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Klinický význam molekul a procesů asociovaných s imunogenní buněčnou apoptózou v terapii nádorových onemocnění

The clinical relevance of immunogenic cell death associated signaling and molecules in cancer therapy

Disertační práce

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Ďakujem!

Klinický význam molekul a procesů asociovaných s imunogenní buněčnou apoptózou v terapii nádorových onemocnění

Schopnost nádorových buněk vyvolat protinádorovou imunitní odpověď závisí na mnoha faktorech, včetně antigenního profilu nádorových buněk a jejich schopnosti poskytovat adjuvantní signály ve formě molekul asociovaných s nebezpečím (DAMPs). Tyto molekuly jsou vystavovány a či uvolňovány během imunogenní buněčné smrti (ICD) a za určitých okolností mohou vést k aktivaci vrozené a adaptivní protinádorové imunitní odpovědi. Schopnost aktivovat ICD mají některá chemoterapeutika, fyzikální modality, cílená protinádorová léčba či radioterapie, které kromě vlastní cytotoxické aktivity na nádorové buňky mohou aktivovat klinicky relevantní protinádorovou imunitní odpověď. Z tohoto důvodu u pacientů s vrozenou poruchou v buněčné signalizaci vedoucí k uvolňování DAMPs může docházet ke snížené odpovědi na uvedený typ léčby. Předložená disertační práce se zejména zaměřuje na klinický význam procesů, které souvisí s imunogenní buněčnou smrtí a zkoumá vliv ICD na rozvoj vrozené protinádorové imunitní odpovědi se zaměřením na NK (z angl. Natural killer cells) buňky. Výsledky naší práce ukazují, že kalretikulin (ekto-CALR), jako klíčový ukazatel ICD, vystavený na povrchu nádorových buněk pozitivně ovlivňuje přítomnost a cytotoxicitu NK buněk v periferní krvi pacientů s akutní myeloidní leukémií (AML). Výsledky našich studií současně podporují předchozí experimentální a klinické pozorování, která naznačují, že aktivní signalizace nebezpečí a uvolňování DAMPs může u onkologických pacientů zvýšit klinicky relevantní odpovědi na standardní léčbu a imunoterapii. Významnou kapitolou klinické aplikace induktorů ICD je příprava protinádorové buněčné terapie založené na dendritických buňkách (DC), konjugátů protilátek či cílené terapie se slibnými klinickými výsledky. Identifikace spolehlivých prediktivních ukazatelů by proto mohla dále podpořit klinický vývoj a následnou aplikaci této terapie v léčbě pacientů. Za tímto účelem jsme identifikovali prediktivní genový profil, který napomáhá identifikovat skupinu pacientů profitujících z buněčné terapie na bázi DC. Závěrem lze říci, že koncept ICD je v současné době považován za významný nástroj v klinicky relevantní aktivaci dlouhodobé protinádorové imunitní odpovědi, která do značné míry určuje výsledek protinádorové léčby.

Klíčová slova: Imunogenní buněčná smrt, molekuly asociované s buněčným poškozením (DAMPs), protinádorová imunintní odpověď, kombinovaná léčba, buněčná terapie onkologických onemocnění.

The clinical relevance of immunogenic cell death-associated signaling and molecules in cancer therapy

The capacity of cancer cells to induce anticancer immune responses relies on multiple factors, including the antigenic repertoire of cancer cells and their ability to provide adjuvant signals, as represented by danger-associated molecular patterns (DAMPs), which are exposed and released by malignant cells during immunogenic cell death (ICD). The release and secretion of DAMPs can orchestrate the activation of innate and adaptive tumor-targeting immunity, resulting in tumor regression. Various chemotherapies, radiation therapy, physical modalities, and targeted anticancer agents have been described as potent ICD inducers, which besides being directly cytotoxic, can activate clinically relevant anticancer immune responses. Therefore, patients whose tumor microenvironment (TME) is shows defective DAMP release or downstream DAMP-sensing signaling pathways do not fully benefit from ICD-inducing treatments, which can lead to overall therapeutic failure. My dissertation contributes to this field by exploring the impact of ICD on the development of innate anticancer immune responses, with a particular focus on natural killer (NK) cells, showing that surface-exposed calreticulin (ecto-CALR) positively impacts the frequency and cytotoxicity of NK cells in peripheral blood of acute myeloid leukemia (AML) patients. Additionally, our findings support the previous observation that active danger signaling, and the release of DAMPs, can enhance the clinically relevant responses to standard of care therapy and immunotherapy in cancer patients. Moreover, ICD inducers have been harnessed in the preparation of dendritic cell (DC)-based vaccines, such as DCVAC, antibody–drug conjugates, and other therapies with promising clinical results. Therefore, the identification of reliable predictive biomarkers could further support the clinical development of such personalized treatments. Finally, to this end, we identified a predictive gene signature for future clinical management of DCVAC therapy in prostate, lung, and ovarian cancer patients. In conclusion, the concept of ICD is currently regarded as a prominent pathway for activation of long-lasting anticancer immune responses, which to a significant degree determines the outcome of anticancer therapies.

Key words: Immunogenic cell death, danger associated molecular patterns (DAMPs), anticancer immune response, combined treatment, anticancer cell therapy

SEZNAM ZKRATEK

ACD	accidental cell death
ARA-C	cytarabine
Ag	antigen
AML	acute myeloid leukemia
ANXA1	annexin A1
APC	antigen-presenting cells
ARG1	arginase 1
ATP	adenosine triphosphate
B2M	beta-2-microglobulin
BAK1	BCL2 Antagonist/Killer 1
BAP31	B-cell receptor-associated protein 31
BTK	Bruton tyrosine kinase
BTLA	B- and T-lymphocyte attenuator
CAF	cancer associated fibloblast
CALR	calreticulin
CCL	C-C motif chemokine
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDK	cyclin-dependent kinase
cDNA	circular DNA
CGAS	cyclic GMP-AMP synthase
CML	chronic myeloid leukemia
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C motif chemokine
CXCR3	C-X-C chemokine receptor 3
DAI	DNA-dependent activator of IRFs
DAMP	danger associated molecular patterns
DC	dendritic cells
DNA	deoxyribonucleic acid
DNR	daunorubicin

dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
eIF2α	eukaryotic translation initiation factor 2α
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immuno sorbent assay
ER	endoplamatic reticulum
FACS	fluorescence activated cell sorting
FasL	fas ligand
FPR1	formyl peptide receptor 1
GBM	glioblastoma
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GZMB	granzyme B
HD	healthy donors
HDAC	DNA methyltransferase, histone deacetylase
HHP	high hydrostatic pressure
HGG	high-grade glioma
HLA-DR	human leukocyte antigen – DR isotype
HMGB1	high mobility group box 1
HSP	heat shock protein
Hyp-PDT	hypericin-based photodynamic therapy
ICIs	immune checkpoint inhibitors
ICD	immunogenic cell death
iDC	immature dendritic cells
IDO	indoleamine 2,3-dioxygenase
IF	immunofluorescence
IFN	interferon
IFNAR2	Interferon-alpha/beta receptor
IHC	immunohistochemistry
IL	interleukin
IL15Rα	interleukin 15 receptor subunit alpha
IRF	interferon regulatory factors
ISGs	IFN stimulated genes
LAG-3	lymphocyte-activation gene 3

LRP1	LDL receptor related protein 1
LSC	leukemic stem cell
M1	M1 polarized macrophages
M2	M2 polarized macrophages
MAMP	microbial associated molecular pattern
mDC	myeloid derived dendritic cells
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MLKL	mixed lineage kinase domain-like
MX1	MX dynamin-like GTPase 1
MyD88	Myeloid differentiation primary response 88
NK	natural killer
NKT	natural killer T cell
NLR	Nod like receptor
OS	overall survival
OV	oncolytic viruses
OXP	oxaliplatin
P2RX7	purinergic receptor P2X7
P2RY2	purinergic receptor P2Y2
PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PDIA3	protein disulfide isomerase family A member 3
pDC	plasmacytoid dendritic cells
PD-1	programmed cell death 1
PD-L1	programmed death-ligand 1
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PPP2R1A	protein phosphatase 2, structural/regulatory subunit alpha
PRF1	perforin 1
PRR	pattern related receptor
qPCR	quantitative polymerase chain reaction
RCD	regulated cell death
RFS	relapse-free survival
RIPK3	receptor-interacting serine/threonine-protein kinase 3
RLR	RIG-I-like receptor

RNA	ribonucleic acid
RNAseq	RNA sequencing
ROS	reactive oxygen species
RUSH	retention using selective hooks
shRNA	small hairpin RNAs
SNV	single nucleotide variant
SoC	standard of care
STING	Stimulator of interferon genes
TAA	tumor associated antigens
TAM	tumor-associated macrophage
TAP	transporter associated with antigen processing
TCGA	the cancer genome atlas
TCR	T-cell receptor
TFAM	transcription factor A, mitochondrial
TGFβ	tumor growth factor beta
Tfh	T follicular helper cells
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TIL	tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptors
TMB	tumor mutation burden
TME	tumor microenvironment
TNF	tumor necrosis factor
TP53	tumor protein 53
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cells
TSA	tumor specific antigens
UPR	unfolded protein response
UVC	ultraviolet C
VEGFα	vascular endothelial growth factor alpha

WB	western blot
ZBP1	Z-DNA binding protein

Contents

1 Introduction

Accumulating preclinical and clinical findings indicate that tumor form, progress and respond to therapy in the context of bidirectional interaction with immune system. Both innate and adaptive immune system components not only directly detect and eliminate malignant and pre-malignant cells from the body but also prevent neoplastic transformations by elimination of viruses and inflammation inducing pathogens, which decrease the risk for viral induced cancers and resolves pro-tumorigenic inflammatory conditions. This direct process of recognition and destruction of malignant cells by innate and adaptive derived mechanisms of immune system is known as cancer immunosurveillance (Swann & Smyth, 2007).

In this context, malignant cells might escape immunosurveillance by losing their antigenicity and adjuvanticity leading to the outgrowth of poorly immunogenic tumor-cell variants (immunoselection) and through subversion of the immune system (immunosubversion). This process is called immunoediting and eventually leads to the emergence of immune-resistant malignant clones, as a dynamic process comprised of three distinct phases: elimination, equilibrium and escape. During the elimination phase, cellular and humoral components of immune system recognize and destroy malignant clones. Second phase of a cancer development represents the establishment of an equilibrium between genetically unstable neoplastic cells and the immune system. Thus, the stage of equilibrium can last many years and decades where cancer cells persist in the body either dormant or they further accumulate mutations and evolve. Cancer cells, at this stage, are under constant selection pressure by immune system eliminating susceptible malignant clones. The final stage or cancer cell and immune system cross talk is an immune escape where tumor growth is no longer under the control due the reduced immunogenicity of malignant cells and built up immunosuppressive microenvironment which leads to clinical manifestation of cancer malignancy (Swann & Smyth, 2007).

In general, for mounting a successful and effective antitumor immune response, the immune system needs to recognize cancer cells as a potential danger. Thus, the final immunogenicity of neoplastic cells is largely driven by their antigenicity, i.e. the exposure of antigens not covered by central thymic and peripheral tolerance and by their adjuvanticity, i.e. the expression and release of danger associated molecular patterns (DAMPs) (Galluzzi, Buque, Kepp, Zitvogel, & Kroemer, 2017) .

1.1 Tumor Immunosurveillance

The development of antitumor immunity depends on both innate and adaptive arms of immune system and their mutual interplay. In general, anti-tumor immune response is initiated by dendritic cells (DCs) or other antigen presenting cells (APCs), which search, process and display tumor antigens to prime and activate effector cytotoxic lymphocytes such as $CD8^+$ T cells to find and destroy malignant cells (Hanahan & Weinberg, 2011; D. S. Chen & Mellman, 2013) (**Figure 1**). In addition, other cytotoxic lymphocytes such γδT cells, NKT and NK cells provide direct cytotoxicity in non-specific manner. Upon the recognition of tumor cells, cytotoxic T cells release cytotoxic proteins and enzymes such perforins and granzymes which penetrate the cellular membrane and subsequently kill the tumor cells. Besides CTLs, $CD4^+$ T cells detect antigen in the context of MHC class II molecules and orchestrate the adaptive arm of the immune system by IFNγ production accompanied by other proinflammatory cytokines produced by activated T_H1 cells, which further mediate chemotactic, pro-inflammatory and immunoprotective properties (**Figure 1**). Thus, the presence of such an active anticancer immunity has been connected with positive prognosis in many cancer types (**Figure 2**). Besides protective anticancer immunity, the cancer development is also associated with strong systemic and intratumoral immunosuppression contributing to disease progression, such as high level of immunomodulatory cytokines, enzymes and metabolites, including interleukin (IL)-6, IL-10, indolamine 2,3-dioxygenase 1 (IDO1) and arginase 1 (ARG1). These factors increase alongside with accumulation of immunosuppressive cell types such as $FOXP3^+$ regulatory T (T_{reg}) cells, tumor-associated macrophages (TAMs), tolerogenic DCs, and myeloid-derived suppressor cells (MDSCs) associated with poor prognosis and aggressive cancer progression (**Figure 1, 2**)

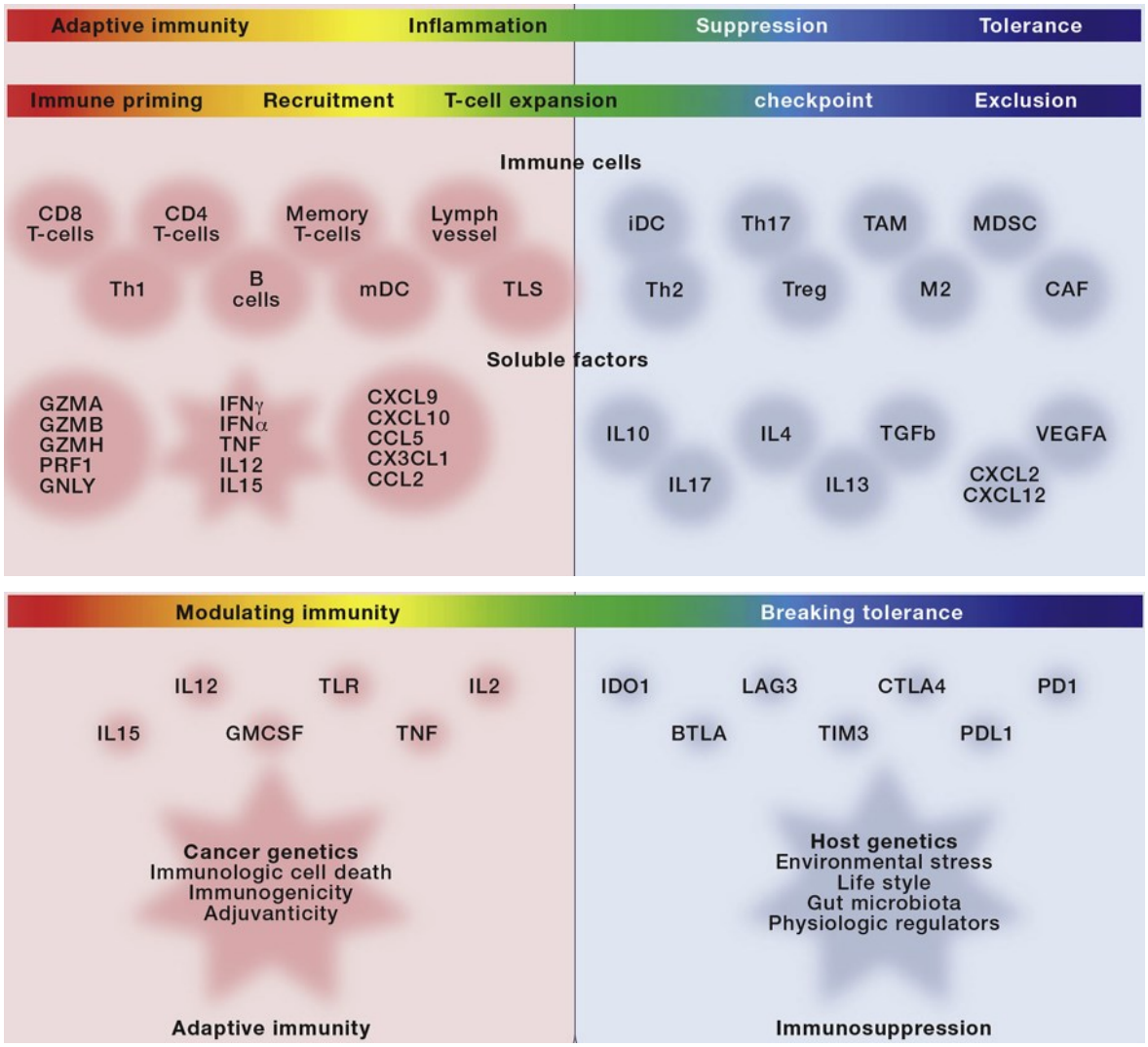

Figure 1. The cellular and humoral components of anti-tumor immunity. The immune contexture of tumor microenvironment largely consists of adaptive immunity either inflamed, immunosuppressive or with tolerogenic phenotype. Adapted from: (Galon & Bruni, 2020)

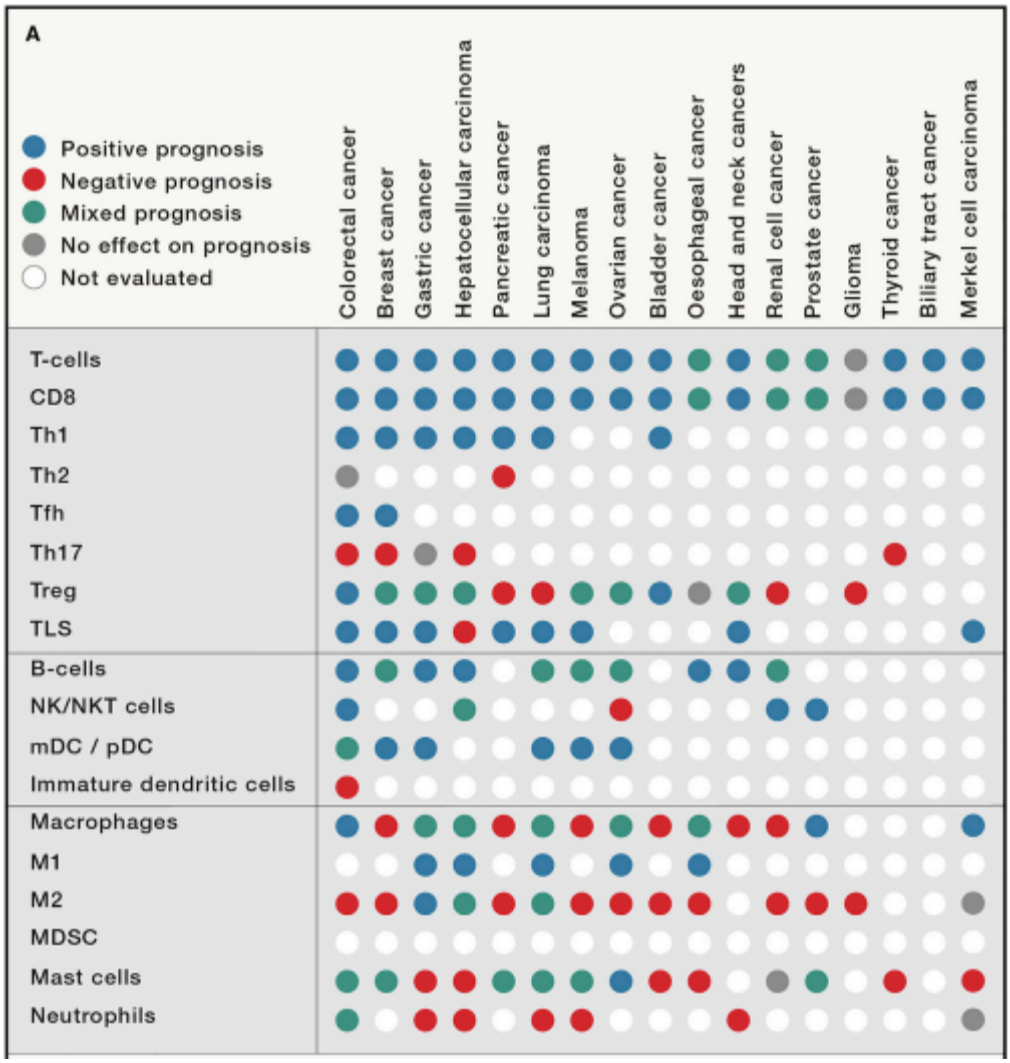

Figure 2. **The prognostic role of cellular immune components in cancer.** Adapted from: (Galon & Bruni, 2020).

1.2 Tumor Antigenicity

The formation of tumors also leads to generation and presentation of mutated peptides, so called neo-antigens, which have not been previously exposed to mechanism of self-tolerance and can therefore be detected by adaptive immune system (Goldszmid, Dzutsev, & Trinchieri, 2014). On top of that, both central and peripheral tolerance are leaky, and they both can be overridden in context of persistent and intensive adjuvanticity. This potential to overcome the peripheral tolerance, opens up a window for recognition of even non-mutated epitopes associated with tumor mass by the immune system (Coulie, Van den Eynde, van der Bruggen, & Boon, 2014; Gilboa, 1999; Schuster et al., 2017).

Tumor cells compensate their increased antigenicity with subsequent decreased expression of specific antigens for example by selective cancer cell clonal expansion, which prefers clones with low to no self-peptide expression, suppressing clones expressing cancer antigens that has already been recognized by immune system or by selecting clones with deregulated/suppressed antigen processing and presenting pathways such as MHCI associated expressing and presenting machinery including MHC Class I molecules, beta-2-microglobulin (B2M), transporter 1, ATP binding cassette subfamily B member (TAP1), TAP2 and proteasomal subunits (Grasso et al., 2018; McGranahan et al., 2016; Rooney, Shukla, Wu, Getz, & Hacohen, 2015).

1.2.1 Tumor specific antigens

Tumor specific antigens (TSA) are cancer-specific proteins which have risen up from the tumorigenesis itself and are hence not covered by central tolerance. Therefore, TSA are perceived by host immune system as foreign entities and can be readily recognized by host immune system. During tumorigenesis TSA are generated by various genetic alterations such as non-synonymous single nucleotide exchange (SNV-neoantigens), frame shift, gene fusion splice variants, endogenous retroelements and others (Smith et al., 2019). However, a majority of TSA comes from unmutated non-canonical transcripts that arise from cancer-specific epigenetic and splicing aberrations (Apavaloaei, Hardy, Thibault, & Perreault, 2020). In general TSA can be divided into two categories as shared and personalized neoantigens whereas former are common across various cancer types and latter being specific for each individual (Sahin & Tureci, 2018; Schumacher & Schreiber, 2015). Even though the emergence of TSA is very important for activation of targeted anti-tumor immunity, the level of TSA expression on cancer cells seems to be the crucial point. It has been shown that a low level of TSA expressing cancer cells might not be sufficient to stimulate the relevant immune response mediated by T cells and a certain threshold level is required for anti-tumor immunity development (Spiotto et al., 2002; Westcott et al., 2021).

1.2.2 Tumor associated antigens

Tumor associated antigens (TAA) are self-antigens that are not exclusively specific to neoplastic cells. TAAs are commonly occurring also on subsets of healthy cells, but due to tumor specific amplification, TAAs are overly or preferentially expressed by tumor cells. Some of the TAA are for example CD19, CD20, and melan-A (MLANA, best known as MART-1) associated with tissue differentiation or ectopically expressed proteins such as carcinoembryonic antigens (CEAs), cancer/testis antigens, prostate specific antigen (PSA), multiple members of the MAGE (melanoma antigen gene) and synovial sarcoma X chromosome breakpoint (SSX) protein families and many others (Coulie et al., 2014; Kirkin et al., 2018; Simpson, Caballero, Jungbluth, Chen, & Old, 2005). As already mentioned, despite TAAs being covered by central tolerance, both central and especially peripheral tolerance can by bypassed in context of highly adjuvant environment resulting in active anti-TAA immune response (Coulie et al., 2014; Gilboa, 1999; Schuster et al., 2017). Although, TAAs are generally weaker at eliciting anticancer immunity as compared to TSAs (Stone, Harris, & Kranz, 2015), they might serve as biomarkers and targets for antibody and cellular mediated immunotherapy approaches (Bezu, Kepp, et al., 2018; Sprooten et al., 2019).

1.2.3 Tumor mutational burden

Since cancer cell mutations are processed into tumor specific neo-antigens, (modified self-antigens that were not subjected to central tolerance mechanism) the overall tumor antigenicity is to some extent determined by complete mutational profile of tumor cells called tumor mutational burden (TMB). TMB varies across various neoplastic diseases and is positively linked to immune infiltration within tumor microenvironment (TME) (Campbell et al., 2017; Fan et al., 2020; Lawrence et al., 2013; Mi, Xu, Liu, & Wang, 2020; Ricciuti et al., 2022; Z. M. Wang, Xu, Kaul, Ismail, & Badakhshi, 2021). Thus, the response to distinct types of cancer immunotherapy, such as immune checkpoint inhibitors (ICIs) is strongly linked to high TMB in distinct solid carcinomas (Goodman et al., 2020; Hellmann, Callahan, et al., 2018; Hellmann, Ciuleanu, et al., 2018; Chalmers et al., 2017; Jardim, Goodman, de Melo Gagliato, & Kurzrock, 2021; Mandal et al., 2019).

1.3 Tumor adjuvanticity

1.3.1 Immunogenicity of cell death

In general, the adjuvanticity is a capacity of substance to promote, induce or enhance the antigen specific immune response. Living cancer cells, aside from having variable levels of antigenicity, have only limited or no adjuvanticity at all. On top of that cancer cells actively evade direct immune recognition and even promote immune suppressive environment by losing their antigenicity and adjuvanticity. However, as cancer cells die, experiencing stress conditions, they passively release, actively secrete, or expose on the membrane surface various danger associated molecular pattern (DAMP) molecules which to various extent influence the cancer cell capacity to induce immune responses. Apoptosis and necrosis are the most described and the most contrasting forms of cellular demise. For decades, former has been viewed as being strictly physiological, regulated, and non-immunogenic being the major representant of regulated cell death (RCD) and the latter as highly immunogenic being the major representant of accidental cell death (ACD). This view was later demonstrated to be a large oversimplification. Currently, based on accumulating preclinical and clinical data, we accept the concept of immunogenic cell death (ICD), which tells that upon various stressors, apoptosis of malignant cells can be accompanied by release of immunogenic molecules. In 2018, the Nomenclature Committee on Cell Death has defined and described the ICD as a "functionally peculiar form of RCD that is sufficient to activate an adaptive immune response specific for endogenous (cellular) or exogenous (viral) antigens expressed by dying cells" (Galluzzi, Vitale, et al., 2018).

ICD can be triggered by various physical and chemical modalities and it is accompanied by release, secretion and display of plethora immunogenic molecules (**Table 1**) including: adenosine triphosphate (ATP), cellular nucleic acids, the non-histone, nuclear DNA- binding protein high mobility group box 1 (HMGB1), annexin A1 belonging to annexin super family (ANXA1), various cytokines such as type I interferon (IFN), C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 1 (CXCL1) and CXCL10, endoplasmic reticulum (ER) chaperones calreticulin (CALR), protein disulfide isomerase family A member 3 (PDIA3, also known as ERp57), heat shock protein family A (HSP70) member 1A (HSPA1A, best known as HSP70), HSP90 alpha family class A member 1 (HSP90AA1, best known as HSP90), cytosolic components like F-actin, and other mitochondrial products like

DNA, reactive oxygen species (ROS), cardiolipin and transcription factor A, mitochondrial (TFAM) and others (Ahrens et al., 2012; Apetoh et al., 2007; Fucikova et al., 2011; Garg, Vandenberk, et al., 2017; Ghiringhelli et al., 2009; Gorgulho, Romagnoli, Bharthi, & Lotze, 2019; Chiba et al., 2012; Krombach et al., 2019; Krysko et al., 2011; C. Li et al., 2018; Michaud et al., 2011; Obeid et al., 2007; Panaretakis et al., 2008; Rodriguez-Ruiz et al., 2019; Sistigu et al., 2014; Soloff & Lotze, 2019; Vacchelli et al., 2015; Yang et al., 2010; Zhang et al., 2010). Many of these molecules fall into category of DAMPs and are detected by specific pattern related receptors (PRRs) which then take a part in navigating subsequent immune response (Galluzzi et al., 2017). Another clinically relevant hallmarks of ICD are phosphorylation of eukaryotic translation initiation factor 2 subunit-α (EIF2S1, better known as eIF2α), the activation of autophagy, and a global arrest in transcription and translation (Bezu, Sauvat, Humeau, Leduc, et al., 2018; Humeau et al., 2020; Michaud et al., 2011; Rybstein, Bravo-San Pedro, Kroemer, & Galluzzi, 2018).

Table 1. Major DAMPs and cytokines mechanistically linked to ICD in cancer. Retrieved and modified from: (Galluzzi et al., 2020)

Factor	Class	Effect	Main receptor(s)
ANXA1	Surface protein	Directs APCs to dying cells	FPR1
ATP	Nucleotide	Promotes the recruitment, maturation and cross-presentation activity of APCs	P2RX7, P2RY2
CALR	ER chaperone	Promotes the uptake of dying cells and type I IFN secretion by APCs	LRP1
CCL2	Cytokine	Promotes T cell and neutrophil recruitment	CCR2
CXCL1	Cytokine	Promotes T cell and neutrophil recruitment	CXCR2
CXCL10	Cytokine	Promotes T cell and neutrophil recruitment	CXCR3
Cytosolic RNA	Nucleic acid	Promotes the secretion of type I IFN and other proinflammatory factors	MDA5, RIG-I, TLR3
Cytosolic DNA	Nucleic acid	Promotes the secretion of type I IFN and other proinflammatory factors	AIM2, CGAS, ZBP1
ERp57	ER chaperone	Promotes the uptake of dying cells by APCs	LRP1
Extracellular DNA	Nucleic acid	Promotes the recruitment and activation of neutrophils	TLR9
F-actin	Cytoskeletal component	Promotes the uptake of dying cells by APCs	CLEC9A
HMGB1	Nuclear DNA-binding protein	Promotes the maturation and cross-presentation activity of APCs	AGER, TLR2 TLR4
HSP70	ER chaperone	Favors the uptake of dying cells by APCs	LRP1
HSP90	ER chaperone	Favors the uptake of dying cells by APCs	LRP1
TFAM	Transcription factor	Promotes APC maturation and recruitment	AGER
Type I IFN	Cytokine	Promotes APC maturation, cross-presentation, and T cell recruitment	IFNARs

The most prominent PRRs and other receptors involved in ICD signaling are: Toll-like receptors (TLRs), cyclic GMP-AMP synthase (CGAS), a sensor of cytosolic double-stranded DNA (dsDNA), RIG-I-like receptors (RLRs), a group of RNA-specific PRRs named after DExD/H-box helicase 58 (DDX58, best known as RIG- I), Z-DNA binding protein 1 (ZBP1), a nucleic acid sensor also known as DAI, NOD-like receptors (NLRs), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), low density lipoprotein receptor-related protein 1 (LRP1, also known as CD91) and purinergic receptors (P2X7/P2RY2) (**Table 1**) (Ablasser & Chen, 2019; Alarcon et al., 2015; Basu, Binder, Ramalingam, & Srivastava, 2001; Galluzzi, Vanpouille-Box, Bakhoum, & Demaria, 2018; Gay, Symmons, Gangloff, & Bryant, 2014; Chow, Gale, & Loo, 2018; Kawai & Akira, 2011; Kersse, Bertrand, Lamkanfi, & Vandenabeele, 2011; Kuriakose & Kanneganti, 2018; Motta, Soares, Sun, & Philpott, 2015; Vanpouille-Box, Hoffmann, & Galluzzi, 2019). These receptors upon recognition of associated DAMPs activate downstream signaling cascades resulting in production of various cytokines and cell maturation which might eventually culminate in initiation of adaptive immune response (**Figure 3**).

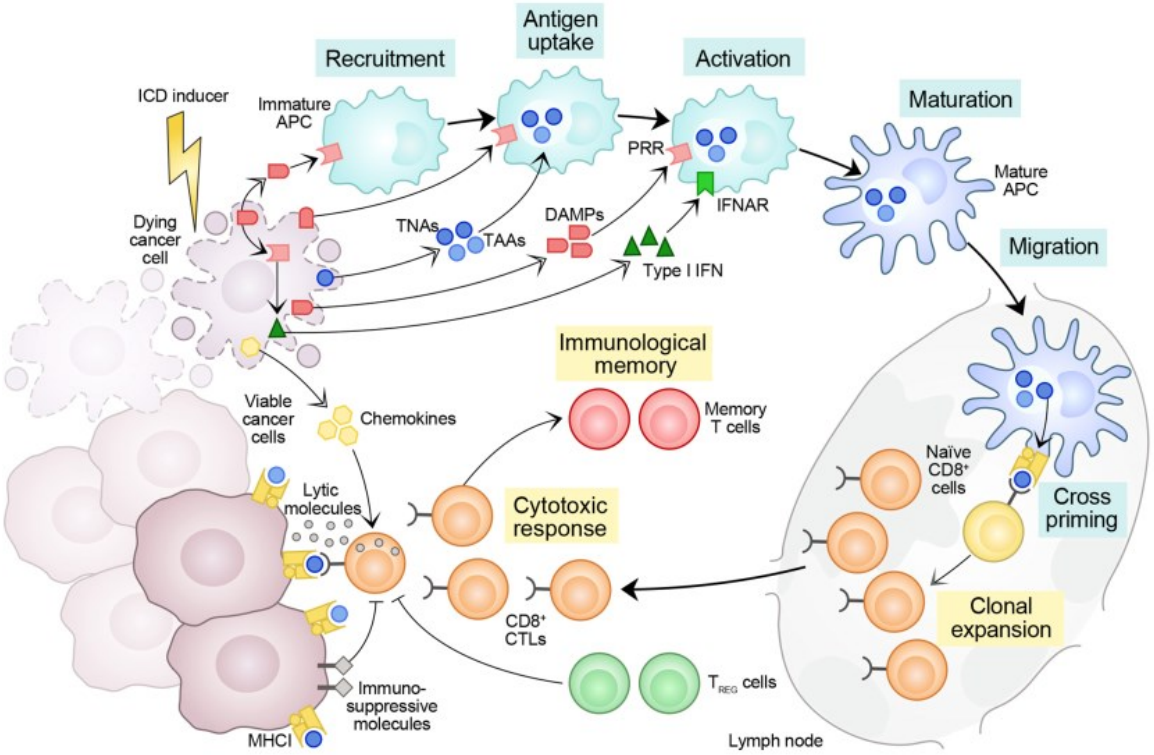

Figure 3. The concept of ICD. Cell undergoing a regulated cell death (RCD) in response to stress can prime an adaptive immune response via panel of immunostimulatory damage-associated molecular patterns (DAMPs) and cytokines that support the recruitment, phagocytic activity, and maturation of antigen-presenting cells (APCs), which prime a cytotoxic T lymphocyte (CTL)-dependent immune response. Adapted from: (Galluzzi et al., 2020).

Even though all cells (most likely) have sufficient levels of DAMP molecules to potentially undergo ICD, it is not always the case. There are three main factors determining the final immunogenicity of a dying cancer cell: (1) cancer cell antigenicity, (2) a capacity of a cell stressor to orchestrate the dynamics and intensity of DAMP release required for initiation of immune response and (3) specificity of a surrounding microenvironment which may either propense or hamper ICD driven adaptive immune response (**Figure 4)** (Hou et al., 2013; Krysko et al., 2012; Rufo, Garg, & Agostinis, 2017; Schumacher & Schreiber, 2015; Vesely, Kershaw, Schreiber, & Smyth, 2011).

