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RIG-I-induced antitumor immunity is driven by immunogenic tumor cell-derived extracellular vesicles

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Abstract

Tumor cell-intrinsic activation of the innate RNA receptor retinoic acid-inducible gene I (RIG-I) has been found to result in tumor cell death; a pathway which is particularly active in malignant cells. Another study revealed that RIG-I activation leads to immunogenic cell death in pancreatic carcinoma cells triggering T cell-based antitumor immune responses. However, whether this is only the case for pancreatic cancer or whether these mechanisms are active in other tumor entities is unknown. Additionally, the dominant immunogenic factors released by tumor cells following RIG-I ligation remain to be determined. This study now demonstrates that immunogenic cell death by RIG-I activation also occurs in malignant melanoma cells. Moreover, tumor-intrinsic RIG-I activation was found to result in increased shuttling of immunogenic nucleic acids within extracellular vesicles (EVs) alongside tumor cell apoptosis induction. Therapeutic application of purified RIG-I-induced EVs resulted in strong T cell priming *in vivo* that was critically dependent on host nucleic acid receptor signaling via MAVS and STING and subsequent type I interferon receptor activation in myeloid antigen-presenting cells. Such EV-mediated expansion of tumor specific T cells resulted in systemic antitumor immunity. In summary, this study demonstrates that melanoma cell-intrinsic RIG-I activation triggers the release of EVs containing immunogenic nucleic acids, which mediate T cell-based antitumor immunity.

Zusammenfassung

Die Aktivierung des RNA Rezeptors *Retinoic acid-inducible gene I* (RIG-I) innerhalb von Tumorzellen führt zu deren Zelltod, vermittelt über eine Signalwegkaskade welche besonders in malignen Zellen aktiv ist. Vorangehende Arbeiten haben gezeigt, dass diese Aktivierung von RIG-I in Pankreaskarzinom-Zellen in der speziellen Form des immunogenen Zelltods resultiert, einhergehend mit der Bildung einer T Zell-basierten Antitumor-Immunantwort. Unklar ist jedoch, ob dieser RIG-I-vermittelte immunogene Zelltod ausschließlich im Pankreaskarzinom auftritt, oder ob er auch in anderen Tumorentitäten durch Aktivierung von RIG-I induziert werden kann. Des Weiteren blieben die dominanten immunstimulatorischen Faktoren, die von RIG-I-aktivierten Tumorzellen abgegeben werden und damit deren Immunogenität vermitteln, bisher unbekannt. Die vorliegende Dissertation zeigt nun, dass auch im malignen Melanom durch die Aktivierung von RIG-I immunogener Zelltod ausgelöst werden kann. Zusätzlich wird im Rahmen dieser Arbeit gezeigt, dass während des RIG-I-vermittelten Tumorzelltods ein erhöhter Transport von immunogenen Nukleinsäuren stattfindet. Diese werden im Inneren sogenannter extrazellulärer Vesikel (EVs) von Tumorzellen sezerniert. Die therapeutische Anwendung aufgereinigter RIG-I-induzierter EVs führte zur Entwicklung einer potenten T-Zell Immunantwort *in vivo*. Diese war in entscheidender Weise abhängig von der Aktivität von Nukleinsäure-Erkennungsrezeptor-Signalwegen via den Adapterproteinen MAVS und STING im Empfängerorganismus. Dies resultierte in der Aktivierung des Typ-I Interferonrezeptors in myeloiden Antigen-präsentierenden Zellen. Die EV-vermittelte Expansion von tumor-spezifischen T Zellen resultierte in systemischer Antitumor-Immunität. Zusammenfassend wird in dieser Dissertation gezeigt, dass die Aktivierung des RNA Rezeptors RIG-I in Melanomzellen zur Abgabe immunogener, Nukleinsäure-tragender EVs führt, welche eine T Zell-basierte Antitumor-Immunität vermitteln können.

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List of abbreviations

°C	Degree Celcius
ACT	Adoptive cell transfer
AP	Alkaline phosphatase
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMDC	Bone marrow-derived dendritic cell
CD	Cluster of differentiation
CDN	Cyclic dinucleotide
CO_2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CRT	Calreticulin
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
Dex	Dendritic cell-derived exosomes
dLN	Draining lymphnodes
DNA	Desoxyribonucleic acid
ds	Doublestranded
DSB	Singlestranded
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FAM	Fluorescein
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanat
G	Gauge
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
H_2O	Water
H_2SO_4	Sulfuric acid
HDR	Homology-directed repair
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
IFN	Interferon
Type I IFN	Type-I interferon
IFNaR	Type-I nterferon receptor
IgG	Immunoglobulin G
IL	Interleukin
iLN	Inguinal lymph node
ILV	Intraluminal vesivles
ip.	Intraperitoneally
IRF	Interferon regulatory factor
ISD	Interferon stimulatory DNA
ISG	Interferon-stimulated genes

iv.	Intravenously
IVT	In vitro transcription
mAB	Monoclonal antibody
MAVS	Mitochondrial antiviral-signaling protein
MHC (-I /-II)	Major histocompatibility complex class I / class II
min	Minute
mL	Milliliter
MVB	Multivesicular body
N	Molar
ng	Nanogram
NHEJ	Non-homologous end joining
NK	Natural killer
nm	Nanometer
NTA	Nanoparticle tracking analysis
OVA	Ovalbumin
Oxa	Oxaliplatin
p(I:C)	Polyinosinic-polycytidynic acid
pAB	Polyclonal antibody
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
pLN	Popliteal lymph node
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RT	Room temperature
sc.	Subcutaneously
sec	Second
ss	Singlestranded
STING	Stimulator of interferon genes
TAA	Tumor-associated antigen
TALEN	Transcription activator-like effector nuclease
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
U	Unit
UC	Ultracentrifuge
VLE	Very low endotoxin
WT	Wild-type
ZFN	Zinc finger nuclease
µg	Microgram
µl	Microliter

1. Introduction

This thesis covers two main areas of research: anti-cancer immunotherapy in terms of immunogenic cell death, as a promising approach to defeat cancer, and tumor-derived extracellular vesicles, as an important communicator between cancer cells and the host's immune system. The first part of the introduction gives an overview of current anticancer immunotherapies and the associated challenges. This section also introduces the concept of immunogenic cell death of cancer cells and the initiation of adaptive immunity. Additionally, innate nucleic acid-sensing receptors and their role in anticancer immunotherapy will be discussed. The second part introduces extracellular vesicles and their role in tumor immunity.

1.1. Anticancer immunotherapy – from theory to final acceptance

For more than one hundred years, immunology and immunotherapy have played an ever-increasing role in the understanding and treatment of cancer. During these years, one of the most controversial questions in immunology was: 'Can the immune system recognize and eliminate malignant tumors?'. The answer to this question has been largely dependent on the prevalent immunological theories at the time. The first indications that the immune system might respond to malignant tissue, dates back to the nineteenth century. In 1891, William Coley, a leading New York surgeon, investigated the phenomenon that feverish infections in cancer patients were occasionally associated with cancer remission (Coley 1991, Wiemann and Starnes 1994). Coley performed intratumoral injections of live or inactivated *Streptococcus pyrogenes* bacteria, provided by Robert Koch, in an effort to reproduce the complete remission of sarcoma observed in a patient who had developed erysipelas, an acute infection with the before mentioned bacteria. In 1893, he published his results describing 'Coley's toxins' as a stimulant of antibacterial phagocytes that might also kill bystander tumor cells. Coley's experiments are considered the first serious attempt of cancer immunotherapy (Coley 1893). Although, Coley's toxin treatment achieved a cure rate of over 10% (Wiemann and Starnes 1994) in the ensuing 40 years, clinical communities of the time did not accept Coley's toxin probably due to the severe fever induced by the treatment and the perceived low cure rates (Parish 2003). Further successes in the field of cancer immunotherapy were sporadic, difficult to reproduce, and not obtained in a scientifically sound fashion. The general feeling amongst immunologists was that it would be impossible for the immune system to recognize and respond to malignant cells (Parish 2003, Mellman *et al.* 2014).

In 1949, Frank M. Burnet published his theory of acquired immunological tolerance. He proposed that lymphocytes able to respond to self-tissues are prenatally deleted during the development of the immune system (Burnet and Fenner 1949). With this model, the view that the immune system is incapable of responding to malignant cells was reinforced, as it was assumed that transformed cells are indistinguishable from healthy self-tissue (Parish 2003). During the 1950s however, E.J. Foley and others demonstrated, that it actually was possible to immunize syngenic (genetically identical; immunologically compatible) animals against their tumors and that indeed there are antigens associated with tumor cells being often called tumor-associated antigens (TAA) or tumor-specific transplantation antigens (TSTA) (Foley 1953, Baldwin 1955, Prehn and Main 1957). Ironically, it was Burnet who in the 1960s changed the common view in favor of cancer immunotherapy. He suggested that lymphocytes are continually patrolling cells that eliminate transformed tumor cells, presumably by recognizing TAAs. He termed this process 'immunosurveillance' (Burnet 1967). However, the then prevailing view on cancer immunotherapy was again short-lived and the concept of immunosurveillance was abandoned until the mid 1990s (Parish 2003). Only then it was shown that auto-reactive T lymphocytes can escape thymic deletion (Arnold *et al.* 1993), and since 1995 the evidence of effective tumor-specific immunity has become convincing.

There have been a large number of studies indicating that dendritic cells (DCs; a specialized form of antigen presenting cells) can effectively elicit tumor-specific T cell immunity, if they are activated in an appropriate manner (Flamand *et al.* 1994, Mayordomo *et al.* 1995, Zitvogel *et al.* 1996). The important role of nucleic-acid detecting receptors in this context is discussed below (see page 16). A number of pilot trials have subsequently been performed and many of these have demonstrated the induction of antitumor immune responses (Brossart *et al.* 2001, Steinman and Dhodapkar 2001). The generation and analyses of immunodeficient knock-out mice, like $RAG^{-/-}$ (Shankaran *et al.* 2001), $STAT1^{-/-}$ (Shankaran *et al.* 2001), and IFN-$\gamma^{-/-}$ mice (Kaplan *et al.* 1998), have further supported the old immunosurveillance hypothesis by showing higher incidences of carcinogen-induced tumors in the absence of a functional immune system. Other studies have shown that natural killer (NK) cells (Smyth *et al.* 2001), natural killer T (NKT) cells (Smyth *et al.* 2000), and $_{\gamma\delta}$T cells (Girardi *et al.* 2001) of the innate immune system play a key role in tumor immunosurveillance. All these studies from 1995 to the present day conclude that many of the tumors that emerge in immunocompetent animals have been selected to evade the host's immune system (Parish 2003).

1.2. Achieving anticancer immunity – a multistep challenge

Research over the last years on the functioning of the immune system identified three key steps that must be achieved, either spontaneously or therapeutically, in order to elicit effective antitumor immunity (Mellman *et al.* 2014).

To initiate an immune response, dendritic cells must process antigens derived from tumor cells, which can be ingested *in situ* or delivered exogenously as part of a therapeutic vaccine. The engulfed antigens might comprise one or more of the many mutated proteins typical of cancer, differentiation antigens associated with the cancer tissue of origin but against which thymic or peripheral tolerance has been incompletely established (e.g. melanosome-associated proteins in melanoma) (Boon *et al.* 2006, Segal *et al.* 2008), or the products of non-mutated genes that are preferentially expressed by cancer cells (e.g. cancer-testis antigens) (Suri *et al.* 2015, Wurz *et al.* 2016). Upon antigen uptake, the DCs also have to receive a suitable activation ('maturation') signal, allowing them to differentiate extensively to promote immunity including enhanced processing and presentation of tumor antigen-derived peptides (Mellman and Steinman 2001, Trombetta and Mellman 2005, Mellman *et al.* 2014). Activation signals that result in the immunogenic maturation of DCs can be therapeutically supplied (exogenously), like agonist antibodies against activating receptors such as CD40 or Toll-like receptor (TLR) agonists. Alternatively, these factors can be supplied endogenously, like the factors released by dying or necrotic tumor cells such as high mobility group proteins or ATP. In addition, dying tumor cells appear to ectopically express ER proteins on the plasma membrane (e.g. ecto-calreticulin) that promote their phagocytosis, enabling capture and presentation of tumor antigens on MHC class I or II molecules (Zitvogel and Kroemer 2009, Mellman *et al.* 2014).

Next, in lymphoid organs like lymph nodes, tumor antigen-loaded DCs must elicit a protective T cell response (Palucka *et al.* 2010). The precise type of T cell responses is not elucidated yet, however certainly they must include the production of $CD8^+$ effector T cells with cytotoxic potential. DCs may also trigger NK/ NKT cell, which can contribute to tumor immunity (Mellman *et al.* 2014). The lymph node environment is thus a second potential site for therapeutic intervention, providing agents that may help forming the T cell response. Again, in order to elicit the desired cytotoxic T cells, DCs must have been matured by a stimulatory adjuvant. Presentation of antigens by DCs that have not received an immunogenic maturation signal promotes tolerance by inducing regulatory T cell (T_{reg}) differentiation, which can potently oppose an antitumor response (Steinman *et al.* 2003, Jiang *et al.* 2007, Darrasse-Jeze *et al.* 2009).

Finally, cancer-specific T cells must enter the tumor microenvironment to perform their cytolytic function. This process is opposed by immune suppression as tumors may prevent immunization or enable local accumulation or expansion of T_{regs}, which would also oppose the activity of effector T cells presumably by skewing DC maturation (Mellman *et al.* 2014). Indeed, accumulation of T_{regs} correlates with poor prognosis in a variety of epithelial tumor types (Curiel *et al.* 2004, Kono *et al.* 2006). In some cases tumors down-regulate the expression of target tumor antigens or display a variety of suppressive molecules like PD-L1 and –L2 on the cell surface, that engage receptors such as PD-1 on activated effector T cells, causing T cell anergy and exhaustion (Kooi *et al.* 1996, Hamanishi *et al.* 2007). Expression of such suppressive ligands can be associated with oncogenic mutations seen in many cancers (e.g. loss of PTEN) (Parsa *et al.* 2007). Additionally, tumors release immunosuppressive molecules, such as indolemaine 2,3-dioxygenase (IDO) which enables them to escape the immune system by limiting T cell function (Munn and Mellor 2004). Myeloid-derived suppressor cells can also be recruited into the tumor bed, and release T cell suppressing enzymes like arginase and nitrous oxide synthase (Marigo *et al.* 2008). A hypoxic state in the tumor microenvironment may promote the generation of adenosine and CCL28, which also inhibits T cell function or attracts the immigration of T_{regs}, respectively (Ohta *et al.* 2006, Facciabene *et al.* 2011). Finally, tumor stroma cells can also suppress the function of effector T cells (Aggarwal and Pittenger 2005). Mesenchymal stem cells for example inhibit T cell proliferation and function, while tumor vascular cells suppress T cell adhesion to tumor endothelium and prevent homing to tumors. This effect is in part mediated by an altered release and expression of vascular endothelial growth factor (VEGF) or the endothelin-B receptor (Bouzin *et al.* 2007, Buckanovich *et al.* 2008), respectively.

Thus, successful anticancer immunity is challenging to achieve. Such approaches must overcome several significant barriers: the fact that tumor-associated antigens are typically closely related to or even identical to self-antigens, making it difficult to separate therapeutic responses from pathological autoimmune responses, both central and peripheral tolerance are prone to deletion or inactivation of the relevant T cell repertoire and, that the tumor bed is inherently immunosuppressive (Mellman *et al.* 2014). Yet, a path to clinical success seems now to be emerging.

1.3. Cancer immunotherapy - current concepts

During the past three decades, the perception of cancer has changed dramatically. It is now appreciated that tumors are not a purely clonal disorder (Dean *et al.* 2005) and it is clear that established neoplasms do not consist only of transformed cells, but contain a heterogeneous non-transformed component, including stromal, endothelial and immune cells (Holzel *et al.* 2013). The metabolism of cancer cells is no longer considered as completely distinct from that of their healthy counterparts (Galluzzi *et al.* 2013b, Green *et al.* 2014). Mechanisms other than intrinsic apoptosis have been discovered that may be harnessed for therapeutic applications such as regulated necrosis (Galluzzi *et al.* 2014a). Finally, evidence is emerging that the host immune system can recognize (and sometimes react against) (pre-) malignant cells as they transform, proliferate, evolve and respond to therapy, founding the theoretical grounds of anticancer immunosurveillance (Schreiber *et al.* 2011, Galluzzi *et al.* 2014b). The acquired knowledge has profound therapeutic implications, some of which have already been translated into clinical realities. Several anticancer agents have recently been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the use in cancer patients. In 2013, the extraordinary clinical success of immunotherapy was acknowledged by the Editors of Science Magazine with the designation of 'Breakthrough of the Year' (Couzin-Frankel 2013). There are now agents available that inhibit tumor-associated angiogenesis (Albini *et al.* 2012) or mediate antineoplastic effects by initiating a new or boosting an existing immune response against cancer cells (Sharma *et al.* 2011, Galluzzi *et al.* 2014b). Today's anticancer therapies are generally classified as 'passive' or 'active' based on their ability to (re-) activate the host immune system against malignant cells (Lesterhuis *et al.* 2011, Galluzzi *et al.* 2014b).

1.3.1. Passive immunotherapy

Tumor-targeting monoclonal antibodies. Tumor-targeting monoclonal antibodies (mAbs) are the best characterized form of anticancer immunotherapy, and the most widely employed in the clinic (Alkan 2004). The name 'tumor-targeting' describes mAbs that (1) specifically alter the signaling functions of receptors expressed on the surface of cancer cells (Kaplan-Lefko *et al.* 2010, Ming Lim *et al.* 2013); (2) bind to, and thus neutralize, trophic signals produced by malignant cells or stromal components of cancerous lesions (Ferrara *et al.* 2004); (3) selectively recognize cancer cells due to the expression of a tumor associated antigen (TAA), resulting in the induction of antibody-dependent cellular (ADCC) and complement-dependent (CDC) cytotoxicity (Cavallo *et al.* 2007).

Adoptive cell transfer. The expression 'adoptive cell transfer' (ACT) refers to a particular variant of cell-based anticancer immunotherapy that involves multiple steps: (1) the collection of circulating or tumor-infiltrating specific lymphocytes; (2) their selection/ modification/ expansion/ activation *ex vivo*; and (3) their (re-) administration to patients (Vacchelli *et al.* 2013a, Galluzzi *et al.* 2014b).

Oncolytic viruses. The term 'oncolytic viruses' refers to non-pathogenic viral strains that specifically infect cancer cells, triggering their death (Vaha-Koskela *et al.* 2007, Russell *et al.* 2012). The anticancer potential of oncolytic viruses can be innate, driven by cytokine release causing migration of immune cells such as natural killer (NK) cells to the site of disease, or adaptive, via a response to TAAs, and originate from a lethal overload of cellular metabolism resulting from a productive viral infection (cytopathic effect) (Boisgerault *et al.* 2013, Turnbull *et al.* 2015). Or, these viruses can mediate an oncolytic activity due to gene products that are potentially lethal for the host cell (Russell *et al.* 2012). Despite robust data from laboratories around the world showing the potential of these agents in initiating tumor cell death (Strong *et al.* 1993, Strong *et al.* 1998), the first phase III trial of a viral-based therapy for melanoma has only just been reported (Andtbacka *et al.* 2015). The field has now expanded to include DNA viruses (e.g. herpes simplex virus (HSV), RNA viruses (e.g. coxsackie virus), and genetically modified viruses such as talimogene laherparepvec (HSV expressing granulocyte-macrophage colony stimulating factor (GM-CSF), known as T-VEC) (Turnbull *et al.* 2015).

1.3.2. Active Immunotherapy

DC-based immunotherapies. Remarkable efforts have been invested in the development of anticancer immunotherapies based on DCs (most often autologous DCs) (Palucka and Banchereau 2012, Coosemans *et al.* 2013). The results of preclinical and clinical investigation reflects the critical role of DCs at the interface between innate and adaptive immunity, and the ability of some DC subsets to prime robust and therapeutically relevant anticancer immune responses (Merad *et al.* 2013). Multiple forms of DC-based immunotherapies have been developed, most of which involve the isolation of patient- or donor-derived circulating monocytes and their expansion/ differentiation *ex vivo* (Galluzzi *et al.* 2014b). For the *ex vivo* maturation maturating agents such as granulocyte macrophage colony-stimulating factor (GM-CSF) have been facilitated (Palucka and Banchereau 2012). This is particularly important due to the fact that immature DCs exert immunosuppressive, rather than immunostimulatory functions, as mentioned above. Most often autologous DCs are re-infused into cancer patients upon exposure to a source of TAAs, including (1) TAA-derived peptides (Mayordomo *et al.* 1995); (2) mRNAs coding for

one or more specific TAAs (Zeis *et al.* 2003); (3) expression vectors coding for one or more specific TAAs (Irvine *et al.* 2000); (4) bulk cancer cell lysates (autologous or heterologous) (Kandalaft *et al.* 2013); or (5) bulk cancer cell-derived mRNA (Garg *et al.* 2013). The common concept behind all these approaches is that DCs are loaded *ex vivo* with TAAs or TAA-coding molecules, thereby acquiring the potential to prime TAA-targeting immune responses upon re-infusion (Galluzzi *et al.* 2014b). Such DC-based interventions should be conceptually differentiated from ACT because infused DCs are not endowed with intrinsic anticancer activity, but act as anticancer vaccines to elicit a tumor-targeting immune response.

Peptide- and DNA-based vaccines. Antigen-presenting cells (APCs), including DCs are also targeted by peptide- and DNA-based anticancer vaccines (Aranda *et al.* 2013, Vacchelli *et al.* 2013a). For the peptide-based vaccine, full-length recombinant TAAs or peptides thereof are administered to cancer patients together with one or more adjuvants, which potently promote DC maturation (Bijker *et al.* 2007, Aruga 2013). The concept behind this approach is that resident DCs and other APCs acquire the ability to present the TAA-derived epitopes while maturing, thus priming a robust TAA-specific T cell-based immune response (Galluzzi *et al.* 2014b). DNA-based cancer vaccines rely on TAA-coding constructs, either naked or delivered by viral particles, non-pathogenic bacteria or yeast cells (Rice *et al.* 2008). In the presence of adequate adjuvants, a DC vaccine prompts resident DCs or other APCs to prime a TAA-targeting immune response (Rice *et al.* 2008). Both, peptide- and DNA-based vaccines have been associated with clinical activity in patients affected by various kinds of cancers (Aranda *et al.* 2013, Senovilla *et al.* 2013).

Immunostimulatory cytokines. The cytokine family regulates numerous biological functions via autocrine, paracrine or endocrine circuits (Tato and Cua 2008a, Tato and Cua 2008b, Tato and Cua 2008c, Tato and Cua 2008d). Various attempts have been made to harness the biological potency of specific cytokines to elicit novel or reinvigorate pre-existent tumor-targeting immune responses (Chen and Balachandran 2013, Vacchelli *et al.* 2013b). Immunostimulatory cytokines are generally employed as adjuvants for other anticancer (immuno-) therapeutics, either as recombinant proteins or encoded by expression vectors (Galluzzi *et al.* 2014b).

Immunomodulatory mAbs. These monoclonal antibodies operate by interacting with, and thus altering the function of, soluble or cellular components of the immune system (Melero *et al.* 2007, Melero *et al.* 2013). They are designed to generate a new or reinforce an existing anticancer immune response. So far, this has been achieved by four general

strategies: (1) the inhibition of immunosuppressive receptors expressed on either activated T lymphocytes, such as cytotoxic T lymphocyte-associated protein-4 (CTLA-4) (Walker and Sansom 2011) and programmed cell death-1 (PD-1) (Munir *et al.* 2013), or NK cells, like various members of the killer cell immunoglobulin-like receptor family (KIR) (Raulet and Guerra 2009); (2) the inhibition of the principal ligands of these receptors, such as the PD-1 ligand-1 CD274, also known as PD-L1 (Munir *et al.* 2013); (3) the activation of co-stimulatory receptors expressed on the surface of immune effector cells, such as tumor necrosis factor receptor superfamily member 4 (TNFRSF4, best known as OX40) (Croft 2009); and (4) the neutralization of immunosuppressive factors released in the tumor microenvironment, such as transforming growth factor β1 (TGFβ1) (Pickup *et al.* 2013). The first of these approaches is referred to as 'checkpoint blockade' and has been shown to induce a robust and durable response in cohorts of patients with a variety of solid tumors (Zitvogel and Kroemer 2012, Mavilio and Lugli 2013, Munir *et al.* 2013).

Inhibitors of immunosuppressive metabolism. The enzyme IDO1 for example, inhibits both, the innate and adaptive immune response by depleting immune effector cells of tryptophan (Trp), resulting in irresponsiveness to an immunological challenge (Munn and Mellor 2004). It has been shown, that both 1-methlytryptophan (an inhibitor of IDO1) and genetic interventions targeting IDO1 mediate anticancer effects while eliciting new or reinvigorate existent anticancer immune responses (Muller *et al.* 2005, Manuel and Diamond 2013).

PRR agonists. Pattern recognition receptors (PRRs) are evolutionary conserved germ-line-encoded proteins involved in the recognition of danger signals (Palm and Medzhitov 2009, Galluzzi *et al.* 2014b). These receptors are key elements of the innate immune system. PRRs include the receptor families of Toll-like receptors (TLRs) (Kawai and Akira 2011), nucleotide-binding oligomerization domain containing (NOD)-like receptors (NLRs) (Saleh 2011), C-type lectin receptors (CLRs) and RIG-I-like receptors (RLRs). The latter receptor family is described in more detail below (see page 19). PRRs can detect a variety of danger signals including exogenous 'microbiome-associated molecular patterns' (MAMPs) like bacterial lipopolysaccharide (LPS) or 'pathogen-associated molecular pattern' (PAMPs like viral RNA or DNA, as well as endogenous 'damage-associated molecular patterns' (DAMPs), like HMGB1 or aberrantly located mitochondrial DNA (Galluzzi *et al.* 2014b). The activation of various PRRs ignites a signal transduction cascade with potent pro-inflammatory outcomes, including the activation of NF-κB (Fitzgerald *et al.* 2001), and the secretion of immunostimulatory cytokines, like type I interferons (IFNs) and TNFα (Honda *et al.* 2005a). Additionally, PRR signaling favors the

maturation of DCs as well as the activation of macrophages and NK cells (Brennan *et al.* 2012).

Immunogenic cell death inducers. Immunogenic cell death (ICD) is described in detail below (see chapter 1.4.). In brief, some chemotherapeutics and few other cell death inducers can kill malignant cells while stimulating them to release specific DAMPs. Such DAMPs can activate APCs, which then acquire the ability to elicit a cancer-specific immune response, in mice often associated with the development of immunological memory (Cirone *et al.* 2012a, Kroemer *et al.* 2013).

1.4. Immunogenic cell death

In a healthy adult human body, every second several million cells succumb to programmed cell death mechanisms and are effectively removed without eliciting a local or systemic inflammatory response. This homeostatic cell death, often occurring through apoptosis, is known to be tolerogenic (promoting tolerance to self-antigens) or silent (exerting no impact on the immune system) (Kroemer *et al.* 2013). However, according to recent cancer research, specific stimuli can promote an immunogenic variant of this regulated cell death (Green *et al.* 2009). These agents induce a cell death modality that does stimulate an immune response against dead-cell antigens, in particular when they derive from cancer cells. The resulting immunogenic cell death, or short ICD, relies on the ability of these specific stimuli to kill cancer cells while provoking changes in the composition of their cell surface as well as the spatiotemporally coordinated release of soluble immunogenic mediators (DAMPs) (Kepp *et al.* 2014).

In-depth biochemical analyses revealed, that ICD is obligatory triggered by two types of stress: endoplasmic reticulum (ER) stress and autophagy (Kroemer *et al.* 2013). As a result of chemotherapy-induced ER stress and autophagy, DAMPs are released. These released DAMPs exert robust immunostimulatory effects upon binding to PRRs expressed by immune cells and subsequently stimulate the subsequent presentation of tumor antigens to T cells (Green *et al.* 2009, Kroemer *et al.* 2013). So far, three DAMPs have been attributed a key role in the immunogenic potential of ICD inducers: the pre-apoptotic exposure of calreticulin (CRT) and other endoplasmic reticulum proteins on the cell surface, the secretion of ATP during the blebbing phase of apoptosis, and the cell death-associated release of the non-histone chromatin protein high mobility group box 1 (HMGB1) (Apetoh *et al.* 2007, Obeid *et al.* 2007b, Kroemer *et al.* 2013). Ecto-CRT binds to CD91 on immature DCs and operates as a potent engulfment signal, thus allowing DCs to engulf debris of stressed and dying tumor cells (Gardai *et al.* 2005). Extracellular ATP

released from dying cells is one of the most prominent chemotactic 'find-me' signals for the recruitment of macrophage and DC precursors, presumably upon its binding to P2RX7 receptors, which are widely expressed on cells of the myeloid lineage (Elliott *et al.* 2009, Kroemer *et al.* 2013). HMGB1 binds to TLR4, thus activating the release of pro-inflammatory cytokines by monocytes/ macrophages (Sims *et al.* 2010). Additionally, HMGB1 augments the expression of pro-IL-1β in TLR4-expressing DCs and avoids the lysosomal degradation of engulfed tumor antigens, which is a major prerequisite for efficient cross-presentation of tumor-associated peptide antigens (Apetoh *et al.* 2007).

Taken together, the spatiotemporally coordinated release of DAMPs promotes and facilitates the recruitment of APCs into the tumor bed (by ATP), the engulfment of dead cell-derived tumor antigens (stimulated by CRT), as well as their capacity to prime an adaptive immune response by promoting an optimal antigen presentation (stimulated by HMGB1). Together, these processes result in a potent IL-1β- and IL-17-dependent immune response involving both, $_{\gamma\delta}$T cells as well as cytotoxic T lymphocytes (Kroemer *et al.* 2013). Cytotoxic T lymphocytes not only mediate direct anticancer effects, mostly by secreting interferon γ (IFN-γ) and via the granzyme-perforin pathway, but also underlie the establishment of a protective immunological memory (Kepp *et al.* 2014). This means, that a patient's dying cancer cells, if killed in this appropriate way, can operate as a '*in situ* vaccine' that stimulates a tumor-specific immune response, which in turn might control and maybe even eradicate residual cancer (stem) cells (**Figure 1**) (Galluzzi *et al.* 2012b, Kroemer *et al.* 2013).

As an operational definition of ICD inducers, G. Kroemer, L. Galluzzi, O. Kepp and L. Zitvogel considered that the induced ICD must meet two criteria (Kroemer *et al.* 2013). (a) Cancer cells succumbing to ICD *in vitr*o and administered in the absence of any adjuvant must elicit an immune response that protects mice against a subsequent challenge with live tumor cells of the same type (Green *et al.* 2009). (b) ICD occurring *in vivo* must drive a local immune response featuring the recruitment of innate and adaptive immune effector cells into the tumor bed and hence result in the inhibition of tumor growth via mechanisms that depend (at least in part) on the immune system.

Chemotherapeutic agents differ in their capacity to induce immunogenic cell death. Back in 2005, the chemotherapeutic agent Doxorubicin was unexpectedly shown to be the first chemotherapeutic agent to induce immunogenic cell death. Researchers found that murine colorectal carcinoma CT26 cells and murine fibrosarcoma MCA205 cells exposed to a lethal dose of doxorubicin *in vitro* can be used to vaccinate syngeneic mice against a

subsequent challenge with living cells of the same type (Casares *et al.* 2005).

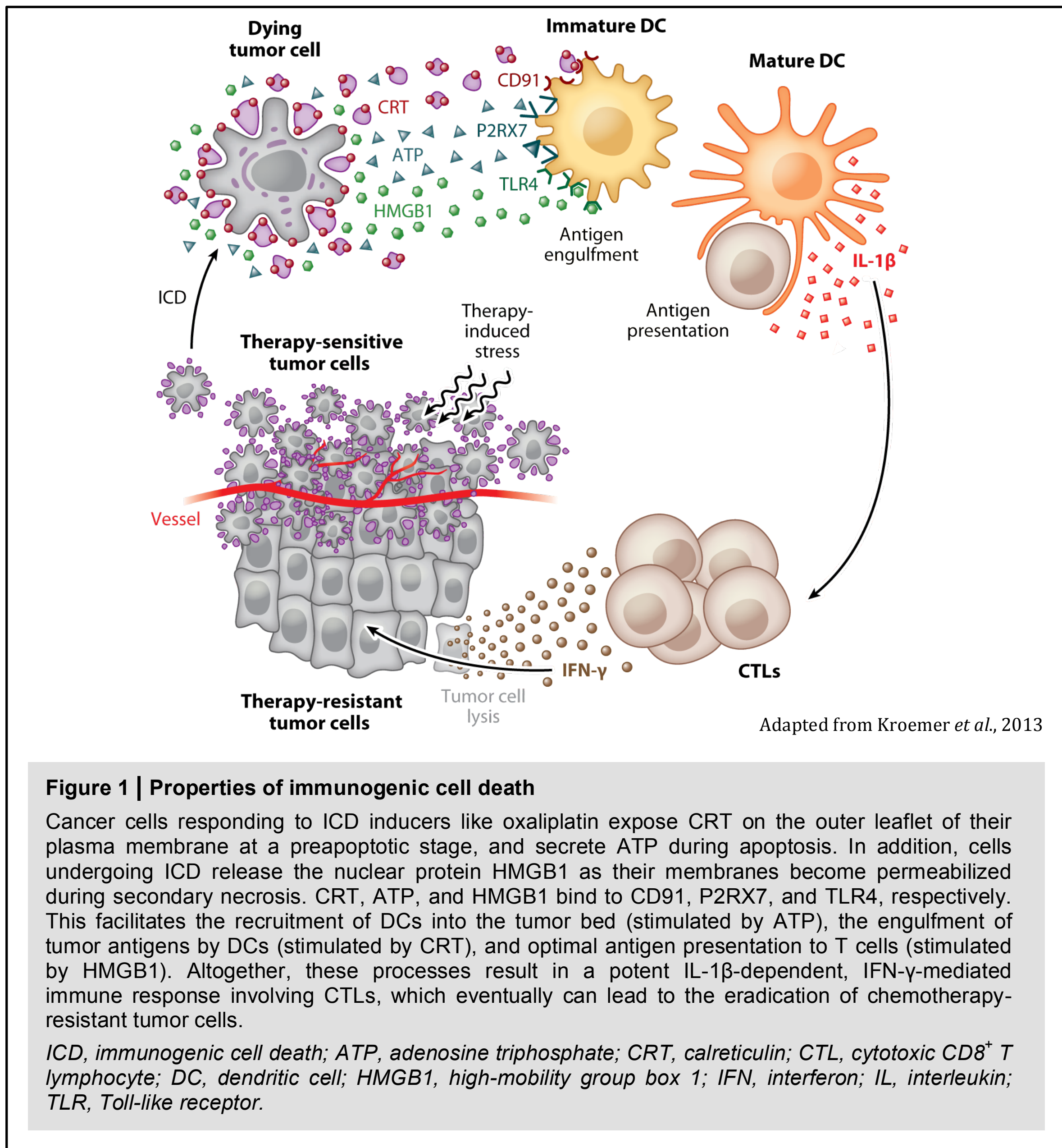

Figure 1 | Properties of immunogenic cell death

Cancer cells responding to ICD inducers like oxaliplatin expose CRT on the outer leaflet of their plasma membrane at a preapoptotic stage, and secrete ATP during apoptosis. In addition, cells undergoing ICD release the nuclear protein HMGB1 as their membranes become permeabilized during secondary necrosis. CRT, ATP, and HMGB1 bind to CD91, P2RX7, and TLR4, respectively. This facilitates the recruitment of DCs into the tumor bed (stimulated by ATP), the engulfment of tumor antigens by DCs (stimulated by CRT), and optimal antigen presentation to T cells (stimulated by HMGB1). Altogether, these processes result in a potent IL-1β-dependent, IFN-γ-mediated immune response involving CTLs, which eventually can lead to the eradication of chemotherapy-resistant tumor cells.

