D**). Light microscopy revealed that LLC cells acquired apoptotic cell morphology characterized by round shape, granularity, and translucent cytoplasm 47 h after infection with either SFV-Ifng or SFV-Tnfa viral particles (**Figure 5E**). The data show that rSFV particles encoding murine IFN-γ or TNF-α efficiently inhibited LLC cell growth at 47 h post-infection.

Mouse Lung Carcinoma Cells Produce and Secrete SFV-Encoded TNF-α and IFN-γ

In the next set of experiments, we sought to determine whether LLC cells secrete cytokines after infection with SFV-Tnfa and SFV-Ifng virus particles. In this experiment, BHK-21 cells were used as a positive control. The LLC and BHK-21 cells were

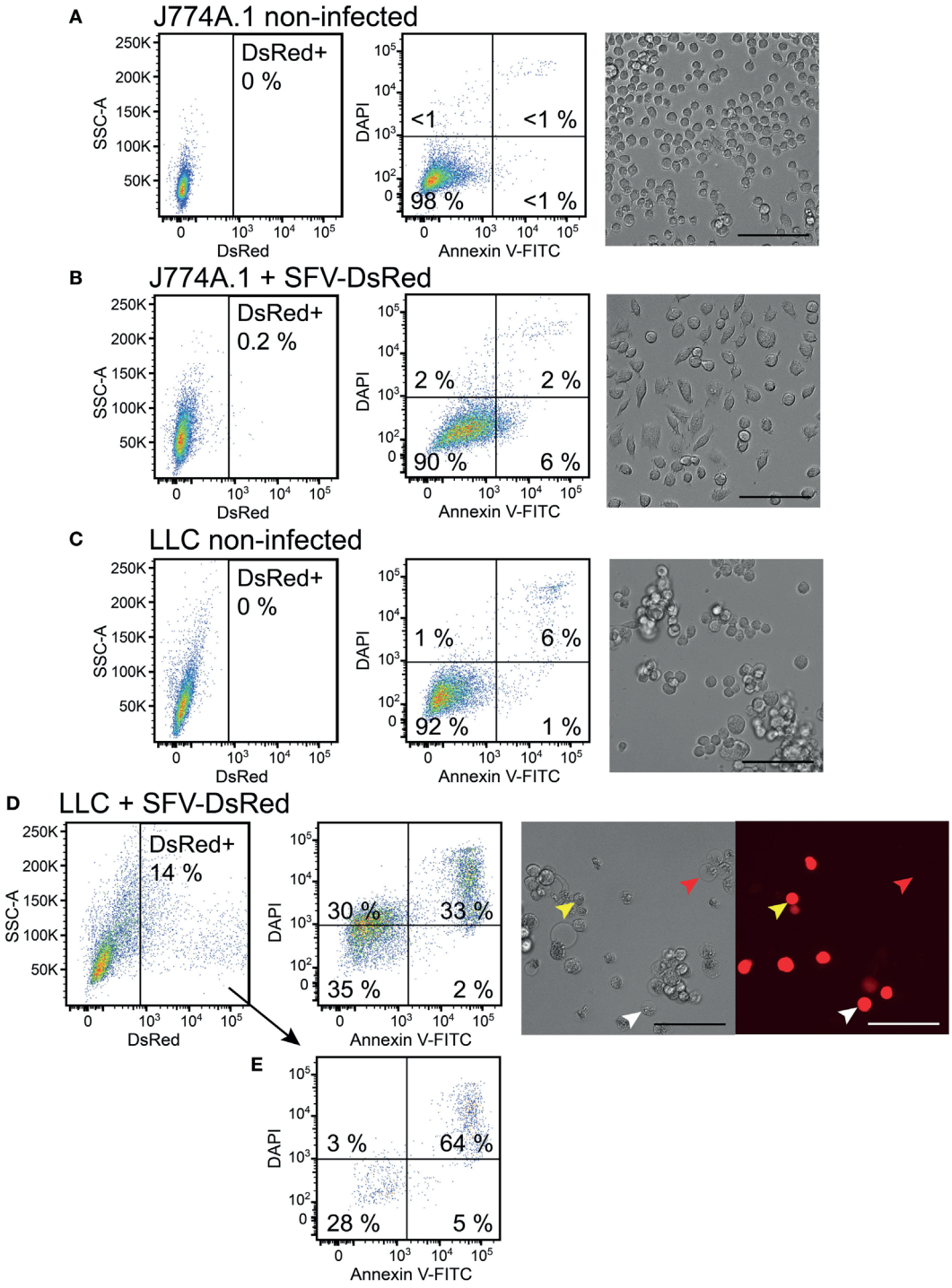

FIGURE 4 | Macrophages remain viable, whereas cancer cells undergo cell death after challenge with Semliki Forest virus (SFV). J774A.1 mouse macrophages and Lewis lung carcinoma (LLC) carcinoma cells were infected with SFV-DsRed particles at MOI = 10 [determined using baby hamster kidney (BHK-21) cells]. DsRed expression (first column, *x*-axis) was evaluated at 48 h post-infection using flow cytometry and fluorescence microscopy (scale bar 100 μM). Cell morphology was observed using bright-field microscopy (scale bar 100 μM) and cell death was quantified 48 h post-infection using flow cytometry analysis after cell staining with annexin V-FITC (second column, *x*-axis) and DAPI (second column, *y*-axis). Annexin V-positive/DAPI-negative cells were regarded as early apoptotic, whereas annexin V-positive/PI-positive cells were regarded as late apoptotic and necrotic. **(A)** Non-infected J774A.1 macrophages were used as a negative control. **(B)** J774A.1 macrophages were resistant to SFV infection, stayed viable but changed their morphology 48 h after challenge with SFV. **(C)** Non-infected LLC cells were used as a negative control. **(D)** LLC cells were susceptible to SFV infection and underwent cell death. DsRed expression was observed using microscopy in both apoptotic (white arrows) and viable (yellow arrows) cells, whereas some apoptotic cells lacked DsRed expression (red arrows). **(E)** The infected DsRed-positive cells were both viable and undergoing cell death. The average of duplicates with SEM <10% from one experiment is shown.