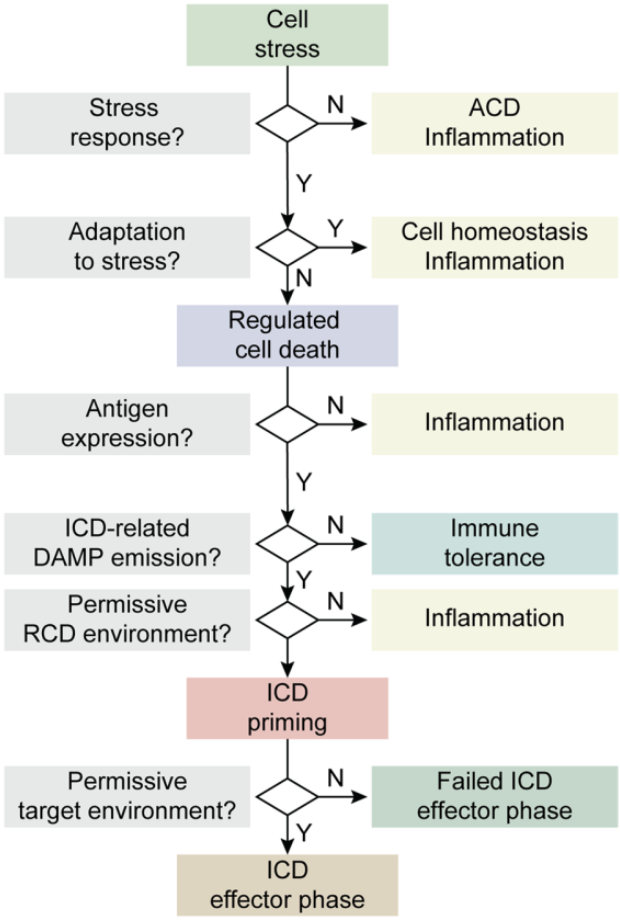

Figure 4. Core requirements for the initiation of adaptive immune responses by dying cells. For cell death to drive bona fide adaptive immune response: (1) cell death must occur in the context of adaptive stress responses; (2) cell death must ultimately occur, as opposed to successful adaptation to stress; (3) dying cells must present antigens that are not cover by thymic tolerance; (4) regulated cell death (RCD) must be accompanied by the emission of endogenous molecules that operate as immunological adjuvants and (5) microenvironmental conditions must be permissive for antigen-presenting cells (APC recruitment, maturation and migration to lymph node (or the sites of antigen presentation), as well as for cytotoxic T lymphocytes (CTL) infiltration and activation. Missing one of the conditions results in failed effector immune response. Retrieved from: (Galluzzi, Kepp, Hett, Kroemer, & Marincola, 2023)

1.3.2 Forms of ICD with encompassing signaling pathways

The first systemic screenings for agents that can induce ICD in cancer cells identified doxorubicin, mitoxantrone and γ-irradiation as efficient inducers (Galluzzi et al., 2020). The capacity of these anticancer agents to induce ICD was shown to be fully dependent on the ER stress induction. Nowadays, no less than four various types of ICD have been described, each depending on release and exposure of unique pattern of DAMPs which are orchestrated by various underlying molecular mechanisms activated by initial stressor (**Figure 5**). The following observations indicate that not all DAMPs are universally required for the engagement of adaptive immune response in various forms of ICD.

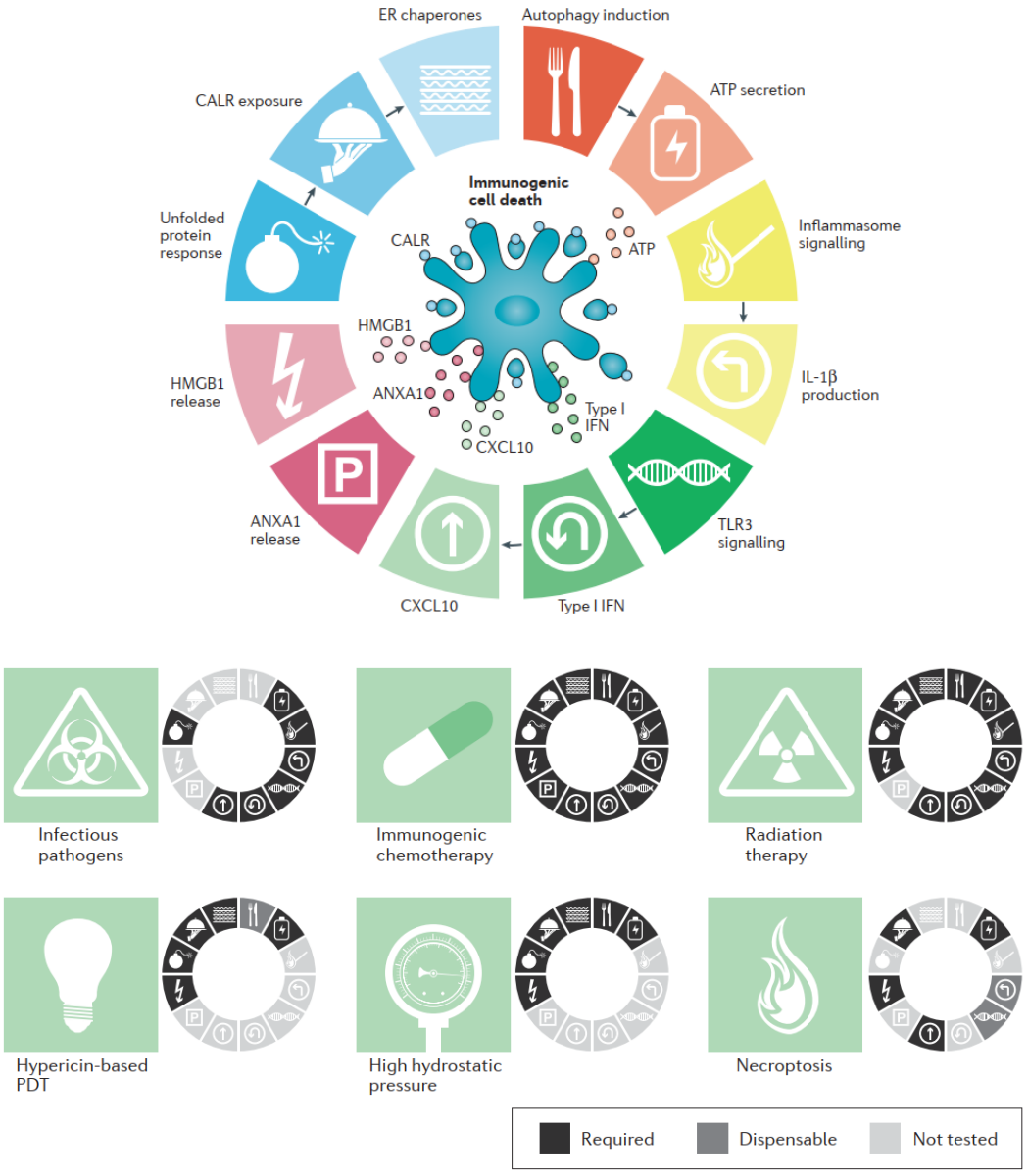

Figure 5. Differential requirements for the immunogenicity of cell death. Processes linked with ICD are unfolded

protein response and consequent exposure of calreticulin (CALR) and other endoplasmic reticulum (ER) chaperones on the cell surface; the activation of autophagy and consequent secretion of ATP; the release of interleukin-1β (IL-1β) upon inflammasome signaling; the activation of Toll-like receptor 3 (TLR3), resulting in a type I interferon (IFN) response that stimulates the production of CXC-chemokine ligand 10 (CXCL10); as well as the release of high-mobility group box 1 (HMGB1) and annexin A1 (ANXA1). Distinct variants of immunogenic cell death depend on (or are associated with) the emission of different sets of danger signals from dying cells. PDT, photodynamic therapy. Adapted from: (Galluzzi et al., 2017).

Pathogen induced ICD

Cell death represents one of the major defense mechanisms against invasion by pathogens. Thus, various microbial and virus-related structures fall within the group of microbial associated molecular patterns (MAMPs) sensed by extracellularly and intracellularly located PRRs resulting in activation of immune response as remnants of dying infected cells are taken up along with foreign microbial antigens (which are not covered by a central tolerance) by resident macrophages. Thus, pathogen and cellular fragments are subsequently presented to $CD4^+$ and $CD8^+$ T cells via MHCI and MHCII molecules-antigen complexes on APCs. Production of proinflammatory cytokines such as tumor necrosis factor (TNF) and type I IFNs, which resulted from PRRs engagement along with microbial peptides presentation by APCs eventually leads to activation of immune response. Initiated danger signaling further activates autophagy and unfolded protein response (UPR), however it has not been yet described to what extent these two mechanisms contribute to the final immunogenicity of pathogen induced cell death (Bettigole & Glimcher, 2015; Sica et al., 2015).

Chemotherapy induced ICD

Besides direct cytostatic and cytotoxic activity, some chemotherapeutics used in the clinics elicit ICD, as demonstrated by comprehensive preclinical and clinical findings. So far, only a few bona fide ICD inducers have been successfully employed in the clinics as cancer therapeutics, including anthracyclines (such as mitoxantrone and doxorubicin), paclitaxel, cyclophosphamide, bortezomib, bleomycin, oxaliplatin and teniposide (Galluzzi et al., 2020). While other conventional chemotherapeutics dispose immunostimulatory effects by mediating off-target effects on immune components as reviewed by our group (Fucikova et al., 2022). In murine models, chemotherapy driven ICD depends on eIF2a mediated exposure of various endoplasmic resident ER chaperones to the outer leaflet of the plasma

membrane, such as s CALR, protein disulfide isomerase family A member 3 (PDIA3; also known as ERp57), HSP70 (also known as HSPA1A), HSP90 (also known as HSP90AA1) (Fucikova et al., 2011; Obeid et al., 2007; Panaretakis et al., 2008). Additionally, chemotherapy driven ICDs is accompanied by autophagy mediated secretion of ATP, release of HMGB1 and ANXA1 (Apetoh et al., 2007; Michaud et al., 2011; Vacchelli et al., 2015). Moreover, the chemotherapy inflicted damage to the cellular components, most probably nucleic acids, results in activation of several intracellular PRRs namely Toll-like receptor 3 (TLR3) or stimulator of interferon genes (STING) and subsequent cancer intrinsic type I IFNs production and following type I IFNs response consisting of CXC-chemokine ligand 10 (CXCL10) production (Apetoh et al., 2007; Sistigu et al., 2014).

Chemotherapeutics that are unable to engage any of these pathways fail to stimulate anti-tumor immune response (Martins et al., 2011). Conversely administration of drugs such as thapsigargin, the activator of UPR response leading to phosphorylation of eIF2α, along with non-ICD chemotherapeutics, supplement the lacking capacity of chemotherapeutics to kill cancer cell immunogenically (Martins et al., 2011).

Besides conventional chemotherapy, several other drugs and chemicals have been shown to stimulate ICD. These are DNA methyltransferase, histone deacetylase (HDAC) and bromo-domain inhibitors; tyrosine kinase inhibitor crizotinib, the epidermal growth factor receptor (EGFR)-specific monoclonal antibody cetuximab, the cyclin-dependent kinase (CDK) inhibitor dinaciclib and the Bruton tyrosine kinase (BTK) inhibitor ibrutinib and other chemicals including the ubiquitin-specific peptidase inhibitor spautin-1, the antibiotic bleomycin, the protein phosphatase- 2A inhibitor LB-100, the herbal components shikonin and capsaicin (Galluzzi et al., 2020).

Physical modalities inducing ICD

So far several modalities such as high hydrostatic pressure (HHP), γ-irradiation, UVC, hypericin-based photodynamic therapy (Hy-PDT), α-irradiation with 213Bi particles, extracorporeal photochemotherapy, near-infrared photoimmunotherapy, cytotoxic heat shock, nanopulse stimulation and electrohyperthermia have been shown to induce ICD in both mice and human tumor cells (Galluzzi et al., 2020). Needlessly to say, frequency, intensity and dose have a very significant impact onto capacity of any treatment to

successfully induce ICD (Deutsch, Chargari, Galluzzi, & Kroemer, 2019; Ko, Benjamin, & Formenti, 2018; Wu & Waxman, 2018). In general, the immunogenicity relies on translocation of HSPs, release of HMGB1, secretion of ATP and production of type I IFNs (Deng et al., 2014; Fucikova et al., 2014; Garg, Krysko, Vandenabeele, & Agostinis, 2012; Garg, Krysko, Verfaillie, et al., 2012; Korbelik, Zhang, & Merchant, 2011). However, the molecular mechanisms that underlie danger signaling in cancer cells that are exposed to various physical cues to some extent differ from each other. As an example, neoplastic cells responding to Hy-PDT translocate CALR independently of eIF2α phosphorylation and caspase 8 activation. Conversely, HHP driven ICD fully dependent on eIF2α phosphorylation (Fucikova et al., 2014).

Necroptotic ICD

Necroptosis is a pro-inflammatory immunogenic form of cell death which unlike necrosis is regulated and executed by receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL) proteins. At the same time, necroptotic ICD is not caused by cellular membrane disintegration and random release of cytosol into surrounding environment, as accidental necrosis caused by freeze-thaw cycles and boiling did not confer such an immunogenic effect (Yang et al., 2016). Employing vaccination experiments it has been shown that accidental necrosis of TC-1 cells caused by freeze-thaw cycles did not instigated cancer protection to follow-up cancer challenge compared to necroptotic TC-1 cells which exposed CALR on its membrane, release HMGB1 and secreted ATP (Yang et al., 2016).

1.3.3 ICD signaling following DAMPs exposure and release by cancer cells

ICD is accompanied by the exposure and release of various DAMPs molecules which then navigate both innate and adaptive immune response against specific tumor antigens. ICD-associated DAMPs include mainly surface-exposed ER-chaperons such as CALR, subsequent active secretion of ATP, ANXA1, type I IFNs and passive release of HMGB1. Additional hallmarks of ICD encompass the phosphorylation of eIF2α.

ER chaperones displayed on the outer cellular membrane of cells, such as HSP70, HSP90 and CARL succumbing ICD serve as an "eat-me" signals to promote dying cells phagocytosis and antigen cross-presentation by DCs to mediate CD8 T cells anti-tumor immune response. The molecular mechanism underlying the chemotherapy driven ICD leading to CALR exposure include the phosphorylation of eIF2α, activation of caspase 8, followed by the cleavage of B-cell receptor-associated protein 31 (BAP31) and aggregation of the pro-apoptotic Bcl-2 family members, protein BAX and BAK1 at the outer mitochondrial membrane (Panaretakis et al., 2008). Ecto-CALR binds mainly to LDL-receptor-related protein 1 (LRP1), whose downregulation abolishes immunogenicity of ICD related CALR (Cirone et al., 2012). Similarly, downregulation of CALR using shRNA also blunts immunogenicity of ICD succumbing cells (Gilardini Montani et al., 2015). However, pro-fagocytic properties of ecto-CALR are antagonized by "don't eat me" signals such as CD47, widely presented on tumor cells. Besides the role of CALR on the initiation of adaptive T-cell-mediated immunity downstream of ICD, we have demonstrated that ecto-CALR is associated with improved innate immunity mediated by NK cells in acute myeloid leukemia patients (AML) (Truxova et al., 2020). Recently, a novel insight into the biology of ICD-driven activation of NK cells, as NKp46 receptor was identified as a receptor for ecto-CALR, directly driving cytolytic functions of NK cells against malignant cells (Sen Santara et al., 2023).

ATP released in an autophagy-dependent manner through the active exocytosis of ATP-containing vesicles via pannexin channels (Martins et al., 2014). Extracellular ATP promote chemotaxis and myeloid cells motility into the sites of active ICD via production of chemo-attractants and other proinflammatory mediators. These effects are mediated by binding extracellular ATP to purinergic receptors P2Y2 (P2RY2) and P2X7 (P2RX7) on APCs triggering chemotaxis and inflammasome mediated activation of caspase-1 resulting in increased production of active IL-1β (Corriden & Insel, 2012; Elliott et al., 2009; Ghiringhelli et al., 2009; Stoffels et al., 2015). IL-1β stimulated by ICD is critical feature for subsequent adaptive immune response orchestrated by $CD8^+$ T cells and IL-17 producing γδT cells (Ma et al., 2011).

The release of **HMGB1** from dying cells is still not completely understood, however its immunogenicity has been well documented. In ICD settings extracellular HMGB1 bind multiple PRRs on myeloid compartment, such as advanced glycosylation end-product-specific receptor (AGER, best known as RAGE) and TLR4, signaling via MYD88. The

signaling through TLR4/MyD88 axis is required for immunogenic cell death, as documented by decreased immunization induced by vaccination in *TLR4*$^{-/-}$ and *MyD88*$^{-/-}$ experimental models (Apetoh et al., 2007). In line with this notion, the knockout of HMGB1 in cancer cells and/or the antibody mediated neutralization of TLR4 abolish the development of anti-tumor immunity in experimental mice models (Apetoh et al., 2007).

ANXA1 is yet another critical molecule necessary for priming adaptive anti-tumor immune response in doxorubicin or oxaliplatin (ICD inducers) therapy. ANXA1 serves as homing factor that mediate the motility of DCs or their precursors to neoplastic cells via formyl peptide receptor 1 (FRP1) (Vacchelli et al., 2015). In line with this notion, the loss-of-function polymorphisms in FRP1 was shown to be associated with poor disease outcome in breast carcinoma after anthracycline-based therapy (Baracco et al., 2016).

All nucleated cells, including malignant cells, comprise the ability to produce and secrete **type I IFNs**. The production by epithelial and fibroblast cells is induced mostly upon viral infection and via autocrine and paracrine signaling. On the other hand, the production of type I IFNs by malignant cells is induced by abundantly present ds/ssDNA self-derived genomic fragments as result of genomic instability and also by chemotherapy- and radiotherapy driven ICD (Sistigu et al., 2014). Moreover, CALR exposure on outer cell membrane seems to drive type I IFNs secretion by APCs in both mouse model and patients (Fucikova et al., 2018; X. Chen, Fosco, Kline, & Kline, 2017). These nucleic fragments subsequently get recognized by PRRs such as TLR3, STING and other nucleic acid sensors which then trigger downstream signaling resulting in type I IFNs production and secretion. The result of type I IFNs binding to IFNAR1/IFNAR2 heterodimeric receptor is a production and secretion of IFN stimulated genes (ISGs), which besides inducing antiviral state in surrounding tissue, make tumor cells more visible by upregulating MHCI molecules and also attract and promote activation of DCs, NK cells, $CD8^+$ T cells (Wang et al., 2017). However, the adjuvanticity of type I IFNs in TME is not direct but rather mediated by cancer cell autonomous signal transduction cascade initiated by TLR3 as demonstrated in experimental mice models of chemotherapy and radiotherapy driven ICD. Binding of type I IFNs to IFNAR on neoplastic cells, type I IFNs trigger autocrine and paracrine circuitries that result in the release of chemokine (C-X-C motif) ligand 10 (CXCL10). Supporting this notion, the therapeutic efficacy of ICD-mediated chemotherapy was significantly reduced in tumors deficient for *Ifnar1*, *Ifnar2*, *Tlr3*, *Cgas*, or *Sting1* or in mouse treated with IFNAR1-blocking antibodies (Deng et al., 2014; Sistigu et al., 2014; Vanpouille-Box et al., 2017). Further

supporting this notion *Ifnar1* deficient murine cancer cells, as in contrast to wild type counterparts, failed to vaccinate syngeneic host upon treatment with ICD inducer, doxorubicin (Sistigu et al., 2014).

1.3.4 ICD subverting strategies

Despite increased antigenicity of tumor cells (to various extents), it is apparent that cancer cell might suppress its overall immunogenicity by evolving mechanisms which target and diminish their adjuvanticity directly or by deregulating associated down- or up-stream processes (release and sensing of DAMPs and UPR or autophagy respectively) (**Figure 6**) (Fucikova et al., 2015; Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; M. O. Li & Rudensky, 2016). Moreover, a redox state of TME and of individual danger molecules may have an impact onto final immunogenicity of regulated cell death. It has been shown that oxidized HMGB1 released by cancer cell during pyroptosis, a form of regulated cell death, promotes expression of inhibitory molecules within TME, thus diminishes anticancer immune response. On the other hand, oxidized mitochondrial DNA triggers activation of inflammasome resulting in secretion of proinflammatory cytokines thus boosting anticancer immunity (Shimada et al., 2012).

Taken together, ICD subverting strategies might not only prevent development of immune-surveillance but also impair effectiveness of various chemo- and radio- and immunotherapy treatments which relies on capacity of cancer cells to emit DAMPs. Therefore, novel clinically relevant strategies are needed to overcome the lack of tumor cell immunogenicity, with particular respect to cancer adjuvanticity (Galluzzi et al., 2017).

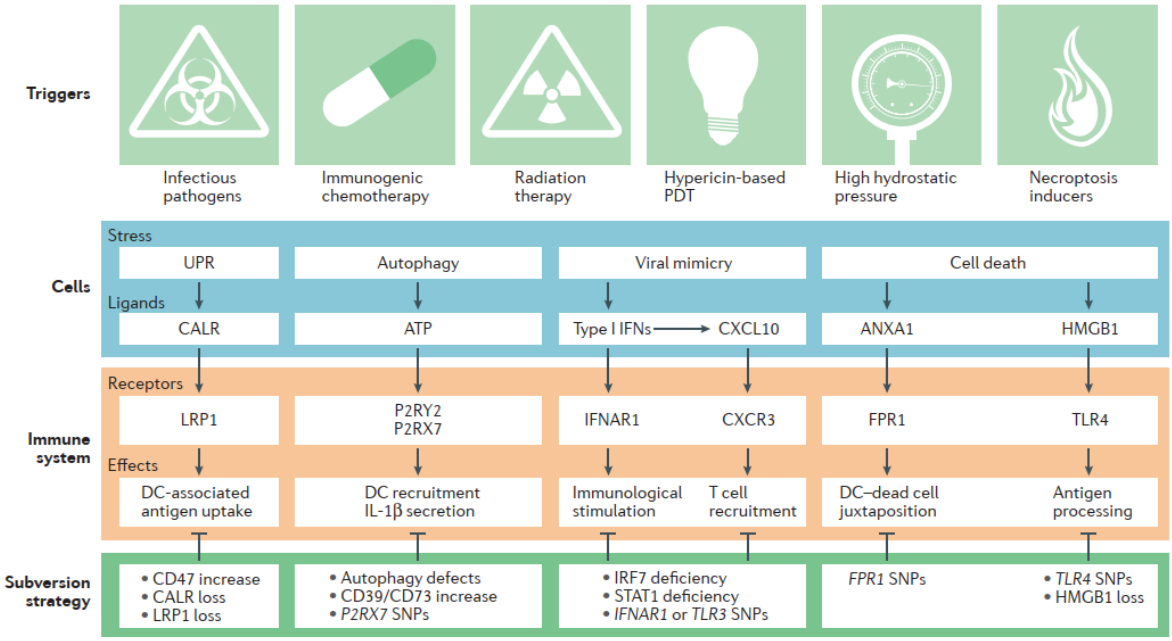

Figure 6. Subversion of danger signaling. As they escape immunosurveillance malignant cells acquire the ability to suppress danger signaling or the consequences thereof. In addition, clinical data obtained in patients with cancer indicate that multiple defects in the perception of damage-associated molecular patterns (DAMPs) by the host are associated with worsened disease outcome. Adapted from: (Galluzzi et al., 2017).

1.3.5 Monitoring of ICD and associated processes in oncological settings

The current gold-standard approach to determine the immunogenicity of dying tumor cells killed by putative ICD inducer relies on vaccination experiments in which dying murine cancer cells are injected into syngeneic immunocompetent mice with subsequent re-challenge with living cancer cells of the same origin (Galluzzi et al., 2020). Percentages of tumor free mice, tumor growth and a tumor size in vaccinated mice compared to unvaccinated ones gives off an approximation of immunogenic capacity of dying cancer cells. The vaccination experiments and other *in vivo* approaches testing ICD-potential of a treatment can be done in various settings (**Figure 7**). However, for apparent reasons, the immunogenicity of cancer cells cannot be tested in such manners in humans. The research in the field of ICD has provided an important knowledge about the mechanism of ICD and not less importantly about its correlates. These correlates are often utilized as surrogate biomarkers for ICD both in patients and in various preclinical models of ICD (**Figure 8**). Based on this knowledge, numbers of methodological approaches for detection of ICD *in vitro* and *ex vivo* were developed for preclinical and clinical practice. Assessment of ICD-

potential in oncological settings is therefore rather determined by evaluation of corresponding biochemical ICD features and associated signaling cascades combined with subsequent evaluation of ICD-mediated anticancer immunity in the tumor microenvironment (Galluzzi et al., 2020).

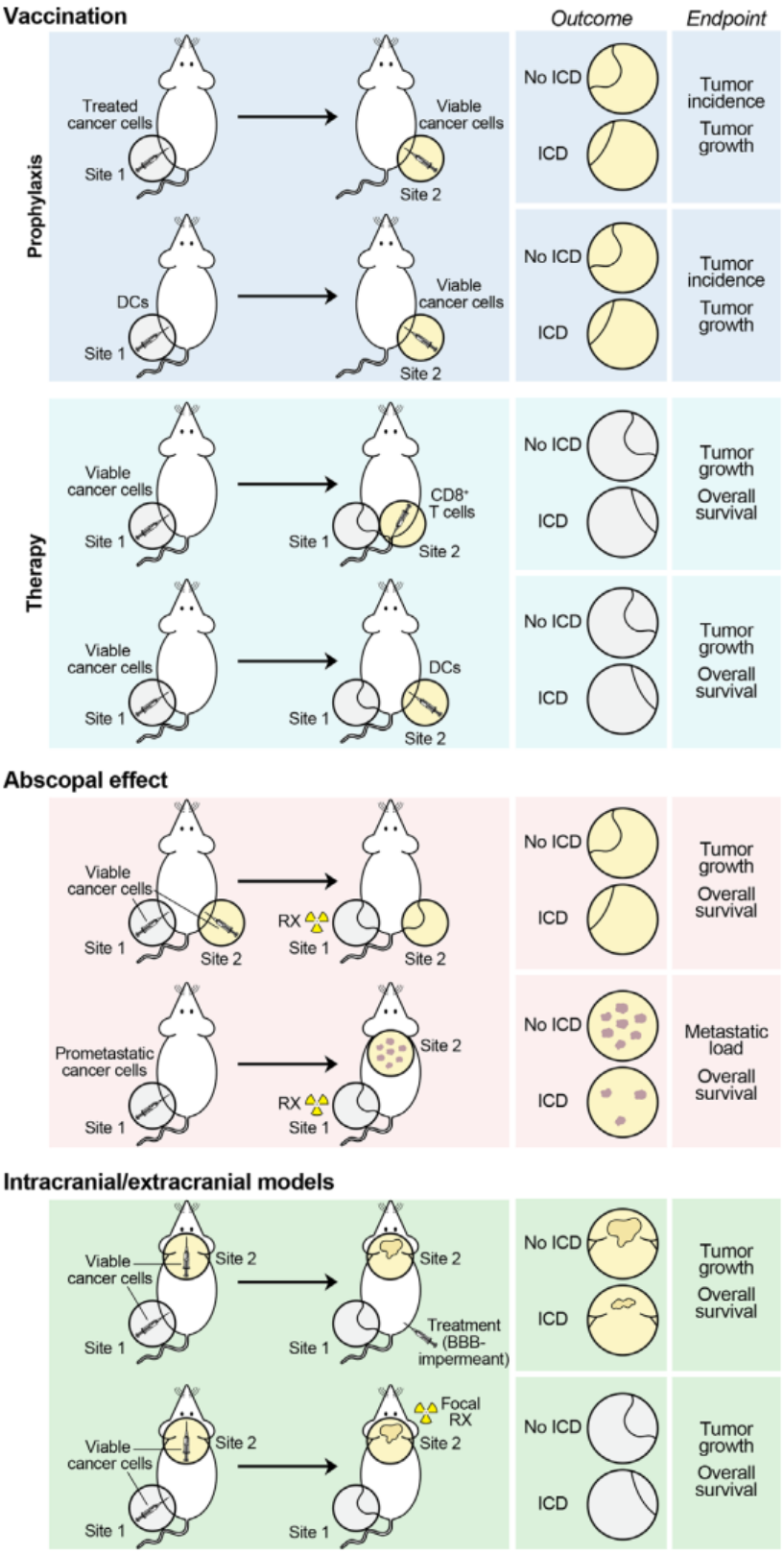

Figure 7. Current methods to assess ICD *in vivo*, in oncological settings. In prophylactic models, mouse cancer cells *succumbing in vitro* to a potential inducer of immunogenic cell death (ICD) are used as a vaccine, either as such, or on loading on immature, syngeneic dendritic cells (DCs). The ability of mice to reject (tumor incidence) or control (tumor growth) a rechallenge with living cancer cells of the same type inoculated 1–2 weeks later is monitored as a sign of protective anticancer immunity. In therapeutic settings, mouse tumors developing in immunocompetent syngeneic hosts are treated with autologous DCs preloaded with cancer cells exposed to a potential ICD inducer *in vitro* (generally in combination with immunological adjuvants), or with autologous $CD8^+$ cytotoxic lymphocytes primed *in vitro* by the same DCs. Tumor control and mouse survival are monitored as indicators of therapeutic anticancer immunity. In abscopal models, mouse cancer cells are harnessed to generate lesions at distant anatomical sites, followed by treatment at only one disease site. Tumor control at the non- treated disease site and mouse survival are monitored as signs of systemic anticancer immunity with therapeutic relevance. Finally, in intracranial/extracranial models, mouse cancer cells are employed to generate one intracranial and one extracranial tumor, only one of which receives treatment. As in abscopal models, tumor control at the non-treated disease site and mouse survival are monitored as indicators of therapeutic anticancer immunity with systemic outreach. In all these models, mice achieving systemic, long-term disease eradication are often rechallenged with cancer cells to monitor durability and specificity (with unrelated, but syngeneic cancer cells). Retrieved from: (Galluzzi et al., 2020)

ISR and HSPs

Detection of ICD associated CALR and other HSPs expression on outer leaflet of a cellular membrane can be monitored by: (1) Flow cytometry (FACS) using specific anti-CALR antibodies and viability dyes, such as 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), or 7-aminoactinomycin D (7-AAD) for exclusion of late apoptotic and necrotic cells with no longer intact outer membrane; (2) Immunoblotting of previously biotinylated cell membrane proteins precipitated by streptavidin; (3) Fluorescent microscopy, which can be used for visualization of CALR upon immunostaining in paraformaldehyde fixed cells (Fucikova, Truxova, et al., 2016; Gao, Zhu, Liu, & Nabi, 2019; Garg, Krysko, Verfaillie, et al., 2012; Pacheco, Merianda, Twiss, & Gallo, 2020) (**Figure 8**).

ICD associated expression of HSPs is orchestrated by complex mechanism called integrated stress response (ISR) culminating in phosphorylation of eiF2a either by protein kinase R (PKR) or protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). Phosphorylation status of eiF2a can be determined by: (1) FACS analyses, (2) immunohistochemistry (IHC), (3) by immunofluorescent labeling (IF) and (4) immunoblotting, whereas FACS, IHC and IF are largely suitable for large scale screening (Bezu, Sauvat, Humeau, Gomes-da-Silva, et al., 2018; Harding, Zhang, & Ron, 1999; Reineke, Dougherty, Pierre, & Lloyd, 2012).

HMGB1 and ANXA1

ICD-associated release of HMGB1 and ANXA1 can be evaluated directly by assessment of extracellular level in cell culture supernatant, sera or other biological fluids using ELISA or by immunoblotting (Apetoh et al., 2007; Garg, Vandenberk, et al., 2017; Melis et al., 2013; Pozzi et al., 2016; Sistigu et al., 2014; Vacchelli et al., 2015). The HMGB1 release can also be determined indirectly by immunoblotting of remaining intracellular HMGB1 (Fucikova et al., 2014; Zhao, Liu, Kepp, & Kroemer, 2019). IHC has also been successfully used in detection of HMGB1 in patient´s samples. Other methods such as retention using selective hooks (RUSH) system, based on nuclear hooks along with GFP tagged HMGB1 cells are quite useful in large scale screening of potential ICD inducers specifically focused on HMGB1 (Zhao et al., 2019) (**Figure 8**).

ATP

For the evaluation of ATP release, both direct and indirect, commercial luminescence-based assays are considered the golden standard (Branchini & Southworth, 2017; Dubyak, 2019). However, the ATP degrading enzymes such CD39, often-times present on cancer cells, might dramatically affect the extracellular concentration of ATP and this limitation needs to be always considered. Alternatively intracellular ATP containing vesicles can be evaluated with the use of a fluorescent fluorochrome quinacrine by FACS or IF. (Marceau, Roy, & Bouthillier, 2014)

Type I IFNs

Secreted levels of type I IFNs might be evaluated by ELISA along with multiplex beads assays. Both methods can be employed for assessment of type I IFNs levels secretion from cultured cell lines, from isolated primary tumors cells or even from various patient´s biological fluids. Other common successfully employed approaches for evaluation of type I IFNs levels are RT-qPCR based measurement of mRNA levels or immunoblotting of cellular lysates (Diamond et al., 2018; Schiavoni et al., 2011; Sistigu et al., 2014; Vanpouille-Box et al., 2017). An indirect approach to evaluate type IFNs is to measure mRNA levels of paramount type I IFNs responding genes downstream of IFNAR receptor (IFN stimulated genes, ISGs) including MX dynamin-like GTPase 1 (MX1) or CXCL10 (Bordignon et al.,

2008; Sistigu et al., 2014). Yet other approaches utilized to visualize type I IFN in mice and patient´s cancer samples combine immunostaining with IHC or IF microscopy (Downey et al., 2017; Sarkar et al., 2018; Takahashi et al., 2018). However it is important to keep in mind that the gene transcription and intracellular protein levels does not always results in IFN secretion and therefore ICD potential based on such data needs to be interpreted cautiously (Gry et al., 2009). Despite that being said, type I IFNs proteins in patient´s samples are very much prone to degradation and therefore the evaluation of mRNA expression levels is often-time the only compatible way to screen large scale patients' cohorts.

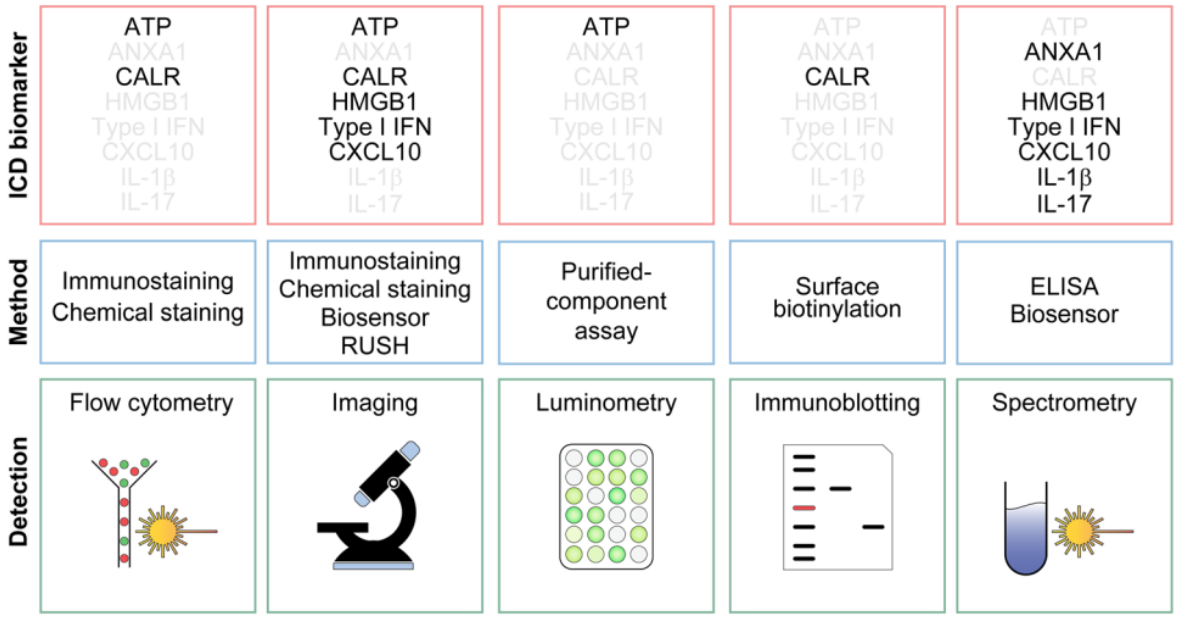

Figure 8. Main methodological approaches to measure ICD biomarkers *in vitro* and *ex vivo*. The main hallmarks of immunogenic cell death (ICD) can be assessed by flow cytometry, (immuno)fluorescence microscopy, immunoblotting, or luminometry, based on a variety of different approaches. Retrieved from (Fucikova et al., 2020)

1.4 Clinical applications of ICD in cancer management

Rationale for employing and exploiting ICD mechanisms in cancer therapy comes from myriad of preclinical and clinical research data indicating that ICD induction is especially important for effectiveness of various standard of cancer therapies as well as cancer-immunotherapies (Yamazaki, Buque, Ames, & Galluzzi, 2020). In various rodent models, it has been shown that plethora of ICD inducers positively synergies with ICIs in otherwise ICIs resistant mice models and that ICD signaling in tumor associates with superior response to clinically employed therapies such as chemotherapy, radiation therapy, ICI-based immunotherapy and photodynamic therapy (Gomes-da-Silva et al., 2018; Liu et al., 2022; Petrazzuolo, Perez-Lanzon, Liu, Maiuri, & Kroemer, 2021; Petroni, Buque, Zitvogel, Kroemer, & Galluzzi, 2021; Pfirschke et al., 2016; Yamazaki, Buque, et al., 2020; Zhou et al., 2022).

Similarly, accumulating findings demonstrate the clinical relevance of ICD in cancer patients. Patients with defects in various elements and stages of ICD signaling such as DAMPs emission, DAMPs sensing or executing mechanism of regulated cell death demonstrate worse prognosis and poor response rate to ICD inducing therapies (**Table 2**) (Apetoh et al., 2007; Garg, More, et al., 2017; Vacchelli et al., 2015). Furthermore, as predicted, the immune compartment of TME of patients treated with ICD inducing agents exhibit activated anticancer immune response compared to patients treated with therapies having rather immunosuppressive effect (Kroemer, Galassi, Zitvogel, & Galluzzi, 2022).