ICD, immunogenic cell death; ATP, adenosine triphosphate; CRT, calreticulin; CTL, cytotoxic CD8$^+$ T lymphocyte; DC, dendritic cell; HMGB1, high-mobility group box 1; IFN, interferon; IL, interleukin; TLR, Toll-like receptor.

The unsuspected ability of doxorubicin (an anthracycline employed for the treatment of various carcinomas) to trigger ICD is shared by a relatively restricted set of lethal triggers (Mattarollo *et al.* 2011, Bracci *et al.* 2014, Vacchelli *et al.* 2014, Pol *et al.* 2015). As of today, these agents employed in the clinic include mitoxantrone and epirubicin (2 other anthracyclines) (Obeid *et al.* 2007b, Fucikova *et al.* 2011), bleomycin, a glycopeptide antibiotic endowed with antineoplastic properties (Bugaut *et al.* 2013), oxaliplatin, a platinum derivative generally employed against colorectal carcinoma (Tesniere *et al.* 2010), cyclophosphamide, an alkylating agent approved for the treatment of neoplastic and autoimmune conditions (Schiavoni *et al.* 2011), bortezomib, a proteasome inhibitor approved for use in subjects with multiple myeloma and mantle cell lymphoma (Cirone *et*

al. 2012b), and idarubicin, an anthracycline currently applied for acute myeloid leukemia (Obeid *et al.* 2007a). Specific forms of irradiation as well as photodynamic therapy, both of which are routinely employed for the treatment of various neoplasms, have also been shown to trigger *bona fide* ICD (Korbelik *et al.* 2011, Galluzzi *et al.* 2013a). Finally, several other so far experimental agents are intrinsically endowed with the capacity to initiate ICD, including, but not limited to, some oncolytic viruses (Donnelly *et al.* 2013, Pol *et al.* 2016), the microtubular inhibitor patupilone (Senovilla *et al.* 2012), and elevated hydrostatic pressures (Fucikova *et al.* 2014).

All of the abovementioned ICD inducers employ the exposure and release of the hallmark DAMPs CRT, ATP and HMGB1. However, it was only recently shown that (anthracycline-induced) ICD also requires type-I interferon (type I IFN) signaling in malignant cells (Sistigu *et al.* 2014). Cancer cells responded to various anthracyclines by activating a TLR3-elicited signal transduction cascade resulting in type I IFN release, autocrine/paracrine type I IFN signaling, and chemokine (C–X–C motif) ligand 10 (CXCL10) secretion; two phenomena that underlie their vaccinating potential. In contrast to their wild-type counterparts, $Tlr3^{-/-}$ and $Ifnar1^{-/-}$ murine cancer cells exposed to anthracyclines fail to vaccinate syngeneic mice against a subsequent injection of living cells of the same type. Additionally, the inability of $Tlr3^{-/-}$ cells to undergo ICD is corrected by the co-administration of recombinant type I IFNs or recombinant CXCL10 (Sistigu *et al.* 2014). Various synthetic TLR3 agonists are available and some of them, including polyinosinic:polycytidylic acid (polyI:C) and its clinical grade analog polyI:polyC12U, also known as rintatolimod and Ampligen™, have been extensively tested as immunostimulants in cancer patients (Aranda *et al.* 2014, Bezu *et al.* 2015). Preclinical studies have shown that poly(I:C) binds not only to TLR3 but also to the cytosolic receptor MDA5. This elicits two distinct antitumor pathways shown for example in prostate cancer cells: one mediated by the TLR3/Src/STAT1 axis, leading to apoptosis, and the other one mediated by MDA5/IRF3, leading to immunoadjuvant IFN-β expression (Palchetti *et al.* 2015). MDA5 is a PRR that belongs to the family of RIG-I-like receptors (RLRs). These cytosolic receptors function as innate nucleic acid sensors, which were recently shown to also induce immunogenic cell death in pancreatic cancer cells (Duewell *et al.* 2014).

1.5. Innate nucleic acid sensors and the activation of adaptive immunity

In vertebrates, two complementary systems have evolved to detect and fight invading microbial pathogens: the innate and adaptive branch of the immune system. As the first line of host defense, the cells of the innate immune system are equipped with a limited number of germ line-encoded receptors called pattern recognition receptors (PRRs) to

detect and respond to the presence of pathogens (Wu and Chen 2014). PRRs recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) that are essential for the life cycle of the pathogen (Kawai and Akira 2011). Many PAMPs, such as lipopolysaccharides, peptidoglycans, and flagellin, are found exclusively in microbes but not in the host, allowing the host to distinguish non-self from self through PRRs.

One apparent exception is the detection of pathogen-derived nucleic acids. In principle, all microbes use DNA and/or RNA as genetic information carriers in their life cycle and could therefore potentially activate host nucleic acid sensors. Innate immune sensors for nucleic acids can be generally divided into two groups on the basis of their subcellular localization and expression pattern. The first group includes several members of the Toll-like receptor (TLR) family that function mostly in immune cells, such as DCs, macrophages, and B cells. These TLRs reside in the endosome and monitor the lumen of endosomes and lysosomes to detect various forms of nucleic acids derived from bacteria or viruses. The second group of receptors, which has not been fully characterized until recently, detects nucleic acids in the cytoplasm of almost all nucleated cell types. These cytosolic nucleic acid sensors include proteins that detect cytoplasmic DNA (cGAS / STING) as well as the RIG-I-like receptor (RLR) family members that detect pathogen-derived RNA in the cytosol (Wu and Chen 2014). Both types of nucleic acid sensors activate a signaling cascade that culminates in the production of type I IFN, as well as proinflammatory cytokines such as tumor necrosis factor α (TNFα) and IL-1β.

Toll-like receptors sense nucleic acids in the endosome. TLRs are type I single transmembrane proteins with ectodomains containing leucine-rich repeats for PAMP recognition and a cytosolic Toll/IL-1 receptor (TIR) domain responsible for signal transduction to downstream adaptors including TRIF and MyD88 (Kawai and Akira 2010). There are 10 and 13 identified TLRs in human and mouse, respectively, of which five are involved in nucleic acid sensing: TLR3, TLR7, TLR8, TLR9, and TLR13. These receptors monitor the endo-/lysosomal lumen for pathogen-derived nucleic acids and function via two signaling pathways: TLR3 activates TRIF, whereas TLR7, TLR8, TLR9, and TLR13 activate MyD88. Both adaptor proteins eventually lead to the activation of NF-κB, whereby Interferon regulatory factor (IRF)3 is facilitated by the TRIF pathway and IRF7 by the MyD88 pathway (Kawai and Akira 2010).

Sensing of nucleic acids in the cytosol. The ligand-binding domain of the nucleic acid-sensing TLRs faces into the lumen of the endosome of immune cells, rendering these

TLRs 'blind' to microbes that have successfully invaded and replicate within the host cytoplasm (Goubau *et al.* 2013). Thus, a cell-intrinsic, cytoplasmic surveillance system must exist to defend against microbes that invade both immune and non-immune cells: the cytosolic nucleic acid-sensing pathways.

1.5.1. Recognition of cytosolic RNA

Infection by RNA viruses such as influenza and hepatitis C virus (HCV) triggers a strong production of type I IFNs. The major PAMP from these viruses that induces type I IFNs is viral RNA, which is delivered to the cytosol in the form of incoming viral genomes or is generated through cytosolic viral RNA replication. In 2004, retinoic acid inducible gene-I (RIG-I) and its homolog melanoma differentiation associated gene 5 (MDA5) were identified as the sensors of cytosolic viral RNA (Yoneyama *et al.* 2004). These landmark discoveries led to an intense research that has yielded a much better understanding of the signaling pathways and mechanisms by which viral RNA in the cytosol triggers the production of type I IFNs (Wu and Chen 2014). Initially identified as a DExD/H-box-containing protein required for intracellular double-stranded (ds)RNA-induced type I IFN production (Yoneyama *et al.* 2004), RIG-I is the eponymous member of the RIG-I-like receptor (RLR) family of cytosolic RNA sensors. The other two members are MDA5 and LGP2 (laboratory of genetics and physiology 2) (Yoneyama *et al.* 2005). All three RLRs share highly conserved domain structures, including a central DExD/H-box helicase core and a C-terminal domain (CTD) that confers part of the ligand specificity (Kolakofsky *et al.* 2012). The N-termini of RIG-I and MDA5, but not that of LGP2, harbor two tandemly linked caspase activation and recruitment domains (CARDs) mediate signaling to downstream adaptor proteins (Jiang *et al.* 2012, Goubau *et al.* 2013). Due to the lack of a CARD domain, LGP2 was considered an inhibitory factor for the RLR signaling pathway (Yoneyama *et al.* 2005). However, later studies suggested that LGP2 may play a positive role in MDA5 signaling (Satoh *et al.* 2010).

RIG-I and MDA5 show distinct preference for RNA ligands (Wu and Chen 2014). Both RIG-I and MDA5 respond to the synthetic dsRNA analog poly(I:C), but with different length restrictions. Long fragments (>4 kb) are preferentially detected by MDA5, whereas shorter fragments generated by enzyme digestion (around 300 base pairs) are recognized by RIG-I (2008). Using different approaches, several groups discovered that the most important molecular feature of RNA for RIG-I recognition is a free triphosphate group at the 5' end (Hornung *et al.* 2006, Pichlmair *et al.* 2006), a molecular motif that is hidden by a cap structure in host mRNAs prior to their shuttling into the cytosol. This serves as a mechanism for self/non-self discrimination by RIG-I. Two follow-up studies further showed

that 5'-triphosphate RNA (or short 3pRNA) requires additional base-paired structures to activate RIG-I (Schlee *et al.* 2009, Schmidt *et al.* 2009). This kind of 5'-triphosphate-bearing panhandle structure is predicted to be present in the genomes of some negative-strand single-stranded (ss) RNA viruses such as influenza A virus (flu) and may function as the *in vivo* ligand for RIG-I (Wu and Chen 2014). Recently, it has been shown that RIG-I is also activated by 5'-diphosphate RNA indicating that the minimal determinant for RIG-I recognition is a base-paired RNA with 5′pp (Goubau *et al.* 2014). RIG-I may also be indirectly activated by cytosolic viral and bacterial dsDNA, as pathogen AT-rich dsDNA can be transcribed by RNA polymerase III to generate dsRNA with 5'-triphosphate ends (Ablasser *et al.* 2009). Compared to RIG-I ligands, the ligand for MDA5 is less well characterized. MDA5 is thought to function as a sensor for long dsRNA, as it could be activated by long poly(I:C) (Kato *et al.* 2008, Lassig and Hopfner 2016). Furthermore, RIG-I and MDA5 may be activated by self RNAs that are cleaved by RNase L (Malathi *et al.* 2007). Very little is known about the nature of RNAs that serve as ligands for LGP2.

In the cytosol, RIG-I and MDA5 signal via mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, IPS1 or VISA) and IRF3/ IRF7 to induce type I IFNs following RNA recognition (Wu and Chen 2014). MAVS comprises a CARD domain at its N-terminus, followed by a proline-rich domain, and a short hydrophobic stretch at its C-terminus, which localizes MAVS to the outer mitochondrial membrane. The homotypic interaction of the CARD domain of MAVS and the CARD domains of RIG-I and MDA5 is essential for signaling, as is the mitochondrial localization of MAVS. Interactions between RLRs and MAVS eventually lead to the activation of the transcription factors IRF1, IRF3, IRF7 and NF-κB, resulting in the production of type I IFNs and pro-inflammatory cytokines (Barbalat *et al.* 2011, Loo and Gale 2011). In addition, RIG-I can interact with the adaptor protein ASC, resulting in inflammasome-dependent caspase 1 activation and the subsequent production of active IL-1β (**Figure 2**) (Poeck *et al.* 2010). Interestingly, only in malignant cells, RIG-I activation leads to the induction of intrinsic apoptosis through Puma and Noxa while non-malignant cells can up-regulate anti-apoptotic Protein BclXL which rescues them from cell death (Poeck *et al.* 2008b).

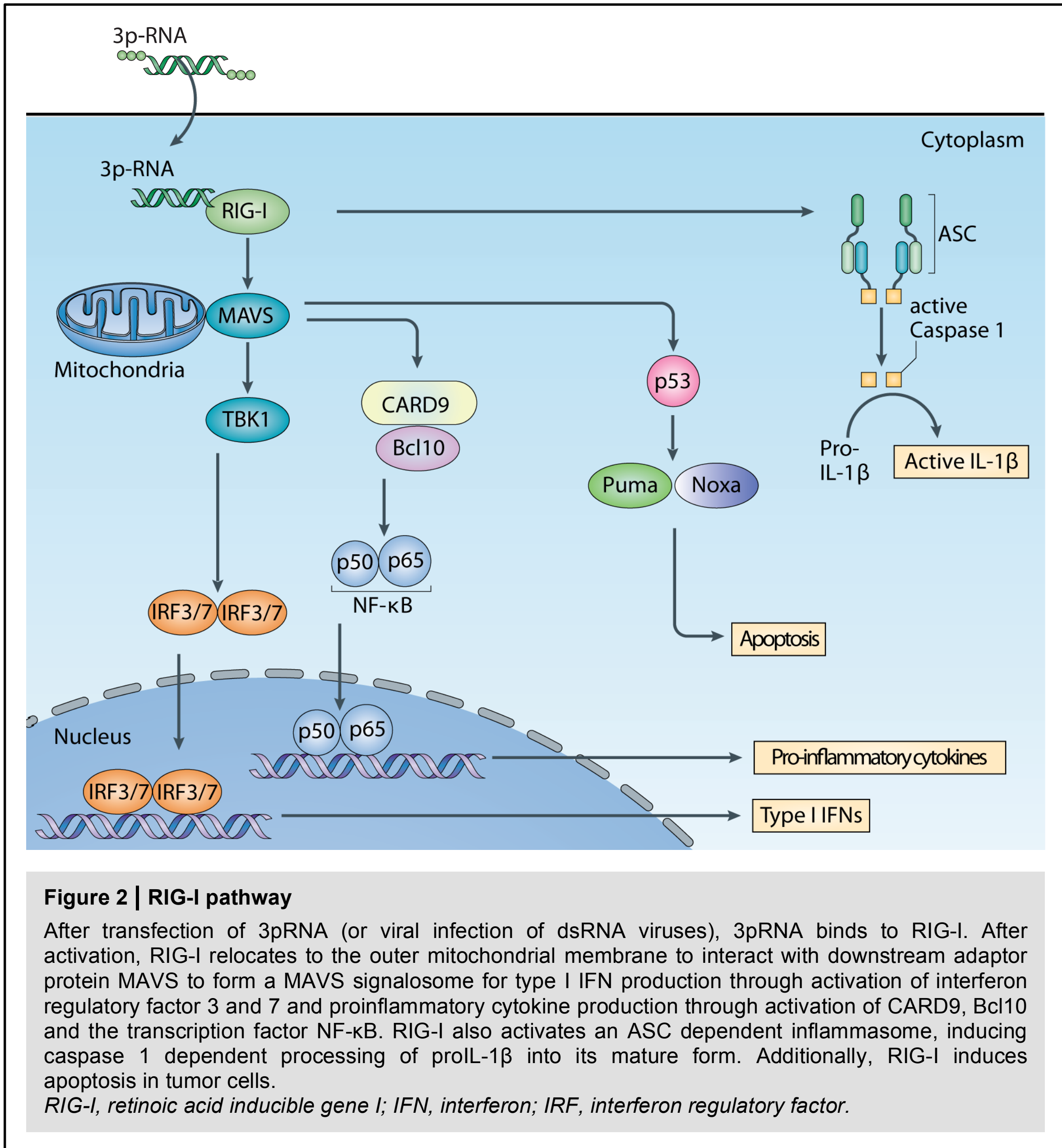

Figure 2 | RIG-I pathway

After transfection of 3pRNA (or viral infection of dsRNA viruses), 3pRNA binds to RIG-I. After activation, RIG-I relocates to the outer mitochondrial membrane to interact with downstream adaptor protein MAVS to form a MAVS signalosome for type I IFN production through activation of interferon regulatory factor 3 and 7 and proinflammatory cytokine production through activation of CARD9, Bcl10 and the transcription factor NF-κB. RIG-I also activates an ASC dependent inflammasome, inducing caspase 1 dependent processing of proIL-1β into its mature form. Additionally, RIG-I induces apoptosis in tumor cells.

RIG-I, retinoic acid inducible gene I; IFN, interferon; IRF, interferon regulatory factor.

1.5.2. Recognition of cytosolic DNA

DNA has been known to potentially trigger immune responses for more than a century (Wu and Chen 2014). However, the underlying mechanism has only been identified very recently. The research in the past few years has led to significant progress toward understanding the mechanism of cytosolic DNA sensing and signaling, culminating in the recent discoveries of the cytosolic DNA sensor, adaptor, and a new second messenger that mediates signal transduction in this pathway.

Several groups identified the protein stimulator of interferon genes (STING, also known as MITA, MPYS, ERIS, and TMEM173) as a crucial signaling adaptor for type I IFN induction following stimulation with cytosolic dsDNA (Ishikawa and Barber 2008, Zhong *et al.* 2008, Sun *et al.* 2009). The STING protein is predominantly localized at the endoplasmic reticulum and has been shown to contain four trans-membrane helices (TM1–TM4), a large cytosolic domain and a folded soluble domain that mediates dimerization (Ishikawa and Barber 2008);(Ouyang *et al.* 2012). Once activated by cytosolic DNA signaling, STING relocalizes from the endoplasmic reticulum to the Golgi complex and assembles into punctate structures that contain the kinase TBK1 (Ishikawa *et al.* 2009). This process stimulates TBK1, resulting in the phosphorylation of IRF3. It was shown that STING deficiency in various cell types, such as mouse embryonic fibroblasts, macrophages, and DCs, abolished IFN-β production after dsDNA stimulation or DNA virus infection (Ishikawa *et al.* 2009). Additionally, STING-deficient mice are highly susceptible to lethal infection with herpes simplex virus 1 (HSV-1), demonstrating that STING is essential for host defense against DNA virus *in vivo* (Ishikawa *et al.* 2009).

Recently, an important study identified STING as a direct sensor for cyclic dinucleotides (CDNs). CDNs are bacterial second messengers including cyclic (3'–5') diguanylate (c-di-GMP) and cyclic (3'–5') diadenylate (c-di-AMP), with a regulatory role in several processes, such as biofilm formation, virulence, and DNA integrity surveillance (Burdette *et al.* 2011). Although it is clear that STING is a direct sensor of CDNs and an important adaptor for type I IFN induction by cytosolic DNA, the identity of the cytosolic DNA sensor was resolved only recently. Using biochemical purification and quantitative mass spectrometry, cyclic GMP-AMP synthase (cGAS) was identified as a sensor for cytosolic DNA (Sun *et al.* 2013, Wu *et al.* 2013). When activated by DNA through direct binding, cGAS catalyzes the production of cyclic 2'-3' GMP-AMP (cGAMP) from ATP and GTP. cGAMP in turn functions as an endogenous second messenger to activate STING (Wu *et al.* 2013). This discovery unified the understanding of the role of STING in cytosolic response to DNA and CDNs (Wu and Chen 2014).

The past decade has witnessed tremendous progress in the understanding of innate recognition of pathogen-derived nucleic acids and their central role in initiating host defense responses. Research in the field of cytosolic nucleic acid sensing has been very prosperous, as represented by the discovery of the RLR-MAVS pathway for cytoplasmic RNA sensing and the cGAS-cGAMP-STING pathway for cytosolic DNA recognition. However, future work will need to uncover new components and regulatory mechanisms of these pathways. It will also be important to gain insights into some yet unresolved

questions, such as the mechanism of MDA5 activation, the role of STING translocation, and the mechanisms by which the RIG-I and cGAS signaling pathways are turned off (Wu and Chen 2014).

1.5.3. Roles of cytosolic nucleic acid sensing pathways in cancer

Type-I interferons have been shown to play a critical role in the immunosurveillance of cancer cells (Dunn *et al.* 2006, Fuertes *et al.* 2011). In addition to their direct cytotoxic effects on cancer cells, type I IFN can also promote the maturation and antigen presentation capacity of DCs, thus linking innate to adaptive immune responses. However, the molecular mechanisms how the innate immune system detects tumor cells for the production of interferons and the role of cytosolic nucleic acid receptors in the context is still a matter of debate.

Self DNA from dying tumor cells has been shown to be an important danger signal that triggers the cGAS–STING pathway to induce type I IFN and thus induces the initiation of spontaneous T cell responses against particular tumor models (Woo *et al.* 2014, Corrales and Gajewski 2016). After the transplantation of immunogenic tumors into syngeneic mice, tumors were found to grow more rapidly in STING-deficient mice (Woo *et al.* 2014). Spontaneous priming of $CD8^+$ T cells against tumor-associated antigens is defective in STING-deficient mice but not in those lacking TLRs, MyD88 or MAVS. STING is also required for the antitumor effects of radiation, but MyD88 and TRIF are not (Deng *et al.* 2014). Intratumoral injection of CDNs leads to substantial inhibition of tumor growth and prolonged survival in mice (Corrales *et al.* 2015, Demaria *et al.* 2015). The combination of CDNs with irradiation or checkpoint inhibitors (antibodies to e.g. PD-1 and PD-L1, described above) produces synergistic antitumor effects, which indicates that the cGAS–STING pathway is important for the sensing of tumors by the innate immune system and has a critical role in intrinsic antitumor immunity (Chen *et al.* 2016).

The RIG-I signaling pathway has also been shown to mediate innate immune responses against tumors, characterized by the production of type I IFN in immune, non-immune, and tumor cells (Yoneyama and Fujita 2009, Chen *et al.* 2013). Furthermore, tumor cells were found to be highly susceptible to a poorly defined, specialized form of RIG-I-induced cell death that is mediated via the activation of the BH3-only proteins Puma and Noxa and thus partly resembles the intrinsic mitochondrial apoptosis pathway. Importantly, nonmalignant cells are rescued from RIG-I-induced apoptosis by their ability to upregulate antiapoptotic Bcl-xL (Besch *et al.* 2009). Thus, 3pRNA as the ligand for RIG-I is a direct tumoricidal agent. When used *in vivo* against melanoma, 3pRNA-mediated RIG-I

activation results in the production of high amounts of IFN-α, IFN-β, and IL-12 and mediates effective antitumor immunity in melanoma and other cancer types (Poeck *et al.* 2008b). RIG-I activation can be combined with gene silencing using siRNAs (what is then called bifunctional small interfering RNAs, e.g., RIG-I activation combined with Bcl2 silencing) which has shown efficacy in a melanoma lung metastasis model (Poeck *et al.* 2008b). Another successfully used bifunctional siRNA is a 3pRNA incorporating a silencing sequence for TGF-β. In murine pancreatic cancer this 3pRNA has been shown to lead to the recruitment of $CD8^+$ cytotoxic T cells into the tumor site along with a reduction of myeloid-derived suppressor cell (MDSC) tumor infiltration, known to install a highly immunosuppressive tumor environment (Ellermeier *et al.* 2013).

A more recent study showed that RLR-mediated cell death of pancreatic cancer cells can have proinflammatory potential, fulfilling the criteria for immunogenic cell death (Duewell *et al.* 2014). This study demonstrated that RIG-I and MDA5 activation leads to the release of proinflammatory cytokines and type I IFN, as well as the induction of tumor cell death. Exposure of $CD8\alpha^+$ DCs to RLR-activated apoptotic tumor cells led to DC maturation, efficient antigen uptake and cross-presentation of tumor-associated antigen to cytotoxic T lymphocytes. In addition, vaccination with MDA5-activated apoptotic tumor cells protected mice from subsequent challenge with viable tumor cells, indicative of the emergence of an adaptive antitumor immune response *in vivo*. Thus, RLR-mediated cell death fulfills the typical criteria defining immunogenic cell death linking innate and adaptive immunity (Duewell *et al.* 2014).

Type I interferons as well as IL-12 induced by cytosolic nucleic acid receptors are crucial cytokines for the activation of naïve, non-activated T cells and $CD8^+$ memory T cells (Raue *et al.* 2013) as well as sustaining effective antitumor $CD8^+$ T cell immunity (Schurich *et al.* 2013). In the case of tumors, not only do compounds such as the RIG-I agonist 3pRNA not only revert MDSC immune suppression, induce tumor cell apoptosis, and liberate tumor-associated antigens for immunity, but also enrich for memory $CD8^+$ T cells residing in or near the tumor site in order to activate their IL-12-IFN-programmed effector functions. Moreover, the $CD8^+$ T cells that are newly raised against the liberated tumor antigens will encounter a tumor cytokine environment that is fully supportive for effector functions and memory development (van den Boorn and Hartmann 2013). Thus, targeting innate nucleic acid sensors expressed in tumor cells, such as RIG-I, allows a switch in the tumor microenvironment from immunosuppressive stroma into a tissue milieu supporting $CD8^+$ T cell infiltration and effector function (van den Boorn and Hartmann).

Regarding type I IFN-mediated tumor immunity, a still unresolved question is by what mechanism DNA released from dying tumor cells can gain access to the cytosol of DCs under physiologic conditions *in vivo*. There are several possible candidate mechanisms of nucleic acid transfer. One of them is the uptake of autophagosomes, which have been shown to contain DNA (Oka *et al.* 2012). Another way is through the release of so-called extracellular vesicles such as exosomes.

1.6. Extracellular Vesicles

The cytoplasm of eukaryotic cells contains several well-described compartments (e.g. the Golgi complex, lysosomes, mitochondria, the endoplasmic reticulum), each performing specific and, in some cases, overlapping functions. Transport of materials (metabolites, lipids, carbohydrates, and proteins) between organelles is mediated by vesicles of about 60–100 nm diameter, moving in a densely populated microenvironment (Balch *et al.* 1984). Likewise, vesicles are used for intercellular transportation and communication, to receive and send signals and bio-information from cell to cell. These vesicles have distinct origins, different sizes and are formed by a variety of mechanisms. It has long been known that apoptotic cells shed large vesicles of about 500–2000 nm in diameter, so called apoptotic bodies, that can be taken up by phagocytic or antigen-presenting cells of the immune system (Kerr *et al.* 1972). However, also healthy cells release vesicles, which are able to mediate intercellular communication (Harding *et al.* 1984). These extracellular vesicles (EVs) are spherical particles enclosed by a phospholipid bilayer and are released by both, eukaryotic and prokaryotic cells (Alenquer and Amorim 2015). The diameter of these EVs characteristically ranges from 30 nm to 1000 nm and thus can be up to three orders of magnitude smaller than the smallest cells.

Although there are many different types of cell-derived vesicles (microvesicles, ectosomes, oncosomes, exosomes, etc.), there is currently no consensus about the nomenclature, partly due to detection difficulties of small-scaled vesicles, the multidisciplinary research field, and different ways of classification (Alenquer and Amorim 2015). For example, cell-derived vesicles have often been named after the cells or tissues, which they originate from, e.g. dexosomes (dendritic cell-derived exosomes). However, such names do not provide a information for classification with regard to the type of vesicles involved. Due to their different way of formation and release, recent reviews classified vesicles into two major different types of extracellular vesicles: ectosomes and exosomes.

1.6.1. Biogenesis of exosomes and ectosomes

Exosomes and ectosomes are assembled by similar mechanisms. Their components are sorted in their membrane of origin into small domains that undergo budding and then pinching off (Cocucci and Meldolesi 2015). In the final step, the two classes of EVs are released to the extracellular space by different processes.

Exosomes are 30–100 nm in diameter, thus comprising the smallest subgroup of extracellular vesicles with respect to vesicle size. Exosomes are initially formed as intraluminal vesicles (ILVs) inside so-called multivesicular bodies (MVBs), and are released upon fusion of the MVB with the plasma membrane (Harding *et al.* 1984, Alenquer and Amorim 2015). Multivesicular bodies or late endosomes are components of the endocytic pathway, which is an intricate web of connected sub-compartments with distinct cell localization, lipid and protein composition, and pH. Cells internalize extracellular components by endocytosis concomitantly with membrane proteins and lipids (Grant and Donaldson 2009). The internalized material (by inward budding) is delivered to early endosomes and sorted to at least three possible destinations. The internalized material can be sent for degradation through maturation into MVBs and fusion with lysosomes, which are acidic compartments containing hydrolytic enzymes able to digest complex macromolecules (Bainton 1981). Alternatively, cargo can be re-routed for recycling or secretion.

The secretion of exosomes requires maturation of early endosomes into MVBs, with associated formation of ILVs driven by the cytosolic endosomal sorting complex required for transport (ESCRT), and subsequent fusion of MVBs with the outer cell surface. ILVs (30–100 nm in diameter) remain trapped within MVBs for a considerable time, resulting in the delayed release of exosomes into the extracellular space. In some MVBs, the ILVs fuse with each other, creating larger, pleiomorphic structures. In addition, some ILVs undergo back fusion, which is the process of reintegrating their membrane with the limiting MVB membrane and discharging their cargo back into the cell cytoplasm (Bissig and Gruenberg). This process is likely to impact the number of exosomes released into the extracellular space. Exocytic fusion of MVBs with the plasma membrane is required for the release of exosomes. Currently the mechanisms governing this process remain largely unknown. A few small GTPases, such as Rab11 and Rab27 have been shown to be involved in the docking of MVBs to the plasma membrane (Ostrowski *et al.* 2010). Once released, ILVs are referred to as exosomes (Simons and Raposo 2009, Mathivanan *et al.* 2010).

Ectosomes measure 100–350 nm in diameter, and are thus of greater size than exosomes (Cocucci and Meldolesi 2015). Compared with the assembly of ILVs, the initial process that leads to the assembly of ectosomes appears largely different. Ectosomes are assembled by the regulated outward budding of small plasma membrane domains (Shifrin *et al.* 2013, Cocucci and Meldolesi 2015). The accumulation of cargo proteins in the ectosome lumen, which is often larger than that of ILVs, occurs by various mechanisms. Recent studies suggest that at least some ESCRT subunits participate in the assembly and budding of ectosomes (Nabhan *et al.* 2012). In addition to ESCRT, the assembly of ectosome luminal cargo requires the binding of cytoplasmic proteins to the plasma membrane. Their binding is based on their plasma membrane anchors (myristoylation, palmitoylation, and others) and high-order polymerization, which concentrate them into the small plasma membrane domains of ectosome budding (Shen *et al.* 2011). In contrast to exosomes, the release of ectosomes does not require exocytosis. Upon pinching off, these larger vesicles are released into the extracellular space at a high rate. In cells with high ectosome turnover (dendritic cells, macrophages, microglia), the release is visible within a few seconds after stimulation by ATP through the P2X7 receptor and proceeds for several minutes, accompanied by retraction and the rearrangement of the cell's shape (Baroni *et al.* 2007, Turola *et al.* 2012, Shifrin *et al.* 2013, Cocucci and Meldolesi 2015).

Most cells can probably release both, PM- and endosome-derived vesicles (Stoorvogel *et al.* 2002). Thus, although in many studies EVs were termed exosomes and were assumed to correspond to ILVs of MVBs, clear evidence for their origin is often lacking, because diverse complementary methods are required, and such evidence is often difficult to obtain. For example, fusion of MVBs with the cell surface is a very dynamic process that is often difficult to catch using electron microscopy.

1.6.2. Uptake of extracellular vesicles

A number of target cells including tumor cells and immune cells can interact with circulating exosomes (Stoorvogel *et al.* 2002). EVs communicate with these cells by either releasing their cargo by directly contacting target cells over short or long distances (Bissig and Gruenberg , Cocucci and Meldolesi 2015). Upon release, some EVs lose their integrity and release their contents into the extracellular space. The released segregated agents, including IL-1β, tissue factors, and various growth factors such as TGFβ, bind their receptors in adjacent cells and activate rapid responses (Dubyak 2012, Cocucci and Meldolesi 2015). The EVs that maintain their structure over longer periods may undergo long-distance trafficking in major fluids, including blood, lymph, and cerebrospinal fluid, impacting the fate of distant target cells (Choi *et al.* 2015). On release, EVs do not interact

with just any cell, but show a preference for certain target cells. For example, vesicles shed from platelets interact with macrophages and endothelial cells but not with neutrophils (Lösche *et al.* 2004). Information about the various proteins exposed on the surface of exosomes, ectosomes, and their target cells that account for these heterogeneous responses remains largely unknown. This is currently an area of active investigation as is the precise mechanism of exosome internalization by recipient cells. However, direct plasma membrane fusion and receptor-mediated endocytosis have been proposed (Thery *et al.* 2009). In terms of endocytosis, it has been shown that exosomes and small ectosomes fit within clathrin vesicles, whereas larger ectosomes employ other processes of internalization such as macropinocytosis and phagocytosis (Tian *et al.* 2014).