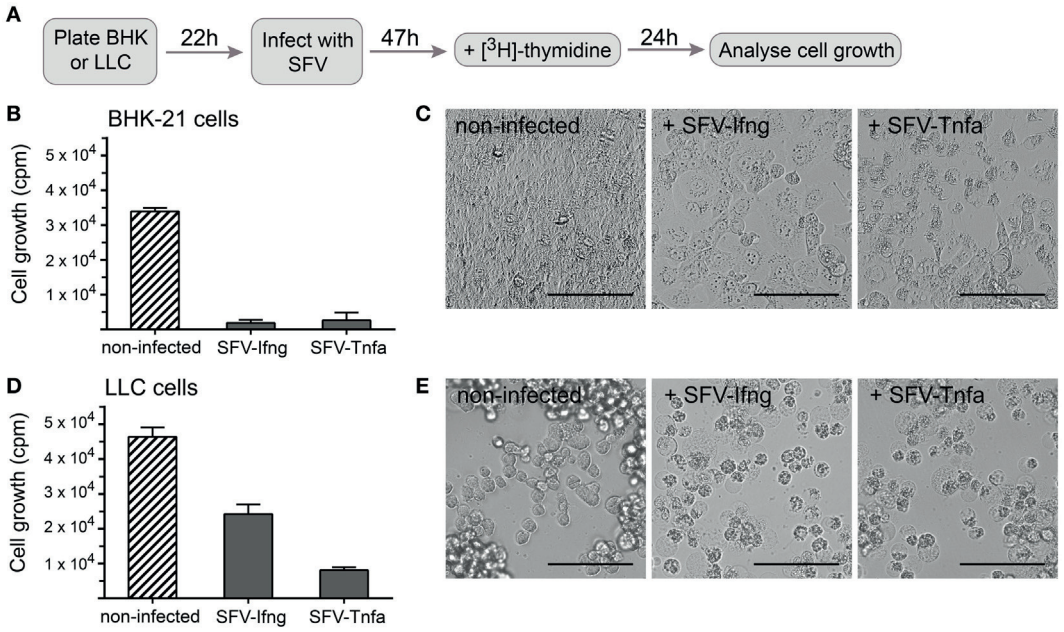

FIGURE 5 | Cancer cell growth is inhibited by infection with Semliki Forest virus (SFV) particles. **(A)** Schematic overview of the experiment. Baby hamster kidney (BHK-21) or Lewis lung carcinoma (LLC) cells were plated and incubated for 22 h before they were infected with SFV-Ifng or SFV-Tnfa particles at MOI = 15. The cells were incubated for 47 h after infection, and [^{3}H]-thymidine was then added to detect proliferating cells. After 24 h, the cells were harvested, and cell growth was determined by measuring the incorporated [^{3}H]-thymidine as counts per minute (cpm, depicted on the *y*-axis). **(B)** As expected, infecting BHK-21 cells with SFV resulted in substantial growth inhibition. **(C)** BHK-21 cells underwent cell death 47 h post-infection as observed using bright-field microscopy (scale bars, 50 μm). **(D)** LLC cell growth was inhibited by infection with different SFV particles. **(E)** LLC cells underwent cell death 47 h post-infection as observed using bright-field microscopy (scale bars, 50 μm). Two experiments were performed in triplicates with SE not exceeding 10% between independent experiments. The bars represent the mean values of triplicates ± SEM from one representative experiment.

cultivated until 50 and 100% confluency, respectively, before they were infected with SFV-Tnfa or SFV-Ifng at a MOI = 1, MOI = 10, or MOI = 40 (calculated according to the titration in BHK-21 cells). After 24 h, the levels of secreted vdIFN-γ and vdTNF-α in the cell cultivation medium were analyzed using Luminex bead-based assay. LLC cells infected with SFV-Tnfa secreted vdTNF-α (range, 10^3–10^4 pg/mL for all tested MOIs) (**Figure 6A**). There was no clear correlation between the MOI of the virus particles and the level of vdTNF-α (**Figure 6A**). The vdTNF-α was not detected in the medium of non-infected cells or in the medium of cells infected with SFV-Ifng, confirming that the secreted vdTNF-α was derived from the *Tnfa* gene that was delivered using SFV (**Figure 6A**).

In LLC cells, infection with SFV-Ifng resulted in the secretion of vdIFN-γ into the growth medium at a concentration ranging from 2.9 to 9.9 × 10^2 ng/mL at 24 h after infection (**Figure 6B**). The concentration of vdIFN-γ appeared to correlate with the concentration of viral particles, and the highest tested MOI resulted in the highest level of vdIFN-γ (**Figure 6B**). The vdIFN-γ was not detected in media obtained from non-infected cells or from cells infected with SFV-Tnfa, indicating that the vdIFN-γ originated from the SFV-delivered cytokine gene (**Figure 6B**). In conclusion, these results show that both BHK-21 and LLC cells produce and secrete vdTNF-α and vdIFN-γ when infected with the rSFV vectors described in this study.

Vector-Derived TNF-α Is Functional in Inducing Cell Death in L929 Murine Fibrosarcoma Cells

To determine whether the vdTNF-α is biologically active, we utilized a known property of TNF-α which is to induce necrotic cell death in the murine fibrosarcoma cell line L929 by binding to TNF receptor-1 (68). In these experiments, L929 cells were cultivated until 90% confluency; we then added rTNF-α or vdTNF-α at concentrations varying from 2.2 to 60 ng/mL to the cells. The culture medium of BHK-21 cells infected with SFV-Tnfa particles was used as the source of vdTNF-α. At 24 h after treatment, the morphology of the L929 cells was evaluated using bright-field microscopy. Then cells were stained with annexin V-FITC to determine phosphatidylserine exposure, whereas staining with PI was used to determine loss of membrane integrity in order to quantify the numbers of viable (Annexin V−PI−), apoptotic (Annexin V+PI−), and necrotic (Annexin V+PI+) cells using flow cytometry (**Figures 7A–J**). Untreated L929 cells were used as a control, and viable cells were defined as those that were annexin V-negative/PI-negative (**Figure 7A**). Treatment with staurosporine was used as the control for apoptotic cell death (65), where annexin V-positive/PI-negative cells were regarded as early apoptotic, whereas annexin V-positive/PI-positive cells were regarded as late apoptotic undergoing secondary necrosis (**Figure 7B**).