Table 2. Cancer cell´s ICD-subversion strategies. Retrieved and modified from (Galluzzi et al., 2017)

Danger signal	*Strategy*	*Setting*	*Notes*
Cancer cells			
UPR and ER chaperone signalling	Improved ER homeostasis	Patients affected by multiple tumours	High levels of GRP78 correlated with worsened disease outcome
	CALR loss	Patients with NSCLC	CALR levels of expression in malignant cells correlated with the phosphorylation of eIF2A and influenced disease outcome
	Limited HSP exposure	Patients with NHL	Limited HSP90 exposure was associated with no clinical responses to autologous cancer cell-based vaccination
	CD47 upregulation	Patients affected by multiple tumours	Low CD47 levels on neoplastic cells correlated with improved disease outcome
	LRP1 downregulation	Patients with melanoma	High LRP1 levels on monocytes were associated with slow progression
Autophagy and ATP signalling	Overexpression of BCL-2-like proteins	Patients affected by multiple tumours	Several cancers are characterized by the overexpression of BCL-2-like proteins, which potently inhibit autophagy
	BECN1 downregulation	Breast cancer patients	Decreased *BECN1* mRNA levels were associated with poor prognosis
	CD39 and/or CD73 overexpression	Patients affected by multiple tumours	High CD39 and/or CD73 levels on malignant or immune cells correlated with worsened disease outcome
	P2RX7 SNPs	Patients with breast cancer	Loss-of-function *P2RX7* mutation was associated with shortened time-to-metastasis
RNA signalling	*TLR3* SNPs	Patients affected by multiple tumours	*TLR3* mutational status influenced disease outcome
	TLR3 downregulation	Patients affected by multiple tumours	High TLR3 mRNA or protein levels were associated with improved disease outcome
	TRIF downregulation	Patients with hepatocellular carcinoma	Robust TRIF expression correlated with increased overall survival
Type I IFN signalling	*IFNAR1* SNPs	Patients with glioma	Loss-of-function *IFNAR1* mutation was associated with worsened disease outcome
	IRF7 downregulation	Patients with breast cancer	Low IRF7 levels have been linked to decreased metastasis-free survival
	STAT1 deficiency	Patients with breast cancer	Approximately 33% of breast cancer biopsies displayed undetectable or extremely reduced STAT1 levels
ANXA1 signalling	*FRP1* SNPs	Patients with breast cancer	Loss-of-function *FPR1* mutation was associated with shortened time-to-metastasis and decreased overall survival
HMGB1 signalling	HMGB1 loss	Patients with breast cancer	Loss of nuclear HMGB1 positively correlated with tumour size
	TLR4 SNPs	Patients with breast cancer	Loss-of-function *TLR4* mutation was associated with shortened time-to-metastasis
Cell death	*TP53* mutations	Patients affected by multiple tumours	Mutations in *TP53* are found in >50% of all human cancers, and are associated with increased resistance to cell death
	Altered expression of BCL-2 family members	Patients affected by multiple tumours	Many cancers overexpress anti-apoptotic BCL-2-like proteins or inactivate their pro-apoptotic counterparts

1.4.1 Prognostic and predictive role of ICD and associated signaling in cancer therapy

As the previous chapter suggests, one of the main potentials for ICD applications lies in stratifying cancer patients based on their ability to undergo ICD. Such stratification could help us to identify patients who are more likely to benefit from both conventional and combined immunotherapies therefore improving the overall survival of patients (OS), lessen the unnecessary toxicities and generally improving the quality of patient´s life.

Supporting this notion, it was shown that high levels of ISR-mediated ecto-CALR in the TME positively corelate with immune infiltration and active anticancer immune response and they also conferred a positive prognostic value in large cohorts of AML, ovarian and lung cancer patients (Fucikova, Becht, et al., 2016; Kasikova et al., 2019; Schardt, Weber, Eyholzer, Mueller, & Pabst, 2009; Wemeau et al., 2010). Another example highlighting the predictive capacity of ICD-related biomarkers comes to a type I IFN–related signature which conferred superior clinical responses to anthracycline-based chemotherapy (ICD inducer) in independent cohorts of patients with breast carcinoma from two independent clinical trials (Sistigu et al., 2014). Furthermore, high intratumoral levels of HMGB1 were shown to predict the successful response to chemoradiotherapy in the cohort of 75 patients with rectal cancer (Hongo et al., 2015). More complex approaches utilize various ICD signaling signatures, based on RNA transcription datasets correlated with protein data from patient´s biopsies. This approach assess patient´s immune landscape and predict responses to immunotherapies, as previously demonstrated for cutaneous melanoma, head and neck squamous cell, breast and gastric cancer (Fu & Ma, 2022; Gan, Tang, & Li, 2023; X. Wang et al., 2022; X. Wang et al., 2021) .

1.4.2 Exploiting ICD in combined anti-cancer therapy

Based on preclinical findings in experimental mice models documenting impact of ICD inducers on anti-tumor immunity development, ICD inducers are currently tested either alone or combined with standard of care therapy and/or immunotherapy in ongoing clinical trials (Garg, More, et al., 2017; Pol et al., 2015). Furthermore, the combination of various therapies might provide an advantage over single approach treatment in terms of increased

efficacy, decreased tumor resistance, shortened treatment period and lower drug doses to minimize side effects. This is also highly applicable for combining various ICD inducers where beside mentioned advantages of combined conventional treatments, combined ICD therapies also potentiate and maximize the long lasting anticancer immune response.

ICD in combination with chemotherapy and targeted anticancer agents

Intact DAMPs sensing pathways and downstream ICD signaling in TME was demonstrated to be essential for efficacy of various standard of care chemotherapeutics in cancer therapy. In addition, ICD signaling associates with superior response to clinically employed therapies such as anthracycline or platinum based therapy, radiation therapy, epidermal growth factor receptor (EGFR) and multitarget tyrosine kinase inhibitors (Liu et al., 2022; Petrazzuolo et al., 2021; Petroni et al., 2021; Yamazaki, Buque, et al., 2020; Yamazaki, Kirchmair, et al., 2020; Zhou et al., 2022).

Furthermore, the combination of cisplatin (non-ICD standalone) and 5-fluorouracil trigger enhanced release of HMGB1 which led to maturation of DC and general improvement of anticancer immune response coupled with favorable prognosis (Nishimura et al., 2021). Similar trend was observed with combined application of sunitinib and paclitaxel, where the synergy of the combination allows for the application of reduced dosage which limits paclitaxel-driven side effects (Qin et al., 2020).

Further understanding of immunological mechanisms driven by immunogenic chemotherapy has potential for further development of synergistic combination regiments with immunotherapy and identification of reliable biomarkers in the field.

ICD in combination with Immunotherapy

Due to well described capacity to re-invigorate anticancer immune response, the field of ICIs is one of the most promising areas for combined immunotherapy with ICD inducers. Accumulating preclinical and clinical findings demonstrate the synergy between, ICD inducers and immunotherapy, mainly ICIs in otherwise ICIs resistant mice models (Pfirschke et al., 2016). Supporting this notion, therapies combining ICD inducing agents alongside with ICIs indeed positively synergies as demonstrated by combined therapy based on paclitaxel and atezolizumab in patients with triple negative breast cancer (TNBC),

carboplatin/etoposide combined with atezolizumab in small cell lung cancer (SCLC) (Mansfield et al., 2020; Saito et al., 2021; Voorwerk et al., 2019).

Another promising approach is to either combine ICD inducers with DC cell-based vaccines and/or harnessing ICD induction for the generation of DC-based vaccines. The latter strategy requires cancer cells killed by ICD-inducers to mature and cross-prime DCs for potent adaptive anticancer immune activation in both prophylactic and therapeutic settings. Various ICD inducers have been investigated for the use in DC vaccines with predominantly positive results and they are also currently tested in clinical trials (Han, Hanlon, Sobolev, Chaudhury, & Edelson, 2019; Wculek et al., 2020). Positive preclinical and clinical responses have been achieved with both therapeutical and prophylactic DC based vaccine pulsed either with cancer cells killed by (1) Hy-PDT in experimental murine model of squamous cell carcinoma, high-grade glioma (HGG) and glioblastoma (GBM) (Doix, Trempolec, Riant, & Feron, 2019; Garg et al., 2016; Vedunova et al., 2022); (2) doxorubicine, shikonin and colchicine in murine models of neuroblastoma, breast cancer and melanoma (H. M. Chen et al., 2012; Komorowski, Tisonczyk, Kolakowska, Drozdz, & Kozbor, 2018; Lin et al., 2018; Wen et al., 2011); (3) HPP in murine models of prostate cancer and also in clinical trials of ovarian and lung cancer patients (Cibula et al., 2021; Mikyskova et al., 2017; Mikyskova et al., 2016; Rob et al., 2022; Zemanova et al., 2021); (4) UV radiation in murine models of squamous cell carcinoma and also partially positive results have been observed in management of head and neck squamous cell carcinoma (Jeong, Lee, Ko, & Son, 2007; Son, Mailliard, Watkins, & Lotze, 2001; Whiteside et al., 2016).

ICD in combination with Radiotherapy

Combination of physical ICD-inducing modalities with chemotherapeutics also demonstrated synergistic and additive effects. Conventional chemotherapeutics, such as oxaliplatin, 5-fluorouracil, cisplatin and adriamycin combined with radiotherapy led to significantly increased levels of CALR expression, HMGB1 and ATP release accompanied by enhanced proliferation of cytotoxic T cells and overall recruitment of immune cells (Petersen, Kua, Nakajima, Yong, & Kono, 2021). In patients, the combination of 5-fluorouracil with radiotherapy resulted in significantly higher response rate, with improved OS of patients (Bains et al., 2020; Golden et al., 2014; Jin et al., 2021; Petersen et al., 2021). In similar lines, combined radiation and heat therapy triggered ICD response, increased

levels of released DAMPs, altered immunosuppressive environment, and boosted anticancer immunity in malignant melanoma (Werthmoller et al., 2016).

Finally, in oncological settings, cancer cells are oftentimes defective in ICD associated signaling pathways, which might eventually diminish or even completely abolish stimulation of anticancer immunity. Currently this issue represents the main obstacle for efficacy of ICD inducing therapies and thus further research needs to be conducted to help overcome this problem.

2 Methodology

Cell lines and *in vitro* assays

Human AML cells KASUMI-1, MOLM-13 and MV4–11 as well as human chronic myeloid leukemia (CML) K562 cells. MOLM-13 and KASUMI-1 cells were grown in RPMI 1640 Medium containing 4 mM L-glutamine and 4500 mg/L glucose (Gibco), and supplemented with 20% fetal bovine serum (FBS, from PAA) and 100 U/mL penicillin plus 100 µg/mL streptomycin (Gibco). MV4-11 and K562 cells were cultured in IMDM containing 4 mM L-glutamine and 4500 mg/L glucose (Gibco) and supplemented with 10% FBS and 100 U/mL penicillin plus 100 µg/mL streptomycin. Peripheral blood mononuclear cells (PBMCs) and $CD33^+$ blasts were cultured in RPMI 1640 Medium containing 4 mM L-glutamine and 4500 mg/L glucose and supplemented with 10% plasma derived human pooled serum (PHS, from Thermo Fisher) and 100 U/mL penicillin plus 100 µg/mL streptomycin. All cell lines were regularly tested for Mycoplasma contamination using the MycoAlert® Mycoplasma Detection Kit (Lonza) and Spark reader (Tecan) to detect luminescence. Cell lines were used for experiments 2 to 5 passages from thawing and were maintained at 30-70% confluency. Polyinosinic:polycytidylic acid (poly(I:C), from InvivoGen), CpG oligodeoxynucleotides (ODN2216, from Invivogen) and TLR3/dsRNA specific complex inhibitor (TLRi, from Merck) were added to culture media to final concentrations of 50 µg/mL, 1,5 µM and 25 µM, respectively, for the indicated time. Recombinant human interferon-alpha (rIFN-α, from Bio-Techne) and recombinant human interferon-beta (rIFN-β, from PeproTech) were used at a final concentration of 500 pg/mL. AML cells were incubated with both rIFN-α and rIFN-β for 7 days, AML primary blasts for 3 days, leukemic stem cells (LSCs) for 5 days and PBMCs/$CD33^+$ cell depleted PBMCs for 24h at 37 °C in 5% CO2 humidified atmosphere before the analysis of phenotype, function and apoptosis by flow cytometry. Human rIFN-α and rIFN-β were re-administered into fresh media every 48 h during the incubation period. Chemotherapeutic drugs commonly used for treatment of AML including daunorubicin (DNR; KASUMI-1: 200 nM, MOLM-13: 150 nM, MV4–11: 400 nM, $CD33^+$ blasts: 500 nM, LSCs: 125 nM) (Sigma-Aldrich) and cytarabine (ARA-C; KASUMI-1: 500 nM, MOLM-13: 1 nM, MV4–11: 500 nM, $CD33^+$: 125 nM, LSCs: 125 nM) (Sigma-Aldrich) were used for the induction of apoptosis over a 24 h course. CD33+ malignant cells were incubated with an IFNAR1-blocking antibody (αIFNAR, from ThermoFisher - MMHAR-2) or isotype control (Iso, from ThermoFisher - PPV-04) at a final concentration of 8 µg/mL

for 24 h, and subsequently at a concentration of 2 μg/mL for 72 h, without culture further replacements in culture medium.

Quantitative real-time PCR (RT-qPCR)

Gene expression levels were evaluated on a CFX 96™ Real-Time System (Bio-Rad) using custom-designed primers and probes (500 nM and 200 nM final concentration, respectively) (Generi Biotech) and KAPA PROBE Fast Master Mix (Kapa Biosystems). Relative gene expression levels were calculated using the ΔΔCt method and were normalized to the expression level of reference gene SURF1 selected by Normfinder (GenEx software, MultiD Analyses). Samples below the detection limit were assigned a relative expression value of 0.

Flow cytometry

PBMCs, malignant blasts, LSCs and cultured tumor cells were stained with multiple panels of fluorescent primary antibodies, appropriate isotype controls and fixable viability dyes to exclude live/dead cells. For the *in vitro* assessment of apoptosis, cells were stained with Annexin V for 20 min at 4 °C and 4′,6-diamidin-2-fenylindol (DAPI) (0.1 μg/ mL) was added to cell suspension shortly prior to sample acquisition. Flow cytometry data were acquired on an LSRFortessa Analyzer (BD Biosciences) and analyzed with FlowJo v10.0 (Tree Star, Inc.).

Degranulation and IFN-γ production after *in vitro* stimulation

To assess NK cell and T cell function in whole PBMCs or $CD33^+$ cell-depleted PBMCs from AML patients, PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, from Sigma Aldrich) plus 1 μg/mL ionomycin or with K562 cells at an effector:target ratio 10:1 in the presence of anti-CD107a antibody (eBioscience) for 1 h, followed by 3h incubation with brefeldin A (BioLegend). Cells were then washed in PBS, stained with antibodies specific for surface markers, fixed in fixation/permeabilization buffer for 15 min (eBioscience), washed with permeabilization buffer and then stained with antibodies targeting a panel of intracellular markers.

Leukemic stem cells (LSCs)

LSCs were isolated from the PBMCs of AML patients as follows. Thawed cell suspensions were depleted of dead cells by magnetic separation using the Dead Cell Removal Kit (Miltenyi Biotech) and subsequently $CD34^+$ cells were isolated using CD34 MicroBead Kit

UltraPure (Miltenyi Biotech), according to the manufacturer's protocols. LSCs were determined as $CD45^{dim}$, Lin^- ($CD3^-CD14^-CD16^-CD19^-CD20^-CD56^-$), $CD34^+$, $CD38^{+/-}$ and $CD123^{+/dim}$ colony forming cells, as determined by flow cytometry and colony-forming assay.

IFNAR2 deletion

KASUMI-$1^{IFNAR2-/-}$ cells were prepared by the CRISPR/Cas9 technology. Briefly, KASUMI-1 cells were electroporated with a mixture of IFNAR2-specific gRNAs incorporated in a pSpCas9(BB)−2A-GFP (PX458) expression vector for dual expression of Cas9 and gRNAs. Two days after electroporation, GFP^+ cells were single sorted into 96-well plates coated with NSG mice bone marrow cells and expanded. Clone selection was performed based on (1) RT-qPCR specific for the *IFNAR2* (2), detection of IFNAR2 by flow cytometry, and (3) sensitivity to daunorubicin.

Statistical analysis

Statistical analyses were performed on GraphPad Prism 8, R v. 3.6.1 and R Studio. 3.6.0. The distributions of data sets were tested by Shapiro-Wilk Test, determining the use of the parametric or non-parametric tests for subsequent analyses. Paired and unpaired Student's t tests, as well as Wilcoxon and Mann-Whitney tests were used to assess differences between two groups. Differences among three or more groups were calculated using one-way ANOVA or Kruskal-Wallis tests corrected for multiple comparison by Holm-Sidak's or Dunn's tests. Pearson or Spearman correlations were used to evaluate the degree of the relationship between variables. Survival analyses were assessed for statistical significance with Log-rank tests. Univariate and multivariate Cox proportional hazard analysis were performed to assess the association of clinicopathological or immunological parameters with RFS and OS. Selected variables used in multivariate Cox regression hazard analysis exhibited no mutual collinearities, calculated by linear and logistic regressions and variance inflation factor (VIF). *p* values are reported and were considered not significant when >0.05.

RNA isolation and reverse transcription

Total RNA was isolated from the PBMCs of healthy donors (HDs) or AML patients (upon depletion of red blood cells using the ACK lysing buffer, from Thermo Fisher Scientific) as well as from isolated $CD33^+$ malignant blasts, using the RNeasy Mini Kit (Qiagen) as per the manufacturer's recommendations. RNA concentration and purity were determined using

a NanoDrop 2000c (Thermo Scientific), while RNA integrity was assessed using an Agilent 2000 Bioanalyzer (Agilent). Purified RNA samples were stored at -80°C, and only samples with an integrity index ≥ 7 were subsequently used for reverse transcription and RNAseq. The cDNA for the quantification by RT-qPCR was synthesized from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad).

Bulk RNA sequencing and analysis.

Sequencing libraries were created using NEBNext Poly(A) mRNA Magnetic isolation Module and NEBNext Ultra II Directional RNA Library Prep kits (both New England BioLabs) and were sequenced by NovaSeq 6000 SP (Illumina). Raw FASTQ sequencing files were aligned to human reference genome (build h19) with bowtie2 (version 2.3.2) and tophat2 (version 2.1). Expression levels as raw "counts" were calculated from aligned reads with mapping quality ≥10 using htseq-count (version 0.6.0). Differential gene expression analyses were performed using DESeq2 (version 1.24.0) in R. Heatmaps were assembled using the ComplexHeatmap package in R. The Cancer Genome Atlas program. The normalized gene expression data for 152 AML patients (after M3 and M7 AML subtypes exclusion) were retrieved from the public database (TCGA) and were used in correlation analyses.

Multiplex assay

Concentration of IFN-α2, IL6, IL10 and IL13 proteins in the sera of AML patients and ovarian patients from study SOV01 were by MAGPIX system (Luminex) using Magnetic Bead Panel HCYTOMAG-60K, 3-plex and 6-plex (Merck) according to manufacturer´s protocol.

ELISA

The concentration of IFN-β in cell culture supernatant was determined using High sensitivity human IFN Beta TCM ELISA (PBL) or by Human IFN-beta DuoSet ELISA (R&D) according to manufacturer's protocol. Sample absorbance was measured by Sunrise Microplate reader (Tecan).

[^{3}H]-thymidine incorporation assay

Inhibition of cell proliferation was determined using the [^{3}H]-thymidine incorporation assay. The cells (2 x 10^4 /well) were seeded into a 96-well flat-bottom tissue culture plate (Nunc)

and were cultivated in growth medium in final volume of 250uL. The plates were incubated in a 5% CO2 atmosphere at 37°C. Triplicates to pentaplets were used for each test condition. At the beginning of the assay cells were incubated with rIFN-α and rIFN-β (500pg/mL) for 7 days and each well was pulsed with 1 μCi (37 kBq) of [^{3}H]-thymidine for the last 6–8 h of total incubation time. The cells were then harvested on glass fiber filters (Perkin Elmer) using a cell harvester (Tomtec) and the radioactive emission was measured in a scintillation counter (1450 Microbeta TriLux, Wallac).

Colony-forming assays

For colony-forming assays, 5 x 10^3 $CD34^+$ enriched cells were cultured in 6-well plates in a mixture of 300 μL IMDM plus 2700 μL of the semi-solid methylcellulose-based media MethoCult™ H4534 Classic Without EPO (StemCell Technologies). Colonies were quantified according to the manufacturer´s guidelines. LSCs were cultured and maintained using a specific medium that preserves the LSC phenotype: StemSpan™ Leukemic Cell Culture Kit (StemCell Technologies).

Preparation of cell lysates and immunoblotting analysis

Cells were harvested and washed with ice-cold PBS and subsequently lysed in sample buffer (300 mM Tris pH 6.8, 5% SDS, 50% Glycerol, 360 mM β-Mercaptoethanol, 0.05% Bromophenol blue). Proteins were separated by 11% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDSPAGE) and then transferred to 0.45μm nitrocellulose membranes (Biorad). Efficiency of the protein transfer was checked by Ponceau-S staining (Abcam). The membranes were blocked in 5% nonfat dry milk in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1h at room temperature and incubated with primary antibody overnight at 4°C. Membranes were washed in TBST buffer and incubated for 1h at room temperature with relevant horseradish peroxidase-conjugated secondary antibodies. SuperSignal West Dura Extended duration detection system (ThermoFisher) was used for protein visualization, performed by imaging system ChemiDoc (Biorad).

Mice and in vivo experiments

$Rag2^{-/-}$ mice on BALB/c background (H-2^d) used for in vivo experiments were 8-20 weeks old and with body weight at least 19 g. Mice were obtained from the Institute of Microbiology of the Czech Academy of Sciences (Prague, Czech Republic) breeding colony.

Food and water were given ad libitum. All animal work strictly followed the protocol approved by the Institutional Animal Care and Use Committee of the Czech Academy of Sciences, with all experiments conducted in compliance with local and European guidelines. $Rag2^{-/-}$ mice were i.v. inoculated with 2.5 x 10^6 KASUMI-1^{WT} or KASUMI-$1^{IFNAR2-/-}$ cells in 300 μL PBS on day 0. Human rIFN-β was i.p. injected on days 13, 14, 15 and 16 twice daily (early morning and late afternoon) in 250 μL PBS using 1.5 μg/dose, i.e. 3 ug per mouse each day. Daunorubicin was administered via tail vein injection on days 15, 17 and 19 in 300 μL PBS using 4 mg/kg per one dose. The body weight of experimental mice was recorded 2-3 times a week throughout the whole experiment.

Preparation of DCVAC

Each DCVAC dose comprises DCs loaded with antigens derived from the EOC cell lines (OV-90 and SK-OV-3) in SOV01, NSCLC cell lines (H522 and H520) in SLU01, and a human prostate adenocarcinoma cell line (LNCaP) in SP005. To prepare DCVAC, the peripheral blood mononuclear cells, obtained via leukapheresis and gradient centrifugation, are first cultured in a medium containing interleukin-4 and granulocyte-macrophage colony-stimulating factor. Immature DCs are separated, co-cultured (pulsed) with high hydrostatic pressure-treated tumor cell lines, and matured using polyino-sinic:polycyticylic acid. The resulting product is cryopreserved at a concentration of approximately 10^7 DCs in 1 mL of CryoStor CS10 (StemCell) per vial.

3 The Aims of the thesis

ICD and danger signaling is accompanied by the exposure and release of various DAMPs, which altogether confer a robust adjuvanticity to dying cancer cells, as they favor the development of clinically relevant adaptive anti-tumor immunity. The aim of this thesis is to investigate the biological and the clinical relevance of ICD, danger signaling and associated molecules in cancer prognosis and response to standard of care therapy and/or immunotherapy.

1. To investigate the biological and clinical relevance of danger signaling and DAMPs exposure on development and activation of innate anti-cancer immunity, with particular focus on NK cells population in cancer patients.

2. To investigate the potential impact of danger signaling and DAMPs exposure on final response to standard of care therapy and immunotherapy in cancer patients.

3. To identify predictive immunological biomarkers that may improve the clinical response to ICD-based autologous DCs immunotherapy of patients with solid carcinomas.

4 Results

Publications *in extenso* as a foundation for dissertation

- Truxova I, Kasikova L, Salek C, Hensler M, Lysak D, **Holicek P**, Bilkova P, Holubova M, Chen X, Mikyskova R, Reinis M, Kovar M, Tomalova B, Kline JP, Galluzzi L, Spisek R, Fucikova J. Calreticulin exposure on malignant blasts correlates with improved natural killer cell-mediated cytotoxicity in acute myeloid leukemia patients. Haematologica. 2020 Jul;105(7):1868-1878. doi: 10.3324/haematol.2019.223933
  IF = 11.047

- **Holicek P**, Truxova I, Rakova J, Salek C, Hensler M, Kovar M, Reinis M, Mikyskova R, Pasulka J, Vosahlikova S, Remesova H, Valentova I, Lysak D, Holubova M, Kaspar P, Prochazka J, Kasikova L, Spisek R, Galluzzi L, Fucikova J. Type I interferon signaling in malignant blasts contributes to treatment efficacy in AML patients. Cell Death Dis. 2023 Mar 24;14(3):209. doi: 10.1038/s41419-023-05728-w.
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List of authors publications with no relation to dissertation

- Rakova J, Truxova I, **Holicek P**, Salek C, Hensler M, Kasikova L, Pasulka J, Holubova M, Kovar M, Lysak D, Kline JP, Racil Z, Galluzzi L, Spisek R, Fucikova J. TIM-3 levels correlate with enhanced NK cell cytotoxicity and improved clinical outcome in AML patients. Oncoimmunology. 2021 Mar 8;10(1):1889822. doi: 10.1080/2162402X.2021.1889822
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4.1 Calreticulin exposure on malignant blasts correlates with improved natural killer cell-mediated cytotoxicity in acute myeloid leukemia patients

ICD is a specific form of apoptosis accompanied by release and exposure of various DAMPs. ICD associated ER stress signaling mediate translocation of CALR on the outer leaflet of cellular plasmatic membrane which together with other DAMPs support the activation of adaptive anti-tumor immune response. Accumulating preclinical data indicates that ecto-CALR expression in the TME associate with superior clinical outcomes in various solid carcinomas. In line with this notion, our research team has previously described the beneficial role of ICD-mediated ecto-CALR expression by malignant blasts in development of adaptive anti-tumor immunity in AML patients. However, the role of CALR in activation of innate immunity and NK cell mediated cytotoxicity is poorly understood.

Here we show, that CALR expression on malignant blasts correlate with higher frequency of circulating NK cells and also indirectly enhance clinically relevant cytotoxic capacity of NK cells, both in AML patients and *in vivo* experimental mice models. Ecto-CALR mediated NK cells cytotoxicity relies on its capacity to promote maturation and activation of $CD11c^{+}CD14^{High}$ cells, as documented by higher expression levels of IL-15Rα, CD86, HLA-DR and CCR7. Ecto-CALR further correlates with high level of type I IFN associated genes. Taken together, our findings document the impact of ecto-CALR on increased cytotoxicity of NK cells associated with improved prognosis in AML patients.

Author´s contribution to the study:

- Patient samples processing
- Patient´s data acquisition by FACS and subsequent analyses
- Performing basic biostatistical tests
- Help with peer review process

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Calreticulin exposure on malignant blasts correlates with improved natural killer cell-mediated cytotoxicity in acute myeloid leukemia patients

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ABSTRACT

In some settings, cancer cells responding to treatment undergo an immunogenic form of cell death that is associated with the abundant emission of danger signals in the form of damage-associated molecular patterns. Accumulating preclinical and clinical evidence indicates that danger signals play a crucial role in the (re-)activation of antitumor immune responses *in vivo*, thus having a major impact on patient prognosis. We have previously demonstrated that the presence of calreticulin on the surface of malignant blasts is a positive prognostic biomarker for patients with acute myeloid leukemia (AML). Calreticulin exposure not only correlated with enhanced T-cell-dependent antitumor immunity in this setting but also affected the number of circulating natural killer (NK) cells upon restoration of normal hematopoiesis. Here, we report that calreticulin exposure on malignant blasts is associated with enhanced NK cell cytotoxic and secretory functions, both in AML patients and *in vivo* in mice. The ability of calreticulin to stimulate NK-cells relies on $CD11c^{+}CD14^{high}$ cells that, upon exposure to CRT, express higher levels of IL-15Rα, maturation markers (CD86 and HLA-DR) and CCR7. CRT exposure on malignant blasts also correlates with the upregulation of genes coding for type I interferon. This suggests that $CD11c^{+}CD14^{high}$ cells have increased capacity to migrate to secondary lymphoid organs, where can efficiently deliver stimulatory signals (IL-15Rα/IL-15) to NK cells. These findings delineate a multipronged, clinically relevant mechanism whereby surface-exposed calreticulin favors NK-cell activation in AML patients.

Introduction

In response to some treatments including anthracycline-based chemotherapy, high hydrostatic pressure or radiation therapy, cancer cells mount unsuccessful adaptive responses to stress that are accompanied by the release of endogenous

molecules that convey danger signals, which are cumulatively known as damage-associated molecular patterns (DAMPs).[1-4] The spatiotemporally regulated emission of DAMPs by cells undergoing immunogenic cell death (ICD) generates a pronounced immunostimulatory milieu that, in the presence of adequate antigenicity (such as that conferred to cancer cells by somatic mutations), supports the initiation of tumor-targeting immunity.[2,5] ICD-relevant DAMPs encompass endoplasmic reticulum (ER) chaperones such as calreticulin (CALR, best known as CRT) and heat-shock proteins (HSPs), nuclear components such as high mobility group box 1 (HMGB1), nucleic acids, as well as small metabolites like ATP.[6,7] In physiological scenarios, DAMPs are mostly intracellular, which prevents their detection by the immune system. Conversely, DAMPs that are secreted into the extracellular space or exposed on the plasma membrane of dying cancer cells can be recognized by the immune system via pattern recognition receptors (PRRs), and hence can drive the activation of therapeutically relevant innate and cognate immune responses.[2,8] In line with this notion, DAMP accumulation in the tumor microenvironment has been correlated with increased infiltration by multiple immune cell subsets, including mature dendritic cells (DCs) and effector memory T cells.[9-12] Moreover, factors linked to danger signaling – including (but not limited to) DAMPs expression levels, PRR expression levels, genetic polymorphisms in DAMP- or PRR-coding genes, and activation of relevant stress responses in cancer cells – have been attributed prognostic values in several cohorts of patients with cancer.[13]

Considerable work has been dedicated to elucidate the mechanisms whereby DAMPs affect the phenotype and function of myeloid cells that operate as antigen-presenting cells (APCs).[2,8] On the contrary, little attention has been given to the effects of DAMPs on cells of the innate lymphoid system, such as natural killer (NK) cells, despite the fact that NK cells are emerging as potent players in the control of metastases.[14] Indeed, surface-exposed HSP family A member 1A (HSPA1A, best known as HSP70) promotes NK-cell-dependent cytotoxicity *in vitro*[15,16] and *in vivo*,[17] while exosome-associated HSP70 can stimulate NK-cell migration and effector functions.[18,19] Similarly, extracellular HMGB1 can stimulate NK-cell activity upon binding to Toll-like receptor 2 (TLR2) and TLR4.[20] Here, we report that CRT exposure on the surface of malignant blasts from acute myeloid leukemia (AML) patients is associated with improved NK-cell secretory and cytotoxic functions. Mechanistic studies revealed that surface-exposed CRT stimulates NK-cell activity indirectly, through the upregulation of IL-15Rα on myeloid $CD11c^{+}CD14^{high}$ cells. Moreover, CRT exposure on AML malignant blasts also correlates with the upregulated expression of genes coding for type I interferon (IFN), which are also involved in the capacity of DCs to enhance NK-cell effector functions.

Table 1. Clinical and biological characteristics of acute myeloid leukemia patients.

Variable	Cohort (n=50)
Age at diagnosis	
< 50 years	23 (46%)
≥ 50 years	27 (54%)
Median (years)	52
Range (years)	21-73
Sex	
Male	23 (46%)
Female	27 (54%)
White blood cell count at diagnosis	
< 30.000/mm^3	42 (84%)
≥ 30.000/mm^3	8 (16%)
Median (10^9 cells/L)	6.9
Range (10^9 cells/L)	0-402.8
Blasts in peripheral blood	
Median (%)	25
Range (%)	0-91
De novo AML	41 (82%)
Secondary AML	9 (18%)
FAB classification	
M0	1 (2%)
M1	10 (20%)
M2	12 (26%)
M4	7 (14%)
M5	10 (20%)
M6	1 (2%)
MDS	8 (16%)
Cytogenetic profile	
Favorable	6 (12%)
Intermediate	29 (58%)
Unfavorable	8 (16%)
Missing data	7 (14%)
Molecular characteristics	
FLT3-ITD	7 (14%)
NPM1 mutated	12 (24%)
CEBPA mutated	2 (4%)
Induction chemotherapy	
Daunorubicin + Ara-C (3+7)	38 (76%)
Idarubicin + Ara-C (3+7)	10 (20%)
FLAG + Idarubicin	1 (2%)
Palliative treatment	1 (2%)
CR	40 (80%)
Consolidation	
Chemotherapy only	14 (28%)
HSCT	30 (60%)
No consolidation	6 (12%)

AML: acute myeloid leukemia; AM1-ETO: acute myeloid leukemia 1-ETO fusion protein; CEBPA: CCAAT/enhancer-binding protein alpha; CR: complete remission; FLAG: fludarabine + high-dose cytarabine + granulocyte colony-stimulating factor (G-CSF); FLT3-ITD: fms-like tyrosine kinase 3-internal tandem duplication; HSCT: hematopoietic stem cell transplantation; MDS: myelodysplastic syndrome; NPM1: nucleophosmin 1. FAB: French-American-British.

Methods

Patients

44 patients diagnosed with AML and treated at the Institute of Hematology and Blood Transfusion in Prague between December 2015 and March 2018 plus six AML patients diagnosed and treated at the Department of Hemato-oncology of the Pilsen Hospital between January 2017 and January 2018 were enrolled in this study. Informed consent was obtained according to the Declaration of Helsinki, and the study was approved by the local ethics committee. The main clinical and biological characteristics of the patients are summarized in Table 1. Induction chemotherapy consisted mainly (96%) of seven days cytarabine plus idarubicin or daunorubicin for the first three days (standard "7+3" regimen).

Flow cytometry

Peripheral blood mononuclear cell (PBMCs) isolated from AML patients or C57BL/6 (B6) mice, as well as mouse splenocytes,

bone-marrow derived DCs and tumor cells were stained with panels of fluorescent antibodies to evaluate the abundance, phenotype and function of immune cell subsets (*Online Supplementary Table S1-2*). Briefly, cells were incubated with primary antibodies or appropriate isotype controls for 20 min at 4 °C. For the analysis of CRT levels on AML blasts, PBMCs were labeled with anti-CD45 PerCP (Exbio) and anti-CD33 PE monoclonal antibodies (BioLegend). Malignant blasts from AML patients were defined as $CD45^{+}$ cells expressing high levels of CD33 ($CD33^{high}$). Surface CRT staining was performed by a three-step procedure: (1) incubation with primary CRT-specific antibody (Enzo Life Sciences), (2) incubation with an APC-conjugated secondary antibody (Jackson Immunoresearch Laboratories) and (3) incubation with Annexin V-FITC (Exbio) and 4′,6-diamidino-2-phenylindole (DAPI, from Molecular Probes) to assess the cell viability. Surface-exposed CRT levels were analyzed only on live ($AnnV^{-}DAPI^{-}$) and dying ($AnnV^{+}/DAPI^{-}$) but not dead ($DAPI^{+}$) cells. Flow cytometry data were acquired on the LSRFortessa analyzer (BD Biosciences) and analyzed with the FlowJo software package (Tree Star, Inc.).

Statistical analysis

Survival analyses were performed by using log-rank tests upon patient stratification into two groups based on the median cutoff of continuous variables. Univariate and multivariate Cox proportional hazard analysis was performed to assess the association of clinicopathological or immunological parameters with relapse-free survival (RFS). Variables that were intrinsically correlated were not included in multivariate Cox regressions. Fisher's exact tests, Student's *t*-tests, and the Wilcoxon and Mann-Whitney tests were used to test for association between variables, *P*-values are reported (considered not significant when >0.05).