1.6.3. Isolation of extracellular vesicles

It is generally accepted that the currently available EV purification methods do not allow for complete separation of exosomes and ectosomes (Raposo and Stoorvogel 2013). Hence, multiple studies have analyzed a mix of both these EV subtypes and have not worked with a pure homogeneous population. As a result of the mentioned challenges in the isolation process of EV subtypes to homogeneity, molecular profiling and functional characterization studies pertaining to EV subtypes are limited. Among the EV subtypes, exosomes have been characterized by multiple groups while ectosomes remain understudied (Keerthikumar *et al.* 2015). Besides their accepted mode of biogenesis and size, very little is known about the buoyant density and the protein composition of ectosomes.

The most commonly used protocol to isolate EVs is based on differential centrifugation, whereby the smallest vesicles (including exosomes) are sedimented by ultracentrifugation at 100.000 x g. Before ultracentrifugation, larger vesicles are eliminated by successive centrifugation steps at increasing speeds to sediment these vesicles without artificially creating small vesicles from large ones by ad hoc high-speed centrifugation (Colombo *et al.* 2014). There are several variations to this method, in any case, ultracentrifugation allows only for the enrichment of subtypes of EVs or exosomes and is not a proper purification procedure, since different vesicles of similar size as well as protein aggregates can cosediment at the same speed. To separate vesicles from protein aggregates a sucrose gradient can be performed, where protein aggregates sediment through sucrose, whereas lipid-containing vesicles float upward to a position of equilibrium buoyant density (Escola *et al.* 1998). Only recently, commercial available methods have been developed, which are both fast and simple and do not require ultracentrifugation. These kits either use

polymer-based precipitation or immune-captureing by antibody-coated beads. The former should precipitate a wider, and the latter conversely a more restricted, range of vesicles when compared to precipitation by ultracentrifugation (Colombo *et al.* 2014).

1.6.4. Visualization of exosomes (and other EVs)

Exosomes can directly be observed using transmission electron microscopy (TEM). Hereby, exosomes appear cup-shaped which is an artifact of the fixation/contrast procedure that induces shrinking of subcellular structures (Colombo *et al.* 2014). In contrast, exosomes observed by cryo-EM show a circular shape (Raposo and Stoorvogel 2013). Another way to measure the size distribution and concentration of EVs is provided by a device, which allows for 'Nanoparticle Tracking Analysis' (NTA) (Dragovic *et al.* 2011). NTA tracks the movement of laser-illuminated individual particles under Brownian motion and subsequently calculates their diameter using statistical methods. This method provides a fast and simple way of analyzing large numbers of particles simultaneously, and at relatively low costs as compared to sophisticated electron microscopes. However, the method does not differentiate a vesicle from a protein aggregate of similar size. So far, most studies have used these two techniques to analyze exosomes and have showed particles of approximately 100 (+/-20) nm in diameter.

1.6.5. Biochemical features of EVs – protein content and nucleic acids

There is a lack of reliable protein markers that allow for the discrimination between exosomes and ectosomes. This impedes the field of EV research, as specific functions could not be attributed to defined populations of EVs. The protein content of EVs derived from different sources has been analyzed by SDS-PAGE followed by protein staining, immunoblotting, or proteomic analysis. The results of these studies on mammalian exosomes were assembled in a database named Exocarta (Mathivanan *et al.* 2012). Exocarta was recently incorporated into a more comprehensive database called Vesiclepedia (Kalra *et al.* 2012), which additionally includes data on nucleic acids and lipids from exosomes but also from other types of EVs and is continuously updated with the help of the scientific community researching EVs.

Highly purified EVs should be devoid of contaminants, such as serum proteins and protein components of intracellular compartments (e.g., the endoplasmic reticulum or mitochondria), that are not in contact with EVs (Raposo and Stoorvogel 2013). EVs contain a specific subset of cellular proteins, some of which depend on the cell type that secretes them, whereas others are found abundantly in most EVs regardless of their originating cell. The latter include proteins form the endosome, the PM, and the cytosol,

whereas proteins from the nucleus, mitochondria, endoplasmic reticulum and the Golgi apparatus are mostly absent. Commonly used protein markers for exosomes are CD63, CD81, Tsg101, Alix, Flotilin and Hsc70 (Bobrie *et al.* 2012). However, recent research showed, that these 'classical' exosome markers are also detected in other types of EVs (Kowal *et al.* 2016). Furthermore, flotillin-1 (FLOT1), the constitutive heat-shock protein HSC70, class II MHC molecules and actin, as well as class I MHC molecules and HSP70 were found not to be exosome-specific markers (Kowal *et al.* 2016). Thus careful deliberation and judicious interpretation is required in the characterization of EVs in the context of protein markers.

A kind of second revolution of EVs happened in 2006/7 when J. Ratajczak and J. Lötvall could independently show that extracellular vesicles carry both miRNA and functional mRNA. This cargo was found to be delivered to other recipient cells and to be functional in this new location (Ratajczak *et al.* 2006, Valadi *et al.* 2007). These two studies on the exosomal transport of micro (mi)RNA and messenger (m)RNA extended the idea that exosomes are 'carriers of information' that can be transferred from one cell type to another – a concept that has enormous implications for human health. Most subsequent studies on genetic material in EVs describe small RNAs, including mRNAs, and miRNAs of various sizes, with low or undetectable levels of 18S and 28S RNA (ribosomal RNA) in purified EVs (Crescitelli *et al.* 2013a). It was also shown, that mRNAs and miRNAs are not randomly secreted in exosomes, but a selection of specific sequences of mRNA and miRNA for extracellular export has been suggested (Batagov *et al.* 2011, Montecalvo *et al.* 2012). Next-generation sequencing techniques have been used to characterize all small RNAs present in mixed EVs released by e.g. DC/T cell co-cultures (Nolte-'t Hoen *et al.* 2012). Several small noncoding RNAs were thus found including vault-RNA (polymerase III transcripts found in vaults), Y-RNA (small non-coding RNA found in Ro60 ribonucleo particles), and selected tRNA (transfer RNA). Many of these exosomal RNAs were enriched relative to cellular RNAs, indicating a specific release of certain species via EVs.

In addition to RNA, genomic DNA has been detected within EVs. However, the incorporation of genomic DNA in EVs is not entirely understood yet. Genomic DNA is found in EVs derived by different tumor cell lines such as glioblastoma, colon and gastric cancers (Lee *et al.* 2011, Iraci *et al.* 2016). In tumor cells, the majority of DNA associated with exosomes is double-stranded and represents a significant fraction of the genomic DNA of the cell of origin, including mutated and amplified oncogenes as well as transposable elements (Thakur *et al.* 2014). Several lines of research are now exploring

the possibility of using exosomal DNA as a circulating biomarker to identify the mutations within parental tumor cells (Thakur *et al.* 2014). Besides cancer, genomic DNA was found to be present in vesicles released from non-malignant cells; for example in prostasomes, the most abundant class of EVs found in seminal fluid and originating from the epithelial cells in the prostate (Olsson and Ronquist 1990, Tannetta *et al.* 2014). Despite the abundance of evidence showing the presence of DNA inside EVs and exosomes, its function still remains unclear; additional studies are needed in order to elucidate its role in physiological and pathological processes.

1.6.6. EV function in immunity and cancer therapy

In the late 1990s, two publications by G. Raposo and L. Zitvogel shed, for the first time, light on the immunogenic function of exosomes (Raposo *et al.* 1996, Zitvogel *et al.* 1998). In 1996, Epstein-Barr virus (EBV)-transformed B cell lines were shown to secrete exosomes enriched in major histocompatibility complex (MHC) class II molecules (Raposo *et al.* 1996). Importantly, in both human and murine models, exosomes released by B lymphocytes have the capacity to stimulate specific $CD4^+$ T cell clones *in vitro*, suggesting a possible role of exosomes as vehicles for MHC class II–peptide complexes between cells of the immune system. In 1998, L. Zitvogel took these findings one step further by demonstrating the release of exosomes by human DCs and the ability of tumor peptide–pulsed DC-derived exosomes to suppress the growth of established tumors *in vivo*. These potential roles as mediators of immune responses, and the suggestion of a possible use of exosomes as immunotherapeutic agents, has led to a plethora of articles related to the immune function of exosomes *in vitro* and *in vivo* (Bobrie *et al.* 2011). For instance, it was shown that subsequent to the uptake of tumor-derived exosomes, DCs induce potent $CD8^+$ T cell-dependent antitumor effects on syngeneic and allogeneic established mouse tumors, identifying exosomes as a novel source of tumor-rejection antigens for T cell cross-priming (Wolfers *et al.* 2001). Another more recent study revealed that EVs isolated from RIG-I-stimulated tumor cells can activate natural killer T cells in the recipient host (Daßler-Plenker *et al.* 2016)

Tumor-derived EVs were also shown to play a role in tumorigenesis and angiogenesis and thus the manipulation of the tumor microenvironment leading to cancer development and metastasis (Kalluri 2016). A recent study used a Cre-LoxP system to directly identify tumor cells that take up EVs *in vivo* revealed that EVs released by malignant tumor cells were taken up by less malignant tumor cells located within either the same or distant tumors. Through this process, the less malignant tumor cells that received EVs from malignant tumor cells gained migratory and metastatic behavior, providing experimental

evidence for a key role of EV crosstalk in tumor progression and metastasis (Zomer *et al.* 2015). In general, exosome production by tumor cells has been portrayed as a cancer-promoting mechanism, enabling tumors to modulate and suppress antitumor immune responses. A specific “immunosuppressive content” within tumor-derived exosomes likely dictates the evasion of immunosurveillance (Pitt *et al.* 2016). With both immunostimulatory and immunoinhibitory characteristics, the biological role of tumor-derived exosomes has been a source of much debate.

Regardless, due to the research over the past few years it is now accepted that the bioactive cargoes of EVs do have innate therapeutic potential in diverse areas, not only as cell-free cancer immunotherapy (Viaud *et al.* 2010), but also in regenerative medicine (Biancone *et al.* 2012). In light of their intercellular communication capability, naturally occurring EVs are also being exploited for the delivery of exogenous therapeutic reagents, such as small molecule anti-inflammatory drugs (e.g., curcumin to activated monocytes (Sun *et al.* 2010) and macromolecular drugs such as siRNA (Alvarez-Erviti *et al.* 2011). Since EVs/exosomes resemble their parental cells in terms of their antigenicity (proteins/lipids), profile of miRNAs and mRNAs, but also cytokines and growth factors, the use of exosomes in medicine holds promise. First, EVs overcome many of the limitations of cell-based therapeutics related to safety, manufacturing and availability. Secondly, studies on the bioavailability of EVs have demonstrated that they are capable of crossing the blood-brain barrier, which classically acts as a major hurdle in the administration of therapeutic agents targeting the central nervous system (Zhuang *et al.* 2011). Finally, exosomes have limited immunogenicity as compared to live cells, protect their cargoes from degradation, are highly stable in serum and blood, and can efficiently deliver their cargo to target cells with reduced off-target effects due to a natural tendency to target specificity (Kooijmans *et al.* 2012, Iraci *et al.* 2016).

The preclinical studies led to several phase I clinical trials that investigated the safety of autologous DC-derived exosomes (Dex) pulsed with tumor peptides for the immunization of patients with stage III/IV malignant melanoma or non-small cell lung cancer (Escudier *et al.* 2005, Morse *et al.* 2005). These studies proved for the first time the feasibility of large-scale production of clinically applicable exosomes and showed their safe use in humans. Exosomes were found to lead to enhanced NK cell effector functions in some melanoma patients. However, only minimal increase in peptide-specific T cell activity was observed. Since the beginning of these early phase I Dex trials, new ways to improve Dex as an immunotherapy have been established with hope to enhance the limited Dex-induced T cell responses. An important innovation has been the use of exosomes derived from

TLR4- or IFN-γ-maturated DCs, following preclinical observations that such Dex induce more potent T cell stimulation compared to Dex derived from immature DCs (Segura *et al.* 2005a, Viaud *et al.* 2011). Based on these findings, a 'second generation' of Dex immunotherapy was developed, which showed that IFN-γ-Dex is a very well tolerated immunotherapy and can boost NKp30-dependent NK cell functions. Disappointingly, such enhanced Dex also failed to induce significant antigen-specific T cell responses (Besse *et al.* 2016).

1.7. Introducing the tumor model: B16.OVA murine melanoma

Experimental tumor models are a critical pre-clinical step for the development and evaluation of immunotherapy regimens for cancer. In this dissertation, B16.OVA a genetically modified form of the murine melanoma cell line B16.F10 was applied. B16.F10 is a widely utilized, poorly immunogenic and aggressively growing melanoma cell line. B16 cells were engineered to express different model antigens like ovalbumin (OVA; B16.OVA) or SIYRYYGL (SIY; B16.SIY), which can be recognized by $CD8^+$ T cells in the context of cross-presentation on MHC-I (Kedl *et al.* 2001, Spiotto *et al.* 2002). These modified cell lines allow tracking the development of tumor antigen-specific T cell responses by using MHC-tetramers incorporating these artificial antigens. The artificial expression of such a model antigen renders B16 cells slightly more immunogenic. Due to spontaneous antitumor immunity, the here applied B16.OVA tumors grow less aggressively (but are not rejected) in syngeneic C57BL/6 hosts compared with the parental B16.F10 cell line.

2. Objectives

Despite vast investigations on new approaches to enhance efficacy of antitumor immunotherapies, they still face a variety of challenges including inter-individual physiologic differences and non-responding tumor entities. Immunogenic cell death is one novel and promising approach to further improve the establishment of antitumor immune responses. Hereby, RIG-I is a promising candidate for inducing therapeutic immunogenic tumor cell death.

This project aims to dissect the process of RIG-I-induced immunogenic cell death and to broaden our understanding of how antitumor immune responses are initiated. If the immunogenic factors released by tumor cells, dying under the specific regimen of immunogenic cell death, are elucidated, therapies might profit in terms of efficiency and accuracy. Therefore, this study aims to clarify,

- which factors mediate the immunogenicity of RIG-I-induced tumor cell death?
- what role do tumor cell-derived extracellular vesicles play in RIG-I-mediated ICD
- which signaling pathways within host antigen-presenting cells are involved in subsequent priming of tumor-specific cytotoxic T cells?

3. Materials and methods

3.1. Materials

3.1.1. Reagents

Product description	Company	Branch
1x TBM Substrate Solution	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
2-Mercaptoethanol	Sigma-Aldrich, division of Merck	Munich, Germany
2-Propanol	Carl Roth GmbH	Karlsruhe, Germany
7-AAD Viability Staining Solution	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Albumin Factor V (BSA)	Carl Roth GmbH	Karlsruhe, Germany
Apex™ - heat-labile alkaline phosphatase	Epicentre	Madison, WI, USA
DNase I	Thermo Fischer Scientific	Waltham, MA, USA
DNAzol	Thermo Fischer Scientific	Waltham, MA, USA
Ethanol absolute	Merck Millipore	Darmstadt, Germany
ISD, synthesized	Sigma-Aldrich, division of Merck	Munich, Germany
Milkpowder	Fluka Analytical	Munich, Germany
Mouse Interferon alpha, mammalian rec. protein	PBL Assay Science	Piscataway, NJ, USA
Mouse Interferon beta, mammalian rec. protein	PBL Assay Science	Piscataway, NJ, USA
Pierce™ ECL Western Blotting Substrate	Thermo Fischer Scientific	Waltham, MA, USA
RIPA Lysis and extraction buffer	Pierce, division of Thermo Fischer Scientific	Waltham, MA, USA
Ponceau S solution	Sigma-Aldrich, division of Merck	Munich, Germany
RBC lysis buffer, G-DEXTM II	Intron Biotechnology	Seongnam, South Korea
RNase A	Thermo Fischer Scientific	Waltham, MA, USA
Sodium Chloride	Carl Roth GmbH	Karlsruhe, Germany
Trizma®	Sigma-Aldrich, division of Merck	Munich, Germany
Tween® 20	Sigma-Aldrich, division of Merck	Munich, Germany

3.1.2. Materials

Product description	Company	Branch
BD Plastipak™ syringes Sub-Q 26 G or 27 G	BD Biosciences, division of Becton, Dickinson and Company	New Jersey, USA
Counting chamber	Brand	Wertheim, Germany
Falcon™ Cell Strainers 70 µm or 100 µm	Thermo Fischer Scientific	Waltham, MA, USA
Falcon™ Polystyrene Microplate 12-well	Thermo Fischer Scientific	Waltham, MA, USA
Millex-GV, 0,22 µm syringe filter	Merck Millipore	Darmstadt, Germany
Mini Quick Spin Column	Roche	Mannheim, Germany
Tissue Culture Flasks 25-150 cm^2	TPP	Trasadingen, Switzerland
Tissue Culture Plates (96-well U or Flat bottom)	TPP	Trasadingen, Switzerland
Tubes for flow cytometry (5 mL)	Sarstedt	Nümbrecht, Germany
SafeSeal Micro Tubes (0,5 - 2 mL)	Sarstedt	Nümbrecht, Germany
S-Monovette®, K3 EDTA	Sarstedt	Nümbrecht, Germany
Ultracentrifuge tubes	Seton Scientific Corp.	Petaluma, CA, USA

3.1.3. Cell lines

Cell line	Source	Origin
B16.F10	MSKCC - NY (van den Brink Lab)	melanoma
B16.OVA	MSKCC - NY (van den Brink Lab)	melanoma
IRF3/$7^{-/-}$	Lab intern	melanoma
RIG-$I^{-/-}$	Lab intern	melanoma

3.1.4. CRISPR/Cas9 target sequences

Gene	Target sequence
RIG-I	GGCTGATGAGGATGATGGAGCGG
IRF3	GCATGGAAACCCCGAAACCG
IRF7	CTACGACCGAAATGCTTCCA

3.1.5. Cell culture materials

Product description	Company	Branch
2-Mercaptoethanol (50 mM)	Thermo Fischer Scientific	Waltham, MA, USA
Brefeldin A Solution (1,000X)	Biolegend	San Diego, CA, USA
Dimethyl-Sulfoxid (DMSO)	Sigma-Aldrich, division of Merck	Munich, Germany
FBS Good Forte (VLE)	PAN Biotech	Aidenbach, Germany
Fetal Bovine Serum (FBS)	Capricorn scientific	Ebsdorfergrund, Germany
Gibco® DMEM [+] 4,5 g/ L D-Glucose, L-Glutamine	Thermo Fischer Scientific	Waltham, MA, USA
Gibco® L-Glutamine (200 mM)	Thermo Fischer Scientific	Waltham, MA, USA
Gibco® Pen Strep (10.000U/ mL)	Thermo Fischer Scientific	Waltham, MA, USA
Gibco® RPMI 1640 Medium	Thermo Fischer Scientific	Waltham, MA, USA
Gibco® Trypan Blue Stain 0.4%	Thermo Fischer Scientific	Waltham, MA, USA
Gibco® Trypsin-EDTA (0.05%), phenol red	Thermo Fischer Scientific	Waltham, MA, USA
Ionomycin, Calcium Salt, *Streptomyces conglobatus*	Sigma-Aldrich, division of Merck	Munich, Germany
Lipofectamine® 2000 Transfection Reagent	Thermo Fischer Scientific	Waltham, MA, USA
Phosphate buffered saline (PBS)	Sigma-Aldrich, division of Merck	Munich, Germany
PMA (Phorbol 12-myristate 13-acetate)	Sigma-Aldrich, division of Merck	Munich, Germany
VLE-RPMI 1640 (very low endotoxin) liquid medium	Biochrom GmbH	Berlin, Germany

3.1.6. Kits

Product description	Company	Branch
Annexin V Apoptosis Detection Kit PE	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
ATP assay Kit	Abcam	Cambridge, UK
Fixable Viability Dye eFluor® 506	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Foxp3/ Transcription Factor Staining Buffer Set (Fix/Perm Solution and Permbuffer)	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
HMBG1 ELISA	IBL, division of Tecan	Männedorf, Switzerland
MEGAschortscript™ T7 Transcription Kit	Ambion, division of Thermo Fischer Scientific	Waltham, MA, USA
Mouse IFN gamma ELISA Ready-SET-Go!®	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Mouse IL-6 ELISA Ready-SET-Go!®	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Mouse IL-12/IL-23 total p40 ELISA Ready-SET-Go!®	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Mouse TNF alpha ELISA Ready-SET-Go!®	eBioscience, division of Thermo Fischer Scientific	Waltham, MA, USA
Pierce™ BCA Protein Assay Kit	Thermo Fischer Scientific	Waltham, MA, USA
Pierce™ ECL Western Blotting Substrate	Thermo Fischer Scientific	Waltham, MA, USA
Total Exosome Isolation Reagent (from cell culture media)	Invitrogen, division of Thermo Fischer Scientific	Waltham, MA, USA
Total Exosome RNA Protein Isolation Kit	Invitrogen, division of Thermo Fischer Scientific	Waltham, MA, USA

3.1.7. Antibodies

	Product description	Working dil.	Company	Branch
ELISA	Coating: Rat anti-mouse Interferon alpha (Mab) (clone RMMA-1)	5 µg / mL	PBL Assay Science	Piscataway, NJ, USA
	Coating: Rat anti-mouse Interferon beta (Mab) (clone RMMB-1)	5 µg / mL	PBL Assay Science	Piscataway, NJ, USA
	Detection: Anti-mouse Interferon alpha, rabbit serum, (PAb)	620 ng/ mL, 1:500	PBL Assay Science	Piscataway, NJ, USA
	Detection: Rabbit Pab against mouse Interferon beta	620 ng/ mL, 1:500	PBL Assay Science	Piscataway, NJ, USA
	Analysis: Goat anti-rabbit IgG HRP (Pab)	1:10.000	Abcam	Cambridge, UK
Flow cytometry	Anti-Calreticulin, rabbit polyclonal (ab2907)	1:400	Abcam	Cambridge, UK
	Anti-CD3 FITC (clone 17A2)	1:400	Biolegend	Waltham, MA, USA
	Anti-CD4 PacBlue (clone GK1.5)	1:400	Biolegend	Waltham, MA, USA
	Anti-CD8 APC or PerCP (clone 53-6.7)	1:400	Biolegend	Waltham, MA, USA
	Anti-CD86 PE/Cy7 (clone GL-1)	1:400	eBioscience, div. of Thermo	Waltham, MA, USA
	Anti-IFNγ PE (clone XMG1.2)	1:200	eBioscience, div. Thermo	Waltham, MA, USA
	Anti-mouse H-2Kb bound to SIINFEKL Antibody (clone 25-D1.16)	1:400	eBioscience, div. of Thermo	Waltham, MA, USA
	Anti-Rabbit IgG, APC (#4414)	1:1000	Cell Signaling Technology	Danvers, MA, USA
	iTAgTM MHC-Tetramer H-2kb OVA, SIINFEKL-PE	1:300	Biozol	Eching, Germany

In vivo blocking	InVivoMab anti-mouseCD8Alpha (clone-2.43)	100 µg 1st 50 µg	Bio X Cell	West Lebanon, NH, USA
	InVivoMab anti-mouse IFNAR-1 (clone-MAR1-5A3)	400 µg	Bio X Cell	West Lebanon, NH, USA
	InVivoMab anti-mouse NK1.1 (clone-PK136)	100 µg 1st 50 µg ff	Bio X Cell	West Lebanon, NH, USA
Western blot	Anti-Alix, mouse (ab117600)	1:1000	Abcam	Cambridge, UK
	Anti-β-Aktin, rabbit (#4970)	1:1000	Cell Signaling Technology	Danvers, MA, USA
	Anti-β-Tubulin, rabbit (#2125)	1:1000	Cell Signaling Technology	Danvers, MA, USA
	Anti-Calnexin, rabbit (ab22595)	1:1000	Abcam	Cambridge, UK
	Anti-CD63, rabbit (H-193)	1:500	Santa Cruz Biotechnology	Heidelberg, Germany
	Anti-CD81, rabbit (SAB3500454)	1:1000	Sigma-Aldrich, div. of Merck	Munich, Germany
	Anti-Cytochrom C,mouse (7H8.2C12)	1:500	Pharmingen, div. of BD	New Jersey, USA
	Anti-Flotilin 1, rabbit (ab41927)	1:1000	Abcam	Cambridge, UK
	Anti-gp100, rabbit (ab137078)	1:1000	Abcam	Cambridge, UK
	Anti-HSP70, mouse (ab2787)	1:1000	Abcam	Cambridge, UK
	Anti-IRF7, rabbit (ab109255)	1:1000	Abcam	Cambridge, UK
	Anti-OVA, mouse (#A6075)	1:1000	Sigma-Aldrich, div. of Merck	Munich, Germany
	Anti-Rab27a, rabbit (orb136214)	1:500	Biorbyt	Cambridge, UK
	Anti-RIG-I, mouse (clone SS1A)	1:1000	Enzo	Lörrach, Germany
	Secondary anti mouse IgG, HRP-linked (#7076)	1:2000	Cell Signaling Technology	Danvers, MA, USA
	Secondary anti rabbit IgG, HRP-linked (#7074)	1:2000	Cell Signaling Technology	Danvers, MA, USA

3.1.8. Devices

Product description	Company	Branch
Biological safety cabinet, HERASafe KS	ThermoFischer Scientific	Waltham, USA
Centrifuge 5810R	Eppendorf	Hamburg, Germany
FACSAria III	BD Biosciences, division of Becton, Dickinson and Company	New Jersey, USA
FACSCanto II	BD Biosciences, division of Becton, Dickinson and Company	New Jersey, USA
INTAS	INTAS Science Imaging Instruments GmbH	Göttingen, Germany
Microscope, Axiovert 40C	Zeiss	Aalen, Germany
NanoDrop 1000	ThermoFischer Scientific	Waltham, USA
Nanosight LM 10	Malvern instruments	Malvern, UK
Pipets: Discovery Comfort,variable volumes	HTL Lab Solutions	Warsaw, Poland
Plate reader Sunrise™	Tecan	Männedorf, Switerland
Qubit® Fluorormeter	ThermoFischer Scientific	Waltham, USA
Table top centrifuge 5417R	Eppendorf	Hamburg, Germany
TapeStation System	Agilent	Santa Clara, CA, USA
Thermocycler - PCR machine	Bio-rad	Munich, Germay
Ultracentrifuge Optima™ L-90K	Beckman Coulter	Brea, USA
Vortex-Genie2 vortexer	Scientific Industries	New York, USA

3.1.9. Online database

Product description	Application	Source
murine sgRNA library	murine CRISPR/Cas9 sgRNA sequences	https://www.addgene.org/pooled-library/broadgpp-mouse-knockout-brie/
PubMed	Literature research	http://www.ncbi.nlm.nih.gov/pubmed

3.1.10. Software

Product description	Application	Source
Adobe Illustrator	Image processing	Adobe Systems, Inc., San Jose, USA
ChemoStar Imager	Western blot software	Intas, Göttingen, Germany
EndNote	Literature management	Thomson Reuters, New York City, USA
FACSDiva	Flow cytometry	BD Biosciences, Heidelberg, Germany
FlowJo	Flow cytometry	Tree Star, Inc., Ashland, USA
GraphPad Prism	scientific graphing and biostatistics	GraphPad Software, Inc., La Jolla, USA
Magellan	ELISA software	Tecan, Männedorf, Switzerland
Mendeley	Literature management	Elsevier, Amsterdam, Netherlands
MS Office	Calculations, figures, text	Microsoft Deutschland GmbH, Unterschleißheim, Germany

3.2. Methods

3.2.1. In vitro methods

3.2.1.1. Cell culture

Murine B16.OVA and B16.F10 melanoma cell lines, originating from the C57BL/6 strain, were cultured in dulbecco's modified eagle's medium (Gibco® DMEM [+] 4,5 g/ L D-Glucose, L-Glutamine) supplemented with 10 % (v/v) FBS, 1 % penicillin (100 Units/ mL) and streptomycin (100 µg/ mL), hereafter referred to as B16 medium. The B16.OVA knock-out cell lines RIG-I$^{-/-}$ B16.OVA, IRF3/7$^{-/-}$ B16.OVA, and Rab27a$^{-/-}$ B16.OVA were also cultured in B16 medium. All melanoma cell lines were cultured in tissue-treated culture flasks. The melanoma cells are adherent to the bottom of the culture flask. For splitting the cells, culture medium was aspirated, adherent cells were rinsed with 1x PBS, and Trypsin-EDTA (0,05 %) was added. The Trypsin-EDTA was incubated on the cells for 1-2 min on 37°C for cell detachment. Cells were then harvested by rinsing the flask bottom with fresh B16 medium. Murine primary cells were cultured in Gibco® RPMI 1640 medium supplemented with 10 % FBS, 1 % penicillin (100 units/ mL) and 1 % streptomycin (100 µg/ mL), 1 % L-glutamine (200 mM), 0.1 % β-mercaptoethanol (50 mM), hereafter referred to as complete RPMI (cRPMI). Complete lymph node cells were cultured on tissue-treated 96-well plates. All cells were cultured in a humidified incubator with 5 % CO_2 and 95 % air.

3.2.1.2. *In vitro* transcription of 3pRNA

The double-stranded 5'-triphosphate RNA (3pRNA) for the specific activation of RIG-I was i*n vitro* transcribed of DNA templates following the instructions of the MEGAshortscript™ T7 Transcription Kit (Thermo Fischer). In brief, sense and anti-sense strands of a DNA template were separately transcribed into RNA using the T7 RNA polymerase. Transcribed RNA was isolated using phenol:chlorophorm extraction and alcohol precipitation and was purified using mini Quick Spin Columns (Roche). The template DNA sequence of the sense strand is 5´- TCA AAC AGT CCT CGC ATG CCT ATA GTG AGT CG -3´, the template DNA sequence of the antisense strand template is 5´- GCA TGC GAG GAC TGT TTG ACT ATA GTG AGT CG -3´.

3.2.1.3. Generation of B16.OVA knock-out cell lines by CRISPR/Cas9 gene editing

Prokaryotes have evolved several defense mechanisms to protect themselves from virus infection. One of them, the clustered regularly interspaced short palindromic repeats

(CRISPR) Type II system has been modified and subsequently been used for artificial genome editing (Hsu *et al.* 2014). CRISPR was originally employed to 'knock-out' target genes in various cell types and organisms, but modifications to the Cas9 enzyme have extended the application of CRISPR to selectively activate or repress target genes, for multiplex genome engineering, to purify specific regions of DNA, and even image DNA in live cells using fluorescence microscopy (Brown *et al.* 2017).

CRISPR consists of two components: the guide (g)RNA and the CRISPR-associated endonuclease 9 (Cas9). The gRNA is a short synthetic RNA composed of a 'scaffold' sequence necessary for Cas9 binding, and a user-defined, around 20 nucleotides spanning 'spacer' or 'targeting' sequence which defines the genomic target to be modified. To generate knock-out cell lines the CRISPR/Cas9 complex has to co-express the specific gRNA and the endonuclease Cas9. Thereby the genomic target DNA sequence has to meet two prerequisites: (1) the sequence is unique within the genome; (2) the target is located immediately upstream of a PAM sequence (protospacer adjacent motif) (Sander and Joung 2014). The PAM sequence is absolutely necessary for target binding and the exact PAM sequence depends on the species of Cas9. For example, 5′-NGG-3′ is the PAM sequence for the Cas9 of *Streptococcus pyogenes*. This Cas9 is currently the most commonly used in genome engineering and has also been used for this work. The end result of Cas9-mediated DNA cleavage is a double strand break (DSB) within the target DNA (around 3-4 nucleotides upstream of the PAM sequence) (Gasiunas *et al.* 2012, Nishimasu *et al.* 2014). The resulting DSB is then repaired by one of two general repair pathways, (1) the efficient but error-prone non-homologous end joining (NHEJ) pathway or (2) the less efficient but high-fidelity homology directed repair (HDR) pathway. The NHEJ repair pathway is the most active repair mechanism, which frequently results in small nucleotide insertions or deletions (InDels) at the DSB site leading to in-frame amino acid deletions, insertions, or frameshift mutations and thus to premature stop codons within the open reading frame (ORF) of the target gene. Ideally, the end result is a loss-of-function mutation within the target gene; however, the 'strength' of the knock-out phenotype for a given mutant cell is ultimately determined by the amount of residual gene function (Vartak and Raghavan 2015).

By using this genome editing technique, several knock-out cell lines were generated, originating from the B16.OVA cell line, to study the RIG-I signaling pathway in RIG-I-induced immunogenic cell death.

Vector generation: genome editing was conducted following the protocol of the Feng Zhang lab (Ran *et al.* 2013). See also this protocol for the used materials. In brief, B16.OVA cells were genetically edited using the *Streptococcus pyogenes* nuclease Cas9, together with a single-guide RNA (sgRNA) sequence targeting the desired respective genes (see Table 3.1.4. for sgRNA sequences). The guide RNA was cloned into the bicistronic expression vector pSpCas9(BB)-2A-GFP (pX458, a gift from Feng Zhang; Addgene plasmid #48138). The pX458 vector additionally contains a green fluorescent protein (GFP) marker for later selection of successfully transfected cells.

Transfection of cells and generation of single cell KO-clones: The Vector containing Cas9, GFP, and the sgRNA specific for the desired genomic deletion site was transfected into B16.OVA cells using Lipofectamin® 2000. After 6 hours, the culture medium was removed and cells were provided with fresh medium. 24h after transfection, successfully transfected cells express GFP. The GFP-expressing cells were subsequently sorted. By using flow cytometry, single GFP-expressing cells were sorted each into one well of a flat-bottom culture plate filled with 100 µl of B16 medium. Some of the single cells gave rise to single cell clones after a few weeks of culture.

Validation of knock-out-clones: Using Western blot the genetic deletion of the single cell clones was examined on protein level. The applied Western blot antibodies are listed in table 3.1.7. Validated knock-out clones were subsequently tested on a functional level. In case of RIG-I$^{-/-}$ and IRF3/7$^{-/-}$ clones, the cell clones were transfected with 3pRNA and levels of released type-I interferons α and β (type I IFN) were analyzed after 48 h using ELISA. If the production and release of type I IFN was lost, the respective clone was considered to be a functional KO. The Rab27a$^{-/-}$ clones were tested for the amount of released EVs either measured by BCA according to manufacturers' instructions or by NTA. Additionally, all clones validated for functional knock-out were additionally tested for their proliferation rates. Only clones, which show proliferation rates similar to the wild-type cell line, were selected for later experiments.