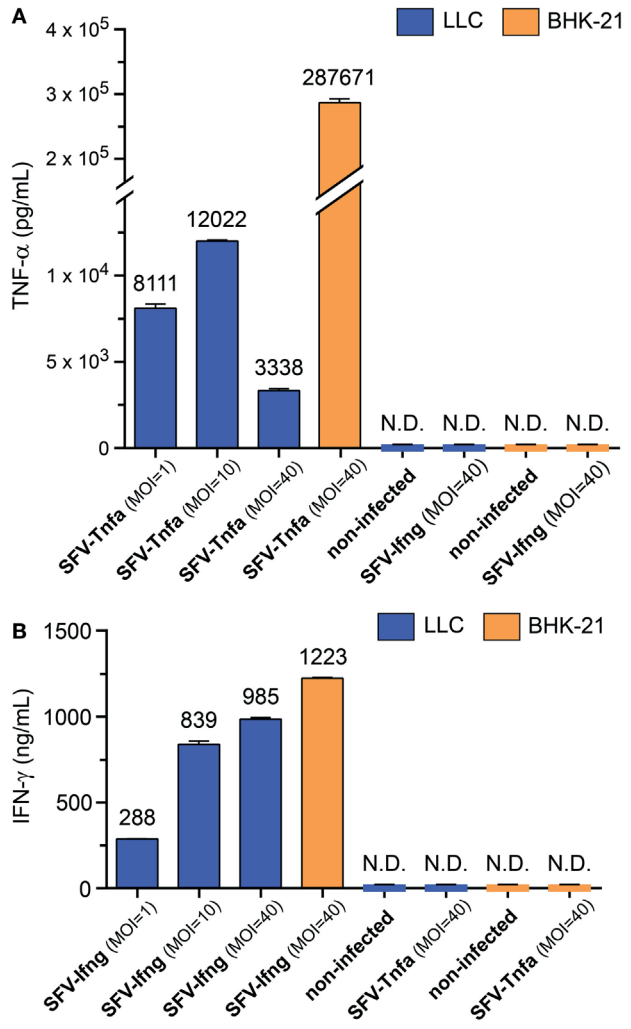

FIGURE 6 | Semliki Forest virus (SFV)-encoded cytokines are produced and secreted by cancer cells. Baby hamster kidney (BHK-21) and Lewis lung carcinoma (LLC) cells were infected with either SFV-Ifng or SFV-Tnfa at MOI = 1, MOI = 10, or MOI = 40 (determined using BHK-21 cells). The level of secreted **(A)** TNF-α or **(B)** interferon (IFN)-γ in the cell culture medium was determined using Luminex bead-based assay at 24 h after infection and is shown on the *y*-axis. The cytokines were undetectable in both the negative controls of non-infected cells and the cultures that were infected with a mismatched SFV that did not encode the cytokine of interest. N.D.—not detectable. Two independent experiments were performed in duplicates. Data from one representative experiment is shown. The bars represent the mean values of duplicates ± SEM.

In the absence of any treatment, the L929 cells reached 100% confluency after 24 h and had a normal fibroblast-like, slightly polygonal cell morphology (**Figure 7A**). The untreated L929 cells were also analyzed using flow cytometry and used to set the gates for the viable (Annexin V−PI−) cell population, which accounted for 99% of all events (**Figure 7A**). After cells were treated with 1 μM staurosporine, cell confluency was lost, and an apoptotic cell morphology characterized by cell shrinkage, a round shape and granularity was observed (**Figure 7B**). Only 11% of the staurosporine-treated cells were viable. The remaining cells were undergoing apoptosis and secondary necrosis (**Figure 7B**). In cells that were incubate with increasing concentrations of rTNF-α or vdTNF-α (2.2, 6.7, 20, or 60 ng/mL), we observed a concentration-dependent increase in the proportion of apoptotic (Annexin V+PI−) and necrotic (Annexin V+PI+) cells (**Figures 7C–J**) in a similar manner between rTNF-α and vdTNF-α. Treatment with vdTNF-α and rTNF-α at the lowest concentration (2.2 ng/mL) induced apoptosis in 9–13% and necrosis in 19–20% of the cells (**Figures 7C,D**), whereas the highest concentrations of rTNF-α and vdTNF-α (60 ng/mL) induced apoptosis in 18–23% and necrosis in 46–62% of the cells (**Figures 7I,J**). Moreover, after treatment with rTNF-α or vdTNF-α, we observed necrotic cell morphology characterized by round, swollen cells with translucent cytoplasm. These data verify the functionality of the vdTNF-α protein because it was able to induce apoptosis and necrosis in L929 cells in a concentration-dependent manner that was similar to that observed for rTNF-α.

Combined Treatment of TNF-α and IFN-γ Induces Cell Death in Mouse Lung Carcinoma Cells

It has been previously shown that LLC cells are resistant to cell death induced by TNF-α (69). Based on previous studies, where IFN-γ was shown to enhance TNF-α receptor expression in cancer cells (51, 52), we wanted to test whether IFN-γ may sensitize LLC cancer cells to TNF-α treatment. Therefore, we tested induction of cell death in LLC cells after combined treatment of recombinant cytokines TNF-α and IFN-γ. LLC cells were single-treated with either TNF-α or IFN-γ for 48 h. LLC cells were treated with TNF-α in combination with IFN-γ for 24 h or 48 h. LLC cells were also pretreated with IFN-γ for 24 h followed by 24 h treatment with TNF-α. L929 fibroblasts, which were treated with TNF-α, were used as a positive control. Untreated cells were used as a negative control. Cell morphology after treatments was evaluated using bright-field microscopy. Cells were stained with annexin V-FITC to determine phosphatidylserine exposure, whereas staining with PI was used to determine loss of membrane integrity in order to quantify the numbers of viable (Annexin V−PI−), apoptotic (Annexin V+PI−), and necrotic (Annexin V+PI+) cells using flow cytometry (**Figures 8A–H**).

In the absence of any treatment for 48 h, 99% of L929 control cells and 94% of LLC cancer cells remained viable and showed normal cell morphology (**Figures 8A,B**). The positive control of L929 cells, which were treated with TNF-α for 48 h, lost their confluency and showed apoptotic morphology characterized by round swollen cells with translucent cytoplasm (**Figure 8C**). Only 26% of the TNF-α-treated L929 cells were viable (**Figure 8C**). By contrast, LLC cells were resistant to treatment with TNF-α for 48 h, showed 86% viability and retained normal cell morphology (**Figure 8D**). Treatment with IFN-γ for 48 h was not cytotoxic to LLC cells, and 90% of the treated LLC cells remained viable (**Figure 8E**). Interestingly, combined treatment of LLC cells with both cytokines for 24 h also did not induce cytotoxicity in LLC cells (**Figure 8F**). However, a prolonged incubation of LLC cells with both cytokines for 48 h induced cytotoxicity in LLC cells resulting in 38% cells being double-positive for Annexin and