Results

CRT exposure on malignant blasts is associated with increased NK-cell frequency and upregulation of ligands for activating NK-cell receptors

We previously demonstrated a link between CRT exposure on malignant blasts and clinically-relevant anticancer immunity in AML patients.[10] To extend these findings, we examined the potential impact of CRT on the plasma membrane (ecto-CRT) of $CD45^{+}CD33^{+}$ malignant blasts on the frequency and phenotype of NK cells from AML patients prior to the initiation of anthracycline-based chemotherapy and at the recovery of normal hematopoiesis. Patients were stratified based on the median percentage of $DAPI^{-}$ecto-CRT^{+} blasts at diagnosis into a CRT^{Hi} and CRT^{Lo} group. In baseline conditions (prior to induction chemotherapy), we were unable to identify statistically significant differences in the frequency and absolute numbers of circulating $CD45^{+}CD3^{-}CD56^{+}$ NK cells between these two groups of patients (Figure 1A-B). Conversely, upon complete remission and recovery of nonmalignant hematopoiesis, CRT^{Hi} AML patients had significantly higher frequency and absolute numbers of $CD45^{+}CD3^{-}CD56^{+}$ NK cells in the circulation as compared to their CRT^{Lo} counterparts (Figure 1A-B). These results are in line with previously published data from our group.[10] Of note, CRT^{Hi} AML patients did not display increased frequency of $CD45^{+}CD3^{-}CD56^{+}$ NK cells in the bone marrow as compared to their CRT^{Lo} counterparts (*Online Supplementary Figure S1A*).

As NK-cell activation is modulated by the balance between stimulatory and inhibitory signals delivered by multiple ligand/receptor interactions,[14] we next analyzed the levels of common activating (NKp30, NKp46, NKp80, NKG2D, DNAM-1 and CD16) and inhibitory (CD158e1, CD158bj, CD158ah, NKG2A, ILT2) NK-cell receptors by flow cytometry. With the exception of $ILT2^{+}$ cells (which were less represented in the circulation of CRT^{Hi} AML patients upon remission), we failed to detect significant differences in the percentage of NK cells staining positively for these receptors between CRT^{Hi} and CRT^{Lo} AML patients, neither prior to induction chemotherapy nor upon complete remission (Figure 1C and *Online Supplementary Figure S1B*). Because CRT exposure relies on ER stress responses,[21] and different stress response pathways may also modulate the expression of ligands for NK-cell receptors,[22] we decided to evaluate the potential connection between CRT exposure and the levels of multiple NK-cell ligands on the surface of $CD45^{+}CD33^{+}$ blasts, namely major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICA), MICB, UL16 binding protein 2 (ULBP2), ULBP5, ULBP6, poliovirus receptor (PVR, also known as CD155), nectin cell adhesion molecule 2 (NECTIN2, also known as CD112 and PVRL2), and B7-H6, by flow cytometry. We found that the percentage of $DAPI^{-}$ecto-CRT^{+} blasts positively correlates with the percentage of AML blasts staining positively for MICA, MICB, CD155 and CD112 (Figure 1D). In the attempt to identify a potential connection between the exposure of NK-cell-activating ligands (NKALs) and ER stress, we retrieved normalized *MICA, ULBP2, PVR* and *NECTIN2* expression levels for 173 AML patients from The Cancer Genome Atlas (TCGA) public database and analyzed their correlation with the expression levels of genes involved in the ER stress response, namely activating transcription factor 4 (*ATF4*), DNA damage inducible transcript 3 (*DDIT3*) and HSP family A (Hsp70) member 5 (*HSPA5*). However, linear regression analysis showed limited degrees of correlation (*Online Supplementary Figure S1C*), suggesting the involvement of other stress response mechanisms in the exposure of NKALs by malignant blasts. Altogether, these findings indicate that malignant blasts from AML patients display different danger signals on their surface, and this influences the abundance of circulating NK cells.

CRT exposure on malignant blasts correlates with improved NK-cell effector functions in AML patients in remission

Since the ability of surface-exposed CRT to deliver activatory signals to NK cells had not been previously investigated, we set out to address this possibility. To this aim, we evaluated degranulation and IFN-γ production by NK cells from CRT^{Hi} and CRT^{Lo} AML patients upon non-specific stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin by flow cytometry (*Online Supplementary Figure S1D*). We failed to detect statistically significant differences in the frequency of NK cells responding to stimulation with IFN-γ production ($IFN\text{-}\gamma^{+}CD45^{+}CD3^{-}CD56^{+}$ cells) and degranulation ($CD107a^{+}GZMB^{+}CD45^{+}CD3^{-}CD56^{+}$ cells) between CRT^{Hi} and CRT^{Lo} AML patients prior to induction chemotherapy (Figure 2A). On the contrary, upon remission and recovery of non-malignant hematopoiesis, CRT^{Hi} patients exhibited significantly improved NK-cell secretory and cytotoxic effector functions compared to

their CRT^{Lo} counterparts (Figure 2B). To evaluate NK-cell effector functions in a more direct manner, we also performed NK-cell cytotoxicity assays using NK cell-sensitive human chronic myelogenous leukemia K562 cells as targets. In general, NK cells isolated from AML patients at recovery had slightly higher cytotoxic functions than NK cells isolated from AML patients prior to induction chemotherapy (Figure 2C). Importantly, while surface-exposed CRT failed to affect the ability of NK cells isolated from AML patients prior to the initiation of treatment

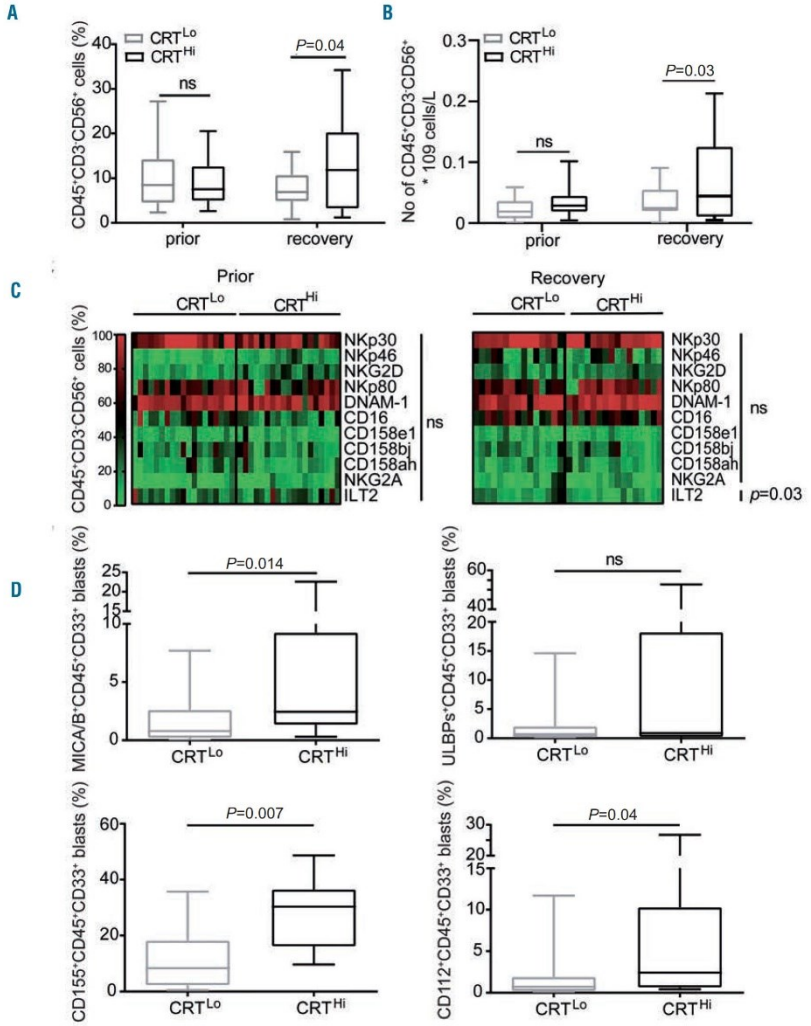

Figure 1. The impact of ecto-CRT on natural killer (NK) cells and the levels of NK-cell ligands present on acute myeloid leukemia blasts. (A) The percentage and (B) absolute numbers of circulating CD45$^+$CD3$^-$CD56$^+$ NK cells in CRT^{Hi} *versus* CRT^{Lo} acute myeloid leukemia (AML) patients before the induction chemotherapy (Prior, n=45) and at re-establishment of normal hematopoiesis (recovery, n=37) determined by flow cytometry. Boxplots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns: not significant. (C) The frequency of CD45$^+$CD3$^-$CD56$^+$ NK cells staining positively for different NK cell receptors (namely NKp30, NKp46, NKG2D, NKp80, DNAM-1, CD16, CD158e1, CD158bj, CD158ah, NKG2A and ILT2) in CRT^{Hi} and CRT^{Lo} AML patients before the induction chemotherapy (prior, n=38) and at re-establishment of normal hematopoiesis (recovery, n=31) determined by flow cytometry. ns: not significant. (D) The percentage of CD45$^-$CD33$^+$ blasts staining positively for NK cell ligands (MICA/B, ULBP, CD155 and CD112) in CRT^{Hi} *versus* CRT^{Lo} AML patients prior to the induction chemotherapy (n=21) determined by flow cytometry. Boxplots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns: not significant. CRT: calreticulin.

to efficiently kill K562 cells (Figure 2D), CRT^{Hi} patients in remission possessed NK cells with superior cytotoxic functions compared to their CRT^{Lo} counterparts (Figure 2E). These data are consistent with the results reported above (Figure 2A-B).

Surface-exposed CRT influences NK-cell effector functions indirectly, by affecting the phenotype of CD11c^{+}CD14^{high} cells

To further evaluate the impact of surface-exposed CRT on NK cells and the mechanisms underlying its NK cell-stimulatory effects, we performed a set of *in vitro* experiments with recombinant CRT (rCRT). Pre-incubation of purified NK cells with rCRT did not affect the capacity of NK cells to release cytotoxic granules containing perforin 1 (PRF1) or secrete IFN-γ in response to either nonspecific stimulation with PMA and ionomycin or exposure to K562 cells (Figure 3A and *Online Supplementary Figure 2A*). Conversely, adding rCRT to whole PBMCs led to significant increase in the percentage of CD45^{+}CD3^{-}CD56^{+} NK cells degranulating in response to PMA plus ionomycin or exposure to K562 cells (Figure 3B), with no effects on IFN-γ secretion (Online Supplementary Figure S2B). We confirmed these results with NK-cell cytotoxicity assays, as

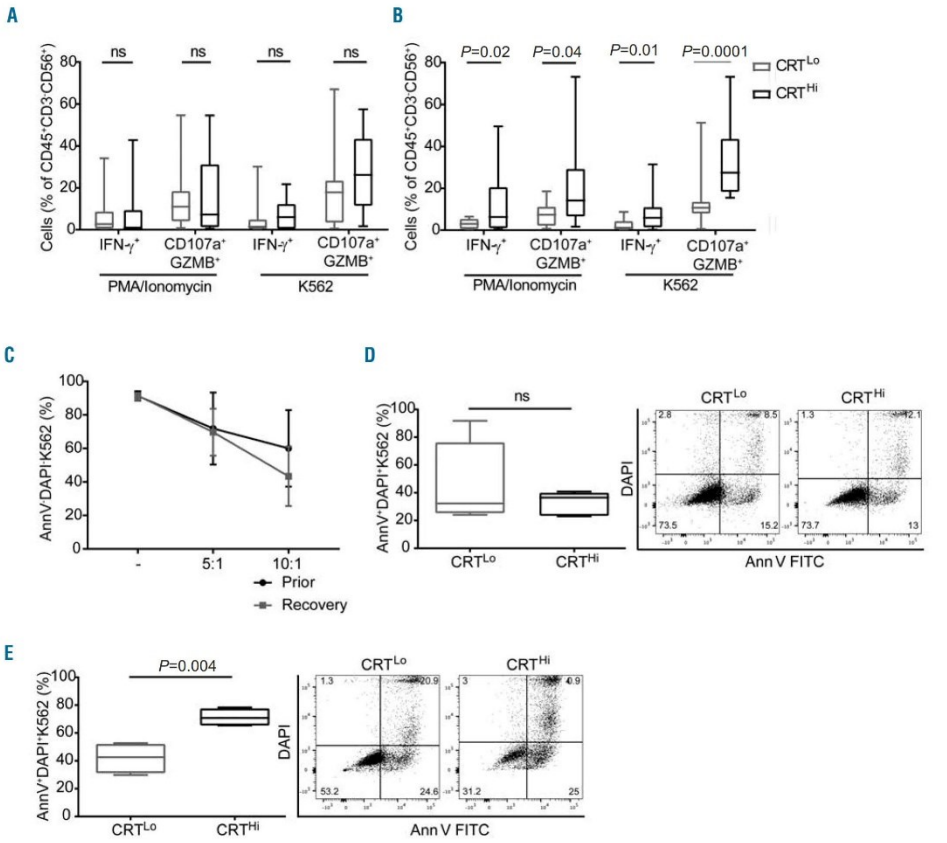

Figure 2. The impact of ecto-CRT on the activation and cytotoxic potential of natural killer cells in acute myeloid leukemia patients. (A, B) The percentage of IFN-γ and degranulating (CD107a^{+}/GZMB^{+}) CD45^{+}CD3^{-}CD56^{+} natural killer (NK) cells upon PMA + Ionomycin or K562 cell line stimulation in 17 CRT^{Lo} and 18 CRT^{Hi} acute myeloid leukemia (AML) patients prior to the induction chemotherapy (A) or in 12 CRT^{Lo} and 12 CRT^{Hi} AML patiens after the restoration of normal hematopoiesis (B). Patient samples were analyzed by flow cytometry. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns: not significant. (C) Cytotoxic potential of NK cells isolated from AML patients before the initiation of chemotherapy (Prior, n=10) versus upon the restoration of normal hematopoiesis (Recovery, n=10). Purified NK cells were tested for their ability to kill target K562 cell line at two different effector:target cell ratios (5:1 and 10:1) and the viability of K562 cells was determined by flow cytometry after 4 hours (h). (D, E) Cytotoxic potential of NK cells isolated from five CRT^{Hi} and 5 CRT^{Lo} AML patients before the initiation of chemotherapy (D) or upon the restoration of normal hematopoiesis (E). Purified NK cells were tested for their ability to kill target K562 cell line at effector:target cell ratio 5:1 and the percentage of dead (AnnV^{+}DAPI^{+}) K562 cells was determined by flow cytometry after 4 h. The representative dot plots of NK cell cytotoxicity assay showing the viability of target K562 cells in CRT^{Hi} *versus* CRT^{Lo} AML patients before the initiation of chemotherapy (D) or upon the restoration of normal hematopoiesis (E) are shown. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns: not significant. CRT: calreticulin.

NK cells isolated from PBMCs pre-incubated with rCRT were able to kill an increased amount of K562 cells compared to NK cells isolated from control PBMC (Figure 3B). These results suggest that CRT stimulate NK cells indirectly, via mechanisms that involve other cellular components of the PBMC mixture.

Previous *in vitro* studies support a role for APCs, mainly DCs, in NK-cell activation.[23] We therefore decided to focus on the phenotype of APCs exposed to rCRT. We found that incubating PBMCs from healthy donors (HD) with rCRT induced the upregulation of the chemotaxis-associated receptor C-C motif chemokine receptor 7 (CCR7) and the maturation-associated molecules CD86 and HLA-DR on $CD11c^{+}CD14^{high}$ cells and increased the frequency of $CD11c^{+}CD14^{high}$ expressing interleukin 15 receptor subunit alpha (IL15RA, best known as IL-15Rα) (Figure 3C),which is crucial for the activatory trans-presentation of IL-15 to NK cells.[24] Inspired by these data, we investigated the relationship between CRT exposed on malignant blasts and the phenotype of APCs in AML patients in remission. We found that CRT^{Hi} patients harbor a significantly higher percentage of $CD11c^{+}CD14^{high}$ cells expressing CCR7 and IL-15Rα compared to their CRT^{Lo} counterparts (Figure 3D), suggesting that these cells have an increased capacity to migrate to secondary lymphoid organs, where they can efficiently activate NK cells. In vitro assays suggested a prominent role for human myeloid over plasmocytoid DCs in NK-cell activation upon exposure to rCRT (*Online Supplementary Figure S3A*). Of note, also mouse PBMCs or bone marrow-derived DCs exposed to rCRT upregulated activation markers including CD54, CD86, and MHC class II molecules, and secreted increased amounts of IL-12 (*Online Supplementary Figure S3B-D*).

We have previously shown that the PMBCs of CRT^{Hi} AML patients who are in complete remission and have

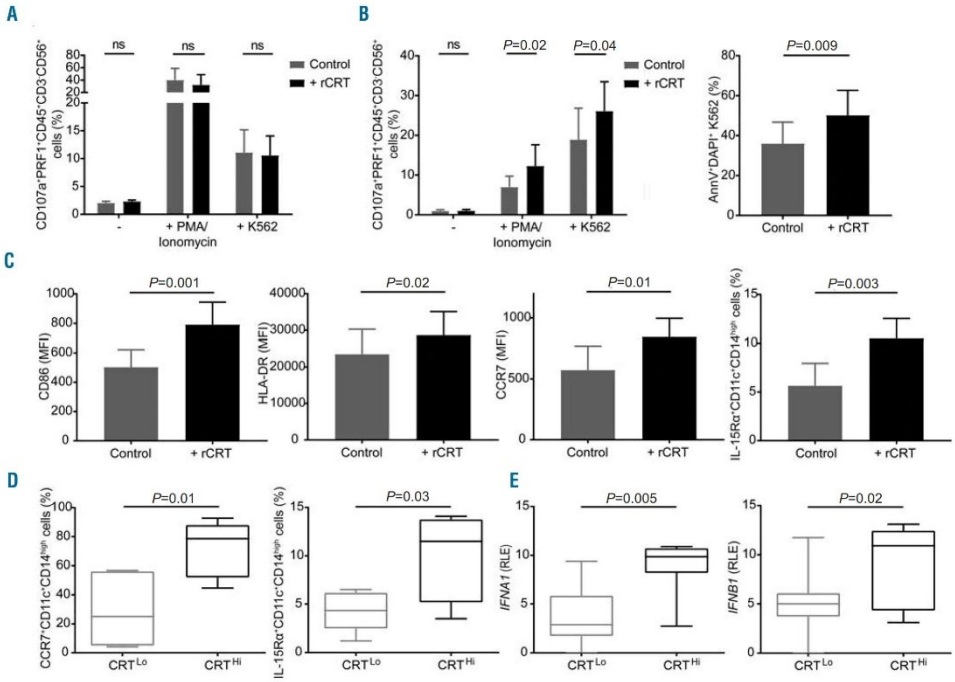

Figure 3. The mechanism of natural killer cell-stimulatory effects of calreticulin and its impact on $CD11c^{+}CD14^{high}$ cell phenotype. (A, B) The effect of recombinant human calreticulin (rCRT, Sino Biological Inc.) on effector functions of natural killer (NK) cells isolated from healthy donors (HDs) (n=8) or NK cells in HD peripheral blood mononuclear cells (PBMC) mixture (n=8). Purified NK cells (A) or whole PBMC (B) were pre-incubated with 5 μg/mL of rCRT overnight and subsequently stimulated by PMA + Ionomycin or K562 cell line for 4 hours (h). The percentage of responding ($CD107a^{+}PRF1^{+}CD45^{+}CD3^{-}CD56^{+}$) NK cells was determined by flow cytometry. Alternatively, NK cells were purified from rCRT-pre-incubated PBMC and their capacity to kill K562 cell line was tested in cytotoxicity assay. The percentage of dead ($AnnV^{+}DAPI^{+}$) K562 cells was determined by flow cytometry after 4 h (B). NK cells/PBMCs without rCRT and unstimulated NK cells/PBMC were used as a negative controls; ns: not significant. (C) The expression of maturation-associated molecules (CD86 and HLA-DR) and CCR7 on $CD11c^{+}CD14^{high}$ cells in HD PBMCs (n=8) incubated with rCRT (5 μg/mL) overnight *versus* control PBMCs without rCRT as determined by flow cytometry. The expression of individual markers is shown as mean fluorescence intensity (MFI). Flow cytometry was also used for the detection of $IL\text{-}15R\alpha^{+}CD11c^{+}CD14^{high}$ cells in rCRT-pre-incubated *versus* control PBMCs. (D) The frequency of CCR7+ and $IL\text{-}15R\alpha^{+}CD11c^{+}CD14^{high}$ cells in CRT^{Hi} *versus* CRT^{Lo} AML patients upon the restoration of normal hematopoiesis (n=16) determined by flow cytometry. Boxplots: lower quartile, median, upper quartile; whiskers, minimum, maximum. (E) Quantitative RT-PCR-assisted quantification of IFNA1 and IFNB1 expression levels in PBMCs from 20 CRT^{Hi} *versus* 21 CRT^{Lo} acute myeloid leukemia (AML) patients at recovery of normal hematopoiesis. Boxplots: lower quartile, median, upper quartile; whiskers, minimum, maximum.

recovered normal, non-malignant hematopoiesis exhibit a remarkable upregulation of genes linked to T_H1 polarization, T-cell activation and cytotoxic immune responses.[10] To confirm and extend these findings, we assessed the expression levels of 46 genes linked to immune function, with particular focus on NK-cell activity, in the PBMCs of 37 AML patients in remission (*Online Supplementary Table S3*). We identified five genes that were differentially expressed in CRT^{Hi} *versus* CRT^{Lo} patients, namely, *IFNA1, IFNB1, CD3E, CD8A* and *CD28* (Figure 3E and *Online Supplementary Figure S4A*). Importantly, type I IFN including the products of *IFNA1* and *IFNB1* are also involved in the capacity of DCs to enhance NK-cell effector functions (23).

Taken together, our results suggest that CRT exposure on the surface of malignant blasts stimulates NK-cell effector functions indirectly, by altering the migratory capacity, surface phenotype, and secretory profile of $CD11c^{+}CD14^{high}$ APCs.

CRT exposure is associated with increased NK- and T-cell responses in mice

To examine the impact of surface-exposed CRT on anticancer immunity *in vivo*, we generated subcutaneous tumors in B6 mice with mouse wild-type (WT) AML C1498 cells (C1498.WT) or C1498 cells constitutively exposing CRT on the plasma membrane (C1498.CRT), and monitored disease progression (*data not shown*) and immune responses. T-cell response was analyzed both in the tumor and spleen (19 days after tumor cell injection) and NK-cell response only in spleen (three days after tumor cell injection) (Figure 4A). Importantly, developing C1498.CRT tumors resulted in an enrichment of activated $CD107a^{+}$ NK cells (defined as $CD45^{+}CD3^{-}NK1.1^{+}$ cells) in the spleen, and enhanced the capacity of NK cells to respond to PMA plus ionomycin stimulation (Figure 4B). In addition, we observed that C1498.CRT tumors are infiltrated by $CD4^{+}$ and $CD8^{+}$ T cells with improved effector functions in response to non-specific stimulation with

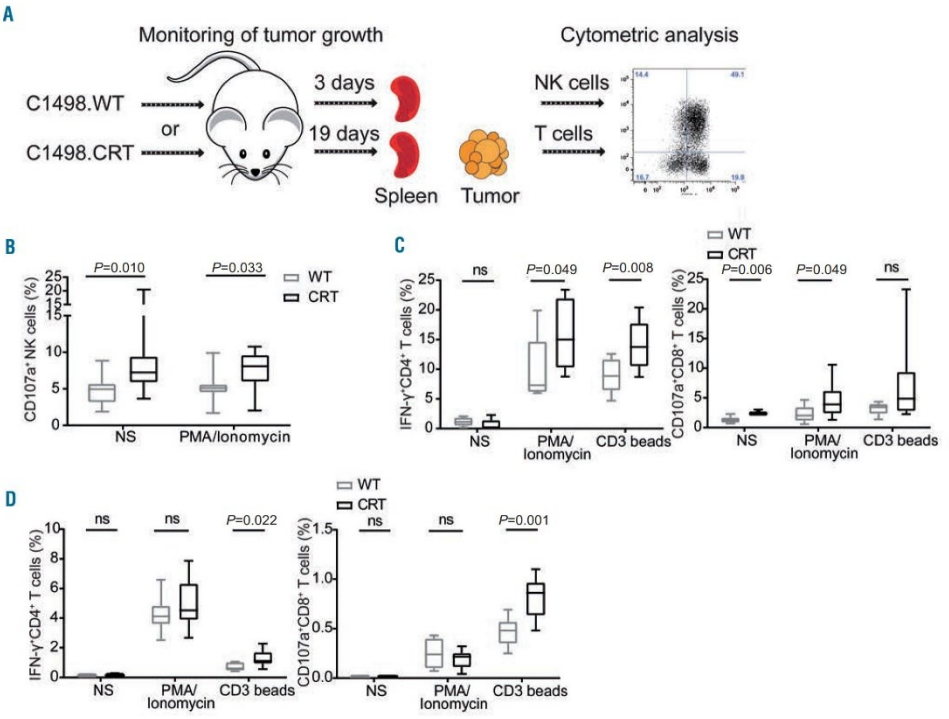

Figure 4. The role of calreticulin in natural killer-cell- and T-cell-based immune response *in vivo* in mice. (A) Schematic representation showing the process of C1498.WT/CRT tumor generation and monitoring the immune responses. To generate tumors *in vivo*, $1x10^{6}$ C1498.WT or C1498.CRT cells were inoculated sub cutaneoulsy into the lower right flank of B6 mice on day 0 (D0). Tumor size was measured every two days by standard laboratory caliper. Mice were sacrificed on D3 or D19 and spleen and tumors were harvested for analysis of functional status of natural killer (NK) cells (spleen on D3) and T cells (both spleen and tumors on D19) by flow cytometry. The experiment was performed three times. (B) The frequency of $CD107a^{+}$ NK cells (defined as $CD45^{+}CD3^{-}NK1.1^{+}$ cells) in spleen harvested from mice injected with C1498.CRT *versus* C1498.WT without further *in vitro* stimulation or upon stimulation with PMA + Ionomycin determined by flow cytometry. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. (C, D) The frequency of activated $IFN\text{-}\gamma^{+}CD4^{+}$ T cells and $CD107a^{+}$ $CD8^{+}$ T cells upon *in vitro* PMA + Ionomycin or anti-CD3 bead stimulation in C1498.CRT *versus* C1498.WT tumors (C) or spleen (D) determined by flow cytometry. Unstimulated cells were used as a controls. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum; ns: not significant. WT: wild-type.

PMA plus ionomycin and/or anti-CD3 beads (Figure 4C). Similarly, splenocytes isolated from C1498.CRT-bearing mice contained $CD4^+$ and $CD8^+$ T cells that were more responsive to stimulation than their counterparts from C1498.WT-bearing mice (Figure 4D).

In line with this notion, PBMCs from CRT^{Hi} AML patients in complete remission contained significantly higher frequencies of both $CD8^+$ and $CD4^+$ T cells responding by IFN-γ secretion to PMA plus ionomycin (*Online Supplementary Figure S4B-C*), with a slightly sub-significant trend towards increased numbers of $CD107a^+GZMB^+CD8^+$ T cells (*Online Supplementary Figure S4D*), compared with their CRT^{Lo} counterparts, comforting previously published data from our group.[10] Quantification of several cytokines essential for NK-cell homeostasis and functions (IL-21, IL-15, IFN-γ and IFN-α2) and for hematopoietic stem cell (HSC) differentiation (IL-3 and IL-7) in the sera of AML patients in remission also revealed higher IFN-γ levels in CRT^{Hi} *versus* CRT^{Lo} patients (*Online Supplementary Figure S4E*).

CRT exposure on malignant blasts and the frequency of NKG2D$^+$ cells correlate with RFS in AML patients

To evaluate the prognostic impact of CRT exposure on malignant blasts and verify our previous results on a larger subgroup of our patients,[10] we investigated RFS upon stratifying AML patients based on the median percentage of DAPI$^-$ blasts staining positively for surface CRT. In line with our previous observations,[10] CRT^{Hi} patients exhibited a significantly improved RFS compared with CRT^{Lo} patients (median: >60 *vs.* 14 months, P=0.027) (Figure 5A). Using a similar cutoff approach based on the median value, we also examined whether the mRNA levels of *KLRK1*, encoding the key NK-cell activating receptor NKG2D, would convey prognostic information in AML patients. We found that patients expressing high levels of *KLRK1* (*KLRK1^{Hi}*) had a significantly lower risk of relapse compared to their *KLRK1^{Lo}* counterparts (median: 39 *vs.* 10 months, P=0.039) (Figure 5B). We validated these findings at the protein level by stratifying a larger group of patients based on the median frequency of $CD45^+CD3^-CD56^+NKG2D^+$ NK cells. Patients with a high frequency of NK cells expressing NKG2D ($NKG2D^{Hi}$) exhibited significantly improved RFS, compared with their $NKG2D^{Lo}$ counterparts (median: >35 *vs.* 24 months, P=0.035) (Figure 5C). However, neither univariate nor multivariate Cox proportional hazard analysis confirmed these findings, potentially reflecting a limited follow-up of this prospectively collected patient cohort, or other confounding factors including disease subtype and inter-individual heterogeneity (Table 2-3). Since both CRT exposure on malignant blasts and NKG2D levels influenced RFS in our cohort of AML patients, we evaluated the combined prognostic value of ecto-CRT^+ blasts and the *KLRK1* mRNA levels or $CD45^+CD3^-CD56^+NKG2D^+$ NK-cell frequency by stratifying the cohort in three groups: CRT^{Hi}/*KLRK11*^{Hi} or CRT^{Hi}/$NKG2D^{Hi}$ patients, CRT^{Lo}/*KLRK1*^{Lo} or CRT^{Lo}/$NKG2D^{Lo}$ patients and patients in which the percentage of CRT^+ blasts was discordant with the *KLRK1* mRNA levels or the frequency of $CD45^+CD3^-CD56^+NKG2D^+$ NK cells (CRT/*KLRK1*^{Mix} or CRT/$NKG2D^{Mix}$). We found that CRT^{Hi}/*KLRK1*^{Hi} or CRT^{Hi}/$NKG2D^{Hi}$ patients had superior RFS as compared with their CRT^{Lo}/*KLRK1*^{Lo} or CRT^{Lo}/$NKG2D^{Lo}$ counterparts (CRT^{Hi}/*KLRK1*^{Hi} *vs.* CRT^{Lo}/*KLRK1*^{Lo}, P=0.050; CRT^{Hi}/$NKG2D^{Hi}$ *vs.* CRT^{Lo}/$NKG2D^{Lo}$, P=0.037) (Figure 5D-E).

Discussion

CRT exposure on cancer cells conveys robust prognostic information in patients with a variety of malignancies, generally reflecting the activation of clinically-relevant tumor-targeting immune responses.[13] Previous work from our group demonstrated that the presence of CRT on the surface of malignant blasts from AML patients correlates not only with an increased frequency of effector memory $CD4^+$ and $CD8^+$ T cells but also with an increased proportion of circulating NK cells, suggesting that CRT exposure is linked to both adaptive and innate immunity.[10] Inspired by accumulating evidence on the key role of NK cells in natural and therapy-driven immunosurveillance,[25-29] we decided to extend these initial observations and characterize the link between surface-exposed CRT and NK-cell activity in AML patients. Indeed, NK cells from patients with high CRT exposure on malignant blasts exhibited improved secretory and cytotoxic effector functions (Figure 2B and E).

As we excluded the possibility that CRT would mediate direct immunostimulatory effects on NK cells (Figure 3A and *Online Supplementary Figure S2A*), we thought that CRT exposure would be linked to increased levels of NKALs on the surface of malignant blasts, because both these processes have been linked to intracellular ER stress signaling.[30] Indeed, the percentage of $CD45^+CD33^+$ malignant blasts staining positively for ecto-CRT^+ correlated with the frequency of blasts staining positively for various NKALs (Figure 1D). However, we were unable to document any correlation between the NKAL expression levels and mRNA abundance of genes involved in the ER stress response (which we and others previously demonstrated to constitutively occur in AML blasts independent of therapy)[10,12] (*Online Supplementary Figure S1C*). These findings suggest that NKALs and CRT are exposed on the surface of AML blasts *via* mechanistically distinct stress response pathways. Replication stress and the consequent DNA damage response stand out as a promising candidate for NKAL exposure in this setting.[30]

We also found that NK-cell activation by CRT involves a population of $CD11c^+CD14^{high}$ cells that, upon exposure to CRT, express maturation markers (CD86 and HLA-DR), acquires improved migratory capacity as a consequence of CCR7 expression, and delivers stimulatory signals to NK cells via IL-15Rα/IL-15 trans-presentation[24] and type I IFN. Consistent observations in peripheral blood of HDs and AML patients, suggest that $CD11c^+CD14^{high}$ cells exposed to CRT have a superior capacity to migrate to secondary lymphoid organs where they can efficiently activate NK cells (Figure 3C-E). Thus, surface-exposed CRT appears to trigger the phenotypic and functional maturation of $CD11c^+CD14^{high}$ cells leading to (i) cell contact-dependent NK-cell activation via trans-presented IL-15, as well as (ii) cell contact-independent NK-cell activation via type I IFNs. Importantly, type I IFN signaling in DCs results not only in a superior ability to drive antigen-specific T-cell priming,[31] but also in IL-15 production,[32] potentially supporting a robust adaptive and innate immune response of therapeutic relevance. Our findings and elegant preclinical data from Chen and colleagues[10,33] lend robust support to

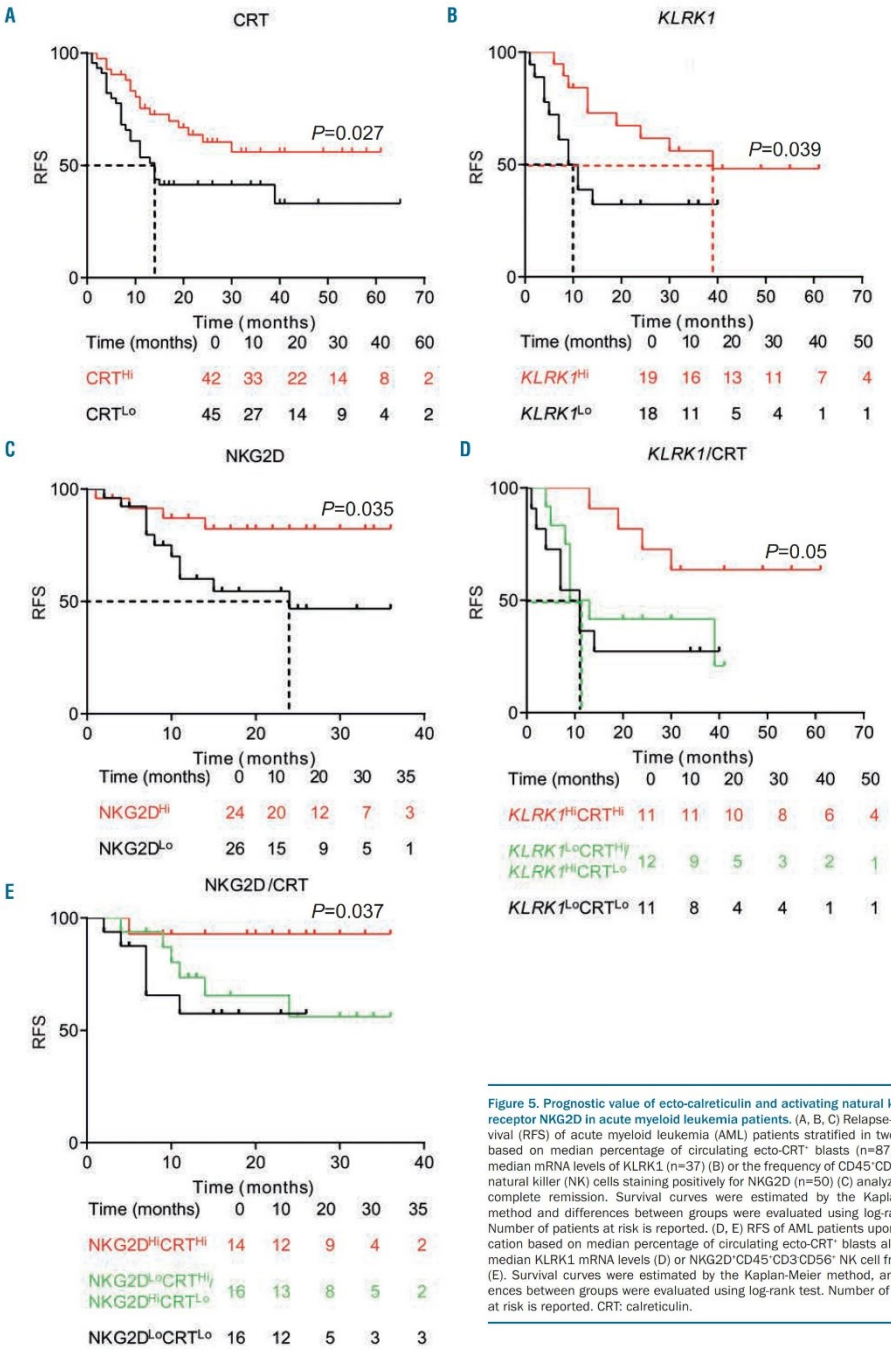

Figure 5. Prognostic value of ecto-calreticulin and activating natural killer cell receptor NKG2D in acute myeloid leukemia patients. (A, B, C) Relapse-free survival (RFS) of acute myeloid leukemia (AML) patients stratified in two groups based on median percentage of circulating ecto-CRT$^+$ blasts (n=87) (A), on median mRNA levels of KLRK1 (n=37) (B) or the frequency of CD45$^+$CD3$^-$CD56$^+$ natural killer (NK) cells staining positively for NKG2D (n=50) (C) analyzed upon complete remission. Survival curves were estimated by the Kaplan-Meier method and differences between groups were evaluated using log-rank test. Number of patients at risk is reported. (D, E) RFS of AML patients upon stratification based on median percentage of circulating ecto-CRT$^+$ blasts along with median KLRK1 mRNA levels (D) or NKG2D$^+$CD45$^+$CD3$^-$CD56$^+$ NK cell frequency (E). Survival curves were estimated by the Kaplan-Meier method, and differences between groups were evaluated using log-rank test. Number of patients at risk is reported. CRT: calreticulin.