3.2.1.4. Melanoma cell killing with 3pRNA or oxaliplatin

B16.OVA cells were harvested from cell culture flasks as described in section 3.1.1 and were re-plated on 12-well non-tissue-treated culture plates in a concentration of 300.000 cells/ mL, 1 mL/ well. The cells were either left untreated or were transfected with 3 µg/ mL 3pRNA or were treated with 30 µg/ mL oxaliplatin, respectively. For transfection, 3pRNA is complexed using Lipofectamine® 2000 transfection reagent (Thermo Fischer) according to the manufacturer's protocol using Gibco® Opti-MEM. The cells were treated

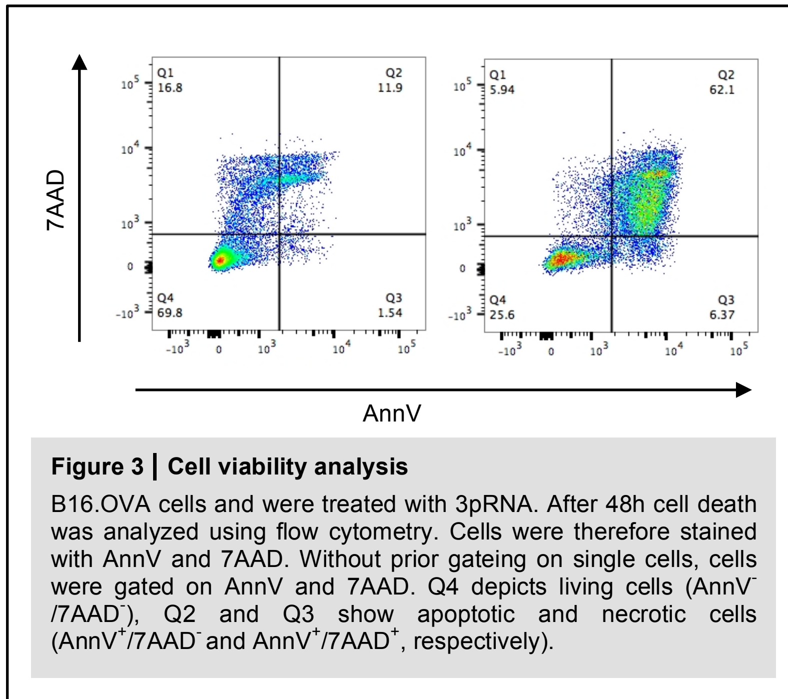

Figure 3 | Cell viability analysis

B16.OVA cells and were treated with 3pRNA. After 48h cell death was analyzed using flow cytometry. Cells were therefore stained with AnnV and 7AAD. Without prior gateing on single cells, cells were gated on AnnV and 7AAD. Q4 depicts living cells (AnnV$^-$/7AAD$^-$), Q2 and Q3 show apoptotic and necrotic cells (AnnV$^+$/7AAD$^-$ and AnnV$^+$/7AAD$^+$, respectively).

with 3pRNA or oxaliplatin for 48 h. B16.OVA cells treated with 3pRNA were named as 3p-B16 cells, B16.OVA cells treated with oxaliplatin were named as Oxa-B16 cells. The described procedure applies similarly for all knock-out cell lines and for B16.F10 cells.

3.2.1.5. B16 cell proliferation assay

Same numbers of WT B16.OVA and knock-out B16.OVA cells were plated in four different wells per cell line at day 0. Each following day, cells of one well per cell line were counted using a cell counting chamber.

3.2.1.6. Analysis of cell death and released ICD hallmark molecules

For dead cell analysis, 3p-B16 or Oxa-B16 cells were resuspended in 1x Annexin V (AnnV)-binding-buffer added with AnnV and 7AAD staining reagent (5 µl reagent/ 100 µl buffer). After 5-10 min incubation on RT in the dark, the cells were analyzed without removing the dyes by flow cytometry using the BD FACSCanto II **(Figure 3)**. Cells positive for either AnnV or positive for both, AnnV and 7AAD, were counted as ‘dead cells’. For the analysis of released ICD hallmark molecules B16.OVA cells were treated with 3pRNA as described above. After 24 h the culture supernatant of RIG-I activated cells was analyzed for the release of ATP using the ATP Assay Kit (abcam) following the manufacturer’s protocol. The release of HMGB1 was assessed via ELISA (IBL). Additionally, the exposure of calreticulin on the outer plasma membrane (ectoCRT) was assessed by flow cytometry.

3.2.1.7. Isolation of extracellular vesicles

FBS itself contains extracellular vesicles from bovine origin. To prevent bovine EV contamination in the isolated murine vesicles, FBS had to be depleted for EVs. Therefore,

FBS was filtered through a 200 nm filter (Merck Millipore) and subsequently centrifuged at 100.000x g for 17 h to pellet bovine EVs. FBS was collected without disturbing the EV Pellet.

For EV isolation from B16.OVA supernatants, the 'Total exosome isolation (from cell media) reagent' (Thermo Fischer), which is based on aprecipitaion reagent was used. The cells were harvested from cell culture flasks and were washed twice with PBS to completely remove any remaining medium. The cells were then re-plated in DMEM containing 10% (v/v) EV-depleted FBS and 1 % penicillin (100 units/ mL) and streptomycin (100 µg/ mL), hereafter referred to as EV-free B16 medium. B16.OVA cells were either treated with 3pRNA (3 µg/ mL), or Oxaliplatin (30 µg/ mL), or Doxorubicin (30 µg/ mL) or left untreated. For treatment, $5x10^5$ cells/ mL were plated on non-tissue-treated 12 well plates. After 48 h, cell supernatant samples were collected and centrifuged at 400x g for 5 min to remove remaining cells (**Figure 4**). The supernatant was collected into a clean sterile Falcon tube. Subsequently, the supernatant was spun at 2,000x g for 30 min to remove cell debris and was transferred to a fresh tube.

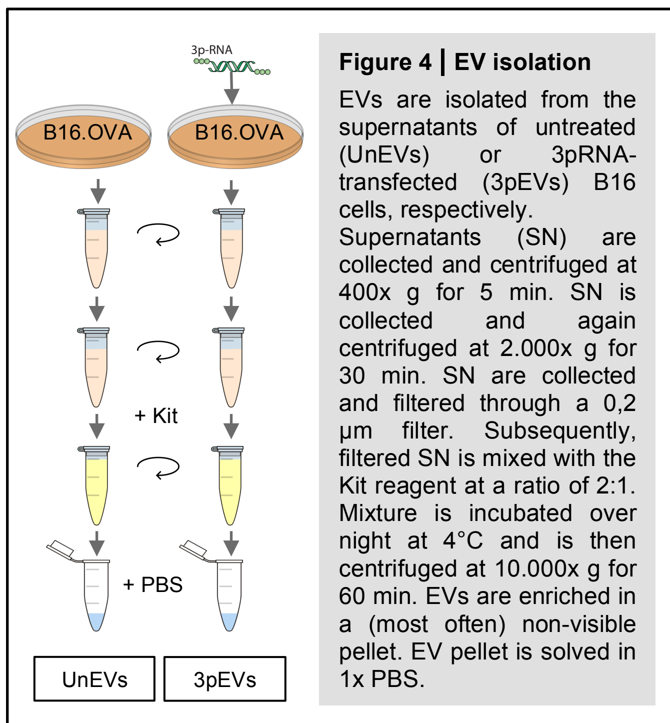

Figure 4 | EV isolation
EVs are isolated from the supernatants of untreated (UnEVs) or 3pRNA-transfected (3pEVs) B16 cells, respectively. Supernatants (SN) are collected and centrifuged at 400x g for 5 min. SN is collected and again centrifuged at 2.000x g for 30 min. SN are collected and filtered through a 0,2 µm filter. Subsequently, filtered SN is mixed with the Kit reagent at a ratio of 2:1. Mixture is incubated over night at 4°C and is then centrifuged at 10.000x g for 60 min. EVs are enriched in a (most often) non-visible pellet. EV pellet is solved in 1x PBS.

The supernatant was additionally filtered through a 200 nm filter to remove any larger protein aggregates. Next, the centrifuged and filtered supernatant was combined with 1/2 volume of 'Total exosome isolation (from cell media) reagent' (Thermo Fischer) and mixed well by vortexing or pipetting up and down until a homogenous solution was formed. Typical cell media volume utilized was 1 mL +0,5 mL Reagent in a 1,5 mL conical Eppendorf tube. The samples were incubated at 4°C overnight and then centrifuged at 4°C at 10,000x g for 60 min. The supernatant was aspirated and discarded, and the (most often invisible) EV pellet was resuspended in PBS. EV pellets were resuspended in a fixed amount of PBS to always obtain the same 'EV stock' concentration. The pellet of 1 mL supernatant was resuspendet in 5 µl PBS. EVs were stored at -80°C. EVs were named after the treatment of the B16 cells. EVs isolated from untreated cells were named 'UnEVs', EVs isolated from 3pRNA-treated, oxaliplatin-treated or doxorubicin-treated cells were named '3pEVs',

'OxaEVs', and 'DoxoEVs', respectively. The described procedure applies as well for all knock-out cell lines and for B16.F10 cells.

3.2.2. In vivo methods

In all *in vivo* experiments, adult mice were at least 6 weeks of age at the onset of experiments. Wild-type (WT) mice of the C57BL/6 strain and were obtained from Janvier Labs with 5 weeks of age and were allowed to rest for at least one week before treatment started. MAVS-deficient (MAVS$^{-/-}$), STING-deficient (STING$^{gt/gt}$ here referred to as STING$^{-/-}$), NLRP3-deficient (NLRP3$^{-/-}$) as well as Asc-deficient (Asc$^{-/-}$) mice were bred under own management. IFNaR1-deficient mice, Itgax-Cre;Ifnar^{fl} mice, and LysM-Cre;Ifnar^{fl} and CD11c-Cre;Ifnar^{fl} mice were a kind gift from Prof. Dr. Ulrich Kalinke, TWINCORE, Hannover. All animals were housed under specific pathogen-free conditions in ventilated cages (Thoren MaxiMiser caging systems or TechniPlast IVC). Studies were conducted in compliance to institutional guidelines and were approved by the local regulatory agency (Regierung von Oberbayern).

3.2.2.1. Immunization with 3p-B16 cells or EVs (boost injection)

3p-B16 cell preparation: Due to the difficulty of counting apoptotic cells, the amount of apoptotic cells injected per mouse was defined before 3pRNA treatment. For each mouse, 1x10^6 B16.OVA cells were treated with 3pRNA. If indicated, the same amount of B16 cells was treated with oxaliplatin. The resulting dying cells detach from the bottom of the well plate. All floating cells (supernatant) and the cells which were easily detached by rinsing the well bottom were collected and centrifuged at 400x g, 4°C for 5 min. 3p-B16 cells (or OxaB16 cells) were washed with PBS. The dead cell pellet of 1x10^6 previously plated B16.OVA cells was resolved in 70 µl of PBS and were carefully injected using a 27G syringe.

EV preparation: The amount of EVs per mouse per injection was adjusted throughout the experiments. One injection consisted of the EVs isolated from the supernatant of 0,5x10^6 B16 cells meaning 5 µl of the 'EV stock'. Therefore, 5 µl of EV stock were diluted in 70 µl 1xPBS and were carefully injected using a 26G syringe.

Immunization (boost injection): For subcutaneous (sc.) immunization, mice were injected sc. in the hock of the right hind leg with either 1x10^6 3p-B16 cells or 5 µl of the exosome stock. The therapy was repeated at day 7 (boost). At day 14, mice were sacrificed and ipsilateral draining lymph nodes (inguinal lymph node - iLN, and popliteal lymph node - pLN) as well as the spleen were isolated.

3.2.2.2. Tumor challenge (therapeutic vaccination)

For the tumor challenge with therapeutic treatment, mice were implanted sc. with $1x10^5$ untreated B16.OVA cells into their right flank on day 0. Calipers were used to measure the growing tumor size. When tumors were readily visible (day x - average tumor size of 50 mm^2), 3p-B16 of $1x10^6$ transfected B16.OVA cells or 5 µl of EV stock were injected sc. into their right hock. Injection was repeated two times on day x+3 and x+6. Tumor growth was monitored over time. In compliance with requirements of the local regulatory agency, mice were sacrificed when the tumors reached 15 mm (1.5 cm) at the largest diameter or when ulceration of tumors occurred regardless of size.

3.2.2.3. Administration of blocking and depleting antibodies

In some experiments, mice were pre-treated intraperitoneally (ip.) with depletion or blocking antibodies. Treatment with 400 µg anti-IFNaR1 antibody (clone MAR1-5A3, BioXcell) was initiated one day prior to the above described immunization and injection was repeated twice a week during the ongoing experiment. The treatment with anti-CD8a (clone 2.43, BioXcell) or anti-NK1.1 (clone PK136, BioXcell) depleting antibodies was initiated two days prior to tumor induction (100 µg ip) and was repeated twice weekly (50 µg ip).

3.2.3. Ex vivo methods

3.2.3.1. Analysis of cytotoxic T cell activation in local draining lymph nodes and spleen

At day 14 of the boost injection, mice were sacrificed and ipsilateral draining lymph nodes (iLN, and pLN) as well as the spleen were isolated. The tissues were separately mashed through a 100 nm strainer to obtain a single cell suspension of complete lymph nodes. The cell suspension obtained from the spleen was subsequently treated with red blood cell lysis buffer to remove erythrocytes. Harvested lymph node cells were counted and seeded at 200.000 cells/ well on a 96-well plate (U-bottom) in a total volume of 200 µl cRPMI. Cells were incubated for 48 h on 37°C. If indicated, cells were restimulated with 1 µg/ mL ovalbumin protein. T cell activation was analyzed after the incubation time by detection of INF-γ using flow cytometry (intracellular stain) and/ or using ELISA on released IFN-γ protein in the supernatant.

Flow cytometric analyses: Lymphocytes were stained with fluorescent antibodies.

For intracellular staining, cells were first restimulated with Brefeldin A (5 ng/ mL), PMA (20 ng/ mL) and Ionomycin (1 µg/ mL) for 4 h on 37°C. Cells were washed two times with

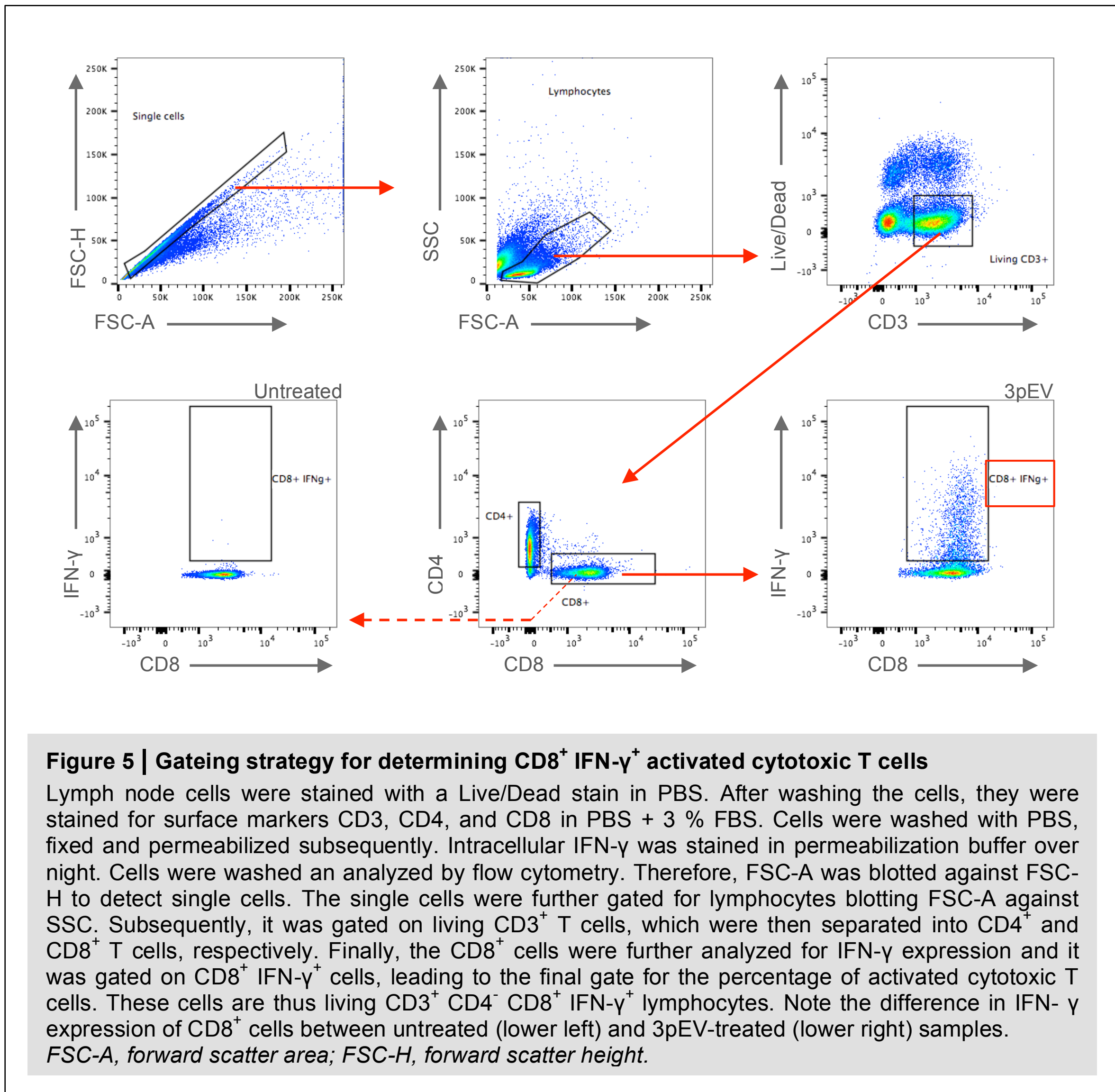

Figure 5 | Gateing strategy for determining CD8$^+$ IFN-γ$^+$ activated cytotoxic T cells
Lymph node cells were stained with a Live/Dead stain in PBS. After washing the cells, they were stained for surface markers CD3, CD4, and CD8 in PBS + 3 % FBS. Cells were washed with PBS, fixed and permeabilized subsequently. Intracellular IFN-γ was stained in permeabilization buffer over night. Cells were washed an analyzed by flow cytometry. Therefore, FSC-A was blotted against FSC-H to detect single cells. The single cells were further gated for lymphocytes blotting FSC-A against SSC. Subsequently, it was gated on living CD3$^+$ T cells, which were then separated into CD4$^+$ and CD8$^+$ T cells, respectively. Finally, the CD8$^+$ cells were further analyzed for IFN-γ expression and it was gated on CD8$^+$ IFN-γ$^+$ cells, leading to the final gate for the percentage of activated cytotoxic T cells. These cells are thus living CD3$^+$ CD4$^-$ CD8$^+$ IFN-γ$^+$ lymphocytes. Note the difference in IFN- γ expression of CD8$^+$ cells between untreated (lower left) and 3pEV-treated (lower right) samples.
FSC-A, forward scatter area; FSC-H, forward scatter height.

1xPBS (by centrifugation at 400x g, 5 min) and were afterwards stained with fluorescent antibodies against cell surface markers CD3 (anti-mouse CD3 - clone 17A2 - FITC), CD4 (anti-mouse CD4 - clone GK1.5 - PacificBlue), and CD8 (anti-mouse CD8 - clone 53-6.7-APC or PerCP) at a dilution of 1:400 in 1xPBS. Antibodies were purchased from Biolegend. Additionally, a fluorescent dye to distinguish live from dead cells was added to the cells (Fixable Viability Dye eFluor® 506, eBioscience). The stain was incubated on the cells for 25 min at 4°C in the dark. After washing the cells two times with FACS buffer (1xPBS + 3 % FBS) they were fixed with the Fixation/Permeabilization concentrate and diluent Kit (eBioscience) for 30 min on 4°C. Subsequently, cells were washed with the diluted 10x Permeabilization Buffer (eBioscience). The anti-mouse IFN-γ - PE (clone XMG1.2) antibody (Biolegend) was diluted (1:200) in 1x Permeabilization Buffer and cells were incubated with the (intracellular) stain for 17 h on 4°C. After this incubation time, cells were washed two times with FACS buffer and were subsequently resuspended in

100-200 µl of FACS buffer. Cells were analyzed by flow cytometry using the BD FACSCanto II. To analyze cytotoxic T cell activation, the gating strategy was as follows: living (Viability Dye$^-$) $CD4^-CD8^+$IFN-γ^+ cells (see Figure 5). To analyze T helper cell activation, the gating strategy was as follows: living (Viability Dye) $CD4^+CD8^-$IFN-γ^+ cells.

3.2.3.2. Generation of bone marrow-derived GM-CSF dendritic cells (BMDCs)

Bone marrow was isolated from tibia and femur of both hind legs of C57BL/6 mice or indicated knock-out mice. With sterile scissors each epiphesis (end of the bone) were cut off. With a sterile syringe (26-28G needle), the bone marrow was flushed with complete RPMI medium and filtered through a sterile 100 µm strainer. Cells were pelleted with 400x g, 5 min on 4°C. Erythrocytes were lysed by resuspending the pellet in 2 mL of red blood cell lysis buffer and a 5 min buffer incubation on room temperature. After addition of 5 mL of cRPMI, cells were centrifuged with 400x g, 5 min on 4°C. The cell pellet was resuspended in cRPMI and cultured to generate BMDCs and BMDMs, respectively.

Bone marrow cells were cultured in DC medium consisting of very low endotoxin (VLE) RPMI, supplemented with 10 % VLE FBS, 1 % penicillin (100 units/ mL) and 1 % streptomycin (100 µg/ mL), 1 % glutamine (200 mM), 0.1 % β-mercaptoethanol (50 mM) and the growth factor GM-CSF (20 ng/ mL). $5x10^6$ cells were plated in 10 mL DC medium on a 10 cm non-tissue-treated bacterial dish. The culture was placed on 37°C for 7 days. At day 3, 10 mL of fresh DC medium was added to the culture. At day 6, 10 mL of culture supernatant (including floating cells) was collected and spun at 400x g, 5 min. Cell pellet was resuspended in 10 mL of fresh DC medium and transferred back into the culture dish. At day 7, cells were harvested using PBS-EDTA. All cells were cultured in a humidified incubator with 5 % CO2 and 95 % air.

3.2.3.3. Stimulation of BMDCs

For DC stimulation, the harvested DCs were plated on tissue culture-treated flat bottom 96-well plates. $2,5x10^4$ cells were plated in 100 µl cRPMI per well. 24 h after plating, DCs were stimulated with the respective stimuli diluted in cRPMI to a total volume of 50 µl (final concentrations: 3pRNA: 1 µg/ mL, ISD: 1 µg/ mL, EVs: 7 µl EV stock in 1 mL, EV-RNA: 800 ng/ mL, EV-DNA: 140 ng/ mL). DC activation was assessed 24 h after stimulation. Therefore, the supernatant was collected and type I IFN release was subsequently analyzed by ELISA or, DCs were collected and stained for cell surface activation markers. If cells were analyzed for expression of cell surface markers, the cells were washed and stained in PBS with the respective antibodies. Before staining, cells were incubated with anti-CD16/CD32 FcRχ block (eBioscience) and stained with Fixable Viability Dye eFluor®

506 to distinguish live from dead cells. Cells were then analyzed for the expression of the co-stimulatory marker CD86 (eBioscience) and cross-presentation of the processed OVA peptide SIINFEKL on MHC-I (H-2k^{b}) (eBioscience). Antibodies were incubated for 25 min on 4°C in the dark. Cells were washed with FACS buffer (1x PBS + 3 % FBS) and analyzed using flow cytometry.

3.2.3.4. Detection of circulating, antigen-specific T lymphocytes

Around 50 µl venous blood was obtained from the facial vein which was collected in a S-Monovette® that ensures mixing of anticoagulant (EDTA) with blood to prevent clotting. 50 µl whole blood was then transferred to a 96 well plate and lysis of red blood cells was performed to enrich the lymphocyte population and to reduce background signals due to erythrocyte contamination. Therefore, blood was mixed with 100 µl red blood cell lysis buffer (Intron Biotechnology) and incubated for 5 min at RT. Lysis was stopped by the addition of 100 µl cRPMI medium and samples were centrifuged at 400x g for 5 min. The lysis step was repeated until all erythrocytes were removed. Cells were subsequently washed twice with PBS and incubated with H-2k^{b}-OVA$_{257\text{-}264}$-Tetramers (Biozol) together with anti-CD4 antibody, anti-CD3 antibody and Fixable Viability Dye eFluor® 506 at RT for 30 minutes. Finally, cells were washed again twice, resuspended in PBS supplemented with 0.5% formaldehyde and stored at 4°C protected from light for a minimum of 1 hour prior to analysis by flow cytometry.

3.2.4. Molecular biology methods and imaging techniques

3.2.4.1. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was performed in collaboration with the University of Ulm, Faculty of Medicine. Sizing and quantification of isolated EVs was performed with the NanoSight® LM10 instrument, following the manufacturer’s protocol, (NanoSight®, Malvern Instruments Ltd, Malvern, UK). The LM10 uses a laser light source to illuminate nanoscale particles (10–1000 nm), which are seen as individual pointscatters moving under Brownian motion. The path of the point scatters, or particles, are calculated over time to determine their velocity, which can be used to calculate their size independent of density. The image analysis NTA software compiles this information and allows the user to automatically track the size distribution and number of the nanoparticles. Therefore, an aliquot (2 µl) of isolated EVs (from the EV stock) was diluted in 1 mL PBS (1:500) to achieve a uniform particle distribution, and 3 sequential measurements (1 min each) at 23 °C (viscosity 0.09 cp) were performed. The instrument settings were: camera level 14, 30 frames/ s; drift correction auto; analysis: blur auto, detection threshold 10 multi, min

track length auto and min expected size auto. At least 900 tracks were recorded per measurement.

3.2.4.2. Transmission electron microscopy

Transmission electron microscopy (TEM) was performed in collaboration with the TUM, Faculty of Chemistry. For electron microscopy, EV samples were diluted 1:50 in PBS. 5 µl of each diluted sample were applied to glow-discharged carbon grids, incubated for 60 sec, blotted, briefly washed with $_{dd}H_2O$ and subsequently stained in 1% w/v uranyl acetate for 40 sec. Images were recorded immediately using a CM200 (Philips) at a nominal magnification of 50,000x on a Tietz4K camera. The pixel size on the specimen level was 0.21 nm.

3.2.4.3. Single EV imaging flow cytometry

Single EV imagint flow cytometry was performed in collaboration with the University Duisburg-Essen, Institute for Transfusion Medicine. André Görgens, PhD (group of Prof. Bernd Giebel) developed a flow cytometry-based technique to visualize single EVs with or without the detection of a fluorochrome (Görgens 2016). Therefore, 3pRNA was fluorescently labeled (FAM) using the Silencer® siRNA Labeling Kit (Thermo Fischer). B16.OVA cells as well as RIG-I$^{-/-}$ B16 were treated with either unlabeled 3pRNA or FAM-labeled 3pRNA. Respective EVs were isolated from the supernatants of cell cultures (3pEVs, FAM-3pEVs WT/ RIG-I$^{-/-}$). The isolated EVs were analyzed for the presence of FAM labeling dye (excitation at 492 nm, emission at 518 nm) using this single EV FACS technique. The post lysis values are based on 3pEV-FAM vesicles, which were lyzed before analysis. With this control it can be analyzed whether FAM-dye is just captured on protein aggregates or whether it is present within a membrane vesicle. Protein aggregates would not be lyzed by the reagent. No fluorescent EVs after lysis indicate that all dye is stored within membrane-surrounded vesicles.

3.2.4.4. Immunoblotting

Cells or EVs isolated from cell culture supernatants were collected in PBS. Protein was extracted using the radioimmunoprecipitation assay (RIPA) buffer (Invitrogen), including complete protease inhibitor cocktail (Roche). Protein yield was measured by BCA assay (Thermo Fischer Scientific) prior to protein separation by SDS-PAGE on 10-12% polyacrylamide gels for 90 min at 80 V. Proteins were blotted to a nitrocellulose blotting membrane (GE Healthcare) for 90 min at 0.3 A and membranes were subsequently blocked for 90 min in 5 % BSA or 5 % milk in 1x TBST (tris-buffered saline and Tween 20). After incubation with primary antibodies (see Table 3.1.7.) in blocking buffer over night at 4°C and 3 washing steps with 1x TBST (each lasting for at least 10 min),

secondary antibodies coupled with horse-radish-peroxidase (HRP) were incubated for 1-2 h at RT. After 3 additional washing steps, signals were visualized using Pierce™ ECL Western Blotting Substrate according to the manufacturers' protocol and the INTAS science imaging system.

3.2.4.5. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used for quantitative determination of proinflammatory cytokines TNF-α, IL-12p40 and IL-6, for HMGB1 as well as for IFN-α, IFN-β, and IFN-γ concentrations in cell culture supernatants. Except for INF-I, the ELISA Kits were bought from eBioscience, the HMGB1 Kit was obtained from IBL international, respectively and conducted according to the procedures provided by the manufacturers.

For the detection on type I IFN (IFN-α and -β), custom ELISA protocols were applied. Flat bottom 96-well plates (Nunc) were pre-coated with 50 µl of 1 µg/ mL of rat anti-mouse IFN-α antibody and rat anti-mouse IFN-β, respectively, in coating buffer derived from the eBioscience Kits mentioned above. This 'coating antibody' was incubated on the plate for 16 h on 4°C. After washing extensively with wash buffer (1xPBS + 0,5% Tween®20), the plate was blocked with blocking buffer (1xPBS + 10 % FBS) for 3 h on room temperature (RT). Afterwards, 50 µl of cell culture supernatant or recombinant mouse IFN-α (1250 ng/ mL top standard) or recombinant mouse IFN-β (1250 ng/ mL top standard) was added to the wells and incubated on 4°C for 24 h. Supernatants were then removed and the plate was washed extensively with wash buffer. The respective detection antibody (anti-mouse IFN-α, or –β, from rabbit serum, 620 ng/ mL) was added and incubated for 3 h at RT. After additional washing, polyclonal goat anti-rabbit IgG coupled with horse radish peroxidase (HRP) was added and incubated for 1 h at RT (56 ng/ mL). After intensive washing, 100 µl of substrate solution (1xTBM Substrate, eBioscience) was added. When the reaction was complete, 100 µl of stop solution (2N H_2SO_4) was added to the substrate solution. Optical density of each well was immediately assessed, using a microplate reader set to 450 nm (Tecan).

3.2.4.6. BCA-Assay

The Thermo Scientific™ Pierce™ BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This kit was used according to the manufacturer's protocol. In brief, cells or EVs were lyzed with RIPA buffer during an incubation time of 30 min on ice. After 1 min of vortexing, lysates were mixed with the kit reagents and incubated for 30 min on 37°C in the dark. The purple-colored reaction product of this assay exhibits a strong

absorbance at 562 nm, which correlates linearly with protein concentrations. The absorbance was measured at or near 562 nm on a plate reader (Tecan).

3.2.4.7. RNase and DNase treatment of EVs

EV surfaces were treated with either RNase A or DNase I to remove nucleic acids attached to their outside membrane. Therefore, isolated EVs were treated with RNase A (Thermo Fischer) with a concentration of 100 µg/ mL for 30 min at RT. RNase A was then inhibited with RNase OUT according to manufacturer’s instructions (Thermo Fischer), or EVs were incubated with DNase I (Thermo Fischer) for 15 min at RT (1 U/ µg RNA). DNase I was inactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and subsequent heating for 10 min at 65°C.

3.2.4.8. Isolation of EV nucleic acids

EV-RNA was obtained by following the instructions of the ‘Total Exosome RNA and Protein Isolation Kit’ (Thermo Fischer). In brief, isolated EVs were lyzed with the kit-included lyzing reagent. Afterwards, RNA was purified using Acid-Phenol:Chloroform extraction followed by a final RNA purification. Therefore, ethanol is added to the samples, which are subsequently passed through a filter cartridge containing a glass-fiber filter, which immobilizes the RNA. The filter is washed, and the RNA is eluted with nuclease free water. RNA concentration was analyzed using the Qubit HS RNA Assay Kit (Thermo Fischer) and RNA quality was assessed using the TapeStation System (Agilent) by applying the manufacturer’s instructions.
EV DNA was obtained using DNAzol® reagent (Thermo Fischer) according to the manufacturer’s protocol. In brief, 1 mL of DNAzol® was added to isolated EVs. EVs are thus lyzed for 30 min at RT and DNA is subsequently precipitated from the lysate with ethanol. Following an additional ethanol wash, DNA is solubilized in water.

3.2.4.9. Alkaline phosphatase treatment of EV-RNA

APex™ is an alkaline phosphatase that dephosphorylates 5′ phosphates from a broad range of substrates including 5’ppp-RNA. EV-RNA was treated with APex™ according to the manufacturer’s guidelines. In brief, 1 µl of APex™ was added to EV-RNA (up to 1 µg) and incubated for 10 min at 37°C. The phosphatase was inactivated by heat inhibition for 5 min at 70°C.

3.2.5. Statistical analysis

All data are presented as mean ± S.E.M. Statistical significance of single experimental findings was assessed with the independent two-tailed student's t-test. For multiple statistical comparison of a data set the one-way ANOVA test with Bonferroni post-test was used. Significance was set at P values < 0.05, $p < 0.01$ and $p < 0.001$ and was then indicated with an asterisk (*, ** and ***). All statistical calculations were performed using Prism (GraphPad Software).

4. Results

Immunogenic cell death (ICD) is a novel therapeutic approach in the battle against cancer and provides a possible way to control tumor growth. The induction of ICD by RIG-I-like receptor activation has been discovered and published only recently (Duewell *et al.* 2014). In parallel to this work, which shows ICD after activation of RIG-I and MDA5 in murine pancreatic carcinoma cells, the present work identified RIG-I activation as an immunogenic cell death inducer in murine melanoma cells. With this observation, it could be shown that ICD after RIG-I activation can be generalized to other entities. The main part of the present work additionally unravels the immunogenic factor of RIG-I induced immunogenic cell death. This dissertation could for the first time show that transferred tumor-derived nucleic acids constitute the immunogenic factor of RIG-I-induced ICD, and that these nucleic acids are shuttled within extracellular vesicles. This study provides detailed *in vivo* data together with intense analyses of the host immune response after administration of these immunogenic extracellular vesicles released by melanoma cells succumbing to RIG-I-triggered ICD *in vitro*.