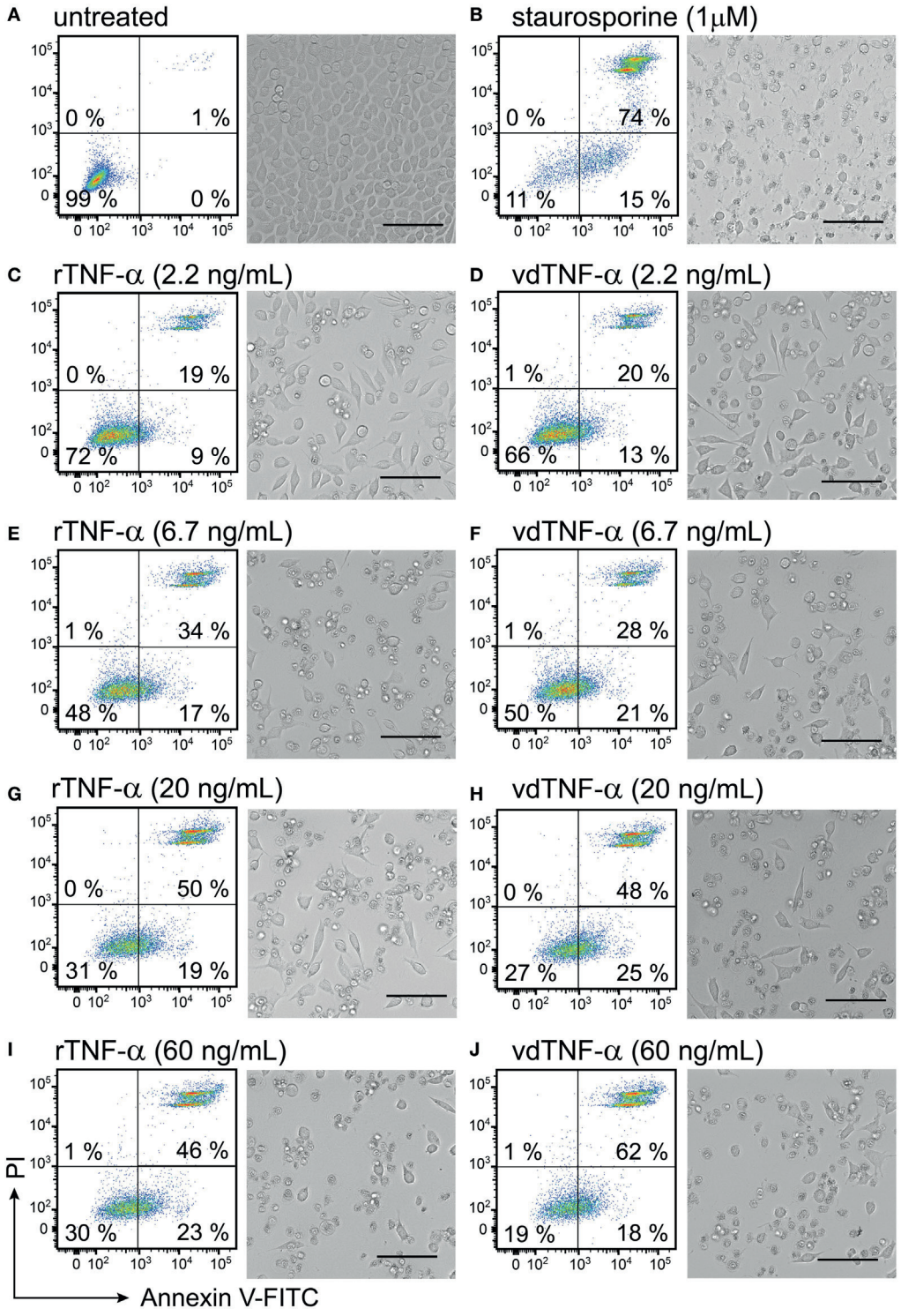

FIGURE 7 | Vector-derived TNF-α induces cell death in L929 fibroblasts. L929 cells were cultured for 24 h until they reached 90% confluency. The cells were then **(A)** left untreated for 24 h and used as a negative control or **(B)** treated with 1 μM staurosporine for 24 h at 37°C and used as a positive control. The remaining cells were treated with rTNF-α or vdTNF-α for 24 h at the following concentrations: **(C,D)** 2.2 ng/mL, **(E,F)** 6.7 ng/mL, **(G,H)** 20 ng/mL or **(I,J)** 60 ng/mL. The resulting cell monolayers were analyzed using bright-field microscopy (scale bar, 50 μM). Cell death was determined using flow cytometry analysis after cell staining with annexin V-FITC (depicted on the *x*-axis) and propidium iodide (PI, depicted on the *y*-axis). Annexin V-positive/PI-negative cells were regarded as apoptotic, whereas annexin V-positive/PI-positive cells were regarded as necrotic. Two independent experiments were performed in duplicates with standard error not exceeding 10% between independent experiments. Data from one representative experiment are shown, where the percentages of the four distinct cell populations represent the averages of duplicates with SEM <10%.