Table 2. Univariate Cox proportional hazard analysis.

Variable	RFS HR (95% CI)	RFS P
Age	1.02 (0.99-1.04)	0.19
Sex	0.79 (0.44-1.43)	0.45
Peripheral blast counts	1.00 (0.99-1.01)	0.26
HSCT	0.76 (0.40-1.44)	0.40
Ecto-CRT$^+$ blasts (%)	0.99 (0.98-1.00)	0.22
KLRK1 expression	0.80 (0.53-1.19)	0.27
NKG2D$^+$ NK cells (%)	0.96 (0.92-1.01)	0.13

CI, 95% confidence interval; HR, hazard ratio; *p< 0.05; RFS, relapse-free survival.

Table 3. Multivariate Cox proportional hazard analysis.

Variable	RFS HR (95% CI)	RFS P
Age	1.07 (1.01-1.13)	0.007*
Sex	1.34 (0.50-3.60)	0.55
Peripheral blast counts	1.00 (0.98-1.02)	0.71
HSCT	0.62 (0.21-1.83)	0.39
Ecto-CRT$^+$ blasts (%)	0.98 (0.96-1.00)	0.10
KLRK1 expression	0.69 (0.44-1.07)	0.10
NKG2D$^+$ NK cells (%)	0.95 (0.90-1.01)	0.11

CI, 95% confidence interval; HR, hazard ratio; *p< 0.05; RFS, relapse-free survival.

this possibility. Indeed, *in vivo* application of C1498 AML cells engineered to constitutively expose CRT on their surface elicited an accumulation of highly functional NK cells and CD4$^+$ and CD8$^+$ T cells in mouse tumors and/or spleen (Figure 4B-D).

Finally, both CRT exposure on malignant blasts and NK cell-related marker NKG2D were associated with improved RFS amongst AML patients (Figure 5A-C), corroborating previously published data.[10,34] Combinatorial assessment of the prognostic value of these parameters identified significantly prolonged RFS in KLRK1^{Hi}CRT^{Hi} and NKG2D^{Hi}CRT^{Hi} subgroup of patients (Figure 5D-E). However, these findings could not be confirmed using univariate and multivariate Cox proportional hazard analysis, potentially reflecting a limited follow-up period, the small size of the patient cohort, disease subset and/or inter-patient heterogeneity. Thus, the precise prognostic value of CRT exposure on AML blasts and NKG2D levels on NK cells remains to be validated in independent patient series.

Taken together, our results support the association of CRT with enhanced activation of the innate and adaptive anticancer immunity. Parallel assessment of CRT exposure on malignant blasts and immune cell parameters, such as NK-cell markers, may provide prognostic information and have therapeutic relevance for AML patients in the future.

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4.2 Type I interferon signaling in malignant blasts contributes to treatment efficacy in AML patients.

Accumulating preclinical and clinical findings document the role of type I IFN in cancer immunosurveillance within tumor microenvironment. In addition, recent studies demonstrate that type I IFNs contribute to the efficacy of standard of care therapy and immunotherapy in both hematological and solid carcinomas. Here we report that cancer cells produce type I IFNs via a Toll-like receptor 3 (TLR3)-dependent mechanism in chemotherapy naïve AML patients. Although, immune-stimulatory capacity of type I IFNs in AML patients was blunted by malignant blasts, type I IFNs turned out to provide direct cytostatic, cytotoxic and chemosensitizing activity in AML patients and AML xenograft model. Furthermore, a genetic signature related to type I IFN signaling positively correlates with favorable prognosis in a cohort of 132 AML patients with potential prognostic and predictive impact in AML therapy.

Author´s contribution to the study:

- Patient samples processing
- Patient´s data acquisition by RT-qPCR, FACS, Western Blotting, Immunoassays, bright field microscopy and subsequent data analyses and data interpretation
- Analyses of Patient´s RNAseq dataset and TCGA database
- Development, optimalisation and data interpretation of all *in vitro* and *ex vivo* tests employed in the study.
- Performing relevant biostatistical tests
- Manuscript preparation

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Type I interferon signaling in malignant blasts contributes to treatment efficacy in AML patients

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While type I interferon (IFN) is best known for its key role against viral infection, accumulating preclinical and clinical data indicate that robust type I IFN production in the tumor microenvironment promotes cancer immunosurveillance and contributes to the efficacy of various antineoplastic agents, notably immunogenic cell death inducers. Here, we report that malignant blasts from patients with acute myeloid leukemia (AML) release type I IFN via a Toll-like receptor 3 (TLR3)-dependent mechanism that is not driven by treatment. While in these patients the ability of type I IFN to stimulate anticancer immune responses was abolished by immunosuppressive mechanisms elicited by malignant blasts, type I IFN turned out to exert direct cytostatic, cytotoxic and chemosensitizing activity in primary AML blasts, leukemic stem cells from AML patients and AML xenograft models. Finally, a genetic signature of type I IFN signaling was found to have independent prognostic value on relapse-free survival and overall survival in a cohort of 132 AML patients. These findings delineate a clinically relevant, therapeutically actionable and prognostically informative mechanism through which type I IFN mediates beneficial effects in patients with AML.

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INTRODUCTION

Type I interferon (IFN) was initially discovered as a key component of a first-line defense system that increases the resistance of mammalian cells to viral pathogens [1–3]. An abundant preclinical and clinical literature emerging over the past decade demonstrate that type I IFN also supports natural and therapy-driven cancer immunosurveillance [4, 5]. In humans, type I IFN is a family of 17 proteins, encompassing 13 isoforms of interferon alpha (IFNA, best known as IFN-α), interferon beta 1 (IFNB1, best known as IFN-β), interferon epsilon (IFNE, best known as IFN-ε), interferon kappa (IFNK, best known as IFN-κ) and interferon omega 1 (IFNW1, best known as IFN-ω) [6, 7]. Type I IFN synthesis and secretion is generally elicited by the activation of pattern recognition receptors (PRRs), which are evolutionary ancient sensors for microbial products and endogenous danger signals commonly known as damage-associated molecular patterns (DAMPs) [8–10].

Type I IFN signals through an ubiquitous heterodimeric receptor consisting of interferon alpha and beta receptor subunit 1 (IFNAR1) and IFNAR2, culminating with the coordinated transactivation of numerous IFN-stimulated genes (ISGs) [3, 7, 11]. ISG synthesis affects a variety of biological processes ranging from resistance against viral infection to angiogenesis and immune activation [3, 7]. With specific respect to cancer, accumulating evidence indicates that while indolent and chronic type I IFN responses may be detrimental and support immunoevasion and tumor progression [12–16], robust and acute type I IFN signaling promotes tumor-targeting immunity by boosting both the priming and effector phase of the response [6, 15–18].

Supporting this notion, the intratumoral abundance of type I IFN or ISGs has been positively correlated with tumor infiltration by effector immune cells, signs of active anticancer immunity and favorable disease outcome in a variety of solid tumors [19, 20]. Conversely, single nucleotide polymorphisms negatively affecting the function of type I IFN-eliciting PRRs (as well as reduced levels of said PRRs or their signal transducers) have been consistently linked with immunosuppression in the tumor microenvironment (TME) and poor disease outcome [21–23]. Moreover, accumulating preclinical and clinical evidence indicates that the efficacy of numerous clinically employed anticancer regimens including conventional chemotherapeutics [4, 24, 25], radiation therapy [17, 26, 27], targeted anticancer agents [5, 28], immunotherapy [29] and non-viral oncolytic agents [30–32] rely on intact type I IFN signaling. Of note, unmodified or pegylated variants of human recombinant IFN-α2a or IFN-α2b have been approved by the US

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Food and Drug Administration (FDA) and other regulatory agencies worldwide for use in patients with various neoplasms, including chronic myelogenous leukemia (CML) [33]. An abundant literature suggests that type I IFN may also be beneficial for (at least some subsets of) patients with acute myeloid leukemia (AML) [34, 35].

Here, we report that peripheral blood mononuclear cells (PBMCs) from patients with AML express higher type I IFN levels that their counterparts from healthy donors, with malignant blasts being the major type I IFN source. While in AML patients with high type I IFN signaling active anticancer immunity is suppressed by malignant cells, type I IFN appears to mediate direct cytostatic, cytotoxic and chemosensitizing effects in multiple models of AML. In line with this notion, genetic signatures of type I IFN signaling were linked with improved relapse-free survival (RFS) and overall survival (OS) in a large cohort of patients with AML ($n = 132$), delineating a clinically actionable pathway with therapeutic and prognostic applications.

MATERIALS AND METHODS

Patients and samples

One hundred and thirty-two patients diagnosed with acute myeloid leukemia (AML) and treated at the Institute of Hematology and Blood Transfusion in Prague between March 2008 and April 2019 were enrolled in retrospective Study Cohort 1 (Table 1). One hundred and fifty-two patients diagnosed with AML from The Cancer Genome Atlas (TCGA) public were used as validation Study Cohort 2. Nine patients diagnosed with AML and treated at the Institute of Hematology and Blood Transfusion in Prague between April 2019 and May 2021 along with seven patients diagnosed with AML and treated at Department of Hemato-oncology of Faculty Hospital Pilsen between April 2019 and December 2021 were enrolled in prospective Study Cohort 3 (Supplemental Table 1). Informed consent was obtained according to the Declaration of Helsinki, and the study was approved by the local ethics committee. Peripheral blood samples obtained before the onset of chemotherapy were drawn into the 9 mL EDTA-coated tubes. Serum was collected and stored at −80 °C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation and used for immediate downstream cell analyses or cryopreserved using CryoStor® CS10 (StemCell Technologies) in liquid nitrogen for later use. An EasySep kit (StemCell Technologies) was employed to separate or deplete $CD33^+$ malignant blasts from PBMCs.

Cell lines and in vitro assays

Human AML KASUMI-1, MOLM-13 and MV4–11 cells as well as human CML K562 cells were a kind gift from Júlia Starková (CLIP - Childhood Leukaemia Investigation Prague). Further details about cell culture are provided in Suppl. Material and Methods. Polyinosinic:polycytidylic acid (poly(I:C), from InvivoGen), CpG oligodeoxynucleotides (ODN2216, from Invivogen) and TLR3/dsRNA specific complex inhibitor (TLRi, from Merck) were added to culture media to final concentrations of 50 µg/mL, 1,5 µM and 25 µM, respectively, for the indicated time. Recombinant human interferon-alpha (rIFN-α, from Bio-Techne) and recombinant human interferon-beta (rIFN-β, from PeproTech) were used at a final concentration of 500 pg/mL. AML cells were incubated with both rIFN-α and rIFN-β for 7 days, AML primary blasts for 3 days, leukemic stem cells (LSCs) for 5 days and PBMCs/$CD33^+$ cell depleted PBMCs for 24 h at 37 °C in 5% CO_2 humidified atmosphere before the analysis of phenotype, function and apoptosis by flow cytometry. Human rIFN-α and rIFN-β were re-administered into fresh media every 48 h during the incubation period. Chemotherapeutic drugs commonly used for treatment of AML including daunorubicin (DNR; KASUMI-1: 200 nM, MOLM-13: 150 nM, MV4–11: 400 nM, $CD33^+$ blasts: 500 nM, LSCs: 125 nM) (Sigma-Aldrich) and cytarabine (Ara-C; KASUMI-1: 500 nM, MOLM-13: 1 nM, MV4–11: 500 nM, $CD33^+$: 125 nM, LSCs: 125 nM) (Sigma-Aldrich) were used for the induction of apoptosis over a 24 h course. $CD33^+$ malignant cells were incubated with an IFNAR1-blocking antibody (αIFNAR, from ThermoFisher - MMHAR-2) or isotype control (Iso, from ThermoFisher - PPV-04) at a final concentration of 8 µg/mL for 24 h, and subsequently at a concentration of 2 µg/mL for 72 h, without culture further replacements in culture medium.

Quantitative real-time PCR (RT-qPCR)

Gene expression levels were evaluated on a CFX 96™ Real-Time System (Bio-Rad) using custom-designed primers and probes (500 nM and 200 nM final concentration, respectively) (Generi Biotech) (Supplemental Table 2) and KAPA PROBE Fast Master Mix (Kapa Biosystems). Relative gene expression levels were calculated using the ΔΔCt method and were normalized to the expression level of reference gene *SURF1* selected by Normfinder (GenEx software, MultiD Analyses). Samples below the detection limit were assigned a relative expression value of 0.

Flow cytometry

PBMCs, malignant blasts, LSCs and cultured tumor cells were stained with multiple panels of fluorescent primary antibodies, appropriate isotype controls and fixable viability dyes to exclude live/dead cells (Supplemental Table 3). For the in vitro assessment of apoptosis, cells were stained with Annexin V for 20 min at 4 °C and 4′,6-diamidin-2-fenylindol (DAPI) (0.1 µg/mL) was added to cell suspension shortly prior to sample acquisition. Flow cytometry data were acquired on an LSRFortessa Analyzer (BD Biosciences) and analyzed with FlowJo v10.0 (Tree Star, Inc.).

Degranulation and IFN-γ production after in vitro stimulation

To assess natural killer (NK) cell and T cell function in whole PBMCs or $CD33^+$ cell-depleted PBMCs from AML patients, PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, from Sigma Aldrich) plus 1 µg/mL ionomycin or with K562 cells at an effector:target ratio 10:1 in the presence of anti-CD107a antibody (eBioscience) for 1 h, followed by 3 h incubation with brefeldin A (BioLegend). Cells were then washed in PBS, stained with antibodies specific for surface markers (Supplemental Table 3), fixed in fixation/permeabilization buffer for 15 min (eBioscience), washed with permeabilization buffer and then stained with antibodies targeting a panel of intracellular markers (Supplemental Table 3).

Leukemic stem cells (LSCs)

LSCs were isolated from the PBMCs of AML patients as follows. Thawed cell suspensions were depleted of dead cells by magnetic separation using the Dead Cell Removal Kit (Miltenyi Biotech) and subsequently $CD34^+$ cells were isolated using CD34 MicroBead Kit UltraPure (Miltenyi Biotech), according to the manufacturer's protocols. LSCs were determined as $CD45^{dim}$, Lin^- ($CD3^-CD14^-CD16^-CD19^-CD20^-CD56^-$), $CD34^+$, $CD38^{+/-}$ and $CD123^{+/dim}$ colony forming cells, as determined by flow cytometry and colony-forming assay (Supplemental Fig. 1A–C).

IFNAR2 deletion

KASUMI-$1^{IFNAR2-/-}$ cells were prepared by the CRISPR/Cas9 technology (Supplemental Fig. 2 and Supplemental Table 4). Briefly, KASUMI-1 cells were electroporated with a mixture of *IFNAR2*-specific gRNAs incorporated in a pSpCas9(BB)−2A-GFP (PX458) expression vector for dual expression of Cas9 and gRNAs. Two days after electroporation, GFP^+ cells were single sorted into 96-well plates coated with NSG mice bone marrow cells and expanded. Clone selection was performed based on (i) RT-qPCR specific for the *IFNAR2* (ii), detection of IFNAR2 by flow cytometry, and (iii) sensitivity to daunorubicin.

Statistical analysis

Statistical analyses were performed on GraphPad Prism 8, R v. 3.6.1 and R Studio. 3.6.0. The distributions of data sets were tested by Shapiro-Wilk Test, determining the use of the parametric or non-parametric tests for subsequent analyses. Paired and unpaired Student's t tests, as well as Wilcoxon and Mann-Whitney tests were used to assess differences between two groups. Differences among three or more groups were calculated using one-way ANOVA or Kruskal-Wallis tests corrected for multiple comparison by Holm-Sidak's or Dunn's tests. Pearson or Spearman correlations were used to evaluate the degree of the relationship between variables. Survival analyses were assessed for statistical significance with Log-rank tests. Univariate and multivariate Cox proportional hazard analysis were performed to assess the association of clinicopathological or immunological parameters with RFS and OS. Selected variables used in multivariate Cox regression hazard analysis exhibited no mutual collinearities, calculated by linear and logistic regressions and variance inflation factor (VIF). p values are reported and were considered not significant when >0.05.

Table 1. Main clinical and biological characteristics of AML patients from Study Cohort 1.

Variable	Study Cohort 1 $n = 132$
Age at diagnosis	
<50 years	60 (45%)
≥50 years	72 (55%)
Median (years)	52
Range (years)	19–68
Sex	
Male	71 (54%)
Female	61 (46%)
Peripheral-blood white cell count	
< 30.000/mm^3	66 (50%)
≥ 30.000/mm^3	66 (50%)
Median (109 cells/l)	30.1
Range (109 cells/l)	0.9–414.12
Blasts peripheral blood	
Median (%)	28
Range (%)	0–99
Blasts bone marrow	
Median (%)	56
Range (%)	2 to 96
De novo AML	113 (86%)
Secondary AML	
MDS/MPN, *n*	4 (3%)
Therapy related, *n*	10 (8%)
Not specified, *n*	9 (7%)
FAB classification	
M0	4 (3%)
M1	27 (21%)
M2	29 (22%)
M4	44 (33%)
M5	23 (17%)
M6	5 (4%)
Cytogenetic profile	
Favorable	14 (10%)
Intermediate	86 (65%)
Adverse	20 (16%)
Missing data	12 (9%)
Molecular characteristics	
DNMT3A	39
FLT3-ITD	38
KMT2A	2
GATA2	3
RUNX1::RUNX1T1	8
CBFB::MYH11	5
NPM1	41
CEBPA	8
Induction chemotherapy	
Daunorubicin + Ara-C (3 + 7)	87 (66%)
Idarubicin + Ara-C (3 + 7)	42 (32%)
BIDFA	1 (>1%)
FLA-IDA	1 (>1%)
HAM	1 (>1%)
Complete remission rate	112 (85%)
Consolidation	
Chemotherapy only	58 (44%)
HSCT	74 (56%)

AML acute myeloid leukemia, *Ara-C* cytarabine, *BIDFA* twice daily fludarabine and cytarabine, *CBFβ::MYH11* core-binding factor subunit beta - myosin heavy chain 11 fusion protein, *CEBPA,* CCAAT enhancer binding protein alpha, *DNMT3A* DNA methyltransferase 3 alpha, *FAB* French-American-British, *FLA-IDA* fludarabine-idarubicin, *FLT3-ITD* fms related receptor tyrosine kinase 3 - internal tandem duplication, *GATA2* GATA Binding Protein 2, *HAM* high-dose cytosine arabinoside and mitoxantrone, *HSCT* hematopoietic stem cell transplantation, *KMT2A* lysine methyltransferase 2A, *MDS* myelodysplastic syndrome, *MPS* myeloproliferative neoplasm, *NPM1* nucleophosmin 1, *RUNX1::RUNX1T1* RUNX family transcription factor 1 - RUNX1 partner transcriptional co-repressor 1 fusion protein.

RESULTS

Cell-autonomous type I interferon (IFN) responses in acute myeloid leukemia (AML) patients

To elucidate the impact of type I IFN in AML immunosurveillance, we determined the expression levels of *IFNA1*, *IFNA2* and *IFNB1* in peripheral blood mononuclear cells (PBMCs) from 132 AML patients (Study Cohort 1; Table 1) by RT-qPCR. Chemotherapy-naïve AML patients exhibited increased levels of *IFNA1*, *IFNA2* and *IFNB1* as compared to healthy donors (HDs) (Fig. 1A). In this setting, we observed rather heterogeneous expression of type I IFN-encoding genes, *IFNB1* being the most abundantly expressed (Fig. 1B; Supplemental Fig. 3A). To validate these findings with an independent technology, we employed multiplex bead assays to quantify IFN-α2 levels in the serum of patients form Study Cohort 1 (Table 1). In line with RT-qPCR findings, the serum levels of IFN-α2 were heterogenous across patients, ranging from undetectable to 559 pg/mL (Fig. 1C). Importantly, we observed a statistically significant correlation between IFN-α2 serum levels and *IFNA2* expression in PBMCs from the AML patients of Study Cohort 1 for which both data points were available ($R = 0.3204$, $p = 0.0011$, $n = 100$) (Fig. 1C). As *IFNA1*, *IFNA2* and *IFNB1* expression exhibited considerable mutual correlation (Fig. 1D), we defined a type I IFN index (IFN-i) as the geometrical average of individual expression values for *IFNA1*, *IFNA2* and *IFNB1* to use in subsequent analyses.

Considering that leukemic blasts make up majority of blood cells in AML patients, we moved onto assessing the cellular source of type I IFN by testing *IFNA1*, *IFNA2*, and *IFNB1* expression levels in isolated CD33$^+$ leukemic blasts *versus* whole PBMCs (including CD33$^+$ blasts). We found that type I IFN expression was comparable in leukemic blasts and whole PBMCs (Fig. 1E, Supplemental Fig. 3A), pointing to the former as the major type I IFN producers in this context. Further corroborating this possibility, we observed a correlation between *IFNB1* levels in isolated CD33$^+$ leukemic blasts and whole PBMCs from 22 AML patients ($R = 0.4243$, $p = 0.0491$) (Supplemental Fig. 3B). These findings indicate that malignant blasts from AML patients produce type I IFN prior to induction chemotherapy.

TLR3 drives type I IFN secretion from AML blasts

To delineate the molecular pathway responsible for type I IFN production in AML patients, we analyzed the expression of genes coding for common DNA/RNA sensors that are known to elicit type I IFN signaling [8, 16], including cyclic GMP–AMP synthase (CGAS), DExD/H-Box Helicase 58 (DDX58; best known as RIG-I), interferon induced with helicase C domain 1 (IFIH1; best known as MDA5), mitochondrial antiviral signaling protein (MAVS), eukaryotic

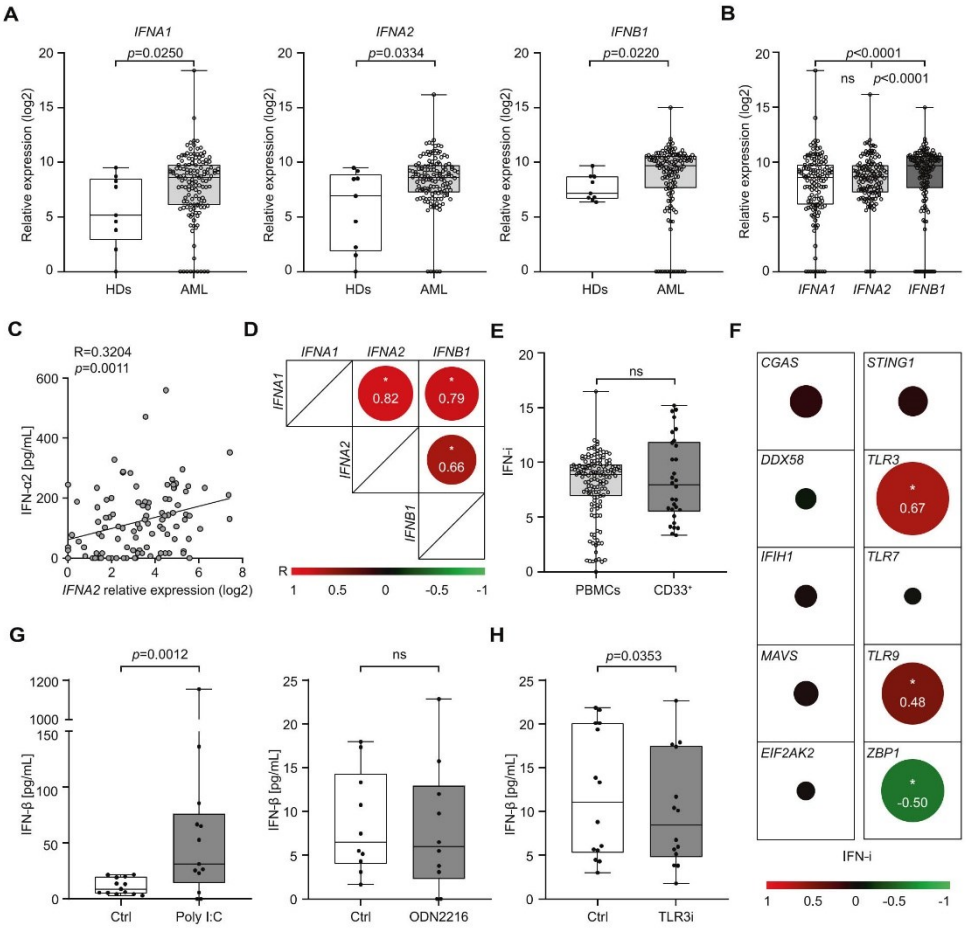
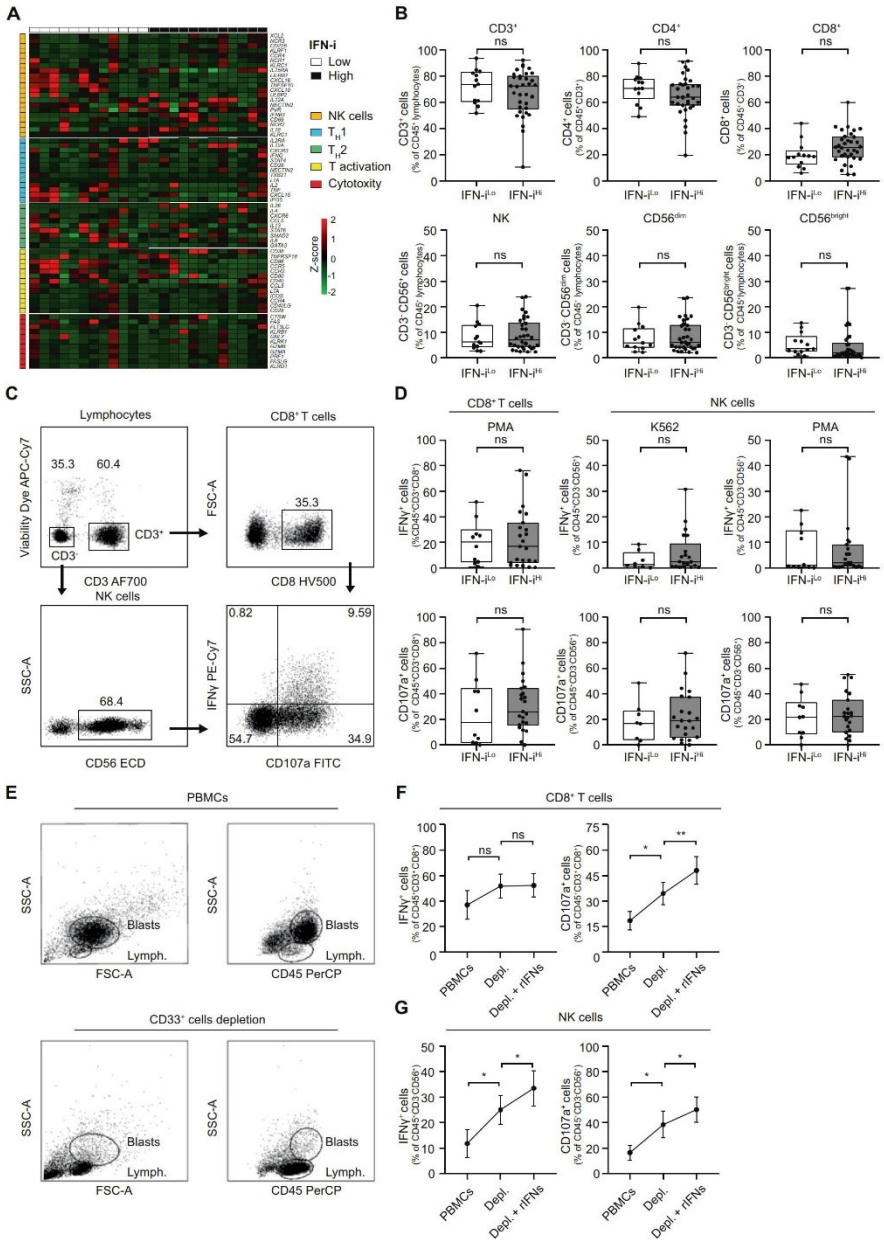


Fig. 1 TLR3 drives type I IFN secretion from AML blasts. A, **B** Relative expression levels of *IFNA1*, *IFNA2*, and *IFNB1* in peripheral blood mononuclear cells (PBMCs) from 9 healthy donors (HDs) and 132 AML patients (Study Cohort 1) prior to induction chemotherapy, as determined by RT-qPCR. Data are presented as median, quartiles and extremes plus individual data points. *p* values are reported (Mann-Whitney test). **C** Correlation between IFNA2 serum levels and *IFNA2* expression in 101 AML patients (Study Cohort 1), as determined by Luminex and RT-qPCR, respectively. Spearman correlation coefficient (R) and associated *p* value are reported. **D** Correlation matrix for *IFNA1*, *IFNA2* and *IFNB1* expression in 132 AML patients from Study Cohort 1. Spearman correlation coefficient (R) is reported; $*p < 0.0001$. **E** Relative expression abundance of type I IFN index (IFN-i) in whole PBMCs ($n = 132$) versus isolated CD33$^+$ malignant blasts ($n = 30$) from AML patients. Data are reported are presented as median, quartiles and extremes plus individual data points. ns, not significant (Mann-Whitney test). **F** Correlation matrix between IFN-i and relative expression levels of *CGAS, DDX58, IFIH1, MAVS, EIF2AK2, STING1, TLR3, TLR7, TLR9* and *ZBP1* in CD33$^+$ leukemic blasts isolated from 30 AML patients (Study Cohort 1). Significant Spearman correlation coefficients (R) are reported; $*p < 0.05$. **G**, **H** IFN-β production by CD33$^+$ blasts 24 h after optional treatment with polyI:C ($n = 10$), ODN2216 (CpG) ($n = 10$) **G** or a TLR3 inhibitor (TLR3i) ($n = 10$) **H**, as determined by ELISA. Data are presented as median, quartiles and extremes plus individual data points. Significant *p* values are reported; ns, not significant (Mann-Whitney test).

translation initiation factor 2-alpha kinase 2 (EIF2AK2; best known as PKR), stimulator of interferon response cGAMP interactor 1 (STING1), TLR3, TLR7, TLR9 and Z-DNA binding protein 1 (ZBP1) on CD33$^+$ malignant blasts isolated from 30 AML patients of Study Cohort 1. We observed a significant positive correlation between IFN-i and the expression levels of *TLR3* ($R = 0.67$; $p < 0.0001$) and *TLR9* ($R = 0.48$; $p = 0.0021$), as well as a negative correlation between IFN-i and *ZBP1* levels ($R = -0.50$; $p = 0.0274$) (Fig. 1F, Supplemental Fig. 3C). These findings were confirmed on the entire Study Cohort 1 using *TLR3* ($R = 0.5610$; $p < 0.0001$) and *TLR9* ($R = 0.3238$; $p = 0.0002$) expression levels in PBMCs (Supplemental Fig. 3D). To corroborate our data in an independent cohort of AML patients, we retrieved normalized *TLR3* expression levels of 152 AML patients from The Cancer Genome Atlas (TCGA) public database (Study Cohort 2), confirming a significant correlation between *IFNB1* and *TLR3* levels ($R = 0.3406$; $p < 0.0001$) (Supplemental Fig. 3E).



To estimate the functional impact of TLR3 and TLR9 signaling on type I IFN production by leukemic blasts, we harnessed ELISA to measure IFN-β synthesis by isolated CD33$^+$ blasts after TLR3 *versus* TLR9 stimulation. We observed a significant increase in IFN-β levels in response to the TLR3 agonist polyinosinic:polycytidylic acid [poly(I:C)], but not to the TLR9 agonist ODN2216 (Fig. 1G). Moreover, IFN-β secretion by otherwise unstimulated CD33$^+$ malignant blasts was significantly reduced upon inhibition of TLR3 with a TLR3/dsRNA-specific complex inhibitor (TLR3i) (Fig. 1H).

Fig. 2 Type I IFN-driven immunostimulation is suppressed by malignant blasts. A Relative expression levels of selected genes associated with NK cells, T_H1 and T_H2 response, T cell activation and cytotoxicity in 12 IFN-i^{Lo} and 12 IFN-i^{Hi} AML patients from Study Cohort 1 as determined RNAseq (see Supplementary Table 4). **B** Percentage of circulating $CD45^+CD3^+$, $CD45^+CD3^+CD4^+$, $CD45^+CD3^+CD8^+$ T cells and $CD45^+CD3^-CD56^+$, $CD45^+CD3^-CD56^{dim}$ and $CD45^+CD3^-CD56^{bright}$ NK cells in 13 IFN-i^{Lo} *versus* 34 IFN-i^{Hi} AML patients from Study Cohort 1 prior to induction chemotherapy, as determined by flow cytometry. Data are presented as median, quartiles and extremes plus individual data points. ns, not significant (Mann-Whitney test). **C** Gating strategy for IFN-γ^+ and $CD107a^+$ $CD45^+CD3^+CD8^+$ T cells and $CD45^+CD3^-CD56^+$ NK cells. The percentage of cells in each gate is reported. **D** Percentage of IFN-γ^+ and $CD107a^+$ $CD45^+CD3^+CD8^+$ T cells and $CD45^+CD3^-CD56^+$ NK cells upon stimulation with PMA plus ionomycin or K562 cells of peripheral blood mononuclear cells (PBMCs) from 10 IFN-i^{Lo} *versus* 23 IFN-i^{Hi} AML patients of Study Cohort 1, as determined by flow cytometry. Data are presented as median, quartiles and extremes plus individual data points. Significant *p* values are reported; ns, not significant (Mann-Whitney test). **E** Representative dot plots showing PBMC composition of one AML patient before and after depletion of $CD33^+$ leukemic blasts. **F, G** Percentage of IFN-γ^+ and $CD107a^+$ $CD8^+$ T cells and NK cells upon stimulation with PMA plus ionomycin of PBMCs optionally depleted of $CD33^+$ blasts and optionally exposed to recombinant IFN-α plus IFN-β (rIFNs) from 7 AML patients of Study Cohort 1, as determined by flow cytometry. Data are reported as means ± SEM. $*p < 0.05$; $**p < 0.01$; ns, not significant (paired t-test).

We next aimed at determining the signal transduction pathways elicited by TLR3 in support of type I IFN secretion in blasts from AML patients. To this aim, we compared the phosphorylation status of the TLR3 signal transducer TANK binding kinase 1 (TBK1) in 3 patients with AML exhibiting lower-than-median IFN-i (IFN-i^{Lo}) vs 3 patients with AML exhibiting higher-than-median IFN-i (IFN-i^{Hi}) from Study Cohort 1 by immunoblot analyses (Supplemental Table 5). We found a trend for TBK1 to be hyperphosphorylated in IFN-i^{Hi} versus IFN-i^{Lo} patients (Supplemental Fig. 4A, Supplemental Fig. 5), suggesting a preferential implication of this pathway. In further support of this notion, there was no difference in the abundance of a gene signature indicative of NF-κB signaling (*i.e., RELA, TRAF6, TAB1, RIPIL1, TNFRSF1B, TNFRSF1A, IL1R1, NFKBIA, MYD88, TNFAIP3, TRADD, TNF, NFKB1, FADD, CHUK, MAP3K1, MAP3K7, IKBKB, IKBKG, MAP3K14*) in 12 IFN-i^{Lo} vs 12 IFN-i^{Hi} patients from our study cohort 1 nor in 76 IFN-i^{Lo} vs 76 IFN-i^{Hi} patients from the TCGA dataset (Study Cohort 2; Supplemental Fig. 4B–D).

Taken together, these findings indicate that AML blasts produce type I IFN via TLR3-TBK1-IRF3 signaling.

Type I IFN-driven immunostimulation is suppressed by malignant blasts

To elucidate the immunostimulatory effects of type I IFN secreted by $CD33^+$ malignant blasts, we first harnessed RNAseq and compared the gene expression profile of PBMCs from 12 IFN-i^{Lo} *versus* 12 IFN-i^{Hi} patients from Study Cohort 1. While we identified a set of 433 differentially expressed genes (DEGs) (Supplemental Fig. 6A, Supplemental Table 6), pathway enrichment analyses failed to determine an association between upregulated DEGs and immune functions such as NK cell infiltration, T_H1 polarization, T_H2 polarization, T cell activation and cytotoxicity (Fig. 2A). These findings were confirmed in Study Cohort 2, suggesting a limited immunostimulatory effect from endogenous type I IFN in patients with AML (Supplemental Fig. 6B).