4.1. Generating knock-out cell lines for the investigation of the RIG-I pathway in melanoma cells using CRISPR/Cas9 gene editing

As a tool to investigate RIG-I signaling in melanoma cells in regards to immunogenic cell death, first several knock-out cell lines deficient for different components of the RIG-I pathway were generated. For the deletion of specific genes the CRISPR/Cas9 gene editing system was applied. The melanoma cell line B16.OVA, which stably expresses the model antigen ovalbumin (OVA), was used for all experiments and for the generation of the respective knock-out cell lines (**Figure 6**). The created B16.OVA knock-out cell lines are deficient for either RIG-I (RIG-I$^{-/-}$ B16.OVA) or Interferon regulatory factors 3 and 7 (IRF3/7$^{-/-}$ B16.OVA) (see also M+M section 3.2.1.3.).

To verify the CRISPR/Cas9-mediated knock-out of the respective genes, the generated genetic deletion was validated using Western blotting **(Figures 6a, b**). The so validated knock-out cell clones were subsequently tested for the disrupted function on protein level. After RIG-I activation with a specific ligand, a short double-stranded RNA with 5'-triphosphorylated ends (3pRNA), B16 cells produce and release high levels of type-I interferon (type I IFN, that is IFN-α and IFN-β). If components of the RIG-I pathway are

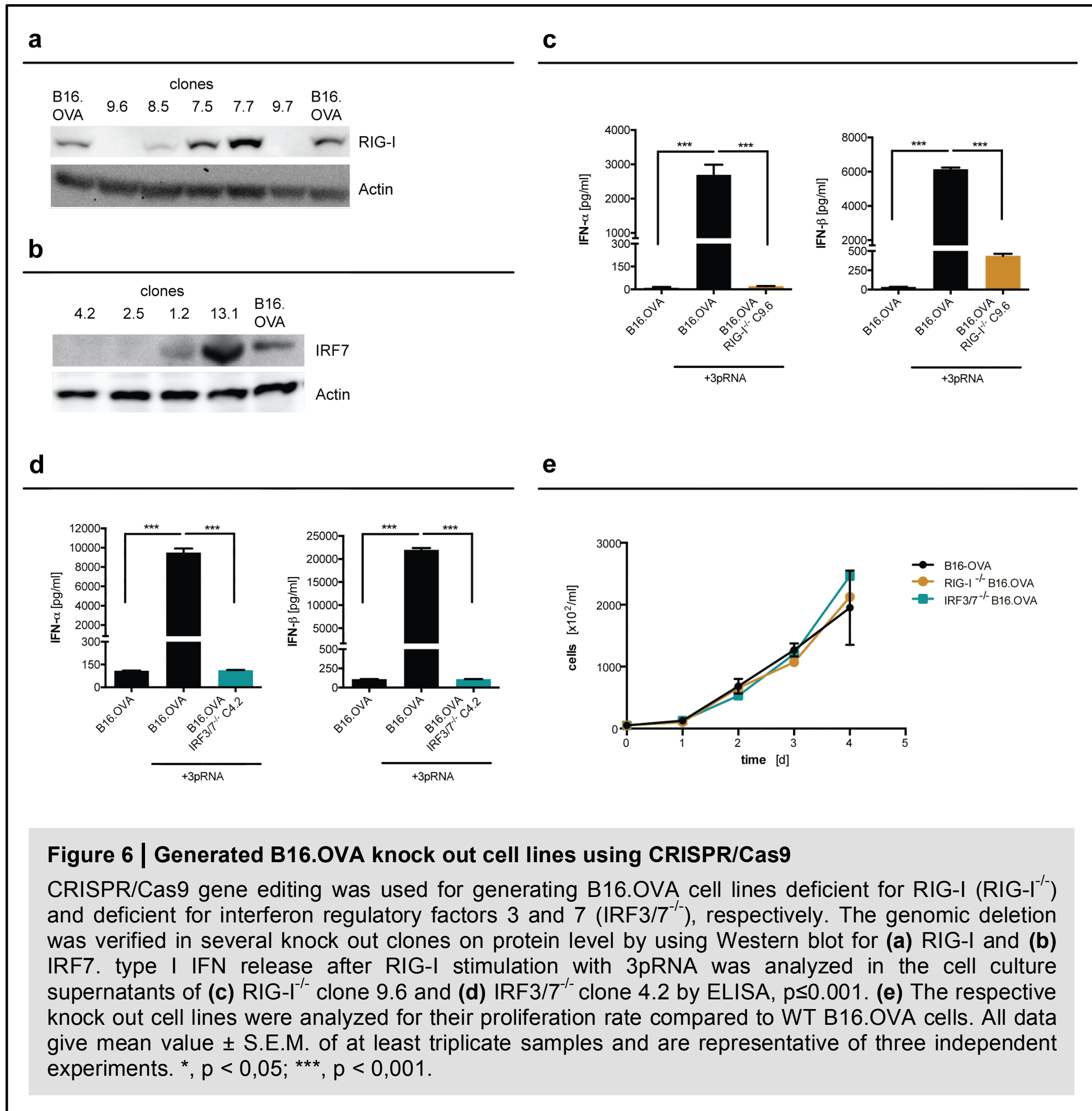

Figure 6 | Generated B16.OVA knock out cell lines using CRISPR/Cas9
CRISPR/Cas9 gene editing was used for generating B16.OVA cell lines deficient for RIG-I (RIG-I$^{-/-}$) and deficient for interferon regulatory factors 3 and 7 (IRF3/7$^{-/-}$), respectively. The genomic deletion was verified in several knock out clones on protein level by using Western blot for **(a)** RIG-I and **(b)** IRF7. type I IFN release after RIG-I stimulation with 3pRNA was analyzed in the cell culture supernatants of **(c)** RIG-I$^{-/-}$ clone 9.6 and **(d)** IRF3/7$^{-/-}$ clone 4.2 by ELISA, p≤0.001. **(e)** The respective knock out cell lines were analyzed for their proliferation rate compared to WT B16.OVA cells. All data give mean value ± S.E.M. of at least triplicate samples and are representative of three independent experiments. *, p < 0,05; ***, p < 0,001.

disrupted due to genetic modification, type I IFN will be no longer released. Thus, the enerated RIG-I$^{-/-}$ and IRF3/7$^{-/-}$ cell lines were tested for the release of type I IFN after transfection with 3pRNA (**Figures 6c, d**). Clones showing no release of type I IFN after 3pRNA treatment were considered as functional knock-out clones. These clones were additionally tested for similar proliferation rates compared to the wild-type B16 cell line (**Figure 6e**). Functional knock-out clones showing the same proliferation rate as wild-type cells were finally selected for in-depth experiments. Shown here are exemplary experiments for the specific knock-out clones chosen for the subsequent experiments of this dissertation (RIG-I$^{-/-}$ clone 9.6 and IRF3/7$^{-/-}$ clone 4.2, respectively).

4.2. Activation of the RIG-I pathway in melanoma cells results in immunogenic cell death

B16.OVA melanoma cells were treated with the receptor-specific ligand 3pRNA and were then analyzed in different settings. The transfection of 3pRNA triggered rapid cell death in melanoma cells. After 48 h of stimulation, around 70% of the cells succumbed to apoptosis (**Figure 7a**). The initiated program of intrinsic apoptosis is dependent on active RIG-I signaling, since the transfection of 3pRNA into RIG-I$^{-/-}$ B16.OVA cells did not induce apoptosis in these cells. WT as well as RIG-I$^{-/-}$ B16.OVA cells were simultaneously treated with the known chemotherapeutic immunogenic cell death (ICD) inducer, oxaliplatin. Concentrations of 3pRNA and oxaliplatin were scaled in order to induce the same rate of cell death after a given time. The indicated concentrations of oxaliplatin induced the same rates of apoptotic cell death in wild-type B16.OVA compared to 3pRNA treatment. In contrast, the RIG-I$^{-/-}$ B16.OVA cells succumbed to the treatment with oxaliplatin.

Both the 3pRNA- and oxaliplatin-treated apoptotic cells (hereafter referred to as 3p-B16 and Oxa-B16, respectively) as well as their culture supernatant were analyzed for ICD hallmarks. The transfection of 3pRNA resulted in the release of all three hallmark molecules known to mediate immunogenic cell death (**Figure 7b**). The amount of released danger associated molecular patterns (DAMPs) was higher when the cells were treated with 3pRNA compared to oxaliplatin. ATP was released into the supernatant, calreticulin (CRT) was exposed on the outer plasma membrane, and HMGB1 was detected in the supernatant. Additional to these ICD hallmark molecules, the proinflammatory cytokines TNF-α, IL-6 and IL-12p40 were secreted into the supernatant after RIG-I activation by 3pRNA transfection (**Figure 7c**). In contrast to oxaliplatin treatment, activation of RIG-I was associated with the potent release of type I IFN (**Figure 7d**).

To test the immunogenicity of the cell death resulting from RIG-I activation in melanoma cells *in vitro*, bone marrow derived dendritic cells (BMDCs) were isolated from syngenic wild-type (WT) C57BL/6 mice or from syngenic mice deficient for type I IFN receptor signaling (IFNaR$^{-/-}$) and subsequently co-cultured 3p-B16 cells or Oxa-B16 cells. Untreated BMDCs served as a control. DC activation in terms of co-stimulatory molecule CD86 up-regulation and presentation of processed OVA peptide SIINFEKL in the context of MHC-I was analyzed after 24 h of co-culture by flow cytometry (**Figure 7e**). It could be observed that to co-culture with 3p-B16 cells activated WT DCs. This activation was

abrogated if DCs lack the receptor for type I IFN. Oxa-B16 cells however, could not activate DCs.

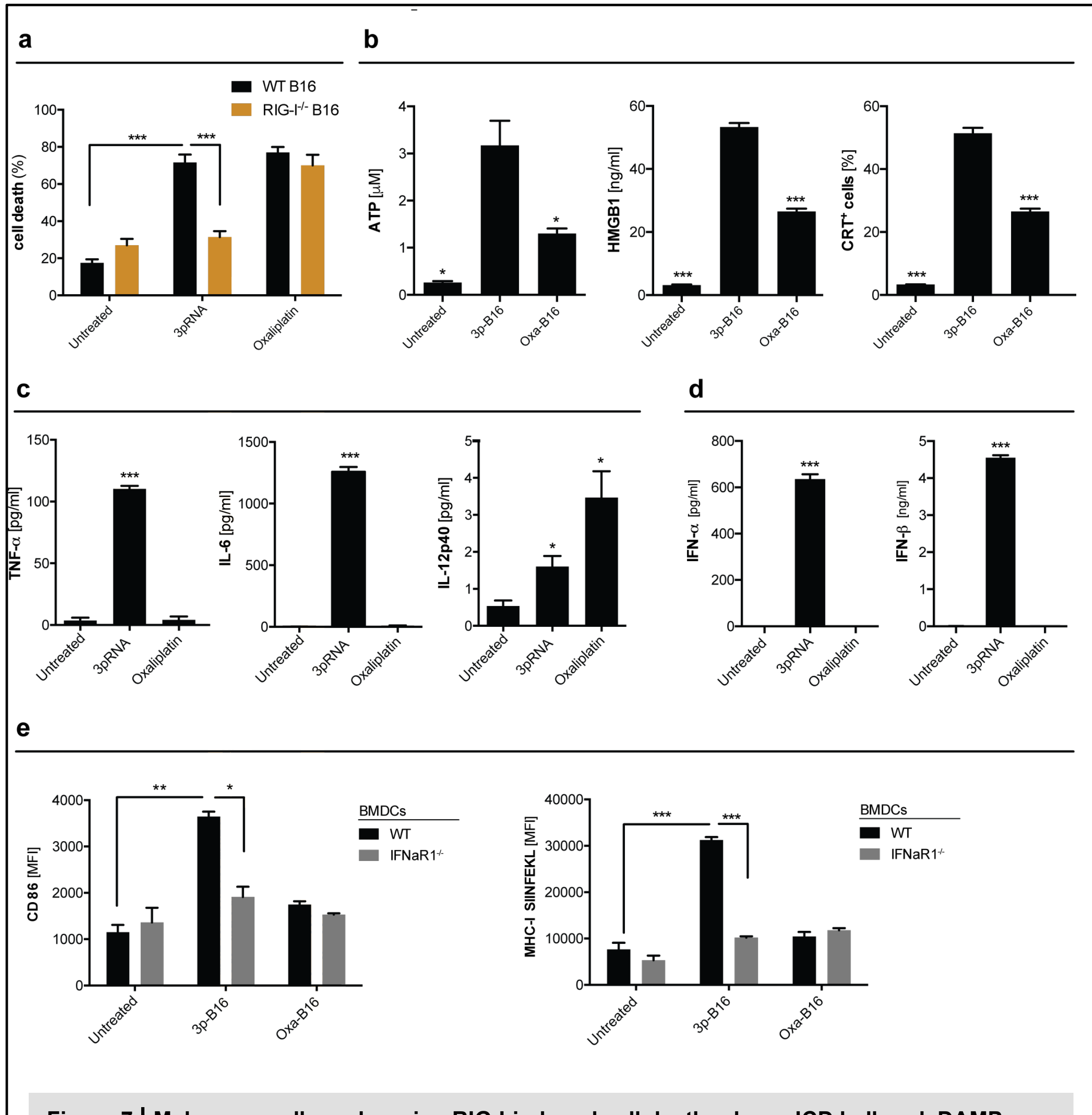

Figure 7 | Melanoma cells undergoing RIG-I-induced cell death release ICD hallmark DAMPs, type I IFN and proinflammatory cytokines

Wild-type (WT) and RIG-I-deficient (RIG-I$^{-/-}$) B16.OVA melanoma cells were treated with the specific RIG-I ligand 3pRNA or oxaliplatin for 48 h. **(a)** Cell viability was assessed by flow cytometry using AnnexinV/7AAD staining. **(b)** Cell culture supernatants were analyzed for the concentration of ICD hallmark DAMPs ATP and HBGB1 by ELISA. The frequency of B16 cells with calreticulin (CRT) exposed on the outer plasma membrane was determined by flow cytometry. Cell culture supernatants were analyzed for the concentration of **(c)** proinflammatory cytokines and **(d)** type I IFN. An asterisk without brackets indicates comparison to untreated cells. **(e)** B16.OVA cells were treated as described above and were subsequently co-cultured with bone marrow-derived dendritic cells (BMDCs) from WT or IFNaR1-deficient (IFNaR$^{-/-}$) donor mice. After 24h exposure to tumor cells CD86 expression and cross-presentation of the processed OVA peptide-epitope SIINFEKL in the context of MHC-I by CD11c^{+} conventional DCs was analyzed by flow cytometry. All data above give mean value ± S.E.M. of at least triplicate samples and are representative of three independent experiments. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$.

These experiments showed that stimulation of RIG-I induced RIG-I dependent melanoma cell death and the release of classical ICD hallmark DAMPs as known for chemotherapeutic ICD inducers like oxaliplatin. Furthermore, only RIG-I activation led to a potent release of type I IFN and provoked BMDC activation *in vitro*.

A prerequisite for an ICD inducer is that cancer cells succumbing to the respective stimulus *in vitro* that are administered in the absence of any adjuvant must drive a potent immune response against dead cell antigens *in vivo* thus protecting mice against a subsequent challenge with live tumor cells of the same type (Kroemer *et al.* 2013). To test whether RIG-I-mediated immunogenic tumor cell death and associated DC maturation translate into cross-priming of cytotoxic T cells *in vivo*, 3p-B16 cells were injected subcutaneously (sc.) into the right hock of wild-type C57BL/6 mice (**Figure 8a**). Control mice were injected with PBS (saline) only. Dead cell injection (and PBS injection) was repeated seven days later in order to elicit a boosted vaccination effect (hereafter referred to as boost injection). At day 14 (seven days after the boost), mice were sacrificed and draining lymph nodes (dLNs), here popliteal and inguinal LNs as well as the spleen were harvested. Complete lymph node cells including dendritic cells and T cells were plated on culture plates and were either left untreated or were *ex vivo* restimulated with soluble ovalbumin to obtain expansion of antigen-specific T cells. Activation of T cells was analyzed in terms of $CD4^+$ and $CD8^+$ IFN-γ^+ cytotoxic T cells (see **Figure 5**). The results show an increased number of IFN-γ^+ $CD4^+$ T cells in the draining LNs as well as in the spleen in 3p-B16-injected mice (**Figure 8b**) as well as an increased number of IFN-γ^+ cytotoxic ($CD8^+$) T cells (**Figure 8c**). Additionally, *ex vivo* restimulated cells show enhanced numbers of activated cytotoxic T cells, suggesting T cell specificity for the ovalbumin antigen. This suggestion could be confirmed by the detection of H-2K^b-$OVA_{257-264}$-$Tetramer^+$ $CD8^+$ T cells in the IFN-γ^+ $CD8^+$ T cell fraction in both, dLNs and the spleen (**Figure 3d**). These activated T cells express a T cell receptor against the OVA peptide SIINFEKL and are thus tumor antigen-specific cytotoxic T cells.

To investigate whether the observed dead cell-induced immune response is potent enough to protect mice already bearing a pre-established tumor, WT mice were challenged with live tumor cells before the therapeutic treatment with 3p-B16 cells (**Figure 8e**). When tumors reached a certain size, mice were repeatedly injected with apoptotic 3p-B16 cells. This tumor model is hereinafter referred to as therapeutic vaccination. The repeated injection of pre-treated B16.OVA melanoma cells undergoing RIG-I-mediated ICD resulted in strong regression of established tumors (**Figure 8f**). The same therapeutic vaccination was performed in mice, which had been injected with deletion antibodies against either CD8a (cytotoxic T cells) or against NK1.1 (natural killer (NK) cells) before

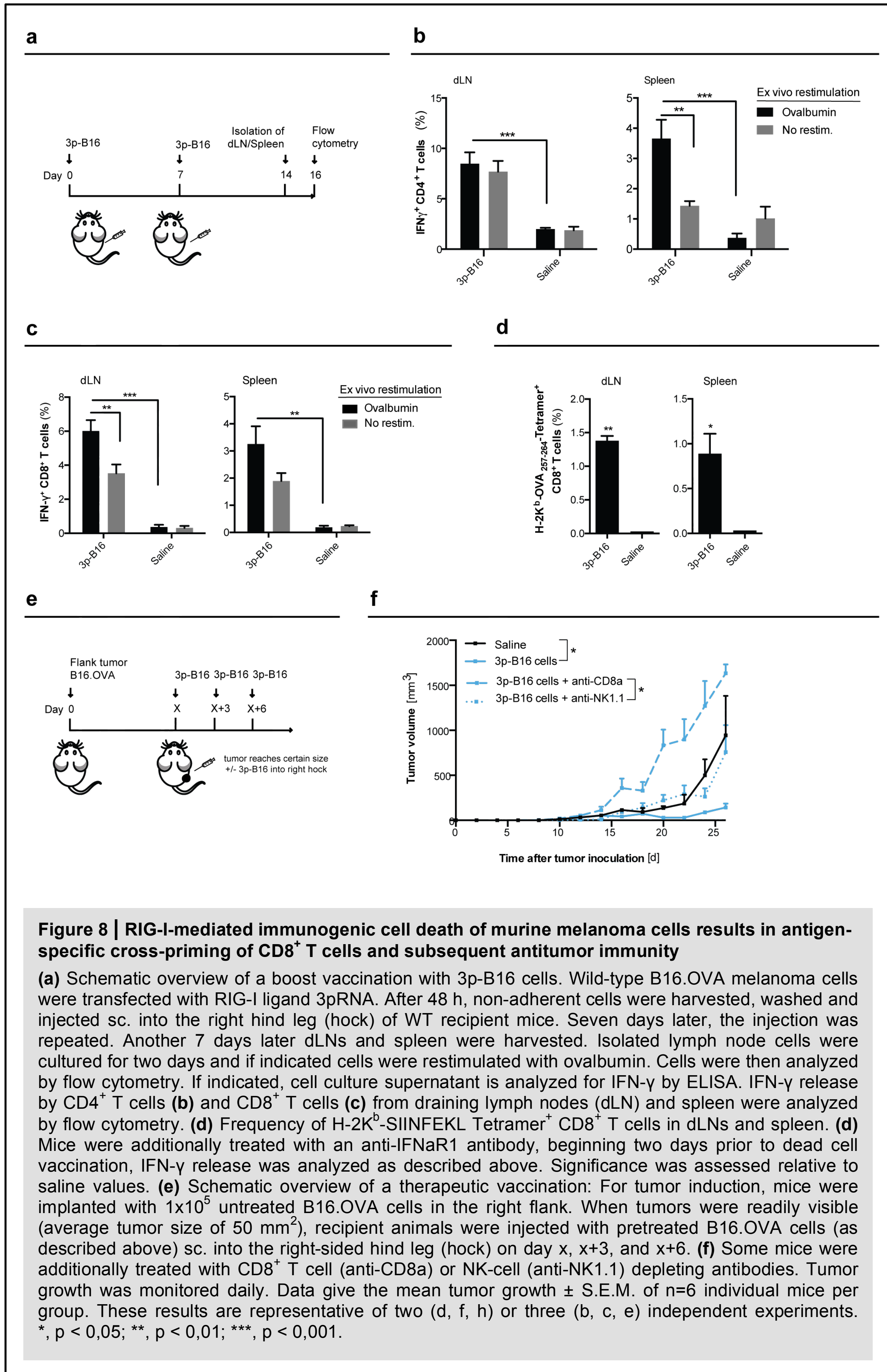

Figure 8 | RIG-I-mediated immunogenic cell death of murine melanoma cells results in antigen-specific cross-priming of CD8$^+$ T cells and subsequent antitumor immunity

(a) Schematic overview of a boost vaccination with 3p-B16 cells. Wild-type B16.OVA melanoma cells were transfected with RIG-I ligand 3pRNA. After 48 h, non-adherent cells were harvested, washed and injected sc. into the right hind leg (hock) of WT recipient mice. Seven days later, the injection was repeated. Another 7 days later dLNs and spleen were harvested. Isolated lymph node cells were cultured for two days and if indicated cells were restimulated with ovalbumin. Cells were then analyzed by flow cytometry. If indicated, cell culture supernatant is analyzed for IFN-γ by ELISA. IFN-γ release by CD4$^+$ T cells **(b)** and CD8$^+$ T cells **(c)** from draining lymph nodes (dLN) and spleen were analyzed by flow cytometry. **(d)** Frequency of H-2K^b-SIINFEKL Tetramer$^+$ CD8$^+$ T cells in dLNs and spleen. **(d)** Mice were additionally treated with an anti-IFNaR1 antibody, beginning two days prior to dead cell vaccination, IFN-γ release was analyzed as described above. Significance was assessed relative to saline values. **(e)** Schematic overview of a therapeutic vaccination: For tumor induction, mice were implanted with 1x10^5 untreated B16.OVA cells in the right flank. When tumors were readily visible (average tumor size of 50 mm^2), recipient animals were injected with pretreated B16.OVA cells (as described above) sc. into the right-sided hind leg (hock) on day x, x+3, and x+6. **(f)** Some mice were additionally treated with CD8$^+$ T cell (anti-CD8a) or NK-cell (anti-NK1.1) depleting antibodies. Tumor growth was monitored daily. Data give the mean tumor growth ± S.E.M. of n=6 individual mice per group. These results are representative of two (d, f, h) or three (b, c, e) independent experiments. *, p < 0,05; **, p < 0,01; ***, p < 0,001.

live tumor administration. Hereby it was ascertained that antitumor immunity after therapeutic 3p-B16 vaccination was mediated by both $CD8^+$ T cells and NK cells.

Taken together, these data show that RIG-I activation in melanoma cells leads to apoptotic cell death. During apoptosis, the RIG-I-activated dying cell release immunogenic factors including all known ICD hallmark molecules as well as relevant levels of type I IFNs and proinflammatory cytokines. Such immunogenic melanoma cell death results in DC maturation and cross-priming of tumor antigen specific $CD8^+$ T cells that translate into potent antitumor immunity *in vivo*. In conclusion, the treatment of tumor cells with the RIG-I ligand 3pRNA leads to cell death and can convert dying cancer cells into a therapeutic vaccine.

4.3. The immunogenicity of RIG-I-mediated B16 melanoma cell death does not follow the immunogenic route of known ICD inducers

Extracellular ATP released from dying cells is one of the most prominent ‘find-me’ signals for macrophage and DC precursors. At least a part of the effects of ATP on DCs are mediated via the P2RX7 receptor. In response to P2RX7 ligation, the efflux of K^+ and Ca^{2+} ions can lead to the activation of the NLRP3 inflammasome, a caspase-1 activation platform, thus stimulating the proteolytic maturation and subsequent secretion of IL-1β and IL-18 (Zitvogel *et al.* 2012, Kroemer *et al.* 2013). Thus, the ATP-elicited production of IL-1β was suggested to be one of the critical factors for the immune system to perceive cell death as immunogenic in the context of anticancer chemotherapeutic ICD inducers.

Since high levels of ATP are released after RIG-I activation in melanoma cells, it was investigated whether the activation of the NLRP3 inflammasome in myeloid APCs is involved in the elicited immunogenicity of 3pRNA-mediated melanoma cell death (**Figure 9a**). Therefore, mice deficient for the NLRP3 inflammasome were boost injected with RIG-I-activated melanoma cells. Control mice were injected with saline. Oxaliplatin-treated melanoma cells served as positive control, since the immunogenic effect of oxaliplatin has been shown to be dependent on NLRP3 activation (Aymeric *et al.* 2010). T cell activation in terms of IFN-γ release was analyzed. Compared to oxaliplatin-treated melanoma cells, which show a NLRP3-dependent induction of T cell activation, the elicited immune response after 3p-B16 cell administration was independent of NLRP3. Additionally, by the use of mice deficient for the inflammasome adaptor protein ASC, it could be shown that also other inflammasome complexes, such as the AIM2-inflammasome had no impact on the activation of the host’s immune system after administration of 3pRNA-treated

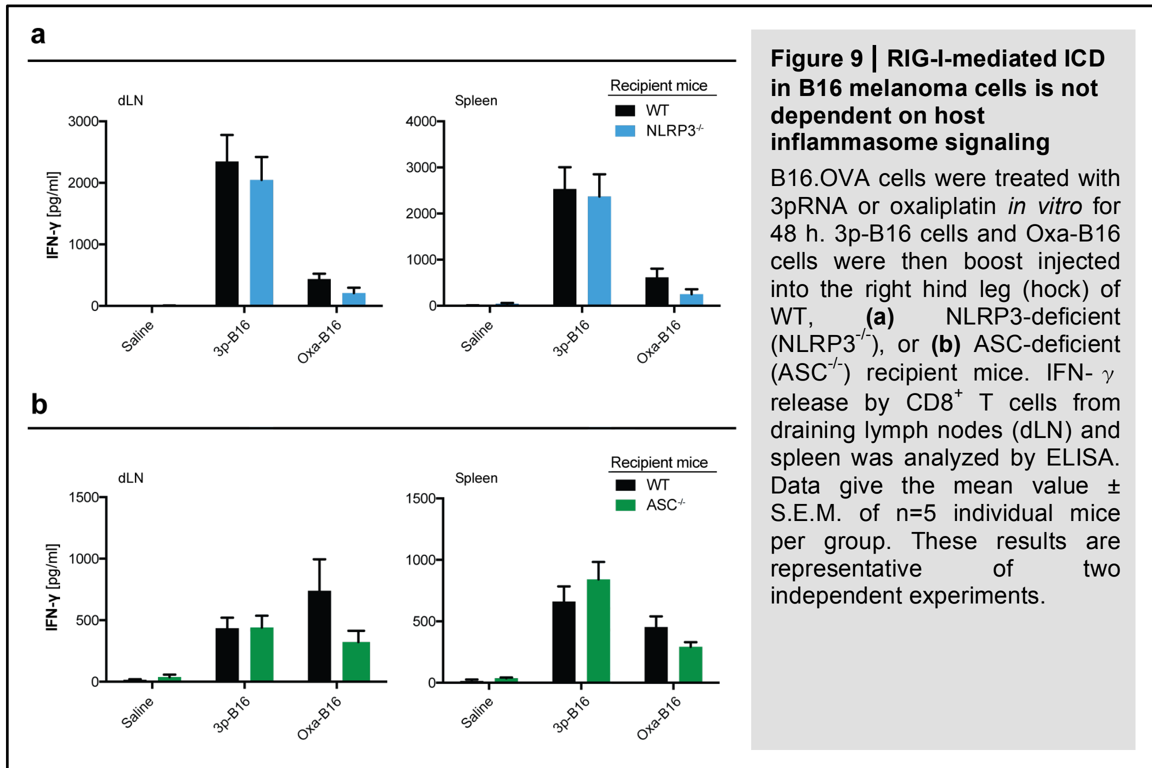

Figure 9 | RIG-I-mediated ICD in B16 melanoma cells is not dependent on host inflammasome signaling

B16.OVA cells were treated with 3pRNA or oxaliplatin *in vitro* for 48 h. 3p-B16 cells and Oxa-B16 cells were then boost injected into the right hind leg (hock) of WT, **(a)** NLRP3-deficient ($NLRP3^{-/-}$), or **(b)** ASC-deficient ($ASC^{-/-}$) recipient mice. IFN-γ release by $CD8^+$ T cells from draining lymph nodes (dLN) and spleen was analyzed by ELISA. Data give the mean value ± S.E.M. of n=5 individual mice per group. These results are representative of two independent experiments.

melanoma cells (**Figure 9b**). In contrast, oxaliplatin-treated melanoma cells showed a dependency on inflammasome formation, however, that was not found to be statistically significant.

CRT exposure, ATP secretion and HMGB1 release are all indispensable for ICD induced by chemotherapeutic agents; the absence of only one of these ICD hallmarks abolishes the efficacy of anthracycline- or oxaliplatin-based chemotherapy in mouse models (Kroemer *et al.* 2013). The observation that ATP is not essentially needed for a proper T cell activation after injection of 3p-B16 cells led to the suggestion, that RIG-I-mediated ICD does not follow the same signaling routes as chemotherapeutic ICD inducers such as oxaliplatin.

4.4. The immunogenicity of RIG-I-mediated B16 melanoma cell death is dependent on host cell nucleic acid receptor and type I IFN signaling

Because RIG-I-mediated cell death has been associated with the release of high levels of type I IFN and 3p-B16-induced BMDC activation is dependent on type I IFN receptor signaling, the role of type I IFN signaling in RIG-I-mediated ICD was examined in more detail.

Since 3pRNA-transfected apoptotic cells were extensively washed in PBS before they are used for co-culture *in vitro* experiments or injected as *in vivo* vaccines in order to get rid of any remaining free 3pRNA, it was analyzed whether these dying cells still release relevant amounts of type I IFN **(Figure 10a)**. 3-B16 cells and Oxa-B16 cells were thus harvested, washed with PBS and re-plated in fresh B16 medium. Released IFN-α was assessed after 24 h by ELISA. Interes-tingly these experiments showed that such RIG-I activated dying B16 cells still release type I IFN even at the time point of apoptotic cell administration *in vivo*. No IFN-α could be measured in the super-natants of re-plated un-treated or oxaliplatin-treated B16. OVA cells.

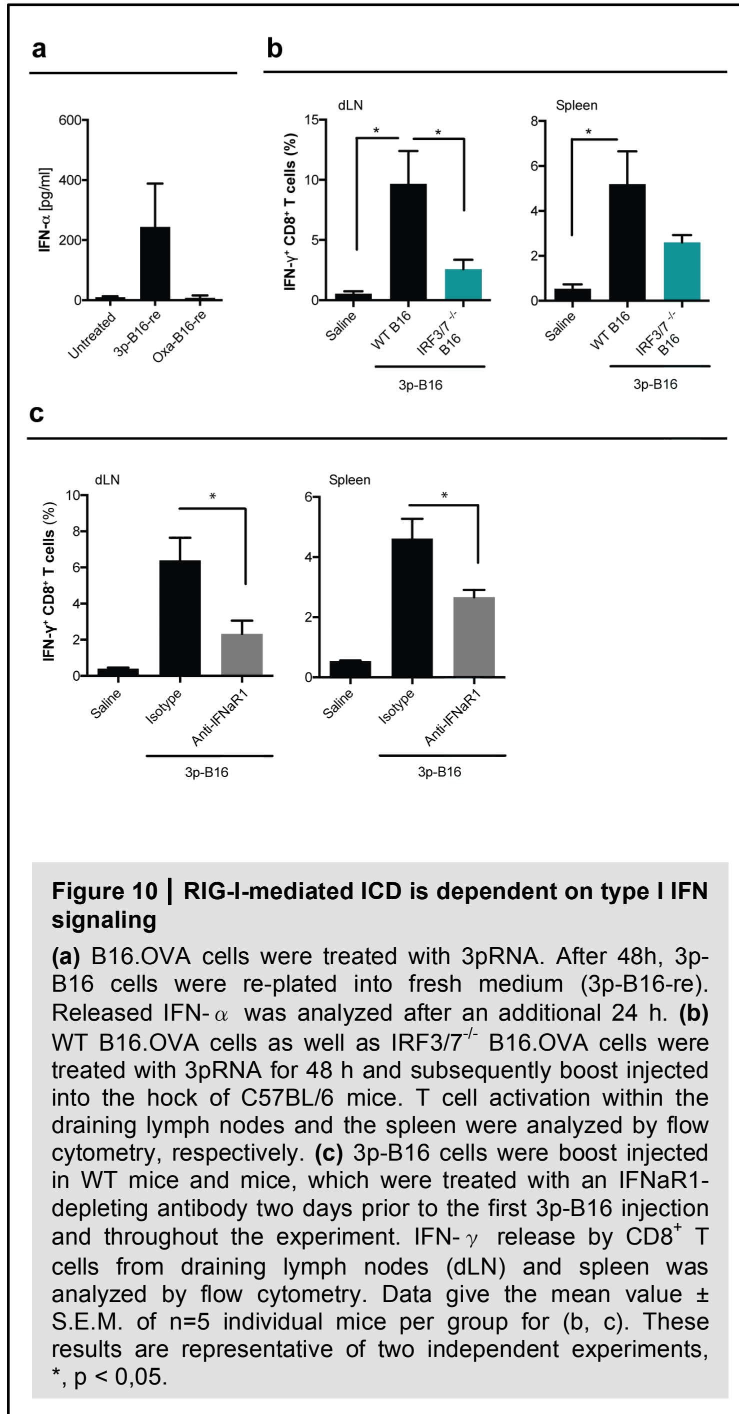

Figure 10 | RIG-I-mediated ICD is dependent on type I IFN signaling

(a) B16.OVA cells were treated with 3pRNA. After 48h, 3p-B16 cells were re-plated into fresh medium (3p-B16-re). Released IFN-α was analyzed after an additional 24 h. **(b)** WT B16.OVA cells as well as IRF3/7$^{-/-}$ B16.OVA cells were treated with 3pRNA for 48 h and subsequently boost injected into the hock of C57BL/6 mice. T cell activation within the draining lymph nodes and the spleen were analyzed by flow cytometry, respectively. **(c)** 3p-B16 cells were boost injected in WT mice and mice, which were treated with an IFNaR1-depleting antibody two days prior to the first 3p-B16 injection and throughout the experiment. IFN-γ release by CD8$^+$ T cells from draining lymph nodes (dLN) and spleen was analyzed by flow cytometry. Data give the mean value ± S.E.M. of n=5 individual mice per group for (b, c). These results are representative of two independent experiments, *, $p < 0,05$.