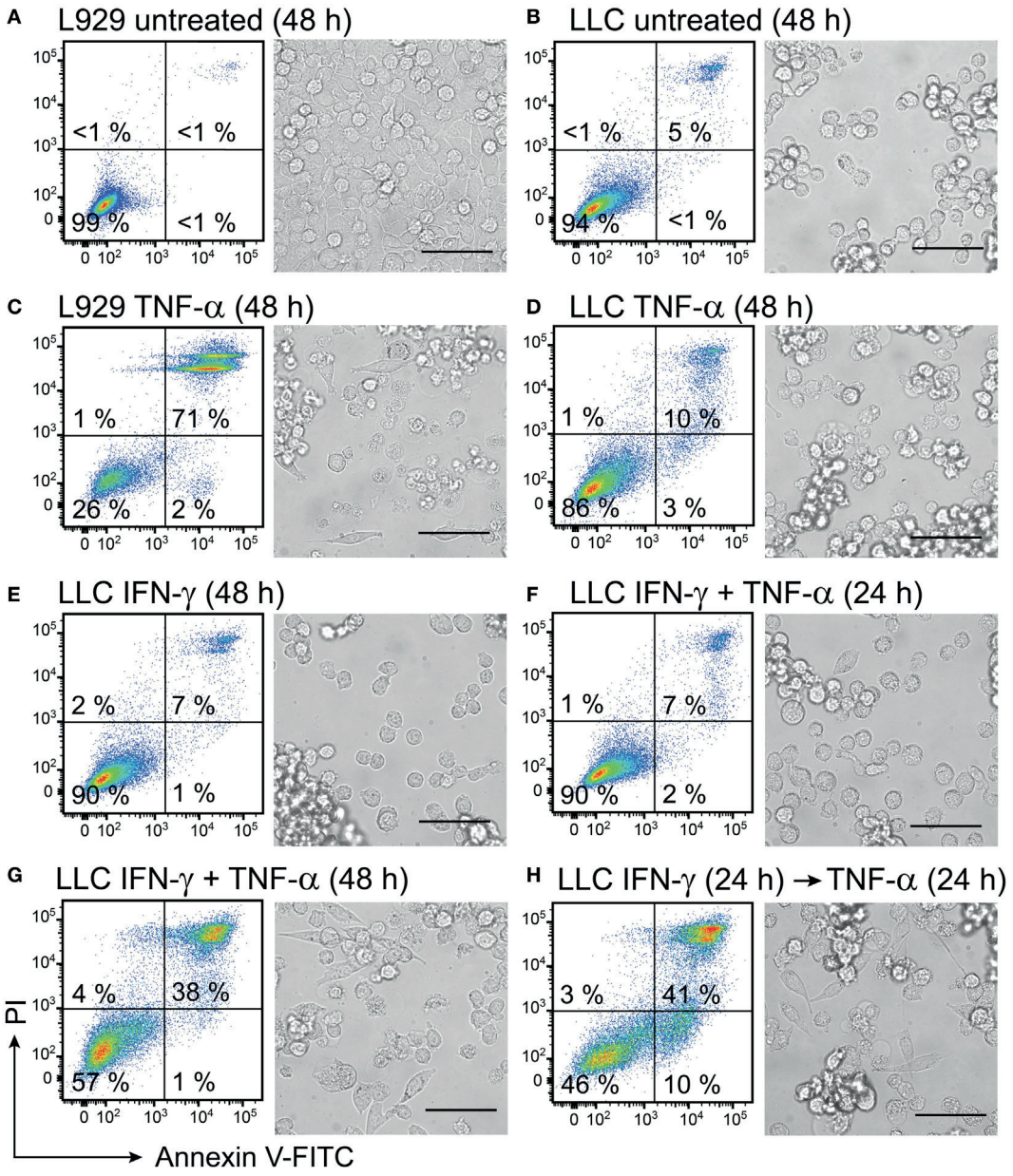

FIGURE 8 | Interferon (IFN)-γ in combination with TNF-α induces cell death in mouse lung carcinoma cells. L929 fibroblasts and Lewis lung carcinoma (LLC) cancer cells were cultured for 24 h before starting treatment with recombinant cytokines. Both L929 and LLC cells were **(A,B)** left untreated for 48 h and used as negative controls or **(C)** L929 cells were treated with 50 ng/mL TNF-α for 48 h and used as a positive control. LLC cell viability was retained after treatment with **(D)** 50 ng/mL TNF-α or **(E)** 100 ng/mL IFN-γ and after **(F)** simultaneous treatment with both 50 ng/mL TNF-α and 100 ng/mL IFN-γ for 24 h. Cell death was induced in LLC cells after **(G)** simultaneous treatment with 100 ng/mL of IFN-γ in combination with 50 ng/mL of TNF-α for 48 h and after **(H)** LLC pretreatment with 100 ng/mL of IFN-γ for 24 h followed by treatment with TNF-α for 24 h. The resulting cell monolayers were analyzed using bright-field microscopy (scale bar, 50 μM). Cell death was determined using flow cytometry analysis after cell staining with annexin V-FITC (depicted on the *x*-axis) and propidium iodide (PI, depicted on the *y*-axis). Annexin V-positive/PI-negative cells were regarded as apoptotic, whereas annexin V-positive/PI-positive cells were regarded as necrotic. Experiment was performed in duplicates and repeated two times with standard error not exceeding 10% between independent experiments. One representative experiment is shown, where the percentages of the four distinct cell populations are averages of duplicates with SEM <5%.

PI (**Figure 8G**). Also pretreatment of LLC cells with IFN-γ for 24 h followed by treatment with TNF-α for 24 h was cytotoxic to LLC cells and induced apoptosis in 10% and necrosis in 41% of the cells (**Figure 8H**). Cell morphology of LLC cells changed after the combined treatments of LLC with both cytokines simultaneously for 48 h or with IFN-γ pretreatment followed by TNF-α (**Figures 8G,H**). After combined treatments with both cytokines, we observed cells with apoptotic cell morphology as well as live cells that had spread in an elongated shapes possibly due to access of free space resulting from cells dying in proximity

(**Figures 8G,H**). All in all, combined treatment with IFN-γ and TNF-α was effective in inducing cell death in LLC cancer cells, which were resistant to single-treatments with these cytokines.

Vector-Derived IFN-γ in Combination with a TLR2/1 Agonist Activates Macrophages toward a Cancer-Suppressive Phenotype

To verify that vdIFN-γ is a functional protein, we used an *in vitro* assay based on cancer cell growth inhibition mediated by activated macrophages. The macrophages were treated with mitomycin C to block their proliferation, and they were activated by applying either vdIFN-γ or rIFN-γ in combination with a second stimulus. Based on previous experiments performed in our lab, we chose to stimulate the BMDMs with IFN-γ and TLR2/1 agonist Pam3 because this combination leads to efficient inhibition of cancer cell growth (70). After the BMDMs were stimulated for 24 h, LLC cells were added, and the co-cultures were cultivated for an additional 20 h. Finally, to enable the detection of proliferating cancer cells, we added radiolabeled thymidine to the cultures at 24 h before they were harvested (**Figure 9A**).

In the first experiment, we treated cells with different concentrations of rIFN-γ in combination with 100 ng/mL Pam3 to identify the lowest effective dose of IFN-γ that is necessary to activate BMDMs toward a cancer-suppressive phenotype (**Figure 9B**). The results revealed that adding rIFN-γ within the range 100–1.56 ng/mL effectively inhibited cancer cell growth (cpm below 750 when the ratio of BMDMs:LLC cells was 20:1) in a concentration-dependent manner. Some growth inhibition was also observed at 0.098–0.39 ng/mL rIFN-γ, but minor or no effect was observed at concentrations ≤0.024 ng/mL (**Figure 9B**).

All controls included in this assay produced the expected results (**Figures 9B,C**). Treating the BMDMs with mitomycin C resulted in counts lower than 650 cpm, confirming that the treatment hindered proliferation (**Figures 9B,C**; first group of bars from the left). Unstimulated macrophages did not inhibit growth in LLC cells since more than 15,000 cpm were measured in the wells (**Figures 9B,C**; second group of bars from the left). These results were comparable to the average cpm in LLC cells that were cultivated alone (**Figures 9B,C**; orange line). Treating cells with either Pam3, rIFN-γ, or vdIFN-γ alone resulted in growth rates similar to those observed in LLC cells cultivated alone (**Figures 9B,C**).

To analyze the functionality of vdIFN-γ, we treated BMDMs with the same concentrations that were used to test rIFN-γ. The vdIFN-γ was produced by infecting BHK-21 cells with SFV-Ifng viral particles and harvesting the cell culture medium to use as a source of vdIFN-γ. The results were similar to what was observed for rIFN-γ in that incubating macrophages with vdIFN-γ in combination with Pam3 resulted in efficient cancer cell growth inhibition in a concentration-dependent manner. The lowest effective concentration of vdIFN-γ was 6.25 ng/mL, which produced less than 1,050 cpm (**Figure 9C**). In all the experiments, the most efficient inhibition of cancer cell growth was observed when the ratio of BMDMs to LLC cells was 20:1 or 10:1 (**Figures 9B,C**). Finally, the pattern of effective IFN-γ doses and the extent of growth inhibition they produced was similar between rIFN-γ and vdIFN-γ (**Figures 9B,C**). Based on these data, we conclude that vdIFN-γ has biological activity and can substitute for rIFN-γ.