To corroborate these findings with an independent technology, we determined the frequency of circulating $CD3^+$ lymphocytes, $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, $CD3^-CD56^+$ NK cells as well as $CD3^-CD56^{dim}$ or $CD3^-CD56^{bright}$ NK cells, plus the phenotypic profile of dendritic cells (DCs) in IFN-i^{Lo} *versus* IFN-i^{Hi} patients from Study Cohort 1 using flow cytometry. In line with our previous observations, we failed to document any statistically significant difference in the abundance or functional profile of the aforementioned cell populations in this setting (Fig. 2B, Supplemental Fig. 6C). Along similar lines, both $CD8^+$ T cells and NK cells from IFN-i^{Lo} patients were equally (and rather poorly) responsive to stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (which non-specifically activate lymphoid cells) or K562 cells (an NK cell target) as their counterparts from IFN-i^{Hi} patients, both in terms of IFN-γ secretion and degranulation (as assessed by CD107a positivity) (Fig. 2C, D).

As malignant blasts are potent drivers of immunosuppression in AML [36], we next assessed the functional capacity of circulating $CD8^+$ T cells and NK cells from 7 AML patients of Study Cohort 3 before and after depletion of $CD33^+$ malignant blasts (Fig. 2E). We found that prior to $CD33^+$ cell depletion, recombinant IFN-α and IFN-β (rIFNs) fails to improve the ability of $CD8^+$ T cells and NK cells from AML patients to respond to PMA plus ionomycin with IFN-γ synthesis and degranulation (Supplemental Fig. 6D, E). Conversely, both $CD8^+$ T cells and NK cells from AML patients were reinvigorated in their ability to respond to PMA plus ionomycin upon depletion of malignant blasts, and even more so depletion of malignant blasts combined with rIFNs administration (Fig. 2F, G), although the effect on IFN-γ synthesis was less pronounced on $CD8^+$ T cells than on NK cells. Of note, the reinvigorated responsiveness of both $CD8^+$ T cells and NK cells to PMA plus ionomycin upon $CD33^+$ malignant blast depletion was compromised by the subsequent re-addition of isolated autologous $CD33^+$ blasts (Supplemental Fig. 6F, G).

Taken together, these findings suggest that $CD33^+$ malignant blasts actively impair baseline and type I IFN-stimulated $CD8^+$ T cell and NK cell effector functions in AML patients.

Recombinant type I IFN mediates direct cytostatic and cytotoxic activity on AML blasts and leukemic stem cells

Type I IFN has previously been suggested to exert direct cytostatic and cytotoxic activity against neoplastic cells, including malignant leukemic blasts [6]. To validate these findings, we tested the effect of rIFNs on human KASUMI-1, MOLM-13 and MV4–11 AML cells, observing a considerable cytostatic activity using a [^{3}H]-thymidine incorporation assay (Supplemental Fig. 7A). Similarly, rIFNs exerted some degree of cytotoxicity against human KASUMI-1, MOLM-13 and MV4–11 AML cells, as determined by flow cytometry (Fig. 3A, B), which could generally build on the effects of standard of care (SOC) chemotherapeutics including daunorubicin (DNR) and/or cytarabine (Ara-C) (Fig. 3B). We next determined the cytotoxic activity of rIFNs on primary blasts from AML patients, also observing direct cytotoxicity from rIFNs and additive effects when rIFNs were delivered along with DNR or Ara-C (Fig. 3C). As disease outcome in AML patients is often determined by the resistance of leukemic stem cells (LSCs) to SOC therapy [37], we next analyzed the impact of rIFNs on LSC viability (Fig. 3D). Importantly, rIFNs also mediated direct cytotoxicity on LSCs, as determined by flow cytometry, an effect was even more pronounced in the presence of DNR or Ara-C (Fig. 3E).

To directly estimate the cytotoxicity of TLR3 signaling in primary AML blasts and obtain insights in the underlying mechanisms, we nest stimulated $CD33^+$ cells isolated from 8 AML patients of Study Cohort 1 with the TLR3 agonist polyI:C in the optional presence of an IFNAR1-blocking antibody. We observed a loss in cellular viability driven by polyI:C comparable to that observed upon exposure of primary AML blasts to rIFNs (Fig. 3C), which could be at least partially counteracted by IFNAR1 blockage (Fig. 3F, G).

Altogether, these findings document the cytostatic and cytotoxic effect of type I IFN (employed at concentrations that are detected in the circulation of AML patients) as secreted

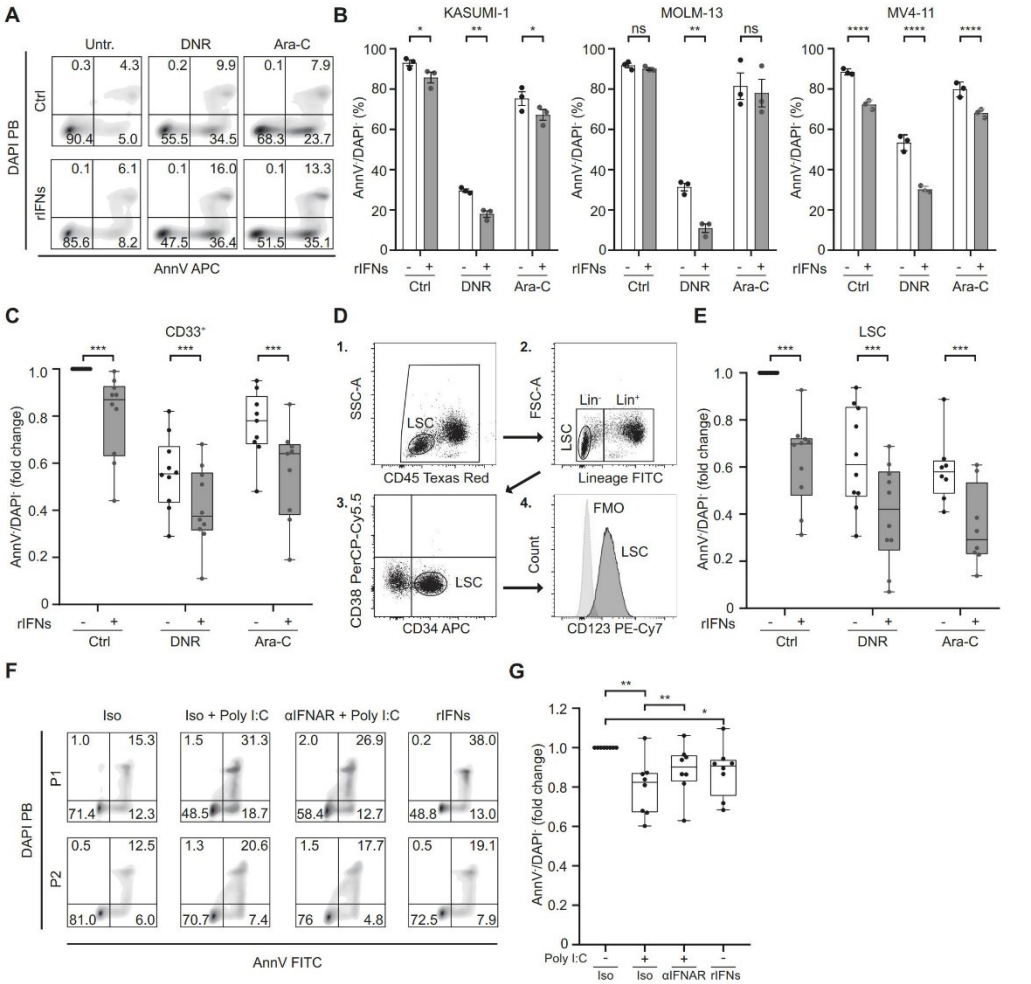
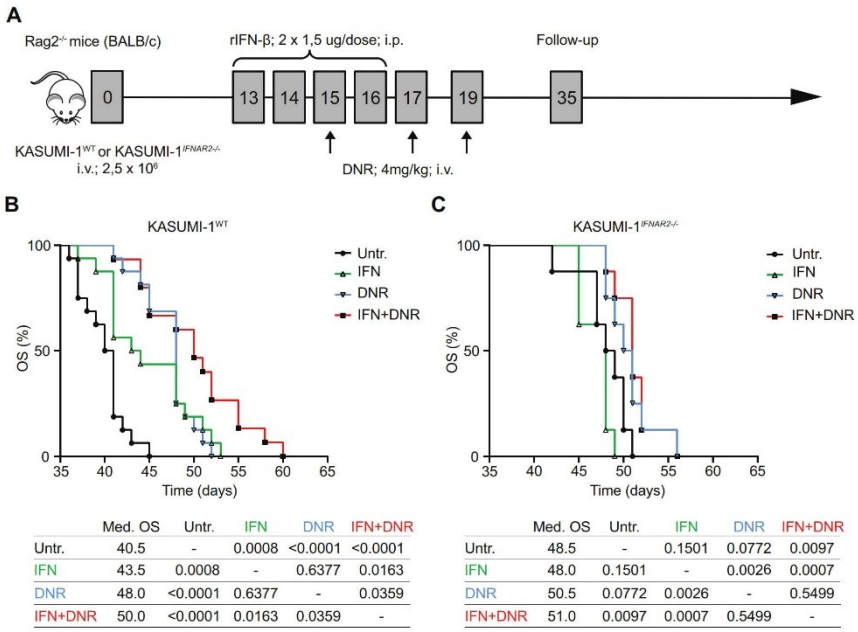


Fig. 3 Recombinant type I IFN mediates direct cytostatic and cytotoxic activity on AML blasts and leukemic stem cells. A, **B** Representative dot plots **A** and percentages **B** of viable (Annexin V^-/$DAPI^-$) KASUMI-1, MOLM-13 and MV4–11 cells after daunorubicin (DNR) and cytarabine (Ara-C) 24 h treatment with optional recombinant IFN-α plus IFN-β (rIFNs) (500 pg/mL) 3 days pre-incubation, as determined by flow cytometry. Data are reported as means ± SD plus individual data points. *$p < 0.05$; **$p < 0.01$, ****$p < 0.0001$, ns: not significant (paired t-test). **C** Relative viability of $CD33^+$ leukemic blasts from 10 AML patients (Study Cohort 3) after DNR or Ara-C treatment with optional 3 days pre-incubation with rIFNs (500 pg/mL). Data are reported as means, quartiles and extremes plus individual data points. ***$p < 0.001$ (paired t-test). **D** Gating strategy for determination of leukemic stem cells (LSCs) in AML patient PBMCs using flow cytometry. **E** Relative viability of LSCs isolated from 10 AML patients (Study Cohort 3) after DNR or Ara-C treatment with optional 3 days pre-incubation with rIFNs (500 pg/mL). Data are reported as means, quartiles and extremes plus individual data points. ***$p < 0.001$ (paired t-test). **F** Relative viability of $CD33^+$ leukemic blasts from 8 AML patients (Study Cohort 3) 96 h upon exposure to polyI:C in the optional presence of an IFNAR1 blocking antibody, or rIFNs, Data are reported as means, quartiles and extremes plus individual data points. *$p < 0.05$, **$p < 0.01$ (paired t-test).

downstream of TLR3 activation on both AML malignant blasts and LSCs.

Chemosensitizing effects of type I IFN in human AML xenografts

To examine the impact of exogenous type I IFN on the efficacy of SOC chemotherapy, we generated AML xenografts by intravenously injecting *Rag2*$^{-/-}$ mice (which lack B and T cells) with 2.5×10^6 human wild-type (WT) KASUMI-1 AML cells (Fig. 4A). Human rIFN-β was optionally administered over 4 consecutive days and 2 days prior to chemotherapy initiation in an attempt to mimic the baseline status of AML patients (Fig. 4A). In line with our in vitro findings, both type I IFN (median OS:43.5 days; $p = 0.0008$) and DNR (median OS:48.0 days; $p < 0.0001$) extended the OS of



	Med. OS	Untr.	IFN	DNR	IFN+DNR
Untr.	40.5	-	0.0008	<0.0001	<0.0001
IFN	43.5	0.0008	-	0.6377	0.0163
DNR	48.0	<0.0001	0.6377	-	0.0359
IFN+DNR	50.0	<0.0001	0.0163	0.0359	-

	Med. OS	Untr.	IFN	DNR	IFN+DNR
Untr.	48.5	-	0.1501	0.0772	0.0097
IFN	48.0	0.1501	-	0.0026	0.0007
DNR	50.5	0.0772	0.0026	-	0.5499
IFN+DNR	51.0	0.0097	0.0007	0.5499	-

Fig. 4 Chemosensitizing effects of type I IFN in human AML xenografts. A Experimental study design of an AML xenograft model using human WT or *IFNAR2*$^{-/-}$ KASUMI-1 cells in *Rag2*$^{-/-}$ mice. **B** Overall survival (OS) of *Rag2*$^{-/-}$ mice xenografted with WT KASUMI-1 cells and optionally treated with human rIFN-β (IFN), daunorubicin (DNR) or IFN + DNR. **C** OS of *Rag2*$^{-/-}$ mice xenografted with *IFNAR2*$^{-/-}$ KASUMI-1 cells and optionally treated with IFN, DNR or IFN + DNR. Survival curves were estimated by the Kaplan-Meier method, and differences between groups were evaluated using log-rank test. Median OS (days) and *p* values are reported.

Rag2$^{-/-}$ mice bearing WT KASUMI-1 cells, as compared to untreated mice (median OS: 40.5 days), an effect that was magnified when type I IFN and DNR were combined (median OS:50.0 days; $p = 0.0359$ *versus* DNR; $p = 0.0163$ *versus* type I IFN) (Fig. 4B). To rule out potential interferences emerging from any cross-reactivity between human rIFN-β and mouse type I IFN receptors, we repeated the same experiments with *IFNAR2*$^{-/-}$ KASUMI-1 cells. Importantly, in the absence of IFNAR2, KASUMI-1 xenografts became irresponsive to human rIFN-β (median OS:48.0 days; $p = 0.1501$) and also poorly responsive to DNR (median OS: 50.5 days; $p = 0.0772$) (Fig. 4C). Along these lines, combining DNR with human rIFN-β offered no survival advantages to mice bearing *IFNAR2*$^{-/-}$ KASUMI-1 xenografts as compared to DNR alone (median OS: 51.0 days; $p = 0.5499$) (Fig. 4C).

These findings extend our previous observations to document a beneficial impact of cancer cell-autologous type I IFN signaling on AML treatment sensitivity.

Type I IFN levels correlate with improved disease outcome in patients with AML

Inspired by our findings on the cytostatic and cytotoxic impact of type I IFN signaling on AML blasts and LSCs, we moved to determine the prognostic role of type I IFN genes in AML patients from Study Cohort 1 ($n = 132$) (Table 1), upon stratifying the entire patient cohort based median *IFNA1, IFNA2, IFNB1* expression level or median IFN-i values into IFN^{Lo} ($n = 66$) and IFN^{Hi} ($n = 66$) groups. IFN^{Hi} patients exhibited significantly improved RFS (*IFNA1*, HR: 0.41, $p = 0.0001$; *IFNA2*, HR: 0.50, $p = 0.0006$; *IFNB1*, HR: 0.49, $p = 0.0009$; IFN-i, HR:0.44; $p < 0.0001$) (Fig. 5A, C) and OS (*IFNA1*, HR: 0.37, $p = 0.0001$; *IFNA2*, HR: 0.44, $p = 0.0017$; *IFNB1*, HR: 0.50, $p = 0.0068$; IFN-i, HR: 0.40; $p = 0.0005$) (Fig. 5B, C) as compared to their IFN^{Lo} counterparts. On the contrary, the relative abundance of leukemic blasts in the peripheral blood or bone marrow failed to confer any prognostic information in this patient cohort (Supplemental Fig. 8A, B). These findings were confirmed by univariate Cox proportional hazard analyses (Table 2). Furthermore, multivariate Cox proportional hazard analysis identified IFN-i as a prognostic biomarker (RFS - HR: 0.91, CI95% 0.85–0.97, $p = 0.003$; OS - HR: 0.91, CI95% 0.84–0.99, $p = 0.021$) independent of clinical characteristics, including age, cytogenetic classification, hematopoietic transplantation, secondary AML and white blood cell (WBC) count (Table 3).

These findings suggest that type I IFN signaling may convey independent prognostic information in patients with AML.

DISCUSSION

Accumulating preclinical and clinical evidence indicates that, beyond a crucial role in curtailing viral infection, type I IFN produced by malignant cells and/or immune components of the TME contributes to clinically relevant cancer immunosurveillance in numerous oncological indications [38]. In line with this notion, type I IFN signaling supports the efficacy of various anticancer regimens including conventional chemotherapeutics [4, 24, 25], radiation therapy [17, 26, 27], targeted anticancer agents [5, 28], immunotherapy [29] and non-viral oncolytic agents [30–32].

Here, we harnessed two independent patient cohorts to define the immunobiology and prognostic relevance of type I IFN in AML. Specifically, we found that malignant blasts from AML patients release type I IFN via a TLR3-dependent mechanism that is not

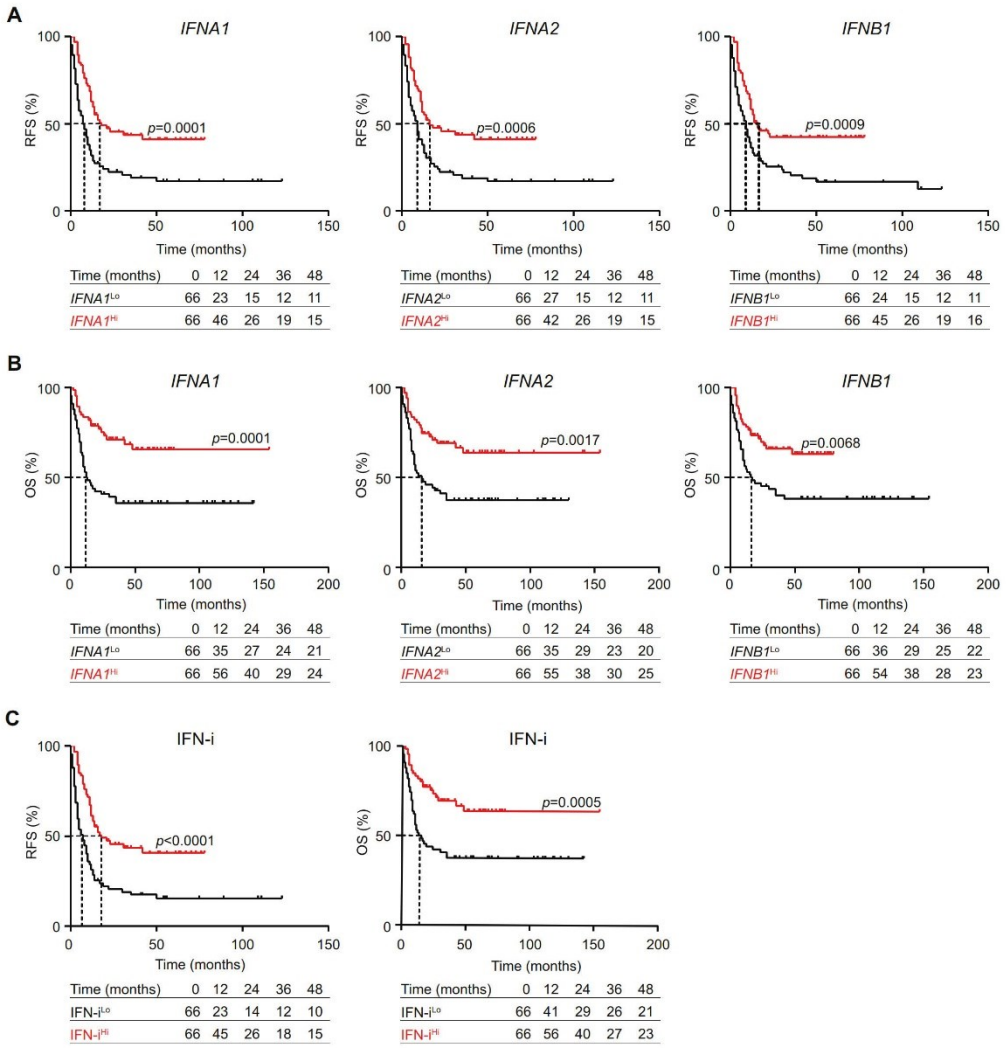


Fig. 5 Type I IFN levels correlate with improved disease outcome in patients with AML. A–C Relapse-free (RFS) and overall survival (OS) of 132 AML patients from Study Cohort 1 upon median stratification based on *IFNA1*, *IFNA2*, *IFNB1* expression or type I IFN index (IFN-i). Survival curves were estimated by the Kaplan-Meier method, and differences between groups were evaluated using log-rank test. Number of patients at risk and *p* values are reported.

induced by treatment (Fig. 1). In this respect, our results extend previous findings documenting a crucial role of TLR3 signaling in type I IFN release by cancer cells [6, 24]. Such a signaling pathway most likely originates from endogenous RNA species released by a fraction of dying malignant blasts, as previously documented in other settings [24, 39].

Despite expectations, type I IFN release failed to correlate with signs of active antitumor immunity as mediated by T_H1 $CD4^+$ T cells, IFN-γ-producing $CD8^+$ cells and NK cells (Fig. 2). Rather, the immunostimulatory function of type I IFN was impaired in AML patients by immunosuppressive mechanisms driven by malignant blasts (Fig. 2). These findings are in line with an ample preclinical and clinical literature demonstrating the potent immunosuppressive activity of leukemic blasts [40–42]. Potential mechanisms at play in this setting include (but may not be limited to): (i) the release of immunosuppressive cytokines like interleukin 10 (IL10) or tumor necrosis factor (TNF) downstream of TNF superfamily member 9 (TNFRSF9, best known as CD137) [40] or TNFSF18 signaling [41] and (ii); the direct inhibition of T cell and NK cell cytotoxic functions via CD200 [42]. Conversely, type I IFN at

Table 2. Univariate Cox proportional hazard analysis.

	OS		RFS	
Variable	**HR (95% CI)**	***p* value**	**HR (95% CI)**	***p* value**
IFNA1	0.86 (0.80–0.92)	<0.0001	0.86 (0.81–0.92)	<0.0001
IFNA2	0.90 (0.82–0.98)	0.011	0.92 (0.86–0.99)	0.018
IFNB1	0.92 (0.87–0.98)	0.014	0.91 (0.86–0.96)	0.00056
IFN-i	0.88 (0.82–0.95)	0.0009	0.88 (0.82–0.93)	<0.0001
Age	1.10 (1.00–1.10)	<0.0001	1.00 (1.00–1.00)	0.12
Leukocytes	1.00 (1.00–1.00)	0.0069	1.00 (1.00–1.00)	0.01
PB - Blasts	1.00 (0.99–1.00)	0.39	1.00 (0.99–1.00)	0.74
BM - Blasts	1.00 (0.99–1.00)	0.59	1.00 (0.99–1.00)	0.59
MRD	1.00 (0.98–1.00)	0.60	0.99 (0.97–1.00)	0.65
HSCT				
	0.27 (0.16–0.40)	<0.0001	0.73 (0.48–1.10)	0.13
Cytogenetics favorable				
	0.62 (0.25–1.60)	0.3100	1.10 (0.63–2.00)	0.67
Cytogenetics intermediate				
	0.53 (0.31–0.92)	0.025	0.51 (0.32–0.80)	0.0032
Cytogenetics adverse				
	2.90 (1.60–5.20)	0.0006	2.70 (1.60–4.60)	<0.0001
Secondary AML				
	2.20 (1.20–3.80)	0.0064	1.20 (0.68–2.00)	0.59
CEBPA				
	0.65 (0.20–2.10)	0.46	0.52 (0.19–1.40)	0.21
DNMT3A				
	0.80 (0.45–1.40)	0.4500	1.20 (0.77–1.90)	0.40
IDH1				
	0.79 (0.28–2.20)	0.65	0.71 (0.31–1.60)	0.43
IDH2				
	1.10 (0.51–2.30)	0.85	0.91 (0.47–1.80)	0.79
FLT3-ITD				
	1.30 (0.75–2.20)	0.36	1.50 (0.98–2.40)	0.059
NPM1				
	1.00 (0.60–1.70)	0.96	0.99 (0.64–1.5)	0.95

BM bone marrow, *CEBPA* CCAAT/enhancer-binding protein alpha, *DNMT3A* DNA (cytosine-5)-methyltransferase 3A, *FLT3-ITD* fms related receptor tyrosine kinase 3 - internal tandem duplication, *HSCT* hematopoietic stem cell transplantation, *IFN-i* type I interferon index, *MRD* minimal residual disease, *NPM1* nucelophosmin 1, *PB* peripheral blood, *IDH1* isocitrate dehydrogenase (NADP(+)) 1, isocitrate dehydrogenase (NADP(+)) 2.

concentrations similar to those detected in AML patients mediated direct cytotoxic effects and cooperated with SOC chemotherapeutics (DNR, Ara-C) against leukemic blasts, in vitro (Fig. 3) and in vivo (Fig. 4). Similar results have previously been obtained with immunodeficient murine AML xenografts subjected to continuous delivery of type I IFN by an adenoviral vector [43].

Supporting the clinical relevance of our findings, type I IFN expression was independently associated with improved RFS and OS in patients with AML (Tables 2, 3). Consistent with this notion, type I IFN expression levels or type I IFN signaling signatures have previously been attributed prognostic value in patients with glioblastoma [44] and breast carcinoma with poor prognosis [24, 45]. That said, signatures of type I IFN signaling have also been linked to poor disease outcome in other cohorts of breast carcinoma patients [12, 13] and colorectal cancer patients [46, 47]. At least in part, such an apparent discrepancy may reflect the differential effect of potent/acute *versus* indolent/chronic type I IFN signaling and/or the overall immunological contexture of the TME [16].

Taken together, our findings corroborate previous preclinical studies documenting the antineoplastic activity of type I IFN in the AML setting [38, 48]. These findings inspired clinical studies investigating recombinant human IFN-α in different therapeutic settings, including (but not limited to): (i) induction (ii), salvage therapy for patients relapsing upon hematopoietic stem cell transplantation (HSCT), and (iii) post-remission consolidation therapy [34, 49] with objective clinical responses observed in all such settings. Our findings suggest that at least part of such a benefit may originate from direct cytotoxicity rather than from the activation of tumor-targeting immunity. Thus, we surmise that monitoring of type I IFN levels might improve the clinical management of AML patients.

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AUTHOR CONTRIBUTIONS

Concept and design: PH, IT, JF; development of methodology: PH, JF, MH, JP, acquisition of data: PH, IT, JR, CS, MH, MK, MR, RM, JP, SV, HR, IV, DL, MH, PK, JP, LK, ZR; analysis and interpretation of data: PH, IT, JR, CS, MH, MK, MR, RM, JP; writing, review, and/or revision of the manuscript: PH, IT, CS, MK, RS, LG, JF; study supervision: RS, LG, JF.

COMPETING INTERESTS

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4.3 Peripheral gene signatures reveal distinct cancer patient immunotypes with therapeutic implications for autologous DC-based vaccines.

Immunotherapy using DCs-based vaccination is currently approved approach for therapy in metastatic hormone refractory cancer, while harnessing the potential of patient´s immune system to eliminate tumor cells. However, the clinical efficacy of DC-based vaccines remains suboptimal, most likely reflecting local and systemic immunosuppression at baseline. An autologous DC-based vaccine, (DCVAC), which harness ICD mechanisms, has recently been shown to improve progression-free survival and OS in randomized clinical trials enrolling patients with lung cancer (SLU01, NCT02470468) or ovarian carcinoma (SOV01, NCT02107937), but not metastatic castration-resistant prostate cancer (SP005, NCT02111577), despite a good safety profile across all cohorts. As exploratory research, we performed biomolecular and cytofluorometric analyses on peripheral blood samples collected prior to immunotherapy from 1000 patients enrolled in these trials, with the objective of identifying immunological biomarkers that may improve the clinical management of DCVAC-treated patients. We found out that sets of genes representing adaptive immunity and T cells activation correlates with improved disease outcomes along with the positive responses to the treatments in patients with lung and prostate cancer but not ovarian carcinoma. Interestingly, in ovarian carcinoma clinical benefits were associated rather with low expression of T_H2-like signature and immunosuppressive regulatory T cells. Furthermore, clinical responses to DCVAC were associated with signs of antitumor immunity in peripheral blood.

Author´s contribution to the study:

- Performing Multiplexed bead immunoassay on patient´s samples
- Performing basic biostatistical tests
- Manuscript preparation

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Peripheral gene signatures reveal distinct cancer patient immunotypes with therapeutic implications for autologous DC-based vaccines

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ABSTRACT

Dendritic cells (DCs) have received considerable attention as potential targets for the development of novel cancer immunotherapies. However, the clinical efficacy of DC-based vaccines remains suboptimal, largely reflecting local and systemic immunosuppression at baseline. An autologous DC-based vaccine (DCVAC) has recently been shown to improve progression-free survival and overall survival in randomized clinical trials enrolling patients with lung cancer (SLU01, NCT02470468) or ovarian carcinoma (SOV01, NCT02107937), but not metastatic castration-resistant prostate cancer (SP005, NCT02111577), despite a good safety profile across all cohorts. We performed biomolecular and cytofluorometric analyses on peripheral blood samples collected prior to immunotherapy from 1000 patients enrolled in these trials, with the objective of identifying immunological biomarkers that may improve the clinical management of DCVAC-treated patients. Gene signatures reflecting adaptive immunity and T cell activation were associated with favorable disease outcomes and responses to DCVAC in patients with prostate and lung cancer, but not ovarian carcinoma. By contrast, the clinical benefits of DCVAC were more pronounced among patients with ovarian carcinoma exhibiting reduced expression of T cell-associated genes, especially those linked to T_{H2}-like signature and immunosuppressive regulatory T (T_{REG}) cells. Clinical responses to DCVAC were accompanied by signs of antitumor immunity in the peripheral blood. Our findings suggest that circulating signatures of antitumor immunity may provide a useful tool for monitoring the potency of autologous DC-based immunotherapy.

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Introduction

Immunotherapy is currently the most rapidly advancing area of clinical oncology and has markedly improved the clinical management of multiple types of cancer.[1] Although, immune checkpoint inhibitors (ICIs) have revolutionized the clinical management of various solid tumors, only about 20% of patients with the most common solid tumors respond to ICIs as standalone therapies, although the proportion varies greatly among different indications.[2,3] Thus, novel strategies are needed alongside the identification of biomarkers that can prospectively identify patients who may benefit from specific immunotherapeutic regimens.[4–6]

Dendritic cells (DCs) have received considerable attention as potential targets for the development of cancer immunotherapies in recent decades.[7] Notably, the activity of DCs is associated with or underlies the efficacy of currently approved cancer therapies, such as ICIs.[8] Therefore, combining DC vaccination with different therapeutic approaches has been proposed. Nonetheless, the clinical efficacy of DC-based vaccines used as monotherapy remains suboptimal, which reflects the baseline level of circulating and/or intratumoral immune responses and the extent of immunosuppression.[9,10]

Although tumor sampling is widely implemented for biomarker identification and analysis, there are several challenges including limited accessibility, heterogeneity of the biopsy site,

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and the patient's condition.[11] Therefore, identification of potential predictive biomarkers using more accessible peripheral blood is critical for the development and clinical utility of biomarkers.[12,13] Recent technological, analytical, and mechanistic advances in immunology have enabled the identification of several circulating cancer biomarkers including, but not limited to: circulating tumor cells in breast and prostate cancer, tumor genomic alterations such as discrete oncogenic variants (e.g. *EGFR, PBRM1* and *JAK1/2*), microsatellite instability, tumor mutational burden-related metrics, peripheral immune-cell function, and analyses of immune-related cytokines and plasma proteins.[14–20] Because personalized DC-based cancer immunotherapy is largely dependent on preexisting circulating immunity, the identification of immune signatures associated with the response to therapy might provide a useful stratification tool.

We recently published the results of three independent open-label, randomized Phase I/II, II and III clinical studies that compared the efficacy of an autologous DC-based vaccine (DCVAC) delivered in the context of standard of care chemotherapy (SOC) versus SOC alone in patients with advanced non-small cell lung carcinoma (NSCLC; SLU01, NCT02470468),[21] epithelial ovarian cancer (EOC; SOV01, NCT02107937),[22,23] or metastatic castration-resistant prostate cancer (mCRPC; SP005, NCT02111577).[24] In these settings, DCVAC was well tolerated and significantly extended the progression-free survival (PFS) and overall survival (OS) of EOC or NSCLC patients.[21,22] In mCRPC patients, DCVAC combined with SOC and continued as maintenance treatment showed a favorable safety profile but did not extend OS.[24]

Here, we performed biomolecular and cytofluorometric analyses using peripheral blood samples collected prior to immunotherapy for 1000 patients enrolled in these trials of DCVAC. We found that a circulating immune-related gene signature associated with adaptive immunity and T cell activation was associated with an improved response to DC-based immunotherapy in mCRPC and NSCLC patients enrolled in SP005 and SLU01, although not in EOC patients enrolled in SOV01. Conversely, the clinical benefit of DCVAC was more pronounced in EOC patients with gene expression levels below median for T_{H2}-like and immunosuppressive gene signatures associated with a low frequency of circulating $CD4^+CD25^+FoxP3^+$ T cells, as determined by molecular and flow cytometry analyses. Pending validation in independent studies, our findings suggest that the circulating immune signature is a potential tool for stratification of patients prior to cellular immunotherapy, largely reflecting the oncologic indication.

Materials and methods

Patient characteristics

In SP005 (NCT02111577), 1182 mCRPC patients were randomized between June 2014 and November 2017 across 177 hospital clinics in Europe and the United States (US). Of these, 787 were assigned to DCVAC and 395 to placebo.[24] Patients in both arms received SOC, and DCVAC was continued as maintenance therapy. In SLU01 (NCT02470468), 112 patients with advanced NSCLC were randomized to one of three arms between January 2015 and November 2016. Patients in arm A received DCVAC/LuCa and chemotherapy (n = 45), patients in arm B received DCVAC/LuCa, chemotherapy and immune enhancers (n = 29), and patients in arm C received chemotherapy alone (arm C, n = 38).[21] In SOV01 (NCT02107937), 99 EOC patients were randomized to one of three arms between November 2013 and May 2015. All patients underwent debulking surgery followed by adjuvant SOC combined with DCVAC administered in parallel with SOC (arm A, n = 34) or sequential to SOC (arm B, n = 34). Patients in arm C (n = 31) received SOC alone.[22] The designs of these studies are briefly described in the Supplemental Materials and Methods. In SP005 and SLU01, the primary endpoint was overall survival (OS) defined as the time from randomization until death due to any cause. In SOV01, the primary efficacy endpoint was PFS. Of 1182 mCRPC patients, 112 NSCLC patients, and 99 EOC patients randomized to treatment, peripheral blood samples and data were available for 804 (68%), 103 (92%), and 93 (96%), respectively. Written informed consent was obtained according to the Declaration of Helsinki, and the study was approved by appropriate Ethical Committees. The results of all three clinical trials have been reported.[21,22,24] The baseline characteristics for patients included in this study were similar across the relevant treatment groups (Supplemental Table 1).

Preparation of DCVAC

Each DCVAC dose comprises DCs loaded with antigens derived from the EOC cell lines (OV-90 and SK-OV-3) in SOV01, NSCLC cell lines (H522 and H520) in SLU01, and a human prostate adenocarcinoma cell line (LNCaP) in SP005. To prepare DCVAC, the peripheral blood mononuclear cells, obtained via leukapheresis and gradient centrifugation, are first cultured in a medium containing interleukin-4 and granulocyte-macrophage colony-stimulating factor. Immature DCs are separated, co-cultured (pulsed) with high hydrostatic pressure-treated tumor cell lines, and matured using polyinosinic:polycyticylic acid.[25,26] The resulting product is cryopreserved at a concentration of approximately 10^7 DCs in 1 mL of CryoStor CS10 (StemCell) per vial.

Isolation of RNA from peripheral blood mononuclear cells (PBMCs) and reverse transcription

Total RNA was isolated with RNeasy Mini Kits (Qiagen). Cell lysates in RLT buffer enriched with 1% 2-mercaptoethanol were quickly thawed and processed according to the manufacturer's instructions, including DNase I digestion. The RNA concentration and purity were determined using a NanoDrop 2000c (Thermo Scientific). Purified RNA samples were stored at −80°C until further use. cDNA for the detection of 93 selected genes associated with the immune system (Supplemental Table 2) was synthesized from 100 ng of total RNA using the TATAA GrandScript cDNA Synthesis Kits (TATAA Biocenter).

cDNA preamplification

Ten microliters of cDNA samples diluted 1:2 was used in a 50 µL preamplification reaction with TATAA PreAmp GrandMaster® Mix and the relevant primers at a final

concentration of 40 nM per primer. Targeted pre-amplification was implemented on a T100 Thermal Cycler (Bio-Rad) with the following conditions: 95°C for 3 min, followed by 14 cycles of amplification (95°C for 20s, 55°C for 3 min and 72°C for 20s). After a final extension step (10 min), the samples were immediately frozen and stored at −20°C until analysis.