To investigate the impact of released type I IFN on B16 cells as autocrine feedback and its role in mediating the host immune response the created knock-out cell line deficient for the interferon regulatory factors 3 and 7 (IRF3/7$^{-/-}$ B16.OVA) was used. This IRF3/7 double knock-out cells are deficient in producing and secreting IFN-α and IFN-β (see **Figure 6**). IRF3/7$^{-/-}$ and WT B16.OVA cells were transfected with 3pRNA and were boost injected subcutaneously into the right hock (**Figure 10b**). Recipient CD8$^+$ T cell proliferation and IFN-γ production in draining LNs and spleen was significantly reduced when animals were injected with the RIG-I activated IRF3/7$^{-/-}$ cells compared to RIG-I activated wild-type B16.OVA cells. Thus,

activation of cytotoxic T cells in terms of IFN-γ expression is dependent on the release of type I IFN from 3pRNA-treated melanoma cells.

Next, the role of type I IFN signaling in the host was assessed by silencing this pathway. Therefore, recipient mice were injected with anti-IFNaR1 blocking antibody or its isotype control prior to boost injection with 3p-B16 cells and during the experiment. Draining LNs and spleen were analyzed for cytotoxic T cell activation (**Figure 10c**). Also here, significantly reduced numbers of activated cytotoxic T cells could be observed. Hence, typeI IFN released by either 3pRNA-transfected B16 melanoma cells or by activated host cells play a key role in mediating the antitumor immune response in the host.

This dependency on the IFN signaling led to the suggestion that immunostimulatory nucleic acids may be the transmitters of the immunogenic signal of 3pRNA-transfected B16.OVA cells. Hereby the question arose, how these nucleic acids are protected and transported to safely reach their target cell. Extracellular vesicles (EVs) were recently shown to be able to transport nucleic acids from cell to cell (Colombo *et al.* 2014).

4.5. Characterization of B16.OVA cell-released extracellular vesicles

To address the role of extracellular vesicles in RIG-I-mediated ICD and resulting antitumor immunity, extracellular vesicles (EVs) were isolated from the supernatants of B16.OVA cultures (see also Figure 4 of the material and method section). All EVs were named after their culture origin, thus vesicles isolated from 3pRNA-transfected B16.OVA cells were named 3pEVs and vesicles isolated from untreated cells were named UnEVs. The isolated EVs were analyzed by electron microscopy (EM) (**Figure 11a**) and quantified by Nanoparticle Tracking Analysis (NTA) using NanoSight (**Figure 11b-d**). EM analysis showed homogenously sized vesicles of 30-40 nm in diameter. NTA measurements showed homogenous distribution of vesicles in samples of UnEVs and 3pEVs, respectively. Here, the average vesicle size was 150 nm. The difference in the measured vesicle size is most likely due to particle fixation and thus particle shrinkage during EM analysis. Additionally, it could be observed that the amount of released vesicles was independent of B16 cell stimulation with 3pRNA.

Endosome-derived, small sized extracellular vesicles are called exosomes and are characterized by the presence of proteins involved in membrane transport and fusion such as flotillin-1, and components of the endosomal sorting complex required for transport (ESCRT) such as Alix, heat shock proteins (HSPs), integrins, and tetraspanins, including

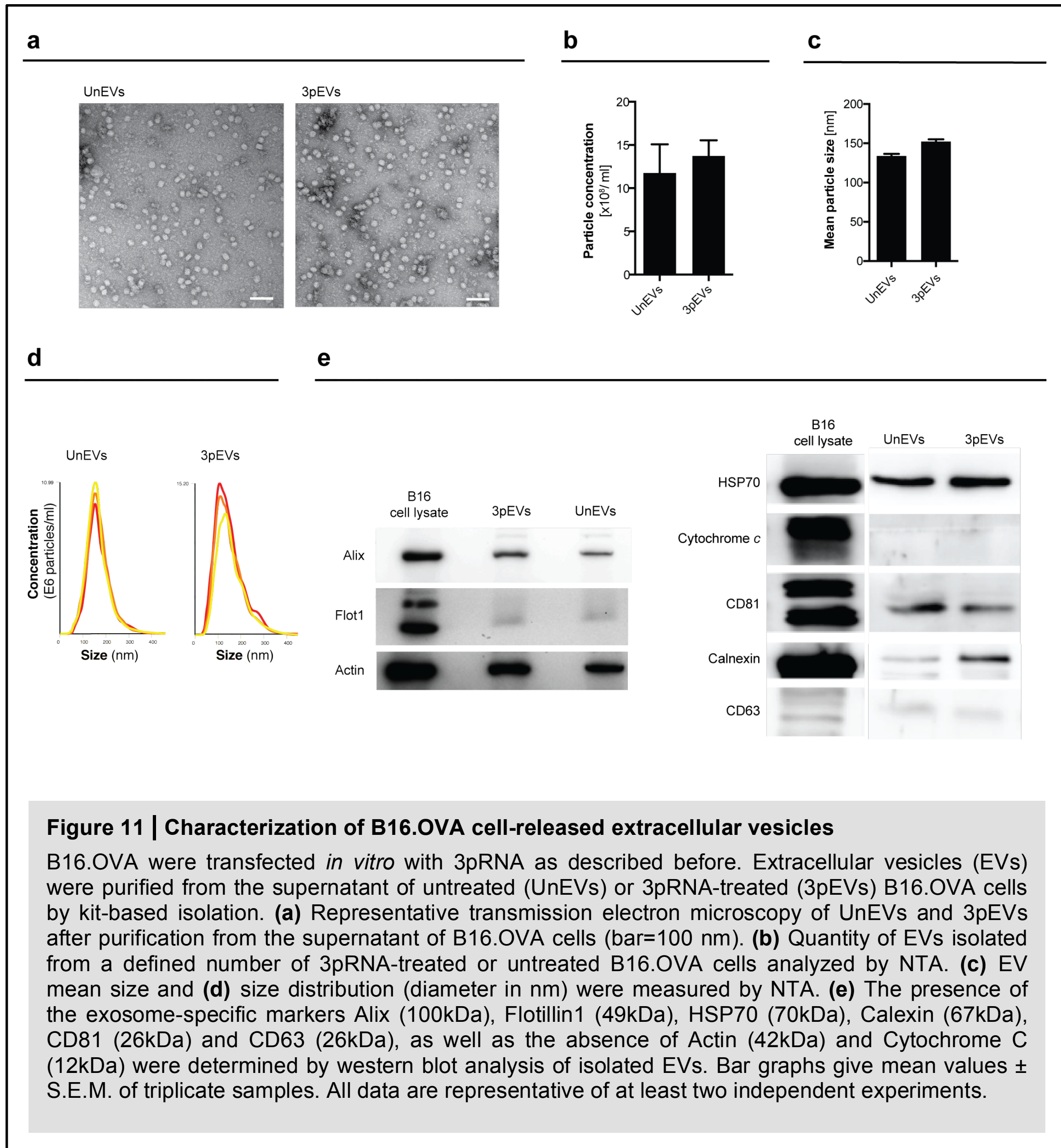

Figure 11 | Characterization of B16.OVA cell-released extracellular vesicles

B16.OVA were transfected *in vitro* with 3pRNA as described before. Extracellular vesicles (EVs) were purified from the supernatant of untreated (UnEVs) or 3pRNA-treated (3pEVs) B16.OVA cells by kit-based isolation. **(a)** Representative transmission electron microscopy of UnEVs and 3pEVs after purification from the supernatant of B16.OVA cells (bar=100 nm). **(b)** Quantity of EVs isolated from a defined number of 3pRNA-treated or untreated B16.OVA cells analyzed by NTA. **(c)** EV mean size and **(d)** size distribution (diameter in nm) were measured by NTA. **(e)** The presence of the exosome-specific markers Alix (100kDa), Flotillin1 (49kDa), HSP70 (70kDa), Calexin (67kDa), CD81 (26kDa) and CD63 (26kDa), as well as the absence of Actin (42kDa) and Cytochrome C (12kDa) were determined by western blot analysis of isolated EVs. Bar graphs give mean values ± S.E.M. of triplicate samples. All data are representative of at least two independent experiments.

CD63, CD81, and CD9 as well as calnexin (Cocucci *et al.* 2009, Simons and Raposo 2009, Thery *et al.* 2009, Bobrie *et al.* 2011). The presence of these known EV surface markers enriched in exosomes was confirmed on the isolated B16 cell-derived EVs by Western blot **(Figure 11e**). Cytochrome *c* is a mitochondrial protein and should not be present in endosomally derived exosomes (Yoshioka *et al.* 2013). The immunoblotting of mitochondrial protein cytochrome c was thus performed as quality control. Cytochrome *c* was readily detectable in the whole cell lysates, but it was completely absent in the purified EV samples indicating that the EV preparations were not contaminated with cellular debris.

Taken together, the isolated EVs showed a homogenous size of 150 nm and carry surface markers characteristic for exosomes. However, classical exosomes are defined by a size ranging from 30 to 120nm. Therefore, despite the presence of canonical exosomal markers, the isolated particles are hereafter generally referred to as extracellular vesicles or short EVs.

4.6. EVs derived from RIG-I activated melanoma cells transduce potent immunogenic information to activate adaptive antitumor immunity

3p-B16 cells induced a strong antitumor immune response in the therapeutic vaccination mouse model (see Figure 8f). To investigate whether 3pEVs released by wild-type B16.OVA cells are also potent enough to control an existing tumor burden, mice were challenged with live B16.OVA cells. When the tumor reached a defined size, mice were treated 3 times with 3pEVs, UnEVs or 3p-B16 cells sc. into the right hock. Control mice were left untreated. The amount of injected EVs was scaled to the number of injected dead cells. In brief, a defined number of cells was treated with 3pRNA for 3pEV purification or left untreated for UnEV purification. The resulting apoptotic cells or the released vesicles (3pEVs, UnEVs) were harvested and injected, respectively. Tumor growth was assessed over time. These experiments showed that 3pEVs exhibit a strong immunogenicity, which translates into potent antitumor activity in this therapeutic application (**Figures 12a**). Repeated injections of 3pEVs resulted in growth arrest of pre-established tumors comparable to the effect of whole dead cell vaccination with B16 cells undergoing RIG-I-mediated ICD. UnEVs however, induced only a weak host immune response against the tumor leading to delayed tumor growth. Only 3pEV or 3p-B16 cell injection led to complete tumor control. In subsequent experiments using depletion antibodies against NK cell and T cell subpopulations, respectively, the 3pEV-induced systemic antitumor immunity was shown to be mediated by both $CD8^+$ T cells and NK cells using the same therapeutic vaccination model (**Figure 12b**). The depletion of one of the two subpopulations largely abrogated tumor control.

To further study 3pEV-mediated immunity, 3pEVs as well as UnEVs were isolated not only from wild-type B16.OVA but also from the culture supernatants of RIG-$I^{-/-}$ B16.OVA cells. With this the role of RIG-I in the release of immunogenic tumor-cell-derived EVs was investigated. The respective EVs were boost injected into the hock of C57BL/6 mice. Draining LNs and spleen were analyzed for activation of $CD8^+$ T cells by flow cytometry (**Figure 13a**). All injected EVs induced low levels of T-cell activation. However, in contrast to steady-state tumor cell-derived particles, treatment with EVs released from melanoma

cells undergoing 3pRNA-induced ICD resulted in significantly enhanced local (dLN) and systemic (spleen) cytotoxic T lymphocyte (CTL) activity in recipient animals. The potent immunogenicity of 3pEVs was critically dependent on tumor cell-intrinsic RIG-I signaling, since their enhanced immunostimulatory capacity was absent in 3pEVs released from RIG-I$^{-/-}$ melanoma cells. Injected UnEVs only showed mild effects on the activation of cytotoxic T cells.

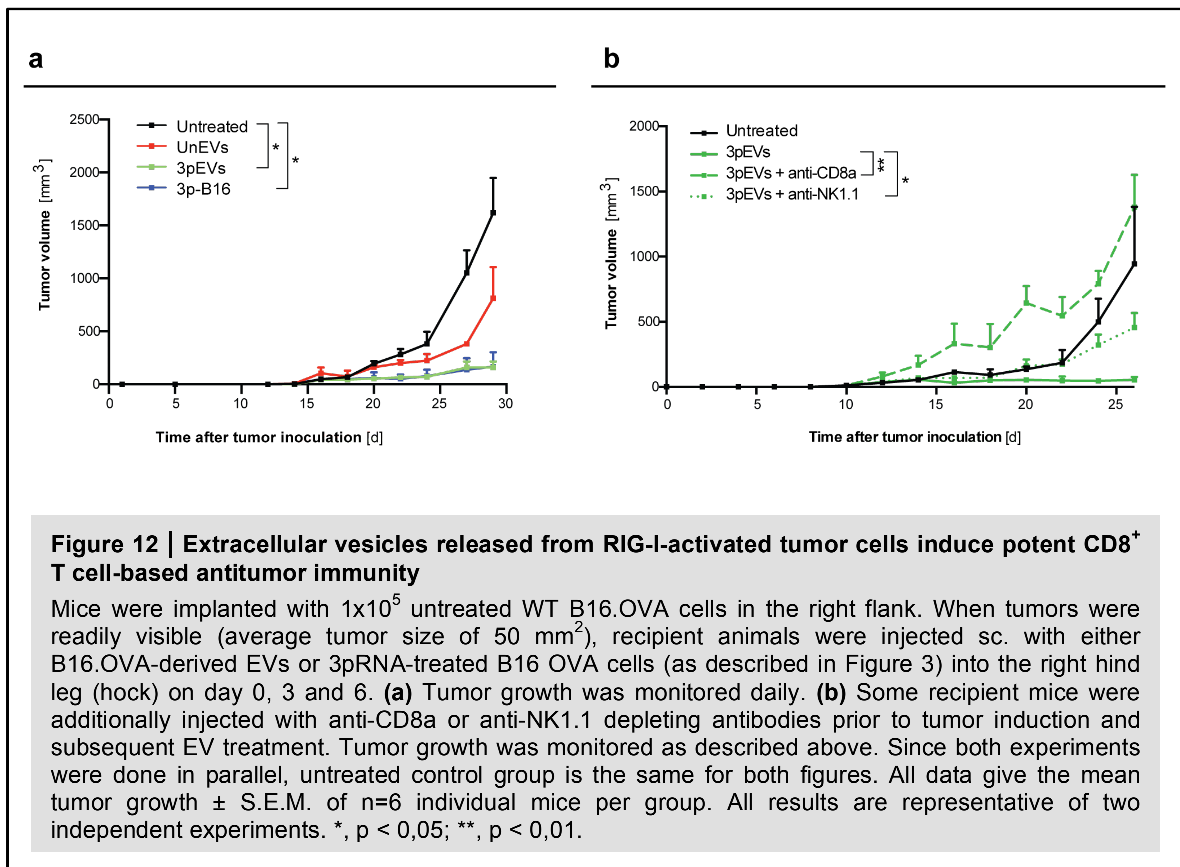

Figure 12 | Extracellular vesicles released from RIG-I-activated tumor cells induce potent CD8$^+$ T cell-based antitumor immunity

Mice were implanted with 1x10^5 untreated WT B16.OVA cells in the right flank. When tumors were readily visible (average tumor size of 50 mm^2), recipient animals were injected sc. with either B16.OVA-derived EVs or 3pRNA-treated B16 OVA cells (as described in Figure 3) into the right hind leg (hock) on day 0, 3 and 6. **(a)** Tumor growth was monitored daily. **(b)** Some recipient mice were additionally injected with anti-CD8a or anti-NK1.1 depleting antibodies prior to tumor induction and subsequent EV treatment. Tumor growth was monitored as described above. Since both experiments were done in parallel, untreated control group is the same for both figures. All data give the mean tumor growth ± S.E.M. of n=6 individual mice per group. All results are representative of two independent experiments. *, $p < 0,05$; **, $p < 0,01$.

Given the important role of type I IFN in RIG-I-mediated ICD, and the subsequent induction of an antitumor immune response, the impact of the type I FN axis within the melanoma cell on the production of immunogenic 3pEVs was assessed. Therefore, mice were boost injected with UnEVs or 3pEVs derived from IRF3/7$^{-/-}$ B16.OVA melanoma cells. Draining LNs and spleen were harvested and analyzed for CD8$^+$ IFN-γ$^+$ CTLs using flow cytometry **(Figure 13b)**. Hereby it was observed that 3pEVs released from IRF3/7$^{-/-}$ melanoma cells failed to induce potent CTL activation. Similar to 3pEVs derived from RIG-I$^{-/-}$ B16, the 3pEVs of IRF3/7$^{-/-}$ B16 did not induce enhanced levels of cytotoxic T cells.

The deletion of RIG-I and IRF3/7 in B16.OVA cells, respectively, results in the loss of type I IFN production and release by 3pRNA-transfected cells as shown in Figure 1 as well as in the loss in producing immunogenic EVs. It was thus assessed, whether the autocrine feedback of released type I IFN (IFN-α and –β) is sufficient enough to stimulate the

production and release of immunogenic EVs. Therefore, B16.OVA cells were treated with type I IFN for 48 h using the same concentrations as observed after 3pRNA-transfection in such cultures. EVs were isolated from the culture supernatant (IFnEVs) and were boost injected into WT recipient mice. As controls, mice were injected with saline, UnEVs or 3pEVs, respectively. Again, cytotoxic T cell activation was analyzed in terms of IFN-γ expression by flow cytometry **(Figure 13c)**. The results showed, that autocrine feedback of type I IFN is not responsible for the release of immunogenic vesicles. IFnEVs were not able to induce T cell activation compared to immunogenic 3pEVs. IFnEVs solely induced a background T cell stimulation as seen for UnEVs. These results indicate that active RIG-I signaling is required for the development and release of immunogenic extracellular vesicles.

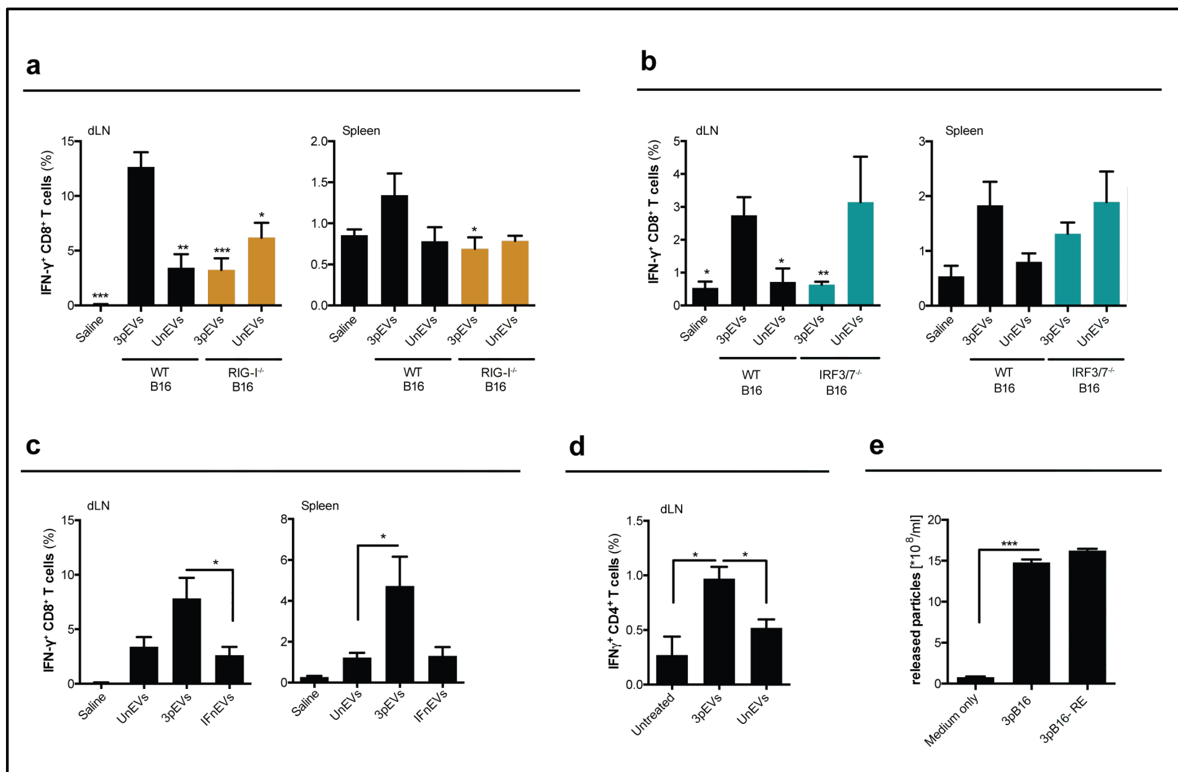

Figure 13 | Tumor-intrinsic RIG-I signaling is a master regulator for the release of immunogenic EVs

WT recipient mice were injected sc. with B16.OVA-derived EVs purified from either untreated (UnEVs) or 3pRNA-treated (3pEVs) cell culture as described before. Herefore, EVs were purified from culture supernatant of WT and either **(a)** RIG-I-deficient (RIG-I$^{-/-}$) or **(b)** IRF3/7-deficient (IRF3/7$^{-/-}$) B16.OVA cells. EV injections were repeated on d7. IFN-γ release by CD8^{+} T cells in draining lymph nodes and spleen was analyzed by flow cytometry. Significance was assessed relative to 3pEV values. **(c)** WT B16.OVA cells were either transfected with 3pRNA as described or were treated with recombinant type I IFN and EVs were subsequently purified. Mice were injected with 3pEVs or IFN-stimulated EVs (IFnEV) as described and IFN-γ release by CD8^{+} T cells was determined. **(d)** IFN-γ release by CD4^{+} T cells in recipient mice that were injected with EVs isolated from *in vitro* pre-treated WT B16.OVA cells. **(e)** B16.cells were transfected with 3pRNA. After 48 h treated cells were re-plated into fresh culture medium. EVs were isolated after additional 24 h from the supernatant. As a control, EVs were isolated from fresh medium only. *In vivo* data give the mean value ± S.E.M. of n = 5 individual mice per group and are representative of two independent experiments. Bar graph (e) give mean values ± S.E.M. of triplicate samples and depicts one of three independent experiments. *, p < 0,05; **, p < 0,01; ***, p < 0,001.

In parallel to cytotoxic T cell activation also activation of CD4$^+$ T cells after 3pEV injection was investigated (**Figure 13d**). It was shown in previous experiments of this dissertation (see Figure 3b) that 3p-B16 cells also induce relevant levels of activated (IFN-γ-expressing) CD4$^+$ T cells. If mice were boost injected with 3pEVs or UnEVs however, this CD4$^+$ T cell activation was less pronounced as compared to 3p-B16 cell injection. Although 3pEVs induce significantly higher amounts of IFN-γ$^+$ CD4* T cells, the percentage of overall activated CD4$^+$ T cells in the draining LN is relatively low.

These results indicate that 3pRNA-transfected B16.OVA cells release immunogenic vesicles dependent on active RIG-I signaling. If these 3pEVs were the immunogenic factor released by 3p-B16 cells leading to potent antitumor immune responses, the 3p-B16 cells must still release these vesicles at the time point of injection into recipient mice. This was investigated by re-plating 3pRNA-transfected B16.OVA cells into fresh medium and isolating the released EVs after additional 24 h. It was observed, that re-plated 3p-B16 cells still release high amounts of extracellular vesicles (**Figure 13e**). With this it is shown that 3pEVs are released from injected 3p-B16 cells are can thus be the immunogenic factor stimulating the antitumor immune response.

Taken together, this set of experiments proofs that the immunogenic factor released by RIG-I activated dying melanoma cells is transported via extracellular vesicles. The released vesicles are enriched in markers known for small sized exosomes of endosomal origin. The injection of such immunogenic 3pEVs is potent enough to stimulate host APCs to drive a local as well as systemic antitumor immune response.

4.7. EVs released from cells undergoing chemotherapy-induced ICD are not immunogenic

In regard to the suggestion that 3pRNA-mediated ICD follows different downstream signaling routes compared to chemotherapeutic agent-mediated ICD, it was further analyzed whether EVs released from cells undergoing chemotherapy-induced ICD are immunogenic. Thus B16.OVA cells were treated with either oxaliplatin or with doxorubicin, both known to be chemotherapeutic ICD inducers. The cells were treated for 48 h with the respective agents, and EVs were isolated from the culture supernatants. EVs isolated from oxaliplatin-treated cells were named Oxa-EVs, EVs isolated from doxorubicin-treated cells were named Doxo-EVs. Both types of EVs were boost injected into C57BL/6 mice, respectively, and T cell activation in draining LNs and spleen was analyzed as described before. Only mice injected with 3pEVs showed increased levels of IFN-γ$^+$ CD8$^+$ cytotoxic

T cells. T cell activation in mice injected with either Oxa-EVs (**Figure 14a**) or Doxo-EVs (**Figure 14b**) was significantly reduced compared to the enhanced levels after 3pEV injection in both, draining LNs and the spleen. UnEV-injection resulted in only low levels of T cell activation. These data confirm that ICD induced by chemotherapeutic agents is not mediated by released extracellular vesicles, and thus follows a different route of host downstream signaling.

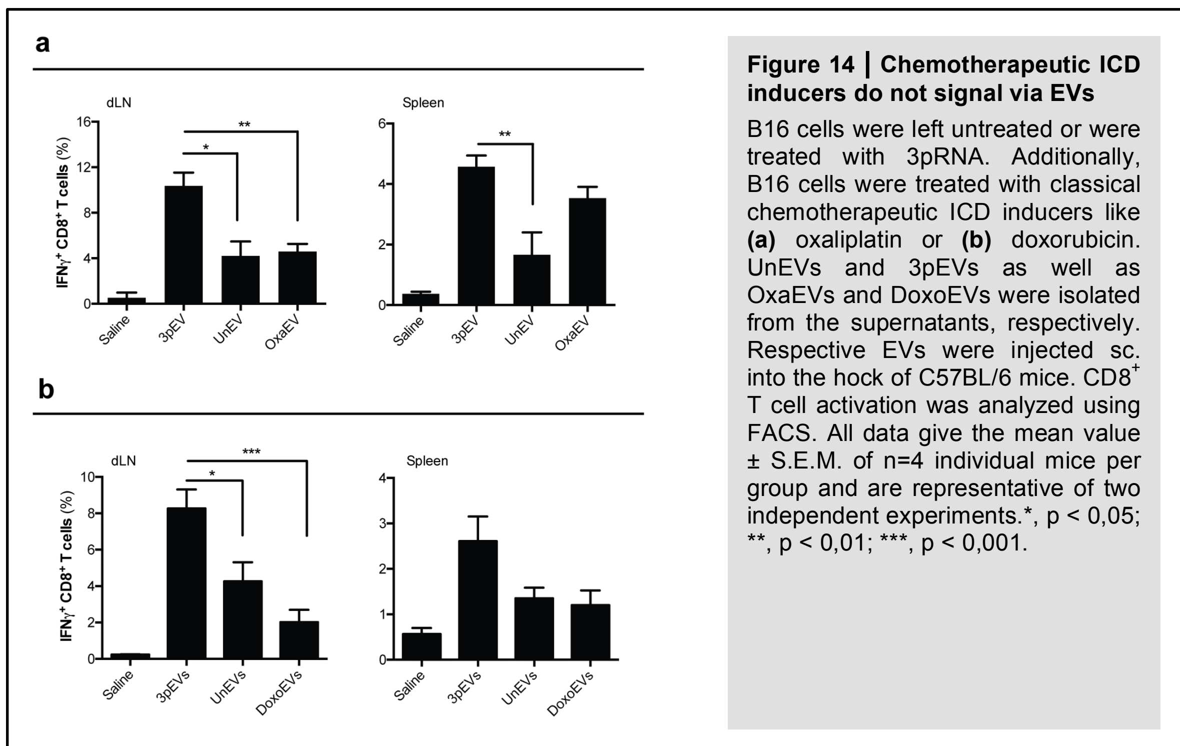

Figure 14 | Chemotherapeutic ICD inducers do not signal via EVs
B16 cells were left untreated or were treated with 3pRNA. Additionally, B16 cells were treated with classical chemotherapeutic ICD inducers like **(a)** oxaliplatin or **(b)** doxorubicin. UnEVs and 3pEVs as well as OxaEVs and DoxoEVs were isolated from the supernatants, respectively. Respective EVs were injected sc. into the hock of C57BL/6 mice. $CD8^+$ T cell activation was analyzed using FACS. All data give the mean value ± S.E.M. of n=4 individual mice per group and are representative of two independent experiments.*, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$.

4.8. Immunogenicity of RIG-I-induced, tumor cell-derived EVs is dependent on host nucleic acid receptor signaling and type I IFN activity in host myeloid antigen-presenting cells

The establishment of a local and systemic immune response after 3p-B16 boost injection was significantly dependent on type I IFN receptor signaling in the recipient host. This observation led to the suggestion, that nucleic acids were transferred by extracellular vesicles. To test, whether this suggestion holds true, T cell activation after 3pEV boost injection in WT mice and in mice pre-treated with anti-IFNaR1 antibodies was examined, respectively (**Figure 15a**). Similar to injected 3p-B16 cells, 3pEVs require host IFN signaling to evolve their immunogenic impact on host immune cells. CTL activation after 3pEV injection was significantly reduced when the receptor for type I IFN signaling was blocked in host cells.

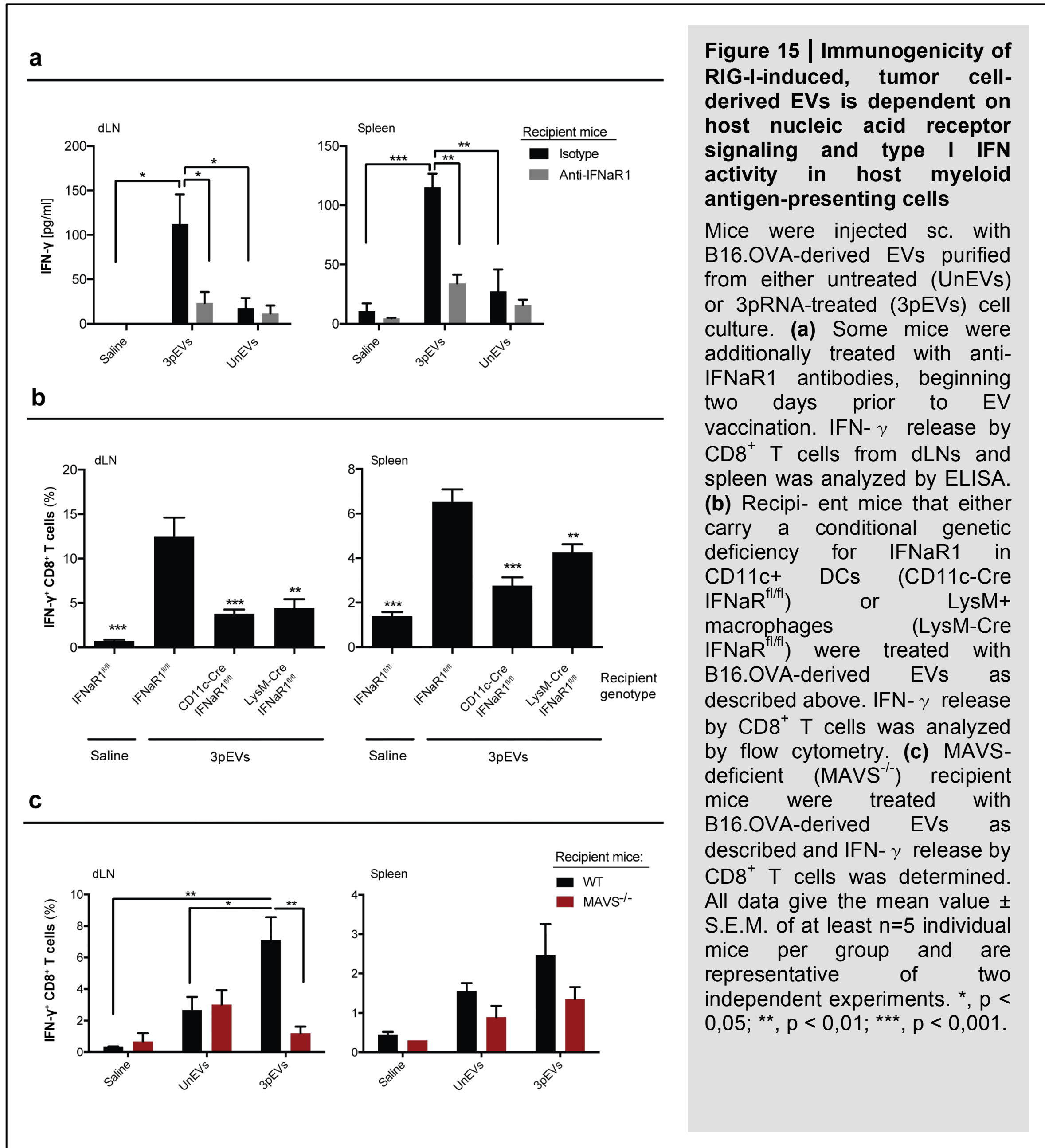

Figure 15 | Immunogenicity of RIG-I-induced, tumor cell-derived EVs is dependent on host nucleic acid receptor signaling and type I IFN activity in host myeloid antigen-presenting cells

Mice were injected sc. with B16.OVA-derived EVs purified from either untreated (UnEVs) or 3pRNA-treated (3pEVs) cell culture. **(a)** Some mice were additionally treated with anti-IFNaR1 antibodies, beginning two days prior to EV vaccination. IFN-γ release by $CD8^+$ T cells from dLNs and spleen was analyzed by ELISA. **(b)** Recipi- ent mice that either carry a conditional genetic deficiency for IFNaR1 in CD11c+ DCs (CD11c-Cre $IFNaR^{fl/fl}$) or LysM+ macrophages (LysM-Cre $IFNaR^{fl/fl}$) were treated with B16.OVA-derived EVs as described above. IFN-γ release by $CD8^+$ T cells was analyzed by flow cytometry. **(c)** MAVS-deficient ($MAVS^{-/-}$) recipient mice were treated with B16.OVA-derived EVs as described and IFN-γ release by $CD8^+$ T cells was determined. All data give the mean value ± S.E.M. of at least n=5 individual mice per group and are representative of two independent experiments. *, p < 0,05; **, p < 0,01; ***, p < 0,001.