Using TLR ligands in combination with IFN-γ provides a classical set of stimuli that activates macrophages toward an M1-phenotype, which can produce NO through inducible NO synthase (70). We, therefore, determined NO production as a readout to verify that vdIFN-γ triggers macrophage activation potentially toward an M1-phenotype. We measured NO_{2^-}, which is the end product of the rapid oxidation of NO, in cell culture media using the Griess test. First, we verified that the BMDMs produced NO in a concentration-dependant manner at 24 h after stimulation with both rIFN-γ and Pam3 (**Figure 10A**). We then stimulated BMDMs with both vdIFN-γ and Pam3, and the results were similar to those observed for rIFN-γ in that the levels of NO_{2^-} varied according to the concentration of vdIFN-γ (**Figure 10B**). The highest levels of NO_{2^-} were observed when rIFN-γ and vdIFN-γ were used in a range from 0.39 to 100 ng/mL. At IFN-γ concentrations ≤0.098 ng/mL, the levels of NO_{2^-} were lower (**Figures 10A,B**). Stimulation with rIFN-γ alone resulted in the production of only small amounts of NO_{2^-} in the cell culture medium (**Figure 10A**). The levels of NO_{2^-} were also low in cultures stimulated with vdIFN-γ alone (**Figure 10B**) or Pam3 alone (**Figures 10A,B**). Thus, IFN-γ and Pam3 had a synergistic effect on the ability of macrophages to produce NO. The higher levels of NO_{2^-} that were observed following stimulation with higher doses of IFN-γ (**Figures 10A,B**) were well-correlated with the enhanced cancer cell growth inhibition that was observed in the growth inhibition experiments (**Figures 9B,C**). Taken together, data from the growth inhibition assay demonstrated that vdIFN-γ is a potent agent at very low concentrations that when combined with Pam3 induces an antitumor phenotype in macrophages that suppresses growth in LLC cells.

DISCUSSION

In this study, we developed two new rSFV vectors encoding either murine TNF-α or IFN-γ and showed by *in vitro* studies that the two rSFV-encoded cytokines are functional. The rSFV efficiently infected mouse and human lung carcinoma cells *in vitro*, whereas murine and human macrophages were resistant to SFV infection. The rSFV inhibited LLC cell growth, induced cancer cell death and simultaneously exploited cancer cells for production of SFV-encoded TNF-α and IFN-γ at levels that are functional *in vitro*. The functionality of SFV-encoded TNF-α was shown *via* cell death induction in L929 cells. The rSFV-encoded IFN-γ activated macrophages toward a tumoricidal phenotype *in vitro*.

Recombinant SFV has been used to express various cytokine genes in experimental tumor models resulting in therapeutic effects (2–15). For example, the same replication-deficient SFV/enh vector used in this study was previously applied for IL-12 expression in mouse breast and colon cancer models, resulting in inhibition of tumor growth upon intratumoral vector inoculation (7). Because of the antitumor potential of TNF-α (40–46) and IFN-γ (28–39) or both in combination (47–50, 53), we subcloned these cytokines in a safe replication-deficient