High-throughput quantitative real-time polymerase chain reaction (qPCR)

High-throughput qPCR was performed on the Biomark HD system (Fluidigm) using the 48.48 Dynamic Array Chip for Gene Expression and probe-based detection. Each reaction sample (5 µL) contained 1 µL of the pre-amplification products (diluted 1:10), 2.74 µL of Probe GrandMaster Mix (TATAA Biocenter), 0.25 µL of 20× GE Sample Loading Reagent (Fluidigm), 0.01 µL of ROX (Life Technologies; final concentration: 50 nM), and DNA/DNAse-free water. The assay reaction mix (5 µL) contained 2.5 µL of Assay Loading Reagent (Fluidigm) and 2.5 µL of a 5 µM mix of the reverse and forward primers plus 2.5 µM probes. Priming and loading of the dynamic array were performed according to the manufacturer's instructions using the IFC controller HX (Fluidigm). The thermal conditions comprised thermal mixing at 50°C for 2 min followed by 70°C for 40 min and 25°C for 10 min, hot-start activation at 95°C for 30s and 40 cycles of amplification (95°C for 10s and 60°C for 60s). Melting curve analysis was performed in the range of 60°C to 95°C with increments of 0.5°C/s. The amplification data were analyzed with Fluidigm Real-Time PCR Analysis software, applying the linear derivative baseline subtraction method and a user-defined global threshold to obtain Cq values.

Flow cytometry

The frequency of $CD4^+CD25^+FoxP3^+$ regulatory T cells was assessed by flow cytometry using standard procedures. Briefly, peripheral blood mononuclear cells (PBMCs) were stained with CD45-HV500 (BD Biosciences) CD3-A700 (Exbio), CD4-ECD (Beckman Coulter), and CD25-PE (Exbio) conjugates plus Aqua Blue Live/Dead cell viability dye (Life Technologies) (Supplemental Table 3). Thereafter, cells were fixed with fixation/permeabilization buffer (BD Bioscience), permeabilized with permeabilization buffer (BD Bioscience), and incubated with FoxP3-A488 (Thermo Fisher Scientific). Flow cytometry was performed on an LSRFortessa Analyzer (BD), and data were analyzed using FlowJo software (Tree Star, Inc). After excluding dead cells, regulatory T cells were determined as $CD45^+CD4^+CD25^+FoxP3^+$ cells (Supplementary Figure 1).

Multiplex assay

The serum levels of IL6, IL10 and IL13 in SOV01 patients were measured using a MAGPIX system (Luminex) with Magnetic Bead Panel HCYTOMAG-60 K, 3-plex (Merck). Samples were stored at −80°C until analyzed.

Statistical analysis

These analyses were conducted in a prospective exploratory manner using data collected from prospective clinical trials. PFS was defined as the time from randomization to the date of the first radiological progression or death, whichever came first. OS was calculated as the time from randomization to death from any cause. Survival analyses were estimated by Cox proportional hazard regression and the Kaplan–Meier method using R survival package, and differences between the groups of patients were calculated using the log-rank test. For log-rank tests, the prognostic value of continuous variables was assessed using cluster stratification or median cutoff for each gene or the frequency of circulating $CD4^+CD25^+FOXP3^+$ regulatory T cells. PCR data were analyzed using GenEx software (MultiD Analyses). The relative gene expression levels were calculated using the ΔΔCt method and were normalized to the expression levels of reference genes selected by Normfinder. Genes for which the expression was below the assay's detection limit were excluded from further analyses (SLU01: *IL4, IL13, NIS2, NCR2, MPPED1, NPR1*; SOV01: *NOS2, NCR2, MPPED1, CCL17, NPR1*; SP005: *IL4, NOS2, NCR2, CCL17, NPR1*). Heatmaps were prepared using ComplexHeatmap R package.[27] The EnrichGo function in ClusterProfiles R package was used to identify enriched GO terms based on hypergeometric distribution.[28] *p* values were adjusted for multiple comparisons using the Benjamini–Hochberg method. Wilcoxon's test was used to compare the frequency of immune markers before and after therapy. Fisher's exact test was used to compare patient distribution across subgroups. All analyses were performed with Prism 8.4.2 (GraphPad), SAS software V.9.4, and R (http://www.r-project. org/). *p* values <0.05 were considered statistically significant.

Results

The immune-related gene signature in peripheral blood predicted survival and the response to DCVAC in mCRPC patients

We first performed biomolecular analyses to compare the gene expression profile associated with the immune system in pre-treatment peripheral blood samples collected from 804 mCRPC patients enrolled in SP005 (Supplemental Table 1A). We focused on the detection of 93 genes classified into 9 clusters reflecting various immune subsets and functions, including (but not limited to): B cells, cytotoxicity, DCs, immune populations, immunosuppression, natural killer (NK) cell function, T cell activation, and T_{H1} vs T_{H2} polarization (Supplementary Table 2). Unsupervised hierarchical clustering identified two main patient clusters, which were well balanced across the study arms (Figure 1a). Cluster 1, a high inflammatory cluster, was significantly enriched with 68 genes compared with cluster 2, a low inflammatory cluster (Supplementary Table 4). Functional studies revealed a significant association between the differentially expressed genes (DEGs), particularly positive regulation of adaptive immune responses, and cytotoxic T cell- and NK cell-mediated immunity (Supplemental Figure 2A).

To assess the prognostic value of the immune-related gene signatures in peripheral blood, we compared OS between the distinct clusters of patients. In both study arms, the high inflammatory cluster was associated with longer OS (p <0.001) compared with the low inflammatory cluster

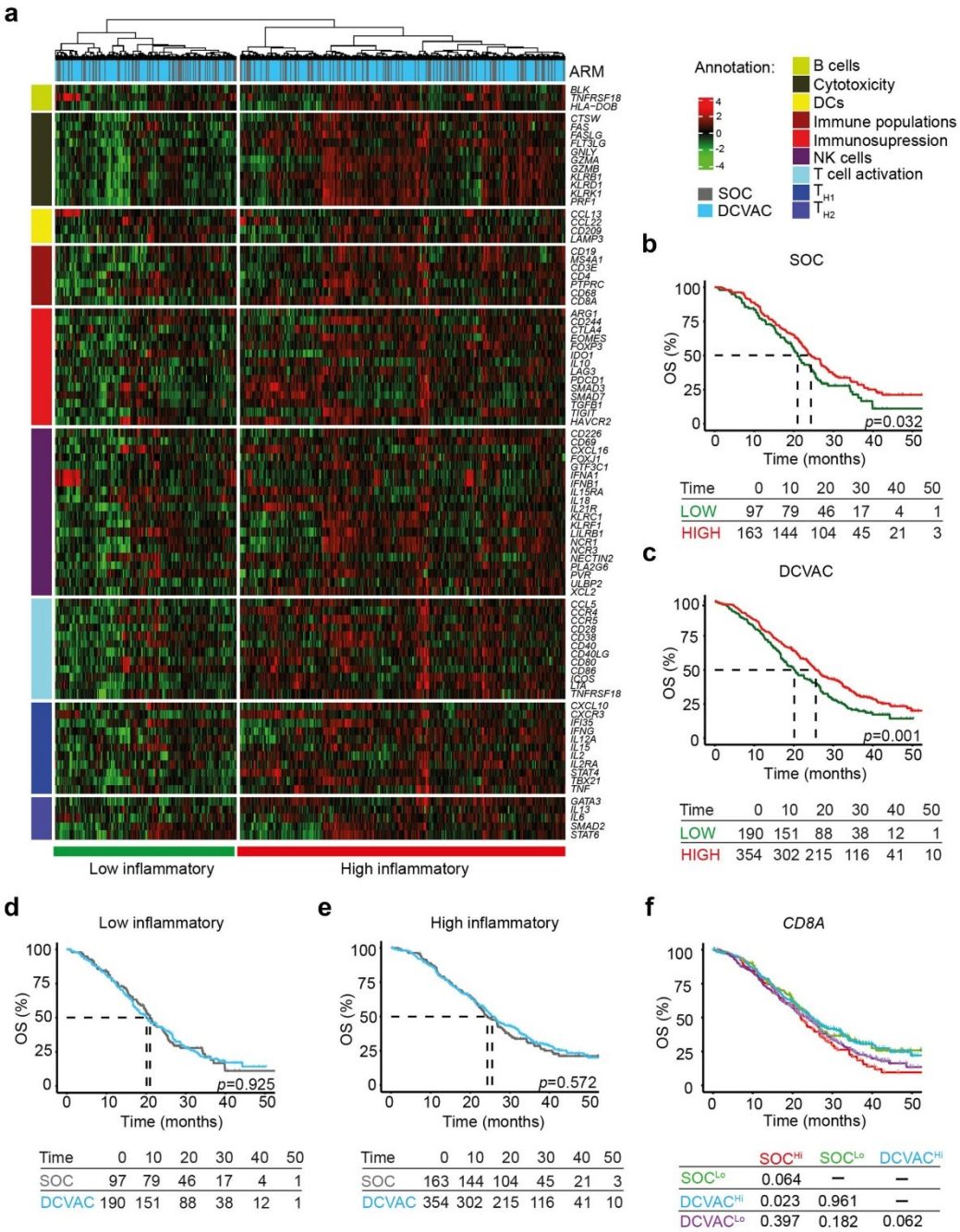


Figure 1. High expression of *CD8A* in peripheral blood is correlated with favorable prognosis and response to DCVAC in mCRPC patients in SP005. (a) Unsupervised hierarchical clustering of 804 mCRPC patients in SP005 based on the expression of 93 genes classified into clusters related to B cells, cytotoxicity, DCs, immune populations, immunosuppression, NK cells, T cell activation, and T_{H1} and T_{H2} signatures. (b, c) OS of 260 patients from the SOC arm (b) and 544 patients from the DCVAC arm (c) following stratification by unsupervised hierarchical clustering into low and high inflammatory clusters. (d, e) Direct comparison of OS of SOC and DCVAC

Table 1. Univariate Cox proportional hazard analyses for OS in mCRPC patients from the SOC and DCVAC arms in SP005.

SOC arm			DCVAC arm		
Variable	HR (95% CI)	*p*-value	Variable	HR (95% CI)	*p*-value
ARG1	1.2 (1.1–1.3)	<0.001	*IL12A*	0.7 (0.61–0.8)	<0.001
IL6	0.8 (0.7–0.9)	<0.001	*MS4A1*	0.79 (0.72–0.87)	<0.001
CD69	0.7 (0.5–0.09)	<0.001	*CCR5*	1.3 (1.2–1.5)	<0.001
GATA3	0.7 (0.6–0.8)	0.001	*CD69*	0.74 (0.65–0.85)	<0.001
CCR5	1.3 (1.1–1.5)	0.009	*CD19*	0.82 (0.75–0.9)	<0.001
KLRB1	0.7 (0.6–0.9)	0.016	*IL6*	0.82 (0.74–0.9)	<0.001
CD209	0.83 (0.7–0.9)	0.016	*CD68*	1.4 (1.2–1.7)	<0.001
CD4	0.7 (0.6–0.9)	0.029	*BLK*	0.84 (0.76–0.92)	<0.001
IL2	0.9 (0.8–1)	0.042	*IL18*	1.4 (1.2–1.7)	<0.001
			IL15	*1.4 (1.1-1.7)*	<0.001
			IFI35	1.2 (1.1–1.4)	<0.001
			CD226	1.3 (1.1–1.4)	<0.001
			CD86	1.3 (1.1–1.6)	<0.001
			TGFB1	1.3 (1.1–1.5)	0.002
			HAVCR	1.3 (1.1–1.5)	0.002
			GNLY	0.83 (0.74–0.93)	0.002
			SMAD2	1.4 (1.1–1.8)	0.002
			LTA	0.8 (0.7–0.92)	0.002
			KLRB1	0.81 (0.7–0.92)	0.002
			ARG1	1.1 (1–1.1)	0.002
			HLA-DOB	0.87 (0.8–0.95)	0.002
			LILRB1	1.3 (1.1–1.5)	0.002
			CD3E	0.83 (0.74–0.94)	0.004
			GATA3	0.83 (0.72–0.95)	0.005
			TBX21	0.85 (0.75–0.96)	0.009
			NECTIN2	1.2 (1–1.3)	0.011
			IL2	0.91 (0.84–0.98)	0.011
			IL15RA	1.2 (1–1.4)	0.014
			IFNG	0.9 (0.83–0.98)	0.016
			PLA2G6	0.79 (0.65–0.96)	0.017
			CXCL16	1.2 (1–1.4)	0.017
			NCR3	0.87 (0.78–0.98)	0.021
			IL10	1.1 (1–1.1)	0.032
			CCL22	1.1 (1–1.2)	0.032
			STAT4	0.88 (0.78–1)	0.043

OS = overall survival; mCRPC = metastatic castration-resistant prostate cancer; SOC = standard of care chemotherapy; DCVAC, dendritic cell-based vaccination; HR = hazard ratio; CI = confidence interval

(Figure 1b,) (SOC: p =0.032; DCVAC: p =0.001). In line with these findings, univariate Cox regression analyses revealed a strong prognostic value of 9 and 35 genes that were mainly associated with adaptive immunity and T cell activation. These genes were significantly overrepresented in the high inflammatory cluster in the SOC and DCVAC arms (Table 1).

To determine the predictive value of the immune-related gene signature in peripheral blood of mCRPC patients, we also compared the OS between the two study arms for the low and high inflammatory cluster separately. However, DCVAC did not show a distinct OS advantage in either cluster (Figure 1d,e). To obtain additional insights into the predictive value of gene-signatures associated with B cells, cytotoxicity, DCs, immune population, immunosuppression, NK cell function, T cell activation, and T_{H1} and T_{H2} on DCVAC efficacy, we directly compared OS among patients stratified by median gene expression levels and study arms. We found, that DCVAC treatment conferred a significant OS advantage to mCRPC patients with high expression of *CD8A* (CD8A: p =0.023), but not to their low counterparts (Figure 1f). Conversely, we failed to identify a predictive impact of gene signatures associated with B cells, DCs, NK cells, or individual T cell subsets and their functional capacity (Supplemental Figure 3A).

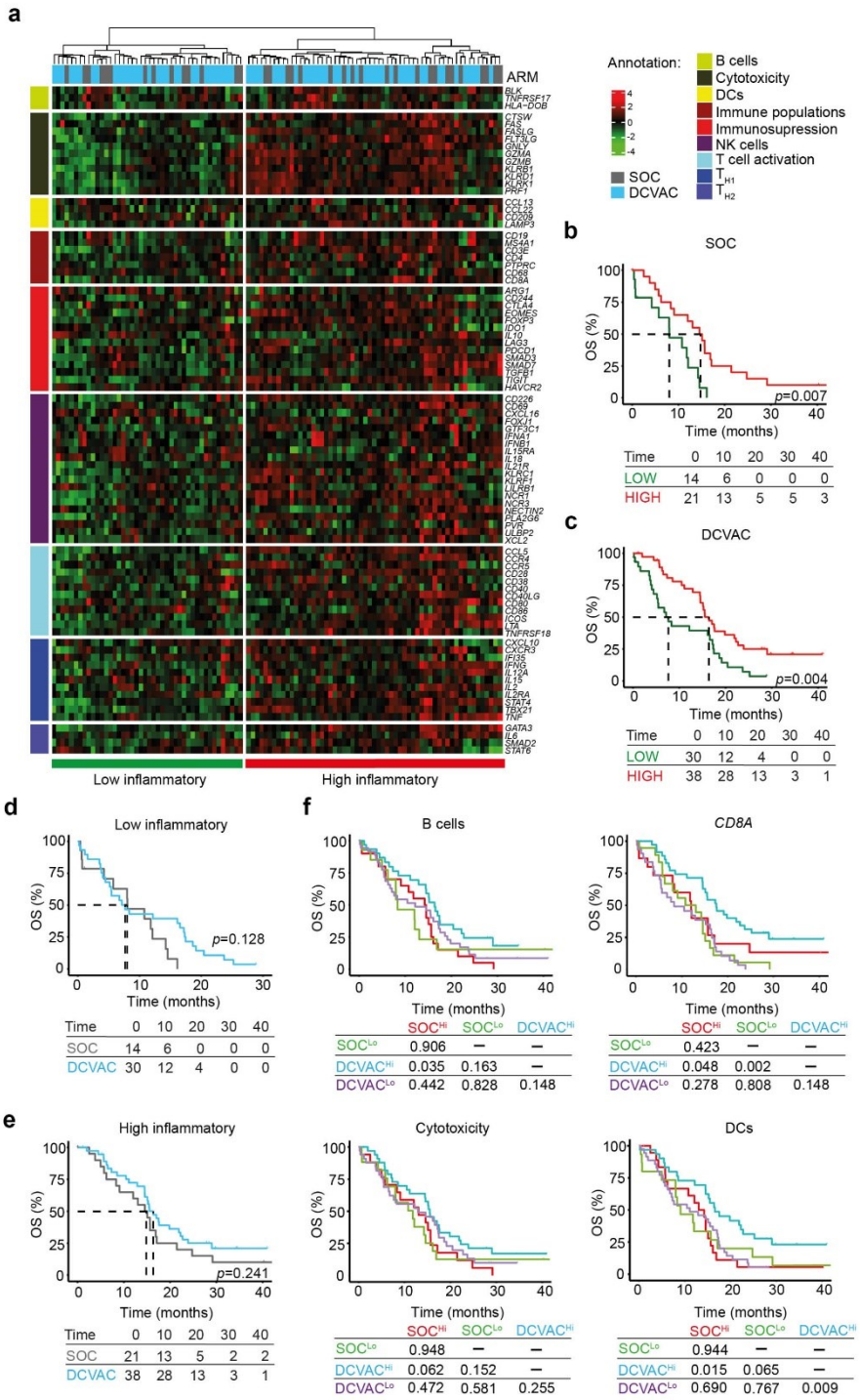
Taken together, these findings indicate that high expression of immune-related genes, especially those related to adaptive immunity, T cells and NK cells, was associated with improved OS in a large cohort of mCRPC patients. However, only high *CD8A* expression in peripheral blood was associated with a significantly improved response to DC-based immunotherapy in mCRPC patients.

The immune-related gene signature in peripheral blood predicted survival and the response to DC-based immunotherapy in NSCLC patients

Inspired by our observation in mCRPC, we then compared the expression profile of the same panel of 93 genes using pre-treatment peripheral blood samples from 103 NSCLC patients enrolled in SLU01 (Supplemental Table 1B). Similar to the results for mCRPC, unsupervised hierarchical clustering identified two main clusters of NSCLC patients that were well balanced across the study arms (Figure 2a). Cluster 1, the high inflammatory cluster, was significantly enriched for 61 genes compared with cluster 2, the low inflammatory cluster (Supplementary Table 4). Similarly, to the findings for mCRPC, functional studies revealed significant associations between the DEGs with positive regulation of the adaptive immune response, and cytotoxic T cell- and NK cell-mediated immunity (Supplemental Figure 2B). To assess the prognostic value of the immune-related gene signatures in peripheral blood, we assess OS in each cluster. In both SOC and DCVAC arms, the low inflammatory cluster was associated with shorter OS compared to high inflammatory cluster (Figure 2b,). Consistent with these findings, univariate Cox regression analyses confirmed a strong prognostic value of 14 and 30 genes, mainly associated with T cell activation, that were significantly overrepresented in the high inflammatory cluster in the SOC and DCVAC arms, respectively (Table 2).

To assess the predictive value of immune-related gene signature in peripheral blood of SLU01 patients, we compared OS between the study arms for the low and high inflammatory clusters separately (Figure 2d, e). Although, there was no advantage of DCVAC in either cluster, we found that DCVAC conferred an OS advantage to patients with high expression levels of gene signatures associated with B cells (p =0.035), *CD8A* (p =0.048), and DCs (p =0.015) (Figure 2f). A similar non-significant trend was also observed for gene signature associated with cytotoxicity (p =0.062) (Figure 2f). Importantly, we failed to observe a negative impact of T_{H2} and *FoxP3* gene signature on the final response to DCVAC in NSCLC patients (Supplemental Figure 3B).

patients following stratification by unsupervised hierarchical clustering into low (d) and high inflammatory clusters. (f) OS of 804 mCRPC patients stratified by the median *CD8A* expression and study arm. Survival curves were estimated using the Kaplan–Meier method and differences between groups were evaluated using the log-rank test. The numbers of patients at risk and p values are reported.


a
ARM
Annotation:
SOC
DCVAC
B cells
Cytotoxicity
DCs
Immune populations
Immunosupression
NK cells
T cell activation
Low inflammatory
High inflammatory
b
SOC
p=0.007
Time (months)
OS (%)
Time 0 10 20 30 40
LOW 14 6 0 0 0
HIGH 21 13 5 5 3
c
DCVAC
p=0.004
Time (months)
OS (%)
Time 0 10 20 30 40
LOW 30 12 4 0 0
HIGH 38 28 13 3 1
d
Low inflammatory
p=0.128
Time (months)
OS (%)
Time 0 10 20 30 40
SOC 14 6 0 0 0
DCVAC 30 12 4 0 0
e
High inflammatory
p=0.241
Time (months)
OS (%)
Time 0 10 20 30 40
SOC 21 13 5 2 2
DCVAC 38 28 13 3 1
f
B cells
Time (months)
OS (%)
SOCHi SOCLo DCVACHi
SOCLo 0.906 – –
DCVACHi 0.035 0.163 –
DCVACLo 0.442 0.828 0.148
CD8A
Time (months)
OS (%)
SOCHi SOCLo DCVACHi
SOCLo 0.423 – –
DCVACHi 0.048 0.002 –
DCVACLo 0.278 0.808 0.148
Cytotoxicity
Time (months)
OS (%)
SOCHi SOCLo DCVACHi
SOCLo 0.948 – –
DCVACHi 0.062 0.152 –
DCVACLo 0.472 0.581 0.255
DCs
Time (months)
OS (%)
SOCHi SOCLo DCVACHi
SOCLo 0.944 – –
DCVACHi 0.015 0.065 –
DCVACLo 0.690 0.767 0.009

Taken together, these findings indicate that, similar to mCRPC, high expression of immune-related genes, particularly those related to adaptive immunity and T cells activation, are associated with improved disease outcome in NSCLC patients. However, a greater clinical benefit of DCVAC was observed in NSCLC patients with high expression levels of genes associated with B cells, effector $CD8^+$ T cells, and DCs.

A low inflammatory gene signature in peripheral blood was correlated with improved PFS in EOC patients treated with DCVAC

Driven by our observations in mCRPC and NSCLC, we also compared the gene expression profile for the same panel of 93 genes in pre-treatment peripheral blood samples of 93 EOC patients enrolled in SOV01 (Supplemental Table 1C). Again, unsupervised hierarchical clustering identified two main patient clusters associated with low and high expression of immune-related genes that were well balanced across the study arms (Figure 3a). The high inflammatory cluster was significantly enriched for 68 genes compare with the low inflammatory cluster (Supplementary Table 4C). Functional studies revealed significant associations between the DEGs, especially positive regulation of adaptive immune responses, as well as cytotoxic T cell- and NK cell-mediated immunity (Supplemental Figure 2C).

To assess the prognostic value of immune gene signatures in peripheral blood, we evaluated PFS in distinct clusters of patients. Importantly, among patients treated with DCVAC, we observed worse PFS in the "high" inflammatory cluster than in the low inflammatory cluster (p =0.049). However, we failed to observe a similar trend in SOC patients (Figure 3b, c). In line with these findings, univariate COX regression analyses confirmed negative prognostic role of 5 genes namely *CD3E, CD4*, forkhead box P3 (*FOXP3*), granzyme A (*GZMA*), granzyme B (*GZMB), HLA-DOB*, and interleukin 4 (*IL4*), which were associated with poor disease outcomes in DCVAC-treated patients (Table 3).

To assess the predictive value of the immune gene signature in peripheral blood of EOC patients in SOV01, we directly compared PFS between the high and low inflammatory clusters of patients in both study arms (Figure 3d, e). Importantly, in the low inflammatory cluster, we found that DCVAC conferred a significant PFS advantage compared with their counterparts in the SOC arm (Figure 3d). By contrast, among patients included in the high inflammatory cluster, PFS was not significantly different between patients treated with SOC and DCVAC (Figure 3e). Consistent with this notion, DCVAC was associated with improved PFS compared with SOC among patients with expression levels below the median for gene signatures associated with B (p =0.039) and *CD3E* (p =0.044), immunosuppression (p =0.041), and T_{H2} response (p =0.048) in peripheral blood (Figure 3f).

Taken together, these findings indicate that low expression levels of T cells-like genes were associated with improved prognosis in EOC patients who received DC-based immunotherapy, opposite to the findings in mCRPC and NSCLC, where high expression levels were associated with improved OS.

High frequency of regulatory T cells in peripheral blood of EOC patients is associated with a poor response to DCVAC

Considering our findings for the individual cancer types, we next compared the expression levels of all 93 genes among mCRPC, NSCLC and EOC patients to examine whether there are differences in the baseline circulating immunity in distinct malignancies. Notably, we found that the expression levels of 8 genes were significantly higher in EOC patients than in mCRPC and NSCLC patients: arginase 1 (*ARG1), FOXP3*, interleukin 6 (*IL6*), interleukin 13 (*IL13*), programmed cell death 1 (*PDCD1*; best known as PD-1), transforming growth factor beta 1 (*TGFB1*), T cell immunoreceptor with Ig and ITIM domains (*TIGIT*), and tumor necrosis factor A (*TNFA*) (Figure 4a,b). These findings indicate higher levels of cellular and humoral immunosuppression in peripheral blood of EOC patients compared with NSCLC and mCRPC patients (Figure 4a,b). Consistent with this notion, we observed increased expression of an immunosuppressive-like gene signature (*FOXP3, HAVCR2, IDO1, IL10, LAG3, PDCD1, TGFB1, TIGIT)* and decreased expression of an immunostimulatory-like gene signature (*GNLY, GZMA, GZMB, IFNG, IL12A, PRF1, TBX21, CD8A)* in EOC patients in SOV01 than in mCRPC and NSCLC patients in SP005 and SLU01 (Figure 4c). Additionally, mCRPC and NSCLC patients with immunostimulatory gene signatures above median levels showed improved responses to DCVAC (mCRPC: p =0.032; NSCLC: p =0.045) (Figure 4d,). However, the gene expression profile of immunosuppressive signature failed to impact disease outcomes (Supplemental Figure 4A, B). By contrast, DCVAC provided a significant benefit to EOC patients with expression levels of the immunosuppressive gene signature below the median (p =0.025) (Figure 4f), but the immunostimulatory gene signature did not have a significant impact on clinical outcomes (Supplemental Figure 4C).

To investigate the potential impact of immunosuppressive soluble factors on DCVAC activity in EOC patients, we measured the serum levels of IL6, IL10 and IL13. Although high levels of IL6 and IL10 were associated with worse PFS in the SOC arm (IL6: p =0.007; IL10: p =0.021), the serum levels of IL6, IL10, and IL13 were not prognostic and predictive factors

Figure 2. High expression gene signatures associated with B cells, *CD8A*, cytotoxicity, and DCs is correlated with favorable prognosis and response to DCVAC in NSCLC patients in SLU01. (a) Unsupervised hierarchical clustering of 103 NSCLC patients in SLU01 based on the expression of 93 genes classified into clusters related to B cells, cytotoxicity, DCs, immune populations, immunosuppression, NK cells, T cell activation, and T_{H1} and T_{H2} signatures. (b, c) OS of 35 patients from the SOC arm (b) and 68 patients from the DCVAC arm (c) following stratification by unsupervised hierarchical clustering into low and high inflammatory clusters. (d, e) Direct comparison of OS of SOC and DCVAC patients following stratification by unsupervised hierarchical clustering into low (d) and high inflammatory clusters (e). (f) OS of 103 NSCLC patients stratified by the median expression of genes associated with B cell signature, *CD8A* expression, cytotoxicity, and DCs, and study arm. Survival curves were estimated using the Kaplan–Meier method, and differences between groups were evaluated using the log-rank test. The numbers of patients at risk and p values are reported.

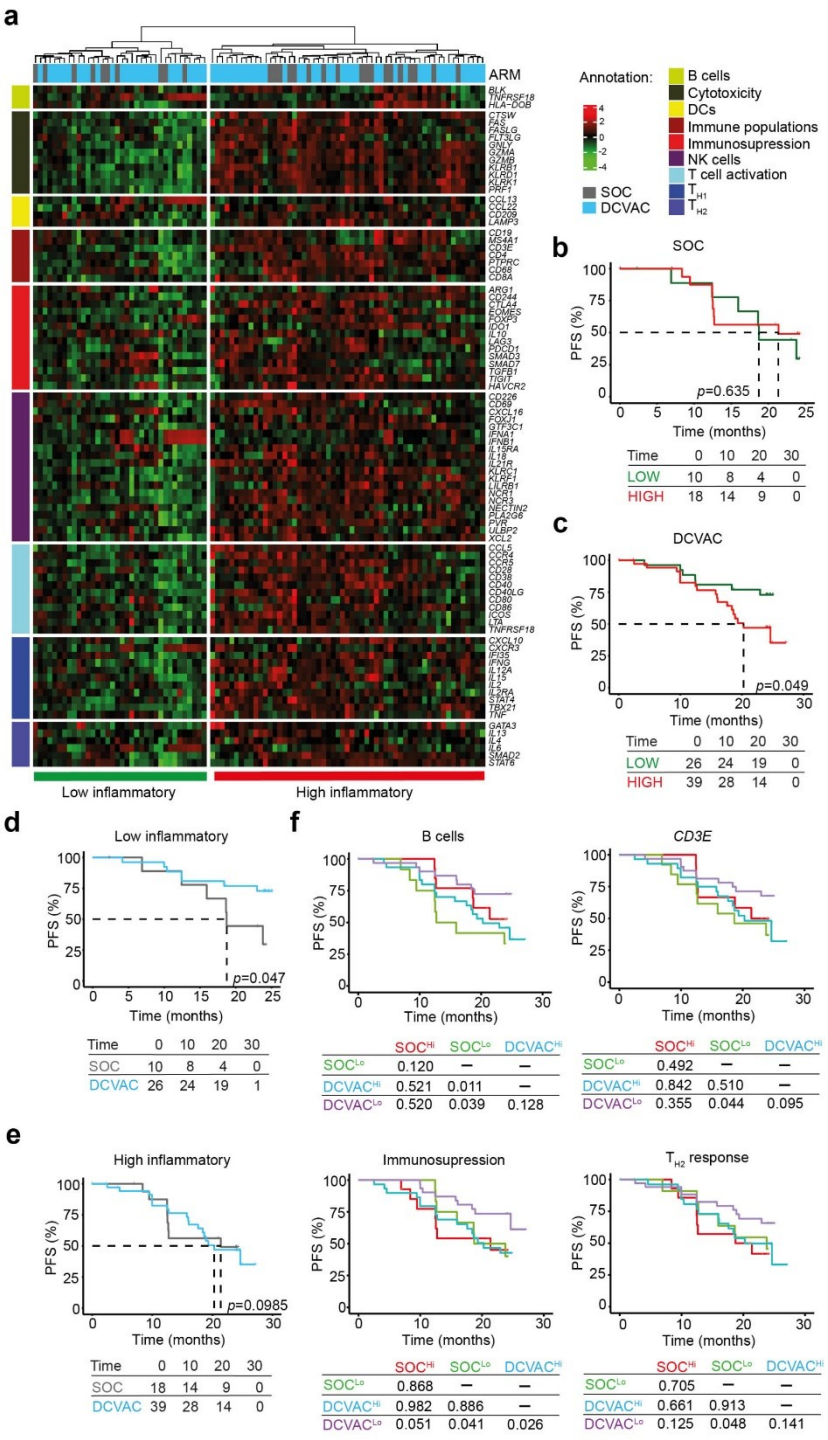

a
ARM
Annotation:
B cells
Cytotoxicity
DCs
Immune populations
Immunosupression
NK cells
T cell activation
SOC
DCVAC
Low inflammatory
High inflammatory
b
SOC
PFS (%)
p=0.635
Time (months)
Time 0 10 20 30
LOW 10 8 4 0
HIGH 18 14 9 0
c
DCVAC
PFS (%)
p=0.049
Time (months)
Time 0 10 20 30
LOW 26 24 19 0
HIGH 39 28 14 0
d
Low inflammatory
PFS (%)
p=0.047
Time (months)
Time 0 10 20 30
SOC 10 8 4 0
DCVAC 26 24 19 1
f
B cells
PFS (%)
Time (months)
SOCHi SOCLo DCVACHi
SOCLo 0.120 – –
DCVACHi 0.521 0.011 –
DCVACLo 0.520 0.039 0.128
CD3E
PFS (%)
Time (months)
SOCHi SOCLo DCVACHi
SOCLo 0.492 – –
DCVACHi 0.842 0.510 –
DCVACLo 0.355 0.044 0.095
e
High inflammatory
PFS (%)
p=0.0985
Time (months)
Time 0 10 20 30
SOC 18 14 9 0
DCVAC 39 28 14 0
Immunosupression
PFS (%)
Time (months)
SOCHi SOCLo DCVACHi
SOCLo 0.868 – –
DCVACHi 0.982 0.886 –
DCVACLo 0.051 0.041 0.026
TH2 response
PFS (%)
Time (months)
SOCHi SOCLo DCVACHi
SOCLo 0.705 – –
DCVACHi 0.661 0.913 –
DCVACLo 0.125 0.048 0.141

Table 2. Univariate Cox proportional hazard analyses for OS in NSCLC patients from the SOC and DCVAC arms in SLU01.

SOC arm			DCVAC arm		
Variable	HR (95% CI)	*p*-value	Variable	HR (95% CI)	*p*-value
PLA2G6	0.1 (0.01–0.5)	0.002	*CD28*	0.4 (0.2–0.6)	<0.001
GATA3	0.45 (0.3–0.8)	0.007	*CD3E*	0.4 (0.2–0.6)	<0.001
LILRB1	2.6 (1.3–5.4)	0.008	*LILRB1*	3.1 (1.7–5.8)	<0.001
LTA	0.5 (0.3–0.9)	0.011	*STAT4*	0.4 (0.2–0.7)	<0.001
IFI35	2 (1.2–3.5)	0.011	*CD8A*	0.6 (0.4–0.8)	<0.001
HAVCR	2.6 (1.2–5.7)	0.019	*FLT3LG*	0.4 (0.2–0.7)	<0.001
CD86	2.8 (1.2–6.7)	0.021	*CD40LG*	0.5 (0.3–0.7)	0.001
RORC	0.7 (0.5–0.9)	0.026	*FOXP3*	0.6 (0.4–0.8)	0.001
KLRB1	0.6 (0.3–0.9)	0.036	*KLRB1*	0.5 (0.3–0.8)	0.002
CD28	0.45 (0.21–0.95)	0.037	*IL15*	3.1 (1.5–6.6)	0.002
CD269	1.4 (1–2)	0.038	*CD68*	1.9 (1.3–3)	0.003
CXCR3	0.6 (0.3–0.9)	0.043	*TIGIT*	0.6 (0.4–0.8)	0.003
CXCL16	2 (1–4)	0.041	*CCL5*	0.6 (0.4–0.8)	0.003
IL2	0.8 (0.6–1)	0.045	*GATA3*	0.5 (0.3–0.8)	0.004
			KLRF1	0.5 (0.3–0.8)	0.006
			PLA2G6	0.4 (0.2–0.8)	0.008
			CTSW	0.6 (0.4–0.9)	0.011
			NCR3	0.6 (0.4–0.9)	0.012
			IL21R	0.5 (0.3–0.9)	0.014
			CTLA4	0.6 (0.4–0.9)	0.014
			IL12A	0.6 (0.5–0.9)	0.014
			STAT6	2.3 (1.2–4.6)	0.017
			SMAD3	0.4 (0.2–0.9)	0.018
			TBX21	0.6 (0.4–0.9)	0.023
			IL10	1.3 (1–1.5)	0.024
			CD86	1.8 (1.1–3.2)	0.027
			CCR4	0.7 (0.4–0.9)	0.028
			IFI35	1.6 (1–2.4)	0.032
			TNFRSF18	0.6 (0.4–0.9)	0.033
			XCL2	0.7 (0.5–1)	0.039

OS = overall survival; NSCLC = non-small cell lung cancer; SOC = standard of care chemotherapy; DCVAC, dendritic cell-based vaccination; HR = hazard ratio; CI = confidence interval

Table 3. Univariate Cox proportional hazard analyses for OS in EOC patients from the SOC and DCVAC arms in SOV01.

SOC arm			DCVAC arm		
Variable	HR (95% CI)	*p*-value	Variable	HR (95% CI)	*p*-value
IL10	2.1 (1.3–3.5)	0.005	*NCR1*	1.9 (1.2–3)	0.007
IL15RA	5.4 (1.6–1.8)	0.005	*GZMA*	1.8 (1.1–2.9)	0.012
CD8A	1.6 (1.1–2.2)	0.009	*IL4*	2.4 (1.2–4.7)	0.015
SMAD3	5.1 (1.3–2)	0.019	*CD4*	2.4 (1.2–5)	0.017
TGFB1	3.6 (1.2–11)	0.023	*CD3E*	2.1 (1.1–4.2)	0.028
HLA-DOB	0.6 (0.3–0.1)	0.033	*HLA-DOB*	1.6 (1–2.6)	0.037
NECTIN2	2.6 (1.1–6.4)	0.033	*GZMB*	1.7 (1–2.9)	0.044
IL6	0.6 (0.4–0.1)	0.046	*FOXP3*	2 (1–3.9)	0.047

PFS = progression-free survival; EOC = epithelial ovarian cancer; SOC = standard of care chemotherapy; DCVAC, dendritic cell-based vaccination; HR = hazard ratio; CI = confidence interval

in the DCVAC arm (Supplemental Figure 5A-C). These findings suggest that humoral immunosuppression is not associated with the response to DCVAC therapy in EOC patients.