Subsequently, the cell type mediating the induction of type I IFN dependent CTL activation was assessed. Mice deficient for IFNaR1 in $CD11c^+$ dendritic cells (Itgax-Cre;$Ifnar^{fl}$ mice) or $LysM^+$ macrophages (LysM-Cre;$Ifnar^{fl}$ mice) were boost injected with 3pEVs and UnEVs, respectively (**Figure 15b**). Compared to the wild-type-like control group ($Ifnar^{fl}$ mice), 3pEV injection in Itgax-Cre;$Ifnar^{fl}$ or LysM-Cre;$Ifnar^{fl}$ mice led to significantly reduced activation of cytotoxic T cells. Hence, type I IFN signaling in both $CD11c^+$ DCs as well as macrophages is involved in mediating the immunogenicity of 3pEVs released from tumor cells that undergo RIG-I-mediated cell death.

Next, T cell activation after EV injection in mice deficient for RIG-I adapter protein MAVS ($MAVS^{-/-}$) was analyzed applying the boost injection setting (**Figure 15c**). 3pEVs induced T cell activation of in WT draining LNs and the spleen. High levels of IFN-γ^{+} CD8^{+} T cells could be analyzed. However, reduced levels of IFN-γ-expressing activated CD8^{+} T cells could be observed in the respective organs of $MAVS^{-/-}$ mice compared to wild-type T cell activation. UnEVs only induced low levels of T cell activation. With this experiments it could be shown, that MAVS is involved in the activation of a cytotoxic T cell response after 3pEVs, which further supports the hypothesis of the presence of immunostimulatory nucleic acids within 3pEVs, More specifically, these experiments suggest the presence of RNA ligands of pattern recognition receptors RIG-I and MDA-5, respectively, which both signal via MAVS.

4.9. Melanoma-derived 3pEVs carry RNA and DNA to activate nucleic acid receptor signaling in host APCs

To further characterize the EV-recipient cells in the host and the 3pEV cargo, syngenic bone marrow-derived dendritic cells (BMDC, or short DC) were cultured in the presence of melanoma-derived EVs. Since STING plays an important role in innate immunity as it works as a direct cytosolic DNA sensor, whereas MAVS is the adaptor protein for signaling cascades of double stranded RNA via RIG-I or MDA-5, BMDCs were isolated from STING- ($STING^{gt/gt}$, hereafter referred to as $STING^{-/-}$) and MAVS-deficient donor mice. To test whether RNA or DNA are transported as EV cargo, respective BMDCs were cultured in the presence of EVs (**Figure 16a, b**). After 24 h, DC activation in terms of released IFN-α. As a control, WT and $MAVS^{-/-}$ DCs were stimulated with 3pRNA, whereas WT and $STING^{-/-}$ DCs were stimulated with a ligand for upstream STING signaling, which is an interferon stimulating DNA (ISD). The results for the controls indicate the respective knock-out. WT BMDCs showed strong activation after the transfection of 3pRNA or ISD, whereas MAVS-/- DCs and STING-/- DCs were not activated after transfection of the specific stimulation, respectively. DC stimulation with UnEVs resulted in all different DC populations in no activation. DC stimulation with 3pEVs resulted in significantly enhanced levels of released IFN-α, thus in DC activation only in WT DCs. Both, DCs deficient for MAVS as well as DCs deficient for STING however, failed to produce relevant levels of IFN-α after stimulation with 3pEVs and are thus not activated by 3pEVs, suggesting that both immunostimulatory RNA as well as DNA are packed into and transferred by EVs released from 3pRNA-transfected melanoma cells.

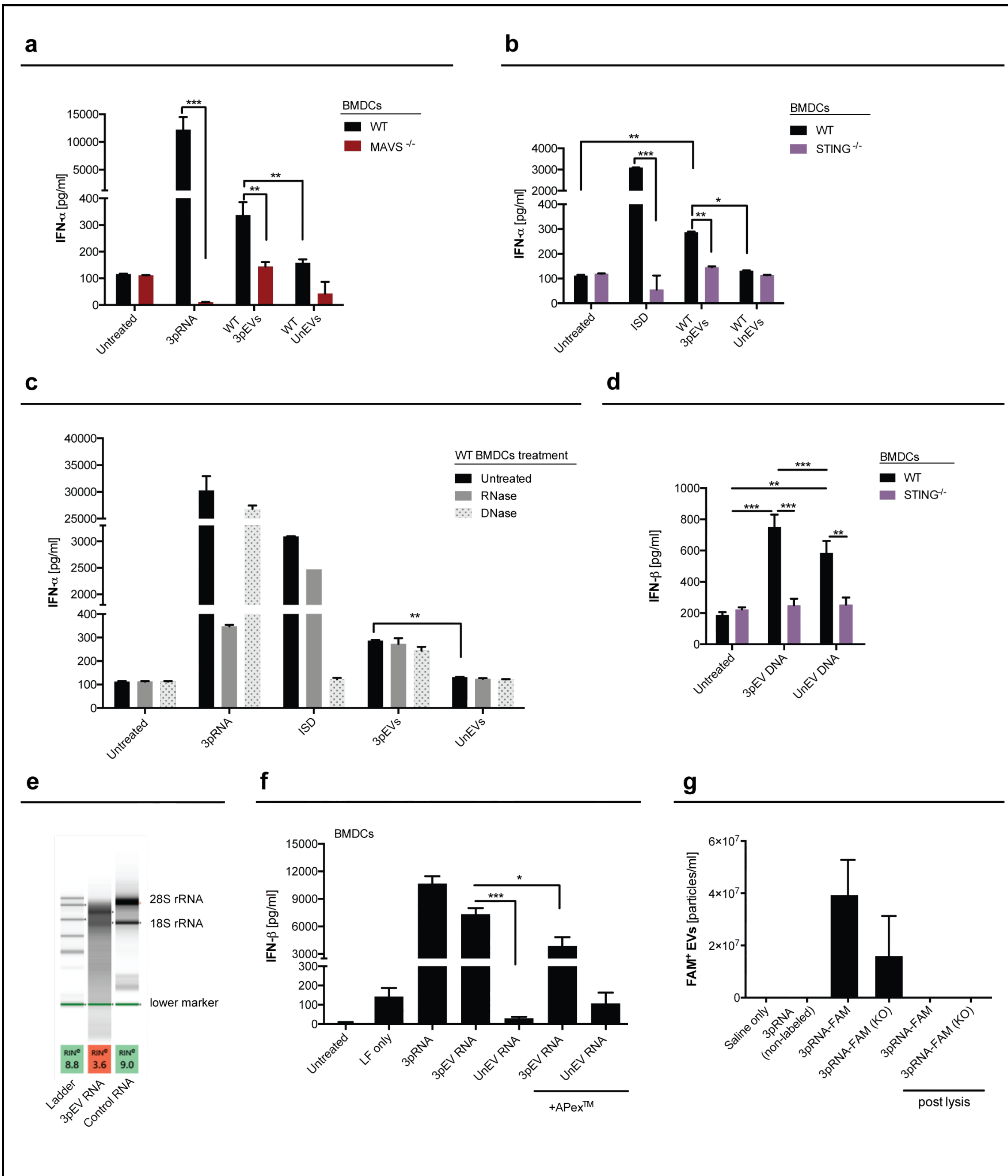

Figure 16 | Tumor intrinsic RIG-I activation mediates shuttling of immunogenic nucleic acids within EVs that induce potent type I IFN production in host DCs

BMDCs derived from WT and **(a)** MAVS-deficient or **(b)** STING-deficient recipient mice were stimulated for 24 h with either 3pRNA/ ISD or EVs derived from B16.OVA. IFN-α release was determined by ELISA. **(c)** EVs derived from WT cells were treated with RNase A or DNase before stimulation of WT BMDCs. Some DCs were stimulated with 3pRNA or interferon-stimulating DNA (ISD). IFN-α release was determined by ELISA. Nucleic acids were extracted from EVs. Their concentrations were analyzed and adjusted. **(d)** UnEV- and 3pEV-DNA was isolated. WT or STING-deficient BMDCs were transfected with either EV-extracted DNA or ISD. IFN-β release was determined by ELISA. **(e)** Gel image of TapeStation (agilent), quality control of 3pEV-derived RNA. **(f)** WT BMDCs were transfected with either EV-extracted RNA or 3pRNA. EV-RNA was additionally treated with an alkaline phosphatase (APex^{TM}). IFN-β release was determined by ELISA. **(g)** 3pRNA was labeled with a fluorescent dye (FAM) and was then transfected into WT or RIG-I$^{-/-}$ B16.OVA cells (KO). EVs were purified and analyzed for FAM signal using single EV flow cytomerty. The post lysis values are based on 3pEV-FAM vesicles, which were lysed before analysis. Protein aggregates would not be lysed by the reagent. No fluorescent EVs after lysis indicate all dye is stored within EVs. The data give the mean value ± S.E.M. of at least triplicate samples and are representative of at least two independent experiments. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$.

It has been shown previously, that EVs can carry high amounts of dsDNA attached to their surface (Thakur *et al.* 2014). To see whether stimulatory RNA or DNA is carried on the EVs surface or is stored within the vesicles, 3pEVs and UnEVs were treated with RNase A, an endoribonuclease digesting ssRNA and dsRNA, and DNase I, an endonuclease that non-specifically digests both ssDNA and dsDNA. Thus nucleic acids attached to EV surfaces were eliminated without affecting nucleic acids within the lumen of EVs. RNase or DNase pre-treated EVs were loaded onto BMDCs and DC activation was analyzed for released INF-α (**Figure 16c**). The result showed no difference in BMDC activation with or without pre-treatment of EVs with RNase A or DNase I, suggesting that the immunostimulatory nucleic acids are transported within the vesicle endoplasm where they are protected from enzymatic digestion.

To verify the presence of nucleic acids within the released vesicles, EV-RNA and -DNA were isolated from 3pEVs as well as from UnEVs. After disruption of the vesicle membrane, it was possible to isolate nucleic acids from the lumen of the vesicle, although concentrations were very low. The EVs isolated from 40 ml of B16.OVA culture medium gave rise to a total amount of around 15 ng of EV-DNA or around 600 ng of EV-RNA, respectively (data not shown). EV-DNA was transfected into BMDCs using the same amount of EV-DNA isolated from UnEVs or 3pEVs (**Figure 16d**). Transfected 3pEV-DNA induced WT DC activation, whereas the same DNA had no effect on DCs derived from $STING^{-/-}$ mice. Although significantly reduced compared to 3pEV-DNA, also transfected UnEV-DNA could activate WT DCs showing that immunostimulatory DNA is present within both types of vesicles.

Next, 3pEV-RNA and UnEV-RNA were also scaled and the equivalent amount of EV-RNA was transfected into BMDCs. Purified and transfected 3pEV-RNA was found to be effective in activating WT BMDCs whereas UnEV-RNA did not show to have any impact on DC activation. The dependency of EV-mediated DC activation on the RNA signaling adapter molecule MAVS (see Figure 10c) rose the question whether 3pRNA was present within 3pEVs. To investigate this, the isolated EV-RNA was further treated with APex™ (Epicentre), an alkaline phosphatase (AP) responsible for removing phosphate groups from many types of molecules including nucleotides. The possibly present 3pRNA was thus dephosphorylated and lost its effect on RIG-I (and thus on signaling via MAVS). BMDCs stimulated with the AP-treated EV-RNA showed significantly reduced DC activation (**Figure 16e**) suggesting that 3pRNA is indeed present within the 3pEVs.

Whether this EV intrinsic 3pRNA is the exogenously applied, *in vitro* transcribed 3pRNA or whether it is an endogenously generated and packed 3pRNA is unclear. To investigate this, exogenously applied 3pRNA was fluorescently labeled (FAM) before transfecting the RNA into WT or RIG-I$^{-/-}$ B16.OVA cells. If this applied RNA is packed into the vesicles, the released 3pEVs should be fluorescently marked. By the use of the 'single EV FACS' technique (see M+M section 3.2.4.3.), single EVs can be analyzed for the presence of fluorescent markers. The FAM-3pEVs isolated from the respective cell lines as well as control 3pEVs generated with non-labeled 3pRNA using wild-type B16.OVA cells were analyzed with this technique (**Figure 16f**). The results clearly showed that some isolated 3pEVs contain exogenously applied 3pRNA. Fluorescent 3pEVs however, could only be detected in vesicles isolated from wild-type B16.OVA. RIG-I$^{-/-}$ B16-derived 3pEVs did not carry the fluorescently labeled 3pRNA.

Taken together, these experiments proved the presence of immunostimulatory DNA and RNA within the released 3pEVs. Moreover, among other short RNA molecules, the exogenously applied 3pRNA was shuttled from the RIG-I-activated tumor cell into the vesicles, which are subsequently released into the extracellular space.

4.10. Tumor-cell-derived EVs carry tumor-associated antigens independent from RIG-I activation

The transferred immunostimulatory nucleic acids alone would not be sufficient to prime an effective adaptive immune response involving antigen specific cytotoxic CD8$^+$ T cells as the host recipient cells also require a second signal in form of antigen. Released 3pEVs were therefore analyzed for the presence of tumor-associated antigens (TAAs). Since Ovalbumin functions as a model TAA within B16.OVA cells, the presence of Ovalbumin protein within the released EVs was analyzed. 3pEVs and UnEVs were therefore isolated from the supernatants of B16.OVA cells and were subsequently lysed for protein analysis by Western blot. As a control, equivalent EVs were isolated from B16.F10 cells, which are basically the same melanoma cells but do not express the ovalbumin protein (**Figure 17a**). The blot demonstrated the presence of OVA protein within EVs isolated from B16.OVA cells. Of note, 3pEVs as well as UnEVs carry the same amount of antigen. EVs isolated from non-OVA-expressing B16.F10 cells did not show the presence of OVA. Additionally, another melanoma-associated antigen gp100, expressed by both B16.OVA and B16.F10, was analyzed for its presence in the isolated EV samples. The protein could be shown to be present within 3pEVs as well as UnEVs from both cell types. For both experiments, β-tubulin was chosen as a loading control, which is actually present in the isolated EVs.

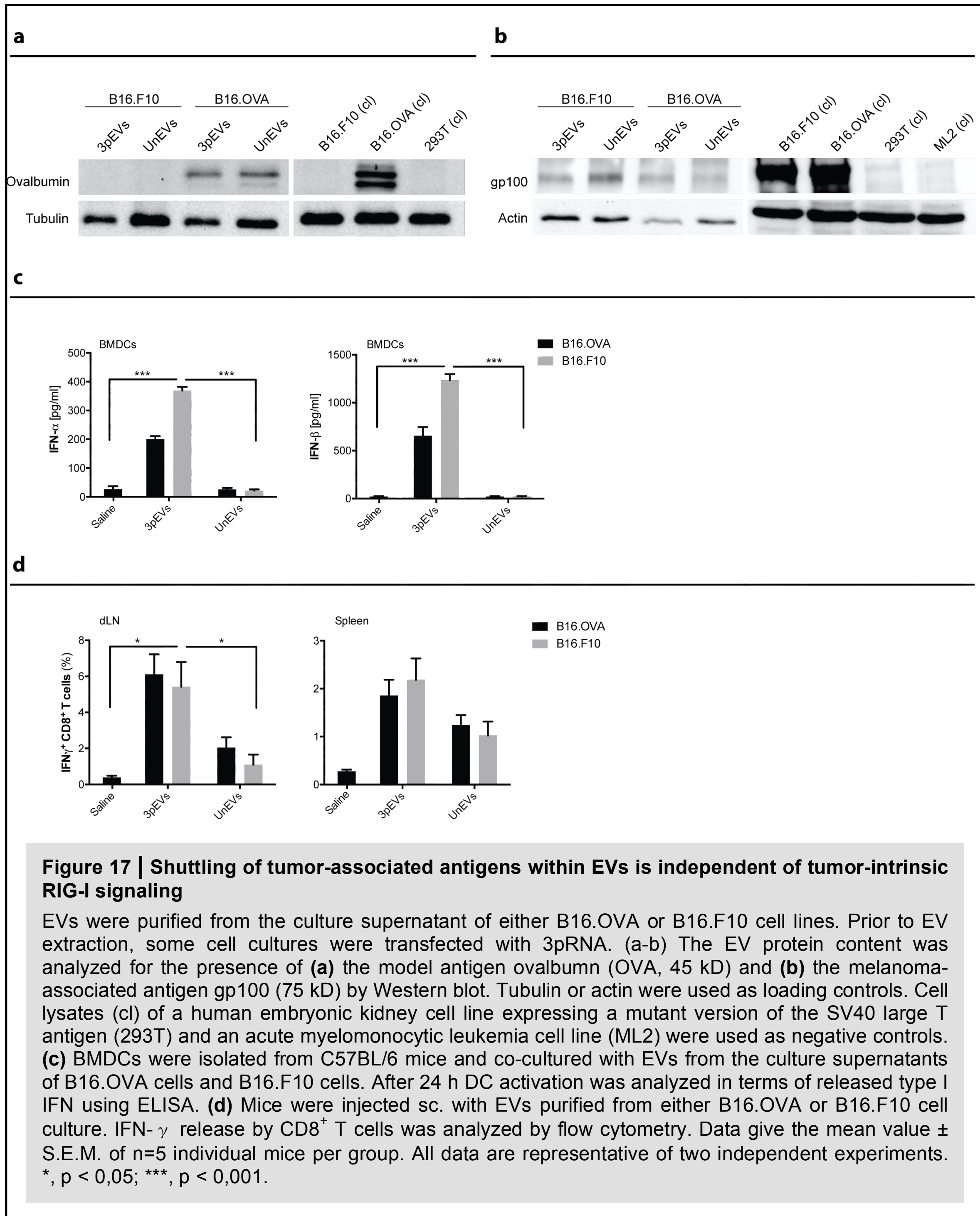

Figure 17 | Shuttling of tumor-associated antigens within EVs is independent of tumor-intrinsic RIG-I signaling

EVs were purified from the culture supernatant of either B16.OVA or B16.F10 cell lines. Prior to EV extraction, some cell cultures were transfected with 3pRNA. (a-b) The EV protein content was analyzed for the presence of **(a)** the model antigen ovalbumn (OVA, 45 kD) and **(b)** the melanoma-associated antigen gp100 (75 kD) by Western blot. Tubulin or actin were used as loading controls. Cell lysates (cl) of a human embryonic kidney cell line expressing a mutant version of the SV40 large T antigen (293T) and an acute myelomonocytic leukemia cell line (ML2) were used as negative controls. **(c)** BMDCs were isolated from C57BL/6 mice and co-cultured with EVs from the culture supernatants of B16.OVA cells and B16.F10 cells. After 24 h DC activation was analyzed in terms of released type I IFN using ELISA. **(d)** Mice were injected sc. with EVs purified from either B16.OVA or B16.F10 cell culture. IFN-γ release by $CD8^+$ T cells was analyzed by flow cytometry. Data give the mean value ± S.E.M. of n=5 individual mice per group. All data are representative of two independent experiments. *, $p < 0,05$; ***, $p < 0,001$.

Since the chicken egg-derived ovalbumin is a strong and artificial antigen, it was investigated whether the immunogenic impact of B16.OVA-derived 3pEVs is driven by the presence of OVA within the 3pEVs. Therefore, 3pEVs and UnEVs were isolated from B16.F10 cells. The EVs were either loaded onto WT BMDCs *in vitro* (**Figure 17b**) or boost injected into WT mice to analyze T cell activation *in vivo* (**Figure 17c**). Both experiments proved that the 3pEV-mediated immune response is not dependent on OVA protein.

Instead, these results suggest that OVA and gp100 are not the only TAAs present within the EVs.

Taken together, these results suggest the presence of several different tumor-associated antigens within the released extracellular vesicle. These TAAs are derived from and thus define the originating mother tumor cell.

In summary, this study shows that tumor-intrinsic RIG-I activation results in immunogenic cell death of melanoma cells, and thus proofs the transferability of 3pRNA-induced ICD in different tumor entities. Additionally, the present data identifies the immunogenic factor released by RIG-I-activated dying tumor cells responsible for the establishment of an antitumor immune response. We could show that the immunogenic factor is shuttled within released extracellular vesicles. Finally, it could be shown, that these extracellular vesicles carry immunostimulatory RNA and DNA together with tumor associated antigens, making these vesicles a powerful tool in the battle against cancer.

5. Discussion

Immunogenic cell death of malignant cells induced by anthracyclines or oxaliplatin has been intensively studied in recent years (Martins *et al.* 2014). Additionally, it was shown only recently, that activation of RIG-I-like helicases in pancreatic adenocarcinoma cells leads to the induction of immunogenic cell death. However, the immunogenic factors released by RIG-I-activated dying tumor cells triggering a tumor-specific immune response have not been elucidated yet. The present work demonstrates that RIG-I-induced ICD also occurs in other malignancies. More importantly, this study led to the identification of the released immunogenic factor solely sufficient in driving potent adaptive immunity involving antigen-specific cytotoxic T lymphocytes. This factor was shown to be extracellular vesicles released by RIG-I-activated melanoma cells containing immunostimulatory nucleic acids as well as tumor-associated antigens and thus all information required for the development of an effective antigen specific immune response. In this section, the critical points of the project are discussed.

5.1. Using CRISPR/Cas9 for the generation of knock-out cell lines

To study the role of RIG-I and its pathway in immunogenic tumor cell death, several knock-out cell lines were generated using CRISPR/Cas9 genome editing. In this study CRISPR/Cas9 was applied to generate RIG-I$^{-/-}$ B16.OVA cells and IRF3/7$^{-/-}$ B16.OVA cells.

Broadly speaking, genome editing refers to the process of making targeted modifications to the genome, its contexts (e.g., epigenetic marks), or its outputs (e.g., transcripts) (Hsu *et al.* 2014). A number of genome editing technologies have emerged in recent years, including zinc-finger nucleases (ZFNs) (Porteus and Baltimore 2003, Sander *et al.* 2011), transcription activator-like effector nucleases (TALENs) (Zhang *et al.* 2011, Sanjana *et al.* 2012) and the RNA-guided CRISPR/Cas nuclease system (Horvath and Barrangou 2010, Cho *et al.* 2013, Cong *et al.* 2013). The first two technologies use a strategy of tethering endonuclease catalytic domains to modular DNA-binding proteins for inducing targeted DNA double-strand breaks (DSBs) at specific genomic loci. In contrast, Cas9 is a nuclease guided by small RNAs which mediate binding to target DNA by Watson-Crick base pairing (Garneau *et al.* 2010, Jinek *et al.* 2012). As with other designer nuclease technologies such as ZFNs and TALENs, Cas9 can facilitate targeted DNA DSBs at

specific loci of interest in the mammalian genome and stimulate genome editing via non-homologous end joining (NHEJ) or homology directed repair (HDR) mechanisms. However, Cas9 offers several potential advantages over ZFNs and TALENs. The Cas9 system is easy to handle and is less time consuming compared to ZFNs or TALENs. Additionally, Cas9 shows higher targeting efficiency and higher specificity then ZFNs or TALENs, and only Cas9 offers the ability to facilitate multiplex genome editing (Ran *et al.* 2013). Thus, for the generation of the B16.OVA knock-out cell lines Cas9 targeting was applied.

Nevertheless, there are also limitations to the Cas9 system. Cas9 can be targeted to specific genomic loci via a 20-nt guide sequence on the sgRNA. The only requirement for the selection of Cas9 target sites is the presence of a PAM sequence directly 3' of the 20-bp target sequence. Each Cas9 ortholog has a unique PAM sequence; for example, SpCas9 – the Cas9 nuclease of *Streptococcus pyogenes*, which was used in this study, requires a 5'-NGG PAM sequence. This PAM requirement does not severely limit the targeting range of SpCas9. In e.g. the human genome, such target sites can be found on average every 8-12 bp (Cong *et al.* 2013, Hsu *et al.* 2013). In addition to the targeting range, another possible limitation is the potential for off-target mutagenesis. Similarly to other nucleases, Cas9 can cleave off-target DNA targets in the genome at reduced frequencies (Fu *et al.* 2013, Hsu *et al.* 2013, Jiang *et al.* 2013). The extent to which a given guide sequence exhibits off-target activity depends on a combination of factors including enzyme concentration and the abundance of similar sequences in the target genome. However, it is now possible to computationally assess the likelihood of a given guide sequence to have off-target sites. These analyses are performed through an exhaustive search in the genome for off-target sequences that are similar to the guide sequence. Comprehensive experimental investigation of the effect of mismatching bases between the sgRNA and its target DNA revealed that mismatch tolerance is (i) position dependent: the 8-14 bp on the 3' end of the guide sequence is less tolerant of mismatches than the 5' bases; (ii) quantity dependent: in general, more than three mismatches are not tolerated; (iii) guide sequence dependent: some guide sequences are less tolerant of mismatches than others; and (iv) concentration dependent: off-target cleavage is highly sensitive to the transfected amounts, as well as relative ratios of Cas9 and sgRNA (Hsu *et al.* 2013, Ran *et al.* 2013). Based on this knowledge, John Doench and colleagues developed fully optimized sgRNA libraries for the human and mouse genomes, named Brunello and Brie, respectively. These libraries consist of sgRNAs of improved on-target activity predictions incorporated with an off-target avoidance metric.

Brunello and Brie thus represent a clear improvement over existing libraries and are available via Addgene (Doench *et al.* 2016).

To maximize the on-target activity and to reduce the degree of off-target genome modification, for this work, sgRNAs were selected from this Brie library in order to generate the respective B16.OVA knock-out cell lines.

5.2. RIG-I-induced immunogenic cell death

5.2.1. Targeting RIG-I in melanoma cells

In this study, RIG-I was targeted in B16.OVA melanoma cells to trigger 1) tumor cell death and 2) the release of immunogenic extracellular vesicles. RIG-I was activated by its specific ligand 3pRNA, which was generated by *in vitro* transcription (IVT) from a DNA template using a T7 RNA polymerase.

It has been reported before that an essential structural feature of the (viral) RNA ligand of RIG-I is a free 5′-triphosphate end that is normally absent from host cytoplasmic RNA due to eukaryotic RNA cap structures (Hornung *et al.* 2006, Schmidt *et al.* 2009). Using short 5′-triphosphate RNAs of 19 to 21 bases produced by IVT, these studies concluded that both single-stranded (ss) and double-stranded (ds) RNAs activate RIG-I as long as they carry the 5′-triphosphate (Hornung *et al.* 2006, Pichlmair *et al.* 2006). The regulatory domain of RIG-I has subsequently been characterized as the structural entity that binds 5′-triphosphorylated RNA and, thus, aids in defining ligand specificity (Cui *et al.* 2008, Takahasi *et al.* 2008). *In vitro* transcribed RNAs have thus been widely used to probe both the ligand requirements for RIG-I signaling in cells and the mechanisms of RIG-I activation *in vitro*. However, the group of Rothenfusser discovered that IVT products must be used with caution. In their study, the functional analysis of IVT products revealed that besides expected 3pRNA transcripts also non-template hairpin RNAs were generated through the RNA-dependent RNA polymerase activity of T7, and that these hairpin structures were immunostimulatory RNA species (Schmidt *et al.* 2009). To circumvent this problem, Schmidt et al. generated chemically synthesized 5′-triphosphate RNA (syn-ppp-RNA). Signaling of chemically synthesized 5′-triphosphate RNAs showed the same RIG-I-dependence seen before with ligands generated by IVT. However, chemical synthesis of 3pRNA is time-consuming and costly. Thus, in this study IVT 3pRNA was used for all experiments having in mind, that non-templated hairpin RNAs may be present and may have an effect on the results.

5.2.2. Receptor pathways involved in the execution of RIG-I-mediated cell death in tumor cells

In order to investigate the immunogenicity of RIG-I activation in melanoma cells, the resulting cell death was compared to the cell death induced after treatment with known chemotherapeutic ICD inducers. ICD is described for only a specific subset of chemotherapeutic agents approved for the treatment of certain malignancies (Pol *et al.* 2015). However, none of the known chemotherapeutic ICD-inducing agents have been approved for the treatment of melanoma. The agent oxaliplatin was chosen as a control for chemotherapeutic ICD in the experiments. Although, oxaliplatin is only approved for the therapy of advanced colorectal carcinomas (Tesniere *et al.* 2010, Galluzzi *et al.* 2012a) several preclinial studies showed significant activity of oxaliplatin against malignant melanoma (Locke *et al.* 2010, Hatch *et al.* 2014).

The results in this study showed that both the treatment with oxaliplatin as well as RIG-I activation resulted in efficient B16.OVA cell death along with the release of ICD hallmark including DAMPs ATP, HMGB1 and CRT (Figure 2b). For the immunogenic effect of tumor cell death after treatment with chemotherapeutic ICD inducers, the release of all three DAMPs have previously been shown to be essential (Kroemer *et al.* 2013). It could be further elucidated that, in response to anthracycline-based chemotherapy, autophagy-competent tumor cells release ATP as they die and hence attract macrophages and DCs to their close proximity. At least part of the effects of ATP on DCs is mediated by P2RX7 receptors. In response to P2RX7 ligation, the efflux of K+ and Ca2+ ions can lead to the activation of the NLRP3 inflammasome, a caspase-1 activation platform, thus stimulating the proteolytic maturation and subsequent secretion of IL-1β and IL-18, as well as a specialized form of inflammation-associated cell death called ‘pyroptosis’ (Zitvogel *et al.* 2012). ICD hallmark DAMPs however, did not appear to have the same impact on immunogenic cell death upon RIG-I activation (Figure 4). It was shown in ASC-deficient mice as well as in NLRP3-deficient mice, that the cytotoxic T cell activation after boost injection of 3p-B16 cells was not dependent on the involvement of the host inflammasome when compared to WT mice, leading to the suggestion that at least the released ATP, in spite of the possible attraction of antigen presenting cells, did not play an essential role.

In addition to these ICD hallmark DAMPs, and only after RIG-I activation, B16.OVA cells released relevant levels of type I IFN (Figure 2d). In the subsequent experiments it was repeatedly shown, that in the absence of type I IFN signaling, the immunogenic effect of RIG-I-activated melanoma cell death is completely abolished. Thereby, both B16.OVA cell

intrinsically generated type I IFN after RIG-I stimulation as well as host type I IFN signaling after inoculating dying B16.OVA cells might be involved in the immunogenic effect. (Duewell *et al.* 2014)

It is known from several previous studies, that initial production of type I IFN is enhanced by a positive feedback loop based on the ability of IFN-β and IFN-α to induce numerous IFN-stimulated genes (ISGs) (Sadler and Williams 2008, Goubau *et al.* 2013). By using IRF3/7-/- B16.OVA cells, it could be identified that this positive feedback, drives not only the immunogenicity of RIG-I-induced tumor cell death but also the level of cell death of the melanoma cells, since levels of cell death were reduced in the knock out cells (data not shown). This is most probably due to the abrogated type I IFN feedback, which would otherwise have led to an enhanced expression of RIG-I and IRF-7, as they are ISGs, and is thus an essential aspect for the amplification of type I IFN signaling (Honda *et al.* 2005b). In mouse models, using IFNAR1-neutralizing monoclonal antibodies, it could be shown that host type I IFN signaling is essential for the therapeutic effect of boost injected 3p-B16 cells (Figure 5c).

5.2.3. Role of host type I IFN

The mechanism by which the host immune system initiates innate immune sensing of tumors and thereby bridges to induction of an adaptive, tumor-specific T cell response has long been unknown. Research on innate immune recognition of tumors *in vivo* forming the bridge to adaptive immunity has pushed the problem further upstream towards the identification of the receptor system and the tumor-derived ligand that mediate this effect. It has been suggested that endogenous adjuvants released from dying cells are capable of initiating innate immune cell activation (Jounai et al., 2012; Kono and Rock, 2008; Marichal et al., 2011; McKee et al., 2013). Spontaneous tumor antigen-specific T cell priming, when it does occur, appears to be dependent on type I IFN signaling in host cells, via a mechanism that involves promotion of cross-presentation by CD8α$^+$ DCs (Diamond et al., 2011; Fuertes et al., 2011). Type I IFNs have a wide range of immune-stimulatory activities, including the augmentation of T helper type 1 cell responses, upregulation of major histocompatibility (MHC) class I molecules, generation of natural killer (NK) cell- and T cell-mediated cytotoxicity, and antitumor activities, including anti-proliferative, anti-angiogenic, and pro-apoptotic effects (Lee *et al.* , Trinchieri 2010). Thus, PRR-mediated cell death and release of type I IFN can cooperatively and synergistically induce both therapeutic and prophylactic cellular immune responses against tumors.

The mechanism by which (dying) tumors induce type I IFN production however, is a topic of active investigation. Infectious disease models have indicated at least three pathways of innate immune sensing that can drive transcription of type I IFNs: TLR signaling through the adaptors MyD88 and TRIF, RIG-I sensing of cytosolic RNA leading to signaling through the adaptor MAVS, and the STING pathway sensing cytosolic DNA (Barber 2011, Gajewski *et al.* 2012). Recent research indicates that the host STING pathway is particularly critical for innate immune sensing of immunogenic tumors, a process that results in APC activation, IFN-β production, and priming of $CD8^+$ T cells against tumor antigens *in vivo,* describing tumor-derived DNA as the likely ligand for this pathway (Woo *et al.* 2014). Another study suggested that some immunogenic chemotherapeutics, including anthracyclines like doxorubicin, promote the activation of TLR3 in mouse and human malignant host cells by cancer cell-derived RNA, which results in the secretion of type I IFN. type I IFN then activated an autocrine or a paracrine IFNAR-dependent circuit that results in the expression of various ISGs, including CXC-chemokine ligand 10 (CXCL10; which is a potent chemoattractant for innate immune cells) and the antiviral factor MX dynamin-like GTPase 1 (MX1) (Casares *et al.* 2005, Zitvogel *et al.* 2013, Zitvogel *et al.* 2015).