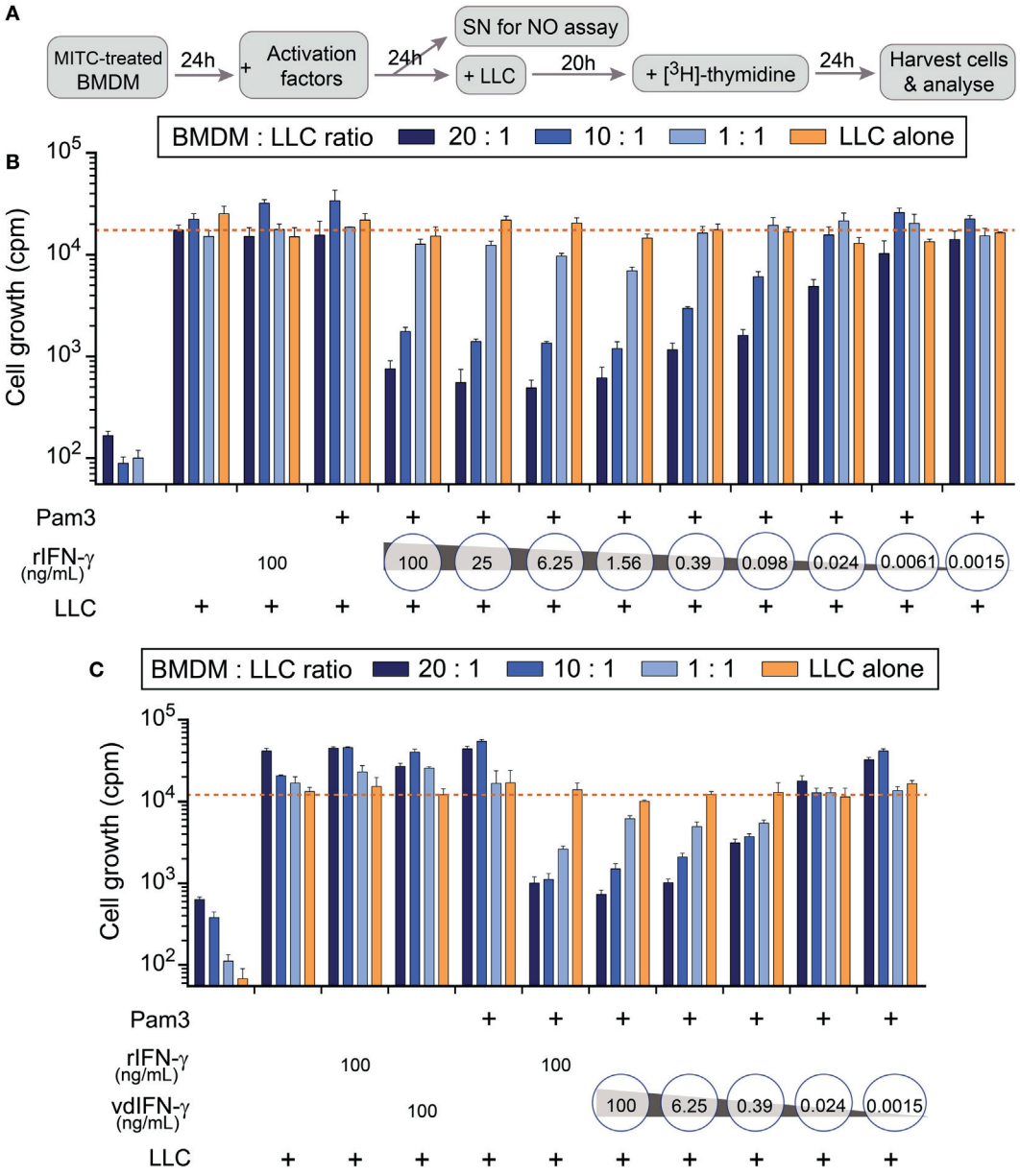

FIGURE 9 | The effect of rIFN-γ or vdIFN-γ in combination with a TLR2/1 ligand on macrophage activation was detected using growth inhibition assay in Lewis lung carcinoma (LLC) cells. **(A)** Schematic timeline of the experiment. Mitomycin C-treated bone marrow-derived macrophages (BMDMs) were plated at three different densities and incubated for 24 h before macrophage-stimulating factors were added. After an additional 24 h, 100 µL of cell culture media was obtained from the wells with the highest macrophage density to measure NO_{2^-} levels. LLC cells were added to the activated BMDMs, and the co-cultures were incubated for an additional 20 h. [^{3}H]-thymidine was then added to the co-cultures, and after 24 h, the LLC cells were harvested to analyze cell growth by measuring the amount of incorporated [^{3}H]-thymidine as counts per minute (cpm, depicted on the *y*-axis). **(B)** LLC cell growth after co-cultivation with BMDMs that were activated with Pam3 (100 ng/mL) in combination with different concentrations of rIFN-γ ranging from 100 to 0.0015 ng/mL in fourfold dilutions. Controls, from left to right: BMDMs cultivated without LLC cells, LLC cells co-cultivated with unstimulated macrophages, and LLC cells co-cultivated with macrophages that were stimulated with either rIFN-γ alone or Pam3 alone. **(C)** LLC cell growth after co-cultivation with BMDMs that were activated with Pam3 (100 ng/mL) in combination with vdIFN-γ ranging from 100 to 0.0015 ng/mL in 16-fold dilutions. Controls, from left to right: BMDMs cultivated without LLC cells, LLC cells co-cultivated with unstimulated macrophages, LLC cells co-cultivated with macrophages that were treated with either rIFN-γ, vdIFN-γ, or Pam3 at a concentration of 100 ng/mL, and LLC cells co-cultivated with BMDMs that were treated with 100 ng/mL of both rIFN-γ and Pam3. Experiments were performed in triplicates and were repeated two times with similar results. The bars represent the mean values of triplicates ± SEM from one representative experiment.

SFV vector, which provides high and transient expression of the transgene without further virus replication (20). In order to form viral particles, we used SFV-Helper1 system (20), which encodes SFV structural proteins. We produced infectious SFV-Tnfa and SFV-Ifng recombinant virus particles in BHK-21 cells at titers 10^7–10^8 IFU/mL.

Various mouse and human cancer cell lines are susceptible to SFV infection although the transduction rates may differ between

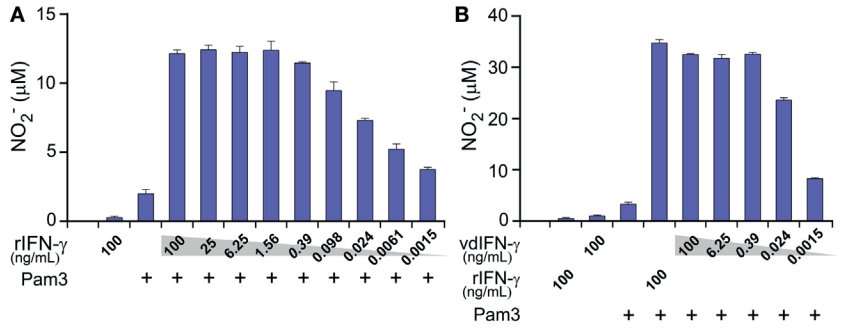

FIGURE 10 | The production of nitric oxide (NO) by macrophages at 24 h after activation was determined by analyzing NO_{2^-} levels in the cell culture medium. Mitomycin C-treated bone marrow-derived macrophages (BMDMs) were cultivated for 24 h before they were activated with the TLR2/1 agonist Pam3 (100 ng/mL) in combination with either **(A)** rIFN-γ at various concentrations ranging from 100 to 0.0015 ng/mL in 4-fold dilutions or **(B)** vdIFN-γ at concentratios ranging from 100 to 0.0015 ng/mL in 16-fold dilutions. Non-stimulated BMDMs and BMDMs that were stimulated with Pam3, rIFN-γ, or vdIFN-γ were used as the negative controls. Nitrite (NO_{2^-}) levels were measured in the macrophage culture medium at 24 h post-stimulation using the Griess test. Experiments were performed in triplicates and were repeated two times with similar results. The bars represent the mean values of triplicates ± SEM from one representative experiment.

cell types (71–73). The hamster kidney fibroblast cell line BHK-21, which is commonly used for production of SFV particles, is efficiently infected by SFV (71). In the current study, the high susceptibility of BHK-21 cells to SFV infection was confirmed. Two cell lines of particular interest for development of lung cancer immunotherapy are mouse and human lung carcinoma cell lines, LLC and A549, respectively, which proved to be susceptible to SFV infection with a comparable transduction level of 41% 24 h post-infection for both cell lines when SFV was used at MOI = 15. This is in agreement with a previous study showing that A549 cells are susceptible to SFV infection (72). Our results encouraged us using LLC cells as target murine cancer cells further because they were successfully infected by rSFV particles and were able to express rSFV-encoded proteins.

In contrast to the tested cancer cell lines, macrophages were found to be resistant to SFV infection. Similar data were obtained with primary mouse and human macrophages, as well as with the mouse macrophage cell line J774A.1. Moreover, macrophages retained their viability after challenge with rSFV particles. The fact that macrophages are not killed by SFV but instead serve as effector cells responding to SFV-encoded factors may be an advantage for immunotherapy based on the activation of tumor-associated macrophages. Cancer cells, due to their susceptibility to SFV infection, may be exploited to produce such SFV-encoded factors. The mechanism by which macrophages remained resistant to SFV infection is unclear. The life cycle of replication-deficient rSFV particles is a several-step process comprising viral entry *via* endocytosis, a pH-dependent release of virus nucleocapsid from the endosome, capsid disassembly, viral RNA replication in the cytoplasm, and subsequent translation of the transgene (74). The suppression in either of the abovementioned steps may have resulted in lack of SFV-encoded transgene expression in macrophages as we observed. Furthermore, fresh isolated peritoneal mouse macrophages were also resistant to infection with the replication-deficient SFV-DsRed virus as was confirmed by absence of fluorescent signal in the infected cells (data not shown). In contrast to our results, a study from 1976 described that a replication-competent SFV was able to infect and multiply at very low yields within peritoneal mouse macrophage cultures *in vitro* (75). The authors tested the presence of SFV within cell growth medium of peritoneal macrophages post-infection by titrating plaque forming units in chick embryo cell cultures. Similarly, another study from 1988 reported low susceptibility of mouse spleen-derived Mac-1-positive cells to infection with replication-competent or first-generation avirulent SFV (76). The differences between the protocols and SFVs used in our study and studies by van der Groen et al. or Wu et al. make it difficult to compare the results. However, a possible explanation for the macrophage low susceptibility toward SFV in the studies mentioned (75, 76) could be that the *ex vivo* isolated peritoneal or spleen cells, which the authors used as a source of macrophages, also contained other cell types that served as host cells for SFV multiplication. The main difference though is that the authors used replication-competent SFVs, including a wt SFV, which was isolated from mosquitos and was produced by replication in chick embryo cell cultures, or first-generation avirulent SFV. Our data based on use of pure macrophage cultures clearly show that mouse and human macrophages are resistant to infection with SFV particles that are based on the replication-deficient rSFV1 vector and that have been produced in BHK-21 cells.

Infection with SFV inhibited proliferation of BHK-21 and LLC cells *in vitro* independent on whether SFV-encoded TNF-α or IFN-γ. The inhibition of cancer cell growth is likely caused by the ability of SFV particles to induce p53-independent apoptosis in infected cells, which is linked to viral RNA replication and appearance of double-stranded RNA intermediates in cell cytoplasm (21). This property is of particular interest for lung cancer therapy since a mutated p53 is one of the key molecular markers in many solid cancers, including human lung cancers (77). In fact, the target LLC cancer cells used in this study were shown to undergo cell death after infection with replication-deficient rSFV particles. The infected cancer cells were shown to express rSFV-encoding transgene while undergoing cell death. Thus, rSFV has therapeutic potential not only as a transgene delivery vehicle but may also elicit a direct oncolytic effect on cancer cells. Moreover, intratumoral injection of rSFV particles has been shown to

inhibit tumor growth in preclinical mice models by induction of apoptosis (78).

Early after infection with rSFV particles the host cells shut down the translation of endogenous cellular proteins (79), whereas the heterologous protein encoded by rSFV is produced at high levels (80). In the present study, we embraced this property in order to produce tumor-suppressive cytokines within cancer cells. We showed that LLC cells can produce and release rSFV-encoded TNF-α and IFN-γ in the cell culture medium at levels that are functional *in vitro*. Whereas vdIFN-γ was present at 100 ng levels in the supernatant, the amount of vdTNF-α was in several nanograms. The relatively low levels of vdTNF-α that we detected might be explained by the lack of translational enhancer in a vector, as well as by the fact that TNF-α is primarily produced as a transmembrane protein (81), and cleavage of the transmembrane form is needed before soluble TNF-α can be released into the cell culture medium (82).

Nevertheless, the levels of vdTNF-α detected were sufficient to elicit a functional response *in vitro* as shown by induction of cell death in mouse fibroblast cell line L929. TNF-α is known to induce either caspase-dependent apoptotic cell death or caspase-independent programmed necrotic cell death (necroptosis) downstream of TNFR1 signalling depending on microenvironment and cell type (83, 84). We induced mainly necrotic cell death in L929 cells in a dose-dependent manner after treatment with either vdTNF-α or rTNF-α, which is in agreement with previous reports showing that L929 cells undergo caspase-independent necroptosis in response to TNF-α treatment (68, 85). In accordance with a previous study (65) we also observed an annexin V-positive/PI-negative cell population after treatment with vdTNF-α, which may represent not only apoptotic but also primary necrotic cells according to studies showing phosphatidylserine exposure during early necrosis (oncosis) before the integrity of the membrane is lost (86, 87). By inducing fibroblast cell death *in vitro*, we verified that SFV-encoded TNF-α is biologically active. However, *in vivo* effects of TNF-α differ and comprise increased vessel permeability used to improve drug penetration (43–45) and destruction of tumor vasculature (41, 42) leading to hemorrhagic tumor necrosis (40, 88). By using recombinant cytokines, we also showed that TNF-α is a cytokine that may induce cytotoxicity in mouse lung carcinoma cells but only when combined with IFN-γ. Such observations can be explained by a previously reported IFN-γ property to enhance TNF-α receptor expression (51, 52), thus sensitizing cancer cells to TNF-α-induced cell death. Furthermore, in the context of cancer immunotherapy TNF-α may serve as an adjuvant in order to enhance T-cell infiltration (46, 89).

In this study, we showed that rSFV-encoded IFN-γ was functional in activating macrophages *in vitro* toward a tumoricidal phenotype, which is consistent with previous studies showing that IFN-γ renders macrophages tumoricidal *in vitro* (34–36). We observed that in order to activate macrophages, presence of both vdIFN-γ and the TLR2/1 agonist Pam3 was required. Also previous studies showed that two signals, IFN-γ in combination with a second signal are required to render macrophages tumoricidal (39) and able to induce NO production (90). It is known that classically activated or M1 macrophages increase the expression of inducible NO synthase and, thus, produce NO (91). We showed that vdIFN-γ synergized with TLR2/1 agonist Pam3 to induce NO production in macrophages in a concentration-dependent manner, similarly as observed by combining rIFN-γ with another TLR agonist, CpG-containing DNA, respectively, in a previous study (90). Our observation that high level of NO, which was produced by activated macrophages, correlated with increased cancer cell growth inhibition is in agreement with the early studies reporting that tumoricidial abilities of macrophages *in vitro* are based on NO being cytotoxic to cancer cells (92, 93).

Macrophages are common immune cells in many tumors and often are considered to inhere a pro-tumoral phenotype associated with angiogenesis, metastasis, and suppression of T cell activation (94). By contrast, M1 macrophages suppress tumor growth and angiogenesis (95) and might be positively associated with patient survival (95–97). Several *in vivo* studies have shown the therapeutic benefit of redirecting tumor-associated macrophages to M1 phenotype by agents such as TLR9 ligand CpG in combination with antibody to IL-10R (88), TLR7/8 agonist 3M-052 (98), or liposomal nanoparticle-encapsulated hydrazinocurcumin (99). Previous studies suggest IFN-γ as a good candidate for tumor-associated macrophage re-education due to the ability of IFN-γ to re-polarize tumor-associated macrophages toward an M1 phenotype *in vitro* (100) and its ability to render macrophages tumor-suppressive in mouse tumor models *in vivo* (31, 37–39). The results from this study suggest that SFV-delivered vdIFN-γ is as efficient as rIFN-γ in rendering macrophages tumoricidal *in vitro*, which along with our finding that macrophages but not cancer cells are resistant to SFV infection encourages a possible application of SFV-Ifng vector for activation of tumor-associated macrophages toward tumor-suppressive M1 phenotype in further studies *in vivo*. Moreover, either IFN-γ or TNF-α delivery by rSFV vector may comprise not only a direct effect of the rSFV-delivered cytokines but may also benefit from the inherent ability of SFV vector to induce p53-independent apoptosis (21) and elicit endogenous type-I IFN responses, which may be required for the therapeutic effect of a vector-encoded cytokine (3).

In summary, the present study shows that rSFV can efficiently infect lung carcinoma cells *in vitro* and exploit them for production of functional rSFV-encoded TNF-α and IFN-γ. The rSFV-encoded IFN-γ was able to activate macrophages toward an antitumor phenotype *in vitro*. Our results set the basis for application of SFV-Tnfa and SFV-Ifng vectors to modulate the cytokine milieu in the tumor microenvironment in further studies using mouse models for lung cancer.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Norwegian Regulation on Animal Experimentation, Norwegian Food and Safety Authority.

AUTHOR CONTRIBUTIONS

AZ and AC conceived the study. All authors contributed to the design of experiments, data analysis, and interpretation of data. BK, EM, PC, IJ, BS, and IØ performed experiments. BK wrote

the manuscript; and IØ, AC, and AZ contributed in writing the manuscript. All authors critically revised the manuscript and approved the final version. AC and AZ contributed equally to this work.

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