Also in the present study, type I IFN signaling was shown to be essential for the establishment of a potent antitumor immune response after administration of 3p-B16 cells. Indeed it could be shown, as discussed below, that this response was mediated through immunostimulatory nucleic acids released from RIG-I-activated tumor cells that were shuttled within extracellular vesicles. *In vitro* data revealed that both, immunogenic RNA and DNA are present within the released vesicles activating the downstream adaptor proteins MAVS and STING in host dendritic cells, respectively. Both pathways lead to induction of type I IFN production and release. Although this study revealed that type I IFN signaling is essential for the establishment of antitumor immunity, the downstream machinery will need further clarification. At the moment, it can be concluded, that these data elucidate further details on RIG-I-induced immunogenic tumor cell death transmitted trough tumor-derived nucleic acids.

5.3. Extracellular vesicles

5.3.1. Precipitation-based isolation of extracelular vesicels

One possible tool for the safe transport of immunostimulatory nucleic acids from dying cancer cells to other recipient cells are extracellular vesicles. Extracellular vesicles released by 3pRNA-treated or untreated B16.OVA cells were thus isolated from cell

culture supernatants using the Total Exosome Isolation Reagent (from cell culture media) (Thermo Fischer).

A critical issue that has to be considered when isolating EVs from conditioned cell culture media is the presence of an additional 'artificial' EV source originating from fetal bovine serum (FBS) (Thery *et al.* 2006, Jeppesen *et al.* 2014). It was shown that the removal of FBS-originated EVs is critical for further downstream experiments, since these vesicles are capable of inducing effects similar to those of EVs isolated from the actual murine cell culture media (Lotvall *et al.* 2014). To counter this problem, FBS was filtered throuh a 0.2 µm filter and subsequently centrifuged for 16 h at 100.000x g using an untracentrifuge before FBS was added to the culture medium. An alternative may be the use of an exosome-free FBS, which is already commercially available, but is still rather expensive.

Another major issue involving EV isolation from conditioned cell culture media regards the culture medium itself. NTA results revealed that cell culture medium contains a trace of particles that resemble EVs in their size(Jeppesen *et al.* 2014, Szatanek *et al.* 2015). The presence of these background particles itself puts tremendous strain on the integrity of the final EV isolation results. It was shown that the storage temperature seems to have an effect on the occurrence of these particles in a cell culture medium. Cell culture medium stored at room temperature showed more background particles than the one stored at 4°C as observed by NTA. According to this studies, the presence of EVs in culture medium itself was analyzed using NTA (**Figure 8e**) which revealed minimal background of particles. Nontheless, culture media were always stored at 4 °C.

Recently, several alternative methods were introduced and utilized for isolation and purification of EVs, including differential ultracentrifugation (UC), antibody-coated magnetic beads, microfluidic devices, precipitation technologies (e.g. the Total Exosome Isolation Reagent), and filtration technologies. However, there is still an urgent need for more efficient, reliable and reproducible EV extraction methods, so that studies in the field of EV research can be more standardized and efficient. A recent study tested and compared isolation methods based on precipitating agents, filtration-based protocols or column-based protocols with classical UC methodology. For downstream analysis, particle yield and size was quantified by NTA and the presence of classical EV protein markers in EV preparations was demonstrated. Thereafter, miRNA extraction yield of each method was assessed. These results point to polyethylene glycol (PEG)-based precipitation as an easy, economic and quick method to enrich EV for a good performance in the subsequent miRNA extraction (Andreu *et al.* 2016).

Because UC of B16.OVA culture supernatant did not lead to a high yield of purified EVs, in this study, EVs were isolated using the Total Exosome Isolation Ragent, which is based on PEG-precipitation.

5.3.2. Characterization of extracellular vesicles

EVs isolated from B16 cultures were further analyzed and characterized by different techniques (**Figure 6**). NTA and electron microscopy were used to define number and size of the isolated EVs. These techniques are widely used and accepted tools for EV size characrerization and quantification (Tatischeff *et al.* 2012, Li *et al.* 2015, Andreu *et al.* 2016). According to most recent proteomic results gathered in the ExoCarta and EVPedia databases (Simpson *et al.* 2012, Kim *et al.* 2013), exosomes, the smallest sized EVs, have a defined protein signature, comprising conserved as well as cell type specific sets of exosomal proteins. In regard of B16.OVA EVs, the most prominent exosomal proteins were analyzed by Western blot (**Figure 6e**). Thus, the 'classical' exosomal markers were shown to be present on the isolated EVs, suggesting that these EVs are at least in part derived from the endosome and thus belong to the group of exosomes.

However, the aspect of exosomal markers has to be taken with caution since many tetraspanins are widely distributed in the plasma membrane, so that they may be present in other subpopulations of vesicles (Andreu and Yáñez-Mó 2014). Studies aimed to distinguish subpopulations of EVs from different cell types based on the presence of several tetraspanins have shown that in some cases this criterion on its own does not permit successful discrimination of exosomes from other EVs. These studies have shown that supposedly classical markers of exosomes, such as CD63 and CD81, are also enriched in vesicles with features of exosomes but which originate through budding from the plasma membrane and could not be distinguished from exosomes (Booth *et al.* 2006, Fang *et al.* 2007, Lenassi *et al.* 2010). CD9 was also found on large vesicles and can thus not be considered as specific components of endosome-derived vesicles (Bobrie *et al.* 2012). Most recently, CD81 and CD63 have been detected by flow cytometry in both microvesicles and exosomes secreted by three different cell lines (Crescitelli *et al.* 2013a).

Hence, although tetraspanins known to be present on endosomal-derived exosomes are detected on the isolated EVs, there might be a heterogenous population of EVs. It would need further detailed analyses to determine the subpopulation of isolated 3pEVs that mediates the immunostimulatiory antitumor effect.

5.3.3. Immunogenic effect of extracellular vesicles in vivo

It was demonstrated that boost injection of 3pEVs resulted in strong local and systemic immune responses (**Figure 8**) which translated into potent antitumor effects leading to tumor growth control (**Figure 7**). In all experiments, the immunogenic impact of 3pEVs was compared to the one gained after the injection of UnEVs. Although the effect of 3pEVs to stimulate a potent cytotoxic T cell response was most often significantly increased compared to UnEV-based T cell stimulation, low levels of T cell activation were always observed after UnEV treatment. The cause of the mild T cell activation by UnEVs in draining lymph nodes still needs to be clarified. There are several suggestions which might be considered regarding this issue. First, it could be shown that RNA and DNA, as well as TAAs are present in UnEVs as well. Although the *in vitro* studies showed, that neither intact UnEVs nor extracted EV-RNA and EV-DNA derived from UnEVs could sufficiently activate DCs or macrophages, this might not resemble the situation *in vivo* (**Figure 11**). Second, by using a precipitation-based EV isolation technique, precipitation of byproducts like protein aggregates is possible. The elevated T cell levels might thus be due to background protein stimulating APCs *in vivo*, which would be also the case regarding 3pEVs. Third, slight traces of precipitation reagent might be present in the isolated EV solution, which might have stimulating effects on *in vivo* APCs. However, although UnEvs stimulate low levels of cytotoyic T cell activity in the local draining LNs, they do not show any significant impact on tumor growth control (**Figure 7**) underscoring the suggestion of non-specific local immune cell activation due to background protein or minimal amounts of precipitation reagent contamination.

5.3.4. Dendritic cells bridging innate and adaptive immunity

Antigen trapping and antigen presentation are primarily a task of dendritic cells in order to activate epitope-specific naive T cells thereby forming the bridging function between innate and adaptive immunity (Banchereau and Steinman 1998). In order to further investigate T cell activation after injection of 3pEVs, GM-SCF BMDCs were analyzed after stimulation with 3pEVs and UnEVs, respectively. It could be shown, that BMDCs were activated only by 3pEVs thus potentially leading to potent T cell responses (**Figure 11a,b**).

Because of their rarity in tissues, much of the biology of DCs has been surmised from studies of cells grown *in vitro* from hematopoietic precursors under the influence of growth factors (Caux *et al.* 1992, Inaba *et al.* 1992, Palucka *et al.* 1998, Naik *et al.* 2007). In particular, the culture of mouse bone marrow (BM) cells with GM-CSF, a cytokine involved

in the development and homeostasis of mononuclear phagocytes, has been used extensively to generate CD11c$^+$ MHC-II$^+$ cells that resemble tissue DCs and are often termed BMDCs as in this dissertation (Inaba *et al.* 1992, Lutz *et al.* 1999). The output from GM-CSF cultures is known to be heterogeneous and comprises granulocytes and macrophages in addition to DCs. The latter are reported to be enriched in the loosely adherent culture fraction and to express CD11c and MHC-II whereas macrophages are shown to be adherent and negative for CD11c and MHC-II (Inaba *et al.* 1992). In this dissertation, BMDCs used for the stimulation with EVs were thus carefully selected from the loosely adherent culture fraction. The gained results of DC activation after 3pEV stimulation should nevertheless be handeled with caution as they might differ from the activation of naturally occurring DC *in vivo*.

5.3.5. 3pEVs as delivery-tool for immunostimulatory RNA and DNA

The present study revealed, that DC activation by 3pEVs is significantly reduced if adaptor proteins of downstream RNA (MAVS) or DNA signaling (STING) are genetically deleted (**Figure 11 a,b**). Further experiments demonstrated the presence of RNA as well as DNA within the lumen of the vesicles (**Figure 11 c-f**). For these experiments, the concentrations of DNA and RNA isolated from 3pEVs or UnEVs, respectively, were adjusted to analyze differences in their quality and immunogenicity. Both, EV-DNA and -RNA derived from UnEVs were less immunogenic then the respective nucleic acids isolated from 3pEVs and failed to induce effective DC activation.

The presence of DNA within EVs is a rather rare event only shown by few studies. One group however coud visualize the presence of DNA in endosomal-derived exosomes of murine B16-F10 melanoma cells. Interestingly, this study revealed that only a subset of around 10% of exosomes contained DNA. High throughput whole-genome sequencing and comparative genomic hybridization analysis revealed the entire genome coverage of exoDNA in an unbiased manner (Thakur *et al.* 2014). The isolation of DNA from 3pEVs or UnEVs was rather challenging. EVs isolated from 40 mL of culture medium gave rise to only around 20 ng total EV-DNA whereas the same amount of isolated EVs led to around 300 ng of EV-RNA. Thus, either the amount of DNA in EVs is very low, or also here, only a subgroup of EVs carry EV-DNA.

The presence of RNA within EVs has been shown repeatedly. EVs secreted by normal or cancer cells have been found to contain functional mRNAs and small ncRNAs, including miRNA (Skog *et al.* 2008, Robbins and Morelli 2014). Some mRNAs and miRNAs are

detected in both EVs and parent cells, whereas others are identified in either EVs or parent cells, which suggests a preferential sorting of certain RNAs sequences into EVs (Nolte-'t Hoen *et al.* 2012). Increasing evidence strongly suggests that EVs not only transfer antigens to APCs, but also signals that may promote activation of the acceptor cells into immunogenic APCs (Robbins and Morelli 2014). The results of this present study on EV-derived RNA showed that 3pEV-RNA is of a more immunogenic quality compared to UnEV-RNA. Only 3pEV-RNA could stimulate dendritic cells and led to their activation. By treating 3pEV with an alkaline phosphatase, the presence of stimulating phosphorylated RNA could be detected. The exact source of RNA however, still needs to be elucidated. miRNA sequencing for instance could give an overview on transported miRNA species and could give information on signalling pathway regulation in the recipient cell.

The presence of 3pRNA within the vesicles was demonstrated by two independent techniques. First, isolated 3pEV-RNA was treated with an alkaline phosphatase which cleaves off any phosphate residues at the 5' end. The treated RNA was shown to be less DC-activating then untreated 3pEV-RNA (**Figure 11f**). This observation led to the suggestion, that there might be 3pRNA inside the lumen of 3pEVs. Second, with "single EV imaging flow cytometry" it could be demonstrated, that indeed exogenously applied, *in vitro* transcribed 3pRNA is present within the 3pEVs (**Figure 11g**) (Görgens 2016). In conclusion, *in vitro* transcribed 3pRNA was found within 3pEVs. However, passivle shuttling of *in vitro* transcribed 3pRNA into EVs is probably not the sole reason of their immunogenicity, because of the following reasons: 1) EVs-derived from RIG-I-deficient B16 cells are not immunogenic, indicating that active tumor-intrinsic RIG-I signaling is a prerequiste for the shuttling of immunogenic nucleic acids within EVs. 2) AP treatment of EV-RNA impairs but does not completely diminish its type I IFN-inducing effect, suggesting the presence of different RNA species, both phosphoraylated and non-phosphorylated. EV-RNA sequencing would give additional information on the RNA portfolio transported via EVs and on the functionality of these different types of RNA.

Current research in the EV field aims to characterise the RNA content of EVs and the details of its delivery *in vitro* and *in vivo*. Although this field has attracted enormous interest spanning basic research, clinics, and industry, understanding of many aspects of the formation and function of RNA-containing EVs remains elusive. A lack of standardisation with regard to EV purification and characterisation of their molecular contents, as well as technical difficulties in unequivocally demonstrating that especially EV-RNA is a causative agent in EV-mediated effects on target cells, are among the

present challenges to this field (Mateescu *et al.* 2017). It is still unknown whether all EVs contain RNA and how diverse the RNA content of different EV subpopulations may be. Various studies indicate that the RNA content of EVs varies among cell types and among EV subpopulations. For example, miR-145 is present at very low levels in HepG2 cell-derived large EVs, whereas the same miRNA is present at significant levels in both large and small EVs derived from A549 cells (Wang *et al.* 2010). Another remarkable example of EV-RNA heterogeneity is the sex difference observed in the miRNA content of urinary EVs (Ben-Dov *et al.* 2016). With regard to the RNA content of different EV subpopulations, it was shown that EV populations that separated into different fractions based on pelleting at different g-forces using ultracentrifugation differed in RNA content (Crescitelli *et al.* 2013b). Even EVs sedimenting at the same g-force are heterogeneous in nature and may be further separated based on differences in migration velocity in density gradients; recent data indicate that EV subpopulations isolated based on this parameter differ in both protein and RNA content (Willms *et al.* 2016). Assumed that all of the detected miRNA species were indeed EV-associated and that EV quantifications were accurate, one explanation for these data is that specific miRNA sequences could be restricted to specific subtypes of EVs. This scenario would be consistent with a high specificity in delivery of RNA molecules to target cells (Mateescu *et al.* 2017).

The presence of extracellular RNA circulating in non-EV-associated forms, for instance in large protein e.g. Argonaute 2 (AGO2) or lipoprotein complexes, adds another layer of complexity to the analysis of EV-RNA. These complexes have been shown to co-isolate with EVs during common isolation procedures such as ultracentrifugation and precipitation (Arroyo *et al.* 2011). Thus it is possible that, of the numerous types of nucleic acids described “in EVs” in the existing literature, some are contained within specific subtypes of EVs and some are perhaps not present in EVs at all but exclusively in other carriers which co-isolate. This urges the need for including control isolates from non-conditioned culture medium in the RNA analysis.

5.3.6. The role of apoptosis in the release of immunogenic extracellular vesicles

Tumor intrinsic RIG-I signaling has been shown to result in both type I IFN production and tumor cell death (Poeck *et al.* 2008a). This tumor cell death has been suggested to be mediated via tumor-intrinsic activation of the mitochondrial apoptosis pathway requiring Apaf-1 and caspase-9 (Besch *et al.* 2009). Only recently, RIG-I activation during viral infection was shown to trigger necroptosis in infected cells (Schock *et al.* 2017). By

targeting RIG-I-like helicases with poly(I:C), a previous study suggested that RIPK1 and NF-κB signaling in dying cells determine cross-priming of $CD8^+$ T cells, independent of the previously activated cell death pathway (Yatim *et al.* 2015). The present study revealed, that RIG-I activation is necessary for the release of immunogenic EVs. The impact of tumor cell intrinsic cell death mechanism resulting from RIG-I stimulation on the machinery involved in EV packaging however, is yet to be defined. The fact that isolated 3pEVs alone mediate antitumor immunity in the therapeutic vaccination model, suggest that DAMPs released during cell death only play subordinate roles. However, the composition of the immunogenic 3pEV content might be dependent on intracellular cell death signaling. To clarify this issue, certain components of apoptosis and/or necrosis pathways should be blocked during RIG-I activation e.g. by gene knock-out using CRISPR/Cas9.

5.3.7. B16.F10 as a model for human melanoma

B16, a spontaneous melanoma derived from a C57BL/6 mouse, has been used in many pre-clinical studies to model human cancer immunotherapy (Ya *et al.* 2015). There are many characteristics of this tumor line that have made it an attractive model. As a model for human tumors, it is important to note similarities and differences between B16 and human melanomas. The most commonly used B16 line is B16.F10, which is highly aggressive and will metastasize from a primary subcutaneous site to the lungs, as well as colonize lungs upon intravenous (iv.) injection.

Similar to human melanomas, B16.F10 express the tumor-associated antigens gp100/pmel 17, MART-1/Melan-A, tyrosinase, TRP-1/gp75, and TRP-2. All of these can be recognized by CTLs from human melanoma patients. The mouse homologs of these genes are all expressed in B16 melanoma. The melanoma-associated antigens, mgp100, mTyr, mTRP-1, and mTRP-2 have been reported to be recognized by mouse CTLs (Bloom *et al.* 1997, Dyall *et al.* 1998, Overwijk *et al.* 1998, Colella *et al.* 2000). Human melanomas however, express variable levels of MHC class I, whereas B16 melanoma normally expresses low levels of MHC Class I (Li *et al.* 1998, Xu *et al.* 1998). Additionally, human melanomas express ongogene mutations such as BRAF V600E which can be targeted by specific inhibitors. The B16 tumor lacks many of these mutations found in human melanomas (Ya *et al.* 2015).

B16 melanoma has classically been described as a non- or low-immunogenic tumor. One reason for this designation is the difficulty in inducing protection against B16 challenge by

injection of e.g. irradiated B16, regardless of the addition of Bacillus Calmette Guérin (BCG) or Corynebacterium parvum (C. parvum), a strategy that can induce reliable protection in many other tumor models (Ya *et al.* 2015). The reason for this low immunogenicity is still unknown, although the low expression of MHC Class I is one obvious candidate. A first step towards the feasibility of clinical translation would be the demonstration of an antitumor effect of immunogenic 3pEVs isolated from human melanoma cell lines.

Another study found that epithelial ovarian cancer (EOC) cells stimulated by RIG-I ligand undergo an immune-activating form of cell death. Monocytes and monocyte-derived dendritic cells that engulfed such apoptotic cancer cells matured and secreted proinflammatory chemokines as well as type I IFN. Thus, RIG-I activation destroys EOC cells while, at the same time, enhancing immune reactivity (Kübler *et al.* 2010).

5.4. The functions and clinical applications of tumor-derived exosomes

5.4.1. Immunosuppressive effects of tumor-derived exosomes

The role of extracellular vesicles in cancer has mostly been studied focussing on exosomes. Recently, exosome transfer from cancer cells to other celltypes was observed *in vivo*. Using a Cre-LoxP-based approach Zomer et al. observed uptake of EVs by tumor cells. Following uptake of EVs of more malignant cells, less malignant cells displayed enhanced migratory behaviour and metastatic capacity (Zomer *et al.* 2015). Thus, malignant cells have the ability to transfer genetic information to other cells within the tumor microenvironment through exosomes. It could be further shown that tumor-derived exosomal miRNAs contribute to cancer cell proliferation, metastatis, dormancy, and drug resistance (Shao *et al.* 2016). Additionally, tumor-derived exsosomes (TEX) can promote T regulator cell expansion leading to the immune escape of tumor cells by inhibition of NK and T cell cytotoxicity (Zhang *et al.* 2016). Other studies have shown that TEX can suppress antigen-specific or non-specific antitumor responses by expressing FasL, TRAIL, and galectin-9, which induce T cell apoptosis (Andreola *et al.* 2002, Huber *et al.* 2005, Klibi *et al.* 2009).

Additionally, the levels of exosomes in blood have been correlated with tumor development. An increase in $CD63^+$ exosomes in melanoma patients compared to healthy donors has been observed (Logozzi *et al.* 2009). In a study of lung adenocarcinoma, both the mean exosome and the miRNA concentrations were higher in lung adenocarcinoma patients compared to healthy controls (Rabinowits *et al.* 2009). Thus, exosome

concentrations in blood may help physicians evaluate the results of surgery and detect relapse in cancer patients. New technologies have been developed for this so-called liquid biopsies to capture circulating exosomes which can serve as tumor markers for personalized diagnostics (An *et al.* 2015, Im *et al.* 2015).

Although tumor-derived exosomes display many tumorigenic and immunosupressive facets, it has been demonstrated by several recent studies, that nontheless, exosomes are a powerful tool in anticancer therapy.

5.4.2. Advantages of extracellular vesicles for cancer therapy

The development for nanoformulations has improved the therapeutic efficiacy of drugs. Unfortunately, none of the nanotechniques avoid toxicity, and the drugs are typically cleared immediately (Peng *et al.* 2013, Shao *et al.* 2016). Unlike synthetic nanoparticels, EVs are more biocompatible and biodegradable, and thus have low toxicity and immunogenicity (Ha *et al.* 2016). Exosomes are stable in biofluids and their small size enables exosomes to easily escape from lung clearence and even pass through the blood-brain barrier (Alvarez-Erviti *et al.* 2011, Kawikova and Askenase 2015). For nucleic acid loading, commercial membrane-permeable reagents such as liposomes have been used for assisting RNA and DNA fragment loading into exosomes. Hewever, these approches have proofed to not be satisfying. Thus, pre-overexpression of candidate RNAs or proteins in donor cells is still considered as the best way to generate candidate protein- and RNA-loaded exosomes (Munoz *et al.* 2013).

Interestingly, the adherence and internalization of exosomes whithin tumor cells is 10-times higher than of liposomes of a similar size, indicating a higher specificity of exosomes for cancer targeting. In addition, due to enhanced mobility and retention effect, nanometric exosomes tend to accumulate in tumor tissues containing abnormally formed blood vessels, thus exosomes can easily reach the bulk of the solid tumors to increase their drug delivery efficiency. Moreover, exosomes can be engineered with tumor-targeting proteins, peptides, or antibodies for precise drug and therapeutic nucleic acid delivery (Wang *et al.* 2016). Taken together, these characteristics make exosomes promising candidates for cancer targeting therapy.

5.4.3. Exosome modification for specific targeting

Synthetic nanoparticle-mediated delivery has low specificity because a very limited number of selective molecules can be used for cell targeting. However, natural cell-produced exosomes were shown to recognize specific cell types via their surface receptors. For example, exosomes with Tspan8 on their membrane surface preferentially bind to CD11b and CD54-positive cells (Rana *et al.* 2012). Thus, the use of engineered donor cells to obtain modified exosomes with particular receptors is under current investigation. For instance, DCs were engineered to express αv integrin-specific iRGD peptide and Lamp2b fusion protein, allowing the engineered DCs to secrete exosomes with iRGD peptide on their surface. These engineered exosomes showed dramatically increased drug delivery efficiency and antitumor effect on αv integrin-positive breast cancer cells in a mouse model (Tian *et al.* 2013).

Protecting drug-loaded exosomes from liver clearance is critical for cancer treatment applications. Researchers blocked scavenger receptor class A family (SR-A), a monocyte/macrophage uptake receptor for exosomes, which dramatically reduced exosome clearance in the liver and enhanced their accumulation in tumor tissue (Watson *et al.* 2016).

5.4.4. Exosome cargo loading for cancer therapy

As a delivery system, exosomes are widely used as vesicles for various tumor therapeutic cargos. The lipid bilayer mambrane of exosomes forms a natural protective barrier and a sustained release capsule for various anti-cancer drugs and cancer gene suppressors, including functional RNAs (Camussi and Quesenberry 2013, Wang *et al.* 2016). There are at least three ways that drugs can be loaded into exosomes for delivery. First, naïve exosomes isolated from parental cells can be loaded *ex vivo* e.g. by using electroporation or sonication. Second, parental cells can be loaded with a drug, which is then released into exosomes, or third, parental cells can be transfected with DNA that encodes therapeutically active compounds, which are then released in exosomes (Shao *et al.* 2016).

Accumulating evidence has shown that exosome-mediated chemotherapeutic delivery has much improved antitumor effects when compared to free drugs in animal tumor models. Paclitaxel for instance, is a widely used antimitotic chemotherapeutic drug for

various tumor therapy (Liu *et al.* 2015). Paclitaxel can be loaded into exosomes by sonication, and these loaded exosomes have 50 times more cytotoxicity than free paclitaxel for drug resistant cancer cells *in vitro*. They can also dramatically block Lewis lung carcinoma pulmonary metastases and reduce tumor size in mouse models (Kim *et al.* 2016). As described above, drug-pretreated donor cells can also produce drug-loaded exosomes. For example, exosomes derived from paclitaxel-treated MSCs exhibited a strong inhibitory effect on human pancreatic adenocarcinoma (Pascucci *et al.* 2014). This is, however, not the case in the present study where it could be demonstrated, that EVs isolated from doxorubicin- or oxaliplatin-treated melanoma cells are not immunogenic. The delivery of chemtherapeutic agents by melanoma-derived EVs thus needs to be investigated in more detail.

Besides anti-cancer therapeutic drugs, exosomes can also deliver various tumor antigens and apoptosis-inducing proteins into cancer cells for targeting therapy. Exosomes derived from peptide-pulsed DCs for instance, can present antigens to T cells to induce their immune response. These exosomes contain MHC-peptide complexes and co-stimulatory molecules on their membrane, which enable them to prolong antigen presentation and boost immunization in mice compared to therapeutically transferred antigen-presenting cells (Luketic *et al.* 2007). Another group worked on survivin, an anti-apoptotic protein, which plays important role in multiple cancer cells to suppress apoptosis activation. Inactive mutation of survivin-T34A impairs the pro-survival activity of survivin and induce caspase activation and apoptosis in cancer cells (Aspe *et al.* 2014). Survivin-T34A-loaded exosomes can induce apoptosis in various pancreatic adenocarcinoma cell lines.

Abundant miRNAs are frequently detected in exosomes. Most of these miRNAs are funcitonally involved in exosome-mediated cell-cell communitaction. Exosomes can also be loaded with specific miRNAs increasing their anticancer potential. For instance, exogenous miRNA-143-loaded exosomes significantly reduced osteosarcoma cell migration (Shimbo *et al.* 2014). Interestingly, in recent years exosomes have been used to silence genes in tumor cells by loading them with siRNAs. For example, delivery of siRNA against RAD51 (which plays central role in homologous recombinational repair) via exosomes dramatically inhibited the proliferation of human breast cancer cells and caused their death *in vitro* (Shtam *et al.* 2013). Exosome-mediated transfer of siRNA against c-Myc can efficiently silence c-Myc and activate the pro-apoptotic protein caspase-3 in mouse lymphoma cells (Lunavat *et al.* 2016).

Taken together, exosomes are advantageous over the so far established nanotechniques in that they can function as both, synthetic nanocarriers and as cell-mediated drug delivery vehicles (Batrakova and Kim 2015). Exosomes are biocompatible, non-cytotoxic, low immunogenic, simple to produce, easy to store, have a long life span, and high cargo loading capacity (Munagala *et al.* 2016, Srivastava *et al.* 2016). These characteristics make exosomes a promising drug carrier for cancer treatment produced as a personalized anti-cancer vaccine derived from individual cells of a single patient.

5.4.5. Extracellular vesicles applied in immunotherapy of cancer

Since many promising results have been achieved in vitro and in animal models, using exosomes/EVs for cancer cell targeting is currently considered to be one of the most hopeful new approaches for cancer treatment. Notably, some clinical trials have already been conducted offering important achievements. As shown in a phase I trial, metastatic melanoma patients were intradermally and subcutaneously given exosomes obtained from autologous DCs loaded with the melanoma-associated antigen MAGE for 4 weeks. Although no significant beneficial outcome has been observed, the safety of exosome administration and feasibility of large-scale exosome-prodaction have been confirmed in these patients (Escudier *et al.* 2005). DC derived-exosomes (DEX) were additionally shown to harbor functional MHC/peptide complexes capable of promoting T cell immune responses and tumor rejection *in vitro* (Andre *et al.* 2004). Another phase I trial showed that the imune response was activated and disease progression was slowed in a small number of DEX-treated patients with non-small cell lung (Morse *et al.* 2005). Since the conduction of these phase I DEX trials, new ways to improve DEX as an immunotherapy have been established with hope to enhance the limited DEX-induced T cell responses. An important innovation here has been the use of exosomes derived from TLR4L- or IFN-γ-maturated DCs, following discoveries that such DEX induce greater T cell stimulation compared to DEX from immature DCs (Segura *et al.* 2005b, Viaud *et al.* 2011). Based on this phase I and preclinical results, a phase II trial was performed which showed that IFN-γ-DC-derived exosomes were capable of boosting NK cell-mediated antitumor immunity in advanced non-small cell lung cancer patients. Thirtytwo percent of participants experienced disease stabilization for more than four months, although the primary endpoint, which was to observe at least 50% of patients with progression-free survival, has not been reached (Besse *et al.* 2016).

Regarding tumor-derived EVs not many clinical trials have been conducted. In one study, EVs were isolated from patient-derived glioma cells. Autologous dendritic and T cells were

obtained from pripheral blood samples. Tumor-derived EVs were loaded onto dendritic cells. These DCs were subsequently shown to elicit a specific $CD8^+$ cytotoxic T-lymphocyte response against autologous tumor cells of patients with malignant glioma. These data demonstrated that tumor exosome-loaded DCs are an effective tool in inducing glioma-specific $CD8^+$ CTLs able to kill autologous glioma cells (Bu *et al.* 2011). Another phase I clinical trial used ascites-derived exosomes (AEX) in combination with GM-CSF to treat 40 patients with advanced colorectal carcinoma (CRC). The majority of the AEX were believed to be derived from colorectal cancer cells. Interestingly, and in contrast with the low-level stimulation of T cell responses in the DEX clinical studies, AEX plus GM-CSF could induce antigen-specific antitumor CTL responses. A greater level of TAAs present in AEX compared with DEX may have also been responsible for the greater T cell responses observed in this study as compared with the three above mentioned DEX phase I trials (Dai *et al.* 2008).

Overall, multiple clinical trials using exosomes in tumor immunotherapy are ongoing. In the future, standardization of exosome isolation, storage, cargo loading, quality control, and efficiacy evaluation procedures will be necessary for wide-spread use of this promising cancer treatment approach.

6. Conclusion

The ongoing development of novel immunotherapies has revolutionized the treatment of many cancers, with the success of immune checkpoint blockade strategies being a prominent example (Chen and Mellman 2013). The goal of cancer immunotherapy is to induce tumor-targeting immunity or to strengthen an ongoing antitumor host immune response that is otherwise ineffective (e.g., following immunosuppression in the tumor microenvironment). Strategies that can harness dendritic cells (DC) or their functions, which drive tumor-associated antigen (TAA)-specific T cell responses, are therefore well positioned to achieve this end. Available anticancer immunotherapies are often difficult to implement in a clinical setting, thus alternative and more effective vaccine strategies targeting the DC axis towards cytotoxic T cell activation are sought. Harnessing immunogenic cell death is one promising approach towards the optimization of immunotherapies.

It was demonstrated before that *in vivo* administration of the RIG-I ligand 3pRNA had a direct effect on tumor cells causing their cell death subsequent to RIG-I activation (Poeck *et al.* 2008b). The present study was set out to analyze the immunogenic potential and mechanism of RIG-I-induced tumor cell death. We hereby found that the RIG-I ligand 3pRNA is an inducer of immunogenic cell death in malignant melanoma cell lines. Furthermore, we discovered that RIG-I-activated melanoma cells release several danger-associated molecular patterns. However, RIG-I-mediated release of immunogenic extracellular vesicles were found to primarly able to trigger an antigen specific antitumor immune response.

In summary, two main conclusions are emphasized. First, RIG-I ligands are inducers of immunogenic melanoma cell death. This is in agreement with a previous study showing that RIG-I-like helicases induce ICD in the pancreatic cancer cell line Panc02 (Duewell *et al.* 2014). Activation of RIG-I in melanoma cells led to the release of the classical ICD hallmark DAMPs ATP and HMGB1, as well as the exposure of calreticulin on the outer plamamembrane. Additionally a set of inflammatory cytokines including IL-6, IL-12p40 and TNF-α were released into extracellular space. Most prominently, type-I interferons were released. An intact type I IFN signaling was shown to be essential for the establishment of an immune response against the tumor. Thereby, tumor-released type I IFN as well as intact receptor signaling of recipient cells was equally important. It could be shown, that RIG-I activated dying cancer cells trigger cytotoxic T cell responses leading to tumor growth retardation.

The second major conclusion is that despite ICD hallmark DAMPs and inflammatory cytokines beeing released after RIG-I activation in melanoma cells, the essential immunogenic factor(s) for cytotoxic T cell activation is shuttled within the lumen of released extracellular vesicles. This study revealed that EVs released from 3pRNA-transfected melanoma cells (3pEVs) stimulate type-I interferon receptor signaling in antigen presenting cells which results in the activation of cytotoxic T lymphocytes. It could be shown that such 3pEVs carry different tumor-associated antigens together with a variety of RNA and DNA molecules. This immunogenic cargo led to two stimulatory signals in DCs which then activate the immunogenic cascade directed against the tumor of which the 3pEVs originated from. Interestingly, this way of transporting immunogenic information to stimulate immune responses is not shared by “classical” chemotherapeutic ICD inducers like oxaliplatin or doxorubicin. Thus, RIG-I-induced ICD differs from the signaling routes of chemotherapeutic ICD inducers.

In summary this study offers novel insights into RIG-I-driven immunogenic cell death. It contributes to our understanding of the concept of this form of ICD initiated by the release of immunogenic extracellular vesicles. The EVs isolated from the supernatants of RIG-I-activated tumor cells carry all necessary factors for the establishment of a potent antitumor immune response involving cytotoxic T lymphocytes. Therefore, this study provides a new and promising approach for the development of improved immunotherapies for anticancer treatment.

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