In terms of cellular immunosuppression, we found that DCVAC-treated *FOXP3*^{Hi} patients did not show a favorable PFS as compared to *FOXP3*^{Lo} counterparts, indicating a negative impact of immunosuppressive circulating regulatory T cells (Supplemental Figure 4D). To validate these findings using an independent approach, we performed flow cytometry to quantify the frequency of circulating $CD4^+CD25^+FoxP3^+$ regulatory T cells in pretreatment peripheral blood samples from EOC patients in SOV01 (Figure 4g). The frequency of circulating $CD4^+CD25^+FoxP3^+$ regulatory T cells was comparable between the DCVAC and SOC arms (Supplemental Figure 4E). To assess the prognostic value of $CD4^+CD25^+FoxP3^+$ cells in EOC patients, we evaluated PFS after stratifying patients based on the median frequency. FoxP3^{Lo} status was associated with improved PFS, but only in DCVAC-treated patients. These findings may indicate that DCVAC provides a significant PFS benefit in EOC patients with a low frequency of $CD4^+CD25^+FoxP3^+$ regulatory T cells compared with patients with a high frequency of these cells (Figure 4h). By comparison, we did not observe a prognostic role of *FOXP3* expression in PBMCs obtained from mCRPC and NSCLC patients (Supplemental Figure 5 F, G).

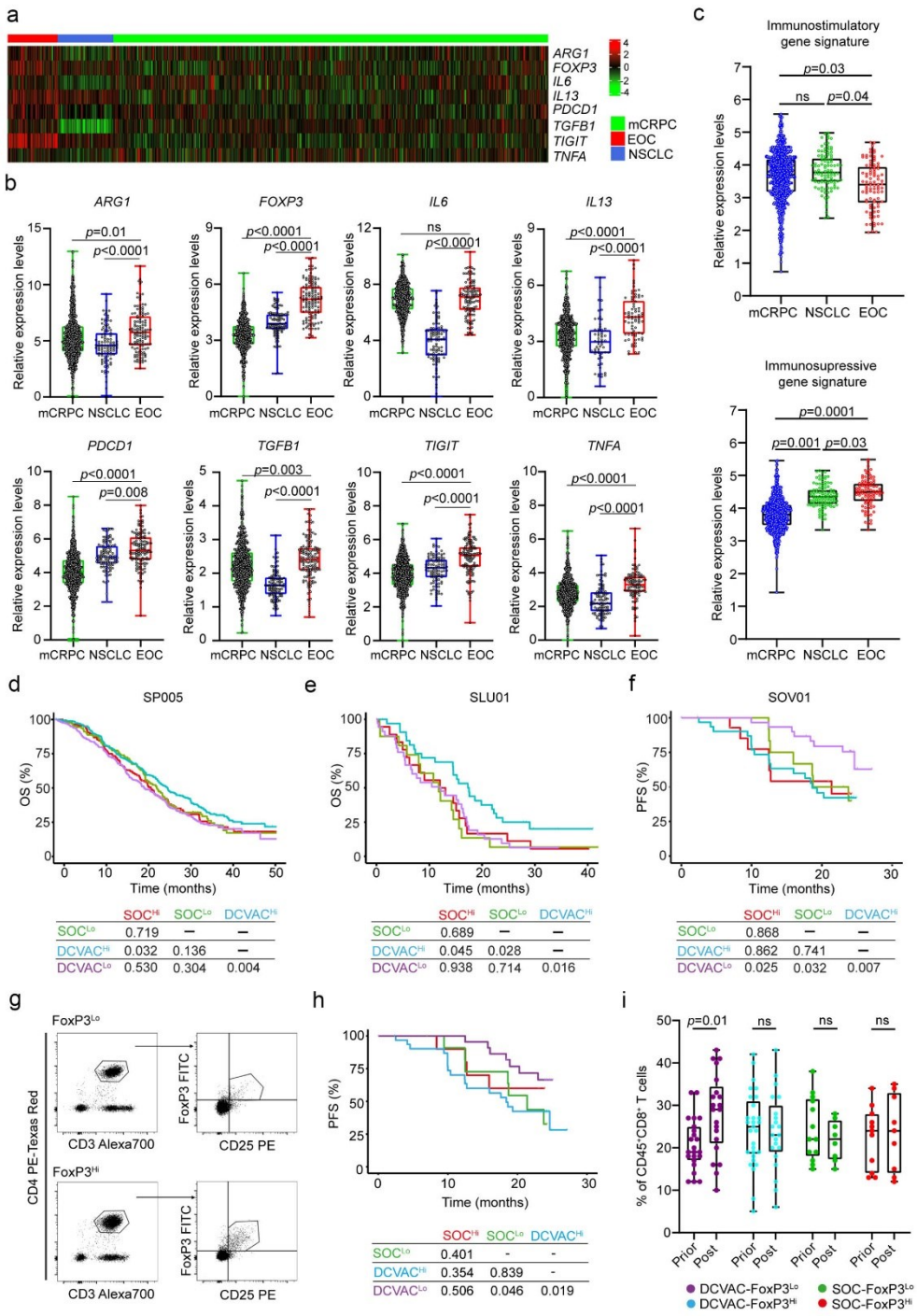
To confirm and extend these findings using another technological approach, we analyzed the circulating biomarkers of immune responses mediated by DCVAC therapy in EOC patients after treatment termination by performing flow cytometry (Supplemental Figure 1A). Confirming our transcriptional findings, we found that, although the frequency of circulating $CD3^+$ T cells remained unchanged before and after DCVAC therapy (Supplemental Figure 5 H), there was a significant increase in the frequency of circulating $CD8^+$ T cells in FoxP3^{Lo} patients following DCVAC therapy (Figure 4i). Overall, these findings indicate that DCVAC improved effector functions in the peripheral blood of EOC patients with a low frequency of regulatory T cells that was associated with a significant PFS benefit compared with patients with a high frequency of these cells.

Although these data need to be confirmed in a larger cohort of DCVAC-treated EOC patients, our findings indicate that DCVAC boosts clinically relevant cytotoxic T lymphocyte (CTL) responses, especially in EOC patients with a low frequency of circulating $FoxP3^+$ cells, which is the patient subset that obtained the greatest clinical benefit of DC-based immunotherapy in SOV01.

Discussion

Over the past decade, several immunotherapies have become available for the routine clinical management of cancer.[1,29] These include (but are not limited to) ICIs targeting cytotoxic T lymphocyte-associated protein 4 (CTLA4), or PD-1 or its ligand PD-L1 in distinct solid cancer malignancies, including

Figure 3. Low expression of genes associated with immunosuppression and T_{H2} signature is correlated with an improved response to DCVAC in EOC patients in SOV01. (a) Unsupervised hierarchical clustering of 93 EOC patients in SOV01 based on the expression of 93 genes classified into clusters related to B cells, cytotoxicity, DCs, immune populations, immunosuppression, NK cells, T cell activation, and T_{H1} and T_{H2} signatures. (b, c) PFS of 28 patients from the SOC arm (b) and 65 patients from the DCVAC arm (c) following stratification by unsupervised hierarchical clustering into low and high inflammatory clusters. (d, e) Direct comparison of PFS of SOC and DCVAC patients following stratification by unsupervised hierarchical clustering into low (d) and high inflammatory clusters (e). (f) PFS of 93 EOC patients upon stratification by the median expression of genes associated with B cell signature, *CD3E*, immunosuppression, and T_{H2} signature, and study arm. Survival curves were estimated using the Kaplan–Meier method, and differences between groups were evaluated using the log-rank test. The numbers of patients at risk and *p* values are reported.


a
ARG1
FOXP3
IL6
IL13
PDCD1
TGFB1
TIGIT
TNFA
mCRPC
EOC
NSCLC
b
ARG1
FOXP3
IL6
IL13
PDCD1
TGFB1
TIGIT
TNFA
Relative expression levels
mCRPC NSCLC EOC
c
Immunostimulatory gene signature
p=0.03
ns
p=0.04
Immunosupressive gene signature
p=0.0001
p=0.001
p=0.03
d
SP005
e
SLU01
f
SOV01
OS (%)
PFS (%)
Time (months)
SOC^Hi SOC^Lo DCVAC^Hi
SOC^Lo 0.719 – –
DCVAC^Hi 0.032 0.136 –
DCVAC^Lo 0.530 0.304 0.004
SOC^Hi SOC^Lo DCVAC^Hi
SOC^Lo 0.689 – –
DCVAC^Hi 0.045 0.028 –
DCVAC^Lo 0.938 0.714 0.016
SOC^Hi SOC^Lo DCVAC^Hi
SOC^Lo 0.868 – –
DCVAC^Hi 0.862 0.741 –
DCVAC^Lo 0.025 0.032 0.007
g
FoxP3^Lo
FoxP3^Hi
CD4 PE-Texas Red
CD3 Alexa700
FoxP3 FITC
CD25 PE
h
PFS (%)
Time (months)
SOC^Hi SOC^Lo DCVAC^Hi
SOC^Lo 0.401 - -
DCVAC^Hi 0.354 0.839 -
DCVAC^Lo 0.506 0.046 0.019
i
% of CD45^+CD8^+ T cells
p=0.01
ns
Prior Post
DCVAC-FoxP3^Lo
DCVAC-FoxP3^Hi
SOC-FoxP3^Lo
SOC-FoxP3^Hi

melanoma, NSCLC and urothelial carcinoma.[2,30,31] Only about 20% of patients with the most common solid tumors respond to ICIs as standalone therapies.[1,2,32] Moreover, some malignancies, particularly prostate and ovarian cancer are insensitive to ICIs as standalone immunotherapies or combined upfront with SOC.[33,34] Thus, strategies to induce anticancer immune responses in patients with limited responses to ICIs as well as biomarkers that improve the decision making with respect to the (immuno)therapeutic approach in solid malignancies are eagerly awaited.[7,35]

DCs are a diverse group of specialized antigen-presenting cells with key roles in the initiation and regulation of innate and adaptive immunity.[7,36] The use of DC vaccines for cancer has been extensively investigated, with more than 200 clinical trials completed to date.[37–39] Many strategies have been developed to target DCs in cancer, including *in situ* vaccination approaches, in which DC antigen uptake and immune recognition of tumors is promoted by immunomodulators, as well as the generation of DC-based vaccines.[7,40,41] The second approach largely depends on loading DCs with tumor antigens *in vitro* followed by administration of those DCs to patients, predominantly with melanoma, prostate cancer, glioblastoma, or renal carcinoma.[24,42–44] Various types of canonical DC-based cancer vaccines have been explored but with limited clinical benefit, with overall response rates of just 8–15%.[37] Thus, strategies to improve the development of anticancer immune responses, implementation of combinatorial immunotherapeutic strategies, and the identification of novel biomarkers for DC-based immunotherapy are needed.[7]

In line with this notion, we recently reported the results of four randomized clinical trials (SOV01, NCT02107937; SLU01, NCT02470468; SOV02, NCT02107950; SP005, NCT02111577) involving more than 1400 cancer patients demonstrating that DC-based immunotherapy DCVAC is well tolerated and significantly extends PFS and OS over SOC in EOC and NSCLC patients.[21,22,45] Despite the favorable safety profile, DCVAC combined with SOC and continued as maintenance treatment did not extend OS in mCRPC patients.[24] Here, using peripheral blood samples from 1000 patients enrolled these DCVAC studies, we have demonstrated that a circulating immune-related gene signature associated with adaptive immunity and T cell activation is associated with good prognosis and improved response to DC-based immunotherapy in mCRPC and NSCLC patients in SP005 and SLU01 (Figures 1 and 2). Although the same was not true for EOC patients in SOV01 (Figure 3), we unexpectedly found that DCVAC provided a significant benefit to the low inflammatory cluster of EOC patients. These unexpected findings might be explained by the fact that EOC, as compared with mCRPC and NSCLC, was associated with the lowest expression of the immunostimulatory-like gene signature. Conversely, the immunosuppressive-like gene signature associated with circulating soluble (*ARG1, IL6, IL13, TGFB1* and *TNFA*) and cellular markers (*FOXP3, PDCD1* and *TIGIT*) is over-represented in EOC patients compared with mCRPC and NSCLC patients, as shown by us and others (Figure 4).[11,46,47] Supporting this perspective, circulating regulatory T cells, in particular, were shown to abolish the potential of DCs and CTLs for mediating anticancer effects through various mechanisms that included but not were limited to immunosuppressive cytokines, adenosine signaling, CTLA-4-dependent downregulation of CD80 and CD86 expression by a process termed trans-endocytosis, LAG-3 engagement of MHC-II molecules, and direct cytolytic effects mediated by GZMB and PRF1 on CTLs and antigen presenting cells.[48–52] Supporting this notion, patients with a low inflammatory immune signature associated with low expression of the immunosuppressive regulatory T cells and T_{H2}-like gene signatures in peripheral blood were shown to be permissive for the effector functions of DCVAC-driven CTLs because systemic immunosuppression has not been established (Figure 4).

These findings demonstrate robust systematic and intratumoral immunosuppression, particularly in EOC, and call for the development of combinatorial treatment strategies.[8,53,54] Overcoming the immunosuppression is crucial for improving the response to immunotherapies, including DC-based immunotherapies. Accumulating preclinical and clinical evidence indicates that chemotherapy regimens and targeted anticancer agents used in the management of various malignancies, including EOC, can induce anticancer immunity by various mechanisms, including (1) selective depletion of immunosuppressive cells; (2) lymphodepletion associated with the renovation of the patient's immunological repertoire; and (3) activation of immune effector cells.[54,55] Therefore, chemotherapy and targeted anticancer agents appear to represent promising partners for combination with immunotherapies, and might improve the clinical benefit of DC-based therapies, particularly in combination with ICIs.[56–58] However, compared to ICIs where several phase III clinical studies are currently evaluated the synergy with SOC, no advanced studies have focused on their potential synergy with DC-based immunotherapies in EOC patients.[54]

Our study has various limitations. First, it was an explorative retrospective study focusing on 93 pre-selected genes related to the circulating immune responses to prior therapy, with no preplanned statistical analysis, which limits the statistical power. Second, post-treatment blood samples were not

Figure 4. High frequency of regulatory T cells in peripheral blood of EOC patients is associated with poor response to DCVAC therapy. (a) Heat map and (b) relative expression levels of the differentially expressed genes (DEGs) *ARG1, FOXP3, IL6, IL13, PDCD1, TGFB1, TIGIT* and *TNFA* in pre-treatment peripheral blood samples among mCRPC, NSCLC, and EOC patients in SP005, SLU01, and SOV01. (c) Relative expression levels of immunostimulatory (*CD8A, GNLY, GZMA, GZMB, IFNG, IL12A, PRF1, TBX21)* and immunosuppressive (*FOXP3, HAVCR2, IDO1, IL10, LAG3, PDCD1, TGFB1, TIGIT)* gene signatures in mCRPC, NSCLC and EOC patients in SP005, SLU01, and SOV01. (d, e) OS of 804 mCRPC (d) and 103 NSCLC (e) patients following stratification by the median expression of the immunostimulatory-like gene signature and study arm. (f) PFS of 93 EOC patients following stratification by the median expression of the immunosuppressive-like gene signature and study arm. Survival curves were estimated using the Kaplan–Meier method, and differences between groups were evaluated using the log-rank test. The numbers of patients at risk and *p* values are reported. (g) Representative dot plots for $CD4^+CD25^+FoxP3^+$ regulatory T cells in low and high EOC patients in SOV01. (h) PFS of EOC patients treated with SOC or DCVAC stratified by the median percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in peripheral blood. Survival curves were estimated using the Kaplan–Meier method, and differences between groups were evaluated using the log-rank test. (i) Percentage of $CD8^+$ T cells in peripheral blood of SOC $FoxP3^{Lo}$, SOC $FoxP3^{Hi}$, DCVAC $FoxP3^{Lo}$ and DCVAC $FoxP3^{Hi}$ patients prior and post DCVAC therapy. Statistical significance was calculated by the Wilcoxon test. *p* values are indicated.

analyzed in the study, which prevented us from investigating the alterations in the anti-tumor immune response elicited by DCVAC.

Because DC-based immunotherapies are promising candidates for management of immunoresistant solid cancers with minimal side effects, additional clinical trials are needed to address the potential value of the immune-related gene signature at baseline to identify biomarkers reflecting the disease origin and potential value of combinatorial approaches that respect the clinical management of individual cancers.[22,45] In particular, DC-based immunotherapies combined with ICIs appear to represent an promising strategy because the transferred DCs may encourage initial antigen-specific effector T cell activation, which is eventually curtailed by the coinhibitory activity that is controlled by ICIs.[8,59] Thus, clinical studies investigating this synergistic approach are urgently needed.

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Contributions

Concept and design: MH, RS, JF; development of methodology: MH, JR, LK, JP, acquisition of data: MH, JR, LK, TL, JP, PH, MH, TH, PK, KS, DR, LS, JD, JL, RH, GH, TB, MH, LR, AL, DC; analysis and interpretation of data: MH, JR, LK, TLS, JP, PH, MH, TH, PK, KS, DR, LS, JD, JL, RH, GH, TB, MH, LR, AL, AC, IV, AG, AL, DC; writing, review, and/or revision of the manuscript: MH, AC, IV, AG, DC, JB, RS, JF; study supervision: MH, JB, RS, JF.

Data availability statement

The data generated in this study are available upon request to the corresponding author.

Disclosure statement

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4.4 Immunological control of ovarian carcinoma by chemotherapy and targeted anticancer agents.

Epithelial ovarian carcinoma is poorly responsive to immunotherapy such as immune checkpoint inhibitors as compared to other solid carcinomas. Accumulating preclinical and clinical data shows that commonly employed chemotherapeutics and targeted anticancer therapies besides direct cytotoxic effect exert also clinically relevant immunostimulatory functions that can be harnessed to inflame tumor microenvironment resulting in at least partially restored sensitivity to ICIs. In this review article we discuss molecular and cellular mechanism underlying immunomodulatory ability of chemotherapies and targeted anticancer agents, emphasizing pathways, such as for examples pathways associated with ICD, that may render EOC particularly sensitive towards ICIs.

Author´s contribution to the review article:

- Data mining
- Help with manuscript preparation

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Review

Immunological control of ovarian carcinoma by chemotherapy and targeted anticancer agents

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At odds with other solid tumors, epithelial ovarian cancer (EOC) is poorly sensitive to immune checkpoint inhibitors (ICIs), largely reflecting active immunosuppression despite CD8$^+$ T cell infiltration at baseline. Accumulating evidence indicates that both conventional chemotherapeutics and targeted anticancer agents commonly used in the clinical management of EOC not only mediate a cytostatic and cytotoxic activity against malignant cells, but also drive therapeutically relevant immunostimulatory or immunosuppressive effects. Here, we discuss such an immunomodulatory activity, with a specific focus on molecular and cellular pathways that can be harnessed to develop superior combinatorial regimens for clinical EOC care.

Highlights

Epithelial ovarian cancer (EOC) is poorly sensitive to immune checkpoint inhibitor (ICI)-based immunotherapy, largely reflecting active immunosuppression despite CD8$^+$ T cell infiltration at baseline.

Various agents routinely used for clinical EOC management mediate therapeutically relevant immunostimulatory or immunosuppressive effects.

Preclinical data indicate that these therapeutics stand out as promising partners for ICI-based immunotherapy in models of EOC.

Clinical evidence in support of this paradigm remains scant, pointing to various, hitherto unresolved, challenges.

Introduction

EOC accounts for more than 95% of ovarian neoplasms, with high-grade serous ovarian carcinoma (HGSOC) being the most common disease subtype [1,2]. While advances in surgical and medical procedures have led to improvements in quality of life and life expectancy in patients with a variety of tumors, survival rates for subjects with EOC have only modestly improved [3]. This prognosis partly reflects the ability of EOC cells to spread via the peritoneum and colonize the omental fat deposits during early stages of the disease [4–6], ultimately establishing an immunosuppressed tumor microenvironment (TME) that supports progression and resistance to therapy [7].

The standard first-line therapy for patients with EOC comprises cytoreductive surgery coupled with chemotherapy, which is generally based on a platinum/taxane doublet [8,9]. While this approach enables complete remission in most patients, such a response is transient and adaptive chemoresistance to platinum derivatives usually emerges to drive disease relapse [10,11]. Homologous recombination (HR) defects imposed by germline or somatic BRCA1 DNA repair-associated (*BRCA1*) or *BRCA2* mutations [12] are key determinants of platinum sensitivity in patients with EOC [13], and provide a robust rationale for maintenance therapies based on poly(ADP-ribose) polymerase (PARP) inhibitors [14–17]. As such, maintenance therapy with PARP inhibitors have extended progression-free survival (PFS) in patients with advanced EOC that has initially responded to platinum irrespective of HR proficiency [18,19]. However, improved overall survival is only seen in individuals with *BRCA1/2* mutations [20]. Nonetheless, more than 50% of all patients with EOC succumb to the disease within 5 years of diagnosis [10], identifying a significant need for improved therapeutic approaches.

The enormous success of modern immunotherapeutic agents, notably ICIs, in the clinical management of various tumors, including melanoma and non-small cell lung carcinoma (NSCLC) [21], has generated considerable expectations around the use of these agents for the management of EOC [22]. However, ICIs are poorly active in individuals with EOC when both used as

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standalone therapeutic agents [23,24] or combined upfront with standard-of-care therapy [25–28]. Patients with melanoma and NSCLC who fail to respond to ICI-based immunotherapy bear so-called 'cold' tumors (i.e., neoplasms that are poorly infiltrated by immune effector cells) [29–31]. Cold tumors are also observed in estrogen receptor (ER)$^+$ breast cancer, which is insensitive to ICIs [32,33], and in EOC [34]. Thus, strategies aimed at converting cold EOCs into 'hot' lesions, which are abundantly infiltrated by immune effector cells, notably mature dendritic cells (DCs), T_H1-polarized helper T (T_H) CD4$^+$ cells, and CD8$^+$ cytotoxic T lymphocytes (CTLs), stand out as promising combinatorial partners for ICI-based immunotherapy in this context [35,36].

Conventional chemotherapeutics [37–39], radiation therapy (RT) [40–43], and targeted anticancer agents [44,45] can be harnessed to inflame the microenvironment of various solid tumors, correlating with partially restored sensitivity to ICIs. In this review, after summarizing key immunological features of the EOC microenvironment, we discuss the molecular and cellular mechanisms underlying the ability of chemotherapy and targeted anticancer agents commonly used in the management of patients with EOC to mediate immunostimulatory or immunosuppressive effects of therapeutic relevance. Specific emphasis is placed on pathways that may be actioned to render EOC sensitive to ICI-based immunotherapy [37,44,46]. Given that patients with EOC generally do not receive RT with curative intents, the immunostimulatory and immunosuppressive effects of RT are not discussed [40,42].

Principles of EOC immunosurveillance

EOC evolves in the context of a highly dynamic interaction network with a variety of non-malignant components of the TME, including endothelial cells, stromal cells, and immune cells [47–49], as well as inert components of the extracellular matrix [50,51]. Such interactions encompass metabolic competition for limited nutritional resources [52–55], bidirectional trophic signaling [56,57], and disease evolution in the context of failing immunosurveillance and accruing intratumoral heterogeneity [31,58].

Similar to other oncological settings, ovarian carcinogenesis is initially restrained by the host immune system in a highly efficient manner [59,60]. The elimination of newly formed malignant cells is largely ensured by CD8$^+$ CTLs and, in some settings, natural killer (NK) cells [61–65]. Over time, however, some malignant cells emerge with the ability to resist eradication by lymphoid cells, and ultimately acquire additional genetic and epigenetic defects that allow them to replicate unaffected by (if not with the support of) the host immune system to form clinically manifest neoplasms that rapidly progress to advanced/metastatic disease [66]. A range of mechanisms through which malignant cells ultimately evade immunosurveillance has been described, many of which are relevant for EOC (Figure 1). Of note, many of these pathways emerge from the same genetic and epigenetic alterations that promote cell-intrinsic aspects of oncogenesis, such as an elevated proliferation rate or an accrued resistance to regulated cell death (RCD) [66–68].

Common mechanisms adopted by EOC cells in support of immune evasion that enables rapid disease progression encompass the loss of MHC Class I molecules [69], rendering malignant cells largely invisible to CD8$^+$ CTLs [60], as well as the establishment of a TME that is rich in immunosuppressive cells, such as CD4$^+$CD25$^+$FOXP3$^+$ regulatory T (T_{REG}) cells [70,71], M2-like tumor-associated macrophages (TAMs) [72–74], interleukin 17A (IL17A)-producing γδ T cells [75], and myeloid-derived suppressor cells (MDSCs) [76,77] coupled to CD274 (also known as PD-L1) expression [76]. Such a microenvironmental configuration reflects the ability of EOC to secrete multiple immunosuppressive factors, such as transforming growth factor beta (TGFβ)

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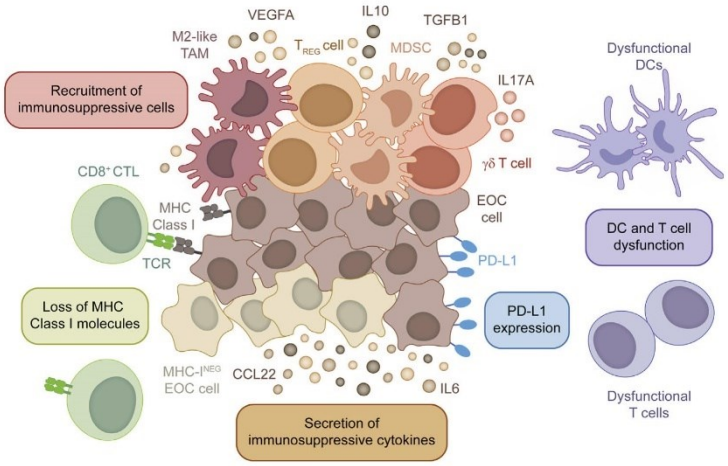

Figure 1. Mechanisms of epithelial ovarian cancer (EOC) immune evasion. EOC evades immune control to form 'cold', clinically manifest neoplasms by a variety of mechanisms, including: (i) loss of MHC Class I molecules and consequent evasion from $CD8^+$ cytotoxic T lymphocyte (CTL) recognition; (ii) PD-L1 upregulation and abundant secretion of immunosuppressive cytokines, resulting in dendritic cell (DC) as well as T cell dysfunction; (iii) recruitment of immunosuppressive cell populations including regulatory T (T_{REG}) cells, M2-like tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and γδ T cells. Abbreviations: IL, interleukin; MHC-I^{NEG}, MHC Class I negative; TGFB1, transforming growth factor beta 1; VEGFA, vascular endothelial growth factor A.

[78,79], IL6 [80], IL10 [80], C-C motif chemokine ligand 22 (CCL22) [70], and vascular endothelial growth factor A (VEGFA) [81]. These immunosuppressive features accumulate in advanced or metastatic EOC compared with their primary counterpart, resulting in profound DC [82] and T cell dysfunction [despite increased intratumoral heterogeneity coupled to the upregulation of various exhaustion markers, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4), programmed cell death 1 (PDCD1, also known as PD-1), and hepatitis A virus cellular receptor 2 (HAVCR2, also known as TIM-3)], which partly underlies the low sensitivity of EOC to ICI-based immunotherapy [83–86].

Corroborating the key impact of the immune contexture on treatment sensitivity and EOC outcome, numerous reports have correlated robust tumor infiltration by functional immune effector cells, including mature DCs, NK cells, and $CD8^+$ T cells, to improved prognosis [34,87–90]. Similarly, high intratumoral levels of immunosuppressive cells, including T_{REG} cells and M2-like TAMs, as well as molecular markers of immune dysfunction, have been consistently linked to poor disease outcome in a variety of cohorts of patients with EOC [34]. Moreover, multiple genetic signatures that have been associated with differential prognosis in patients with EOC encompass various genes involved in innate or adaptive immunity [91,92]. Taken together, these observations explain the scarce therapeutic activity of ICIs in patients with EOC, despite relatively abundant tumor infiltration by $CD8^+$ T cells. It also lends a strong rationale to the identification of strategies to inflame the EOC microenvironment in support of superior ICI sensitivity. Various chemotherapeutics and targeted anticancer agents commonly used for clinical EOC management can be harnessed.

Cytotoxic chemotherapy

Platinum derivatives

Both oxaliplatin and the new platinum-pyrophosphate conjugate PT-1112 can promote immunogenic cell death (ICD), a form of RCD that is sufficient to elicit adaptive immune responses against dead cell-associated antigens. However, carboplatin and cisplatin, both of which are used in patients with EOC, cannot promote ICD [93–95], but can mediate immunostimulatory effects. Carboplatin and cisplatin can suppress signal transducer and activator of transcription 6 (STAT6) signaling in malignant cells, resulting in PD-L1 and programmed cell death 1 ligand 2 (PDCD1LG2, also known as PD-L2) downregulation [96]. Moreover, cisplatin can promote the upregulation of MHC Class I molecules and mannose-6-phosphate receptor, cation-dependent (M6PR) on the surface of malignant cells, rendering them more visible to $CD8^+$ T cells and more susceptible to granzyme B (GZMB)-dependent lysis [97,98]. It can also induce the expression of co-stimulatory molecules, such as CD80 and CD86, on antigen-presenting cells (APCs) [99]. Similarly, carboplatin has been linked to increased MHC Class I expression and accrued tumor infiltration by $CD8^+$ CTLs, coupled with genetic signatures of $CD4^+$ T cell activation and T_{REG} cell depletion in cohorts of patients with EOC [100,101]. Accordingly, both cisplatin and carboplatin can synergize with ICIs targeting CTLA4, PD-1, or PD-L1 in immunocompetent mouse models of EOC, correlating with increased tumor infiltration by $CD8^+$ CTLs coupled with T_{REG} cell and granulocytic MDSC depletion [99,102–105]. Platinum-based chemotherapy has also been linked to accrued expression of PD-L1 on EOC cells and to the upregulation of PD-1 and CTLA4 on tumor-infiltrating lymphocytes [100,101,106], reflecting local NF-κB activation [100,107], and compensatory signaling downstream of interferon gamma (IFNγ) production by $CD8^+$ CTLs [108]. Moreover, carboplatin can promote tumor infiltration by M2-like macrophages and monocytic MDSCs coupled to upregulation of indoleamine 2,3-dioxygenase 1 (IDO1) and arginase 1 (ARG1), at least in preclinical EOC models [104,109]. Such detrimental effects also appear to originate from NF-κB activation and consequent IL6 secretion by EOC cells [104,110]. These effects are partially prevented by combining carboplatin with paclitaxel [104]. Finally, cisplatin can directly boost the ability of TAMs to promote EOC cell migration by favoring the secretion of CCL20 [111]. In summary, both cisplatin and carboplatin can mediate immunostimulatory or immunosuppressive effects of therapeutic relevance that can be used to inflame the EOC microenvironment.

Taxanes

By impairing microtubular function, paclitaxel and docetaxel often promote mitotic dysfunction leading to RCD coupled with polyploidization [112], which is accompanied by the exposure of the endoplasmic reticulum (ER) chaperone calreticulin (CALR) on the cell surface as a consequence of ER stress [113,114]. Surface-exposed CALR is a potent prophagocytic signal that enables the efficient uptake of dying and dead cells by APCs in support of adaptive immune priming [115]. As such, paclitaxel-based chemotherapy has been shown to increase gene signatures of MHC Class I antigen presentation, NF-κB signaling, and $CD8^+$ and $CD4^+$ T cell infiltration in post-treatment biopsies from patients with EOC, correlating with the ability of paclitaxel to promote NF-κB-dependent (rather than IFNγ-dependent) PD-L1 upregulation in human EOC cell lines [100]. Moreover, a circulating signature of NF-κB signaling correlated with poor disease outcome in two cohorts of patients with EOC and poor sensitivity to paclitaxel-based chemotherapy in preclinical EOC models [116]. Paclitaxel also promotes DC maturation by acting as a Toll-like receptor 4 (TLR4) agonist [117], and favors the maturation of MDSCs and the depletion of T_{REG} cells via TLR4-independent mechanisms [118–121]. Finally, paclitaxel resembles cisplatin in its ability to promote M6PR upregulation and consequently sensitizes cancer cells to GZMB-mediated lysis [97]. Accordingly, paclitaxel synergized with ICIs targeting PD-1 or PD-L1 [optionally in the context of chimeric antigen receptor (CAR) T cell therapy] in the control of mouse ovarian carcinoma ID8 cells established in immunocompetent syngeneic mice [100,105,122].

Paclitaxel also appears to recruit M2-like TAMs by enabling the secretion of CCL2 by EOC cells [123] while decreasing the abundance of intratumoral M1-like TAMs [104]. The latter effect appears to be fully antagonized by the co-administration of carboplatin [104], potentially contributing to the clinical effects of platinum/taxane doublets. Accordingly, post-treatment biopsies from patients with EOC receiving platinum/taxane-based neoadjuvant chemotherapy (NACT) reveal accrued tumor infiltration of multiple immune effector cell subsets, including $CD3^+$, $CD4^+$, and $CD8^+$ T cells, as well as $CD20^+$ B lymphocytes, but generally no decrease in the intratumoral abundance of T_{REG} cells and other immunosuppressive cells [106,124,125], at least in some settings in the context of PD-L1 and PD-1 upregulation [106,125]. Of note, both baseline and post-treatment stromal tumor-infiltrating lymphocyte (TIL) levels, as well as post-treatment $GZMB^+$ cell/T_{REG} cell ratios, correlated with improved disease outcome in these cohorts [106,124]. Along similar lines, circulating $CD8^+$ CTL responsiveness to viral peptides negatively correlated with circulating CA125 levels, which are commonly used to monitor tumor load and progression in patients with EOC [126]. Moreover, 2 weeks after treatment initiation, patients with EOC receiving platinum/taxane-based NACT exhibited increased circulating levels of T_H1-polarized $CD4^+$ cells, $CD45RO^+$ memory T cells, NKT cells, and $IFN\gamma^+CD8^+$ T cells, correlating with decreased abundance of soluble IL10, TGFβ, VEGFA and ARG1 [127,128]. While these changes can be temporary, patients with EOC undergoing remission after NACT appear to preserve an increase in circulating $CD8^+$ CTL functions compared with baseline [126]. Taken together, these observations document the ability of platinum/taxane-based NACT to successfully engage the host immune system against EOC cells, at least in some patients shortly, after treatment initiation. Recent data from patients with triple-negative breast cancer (TNBC) indicate that paclitaxel monotherapy depletes intratumoral $CXCL13^+$ T cells, which appear to be important, together with specific B cell and DC subsets, for these patients to respond to paclitaxel in combination with the PD-L1 blocker atezolizumab [129]. Whether the same holds true in patients with EOC and any potential influence from HR proficiency remain to be investigated.

Other chemotherapeutics

Pegylated liposomal doxorubicin (PLD), low-dose cyclophosphamide, and gemcitabine can also be used for the clinical management of EOC [130]. Similar to other anthracyclines, doxorubicin *per se* is a potent ICD inducer in various preclinical tumor models [131], including immunocompetent models of EOC [132,133], especially when EOC cells lack *BRCA1* [134]. As such, when paclitaxel and doxorubicin were administered sequentially as a therapeutic vaccine to murine ovarian surface epithelial cell (MOSEC)-bearing immunocompetent mice *in vitro*, paclitaxel-resistant MOSECs elicited superior levels of $CD4^+$ T cells and had improved survival-extension capacity compared with paclitaxel-treated MOSECs [135]. Moreover, bone marrow-derived DCs (BMDCs) exposed to MOSECs sequentially treated with paclitaxel and doxorubicin exhibited increased levels of co-stimulatory molecules and secreted abundant IL12 compared with BMDCs exposed to MOSECs treated with paclitaxel only, correlating with superior activation of MYD88 innate immune signal transduction adaptor (MYD88) [135]. In preclinical models of breast cancer, PLD promotes the upregulation of MHC Class I molecules, M6PR, as well as Fas cell surface death receptor (FAS) on the surface of EOC cells, rendering them more susceptible to recognition and lysis by CTLs [97,136], and to MDSC depletion [137]. Whether this latter observation holds true in EOC models remains to be assessed. Moreover, doxorubicin appears to deplete M1-like TAMs [104], which may ultimately limit its therapeutic benefits.

While high-dose cyclophosphamide can induce ICD [138], it is also highly immunosuppressive when delivered systemically, reflecting its elevated myelo- and lymphotoxic potential [139]. Metronomic schedules enable robust cyclophosphamide-driven immunostimulation in multiple preclinical models of nongynecological malignancies [37]. Specifically, metronomic cyclophosphamide

depletes T_{REG} cells in patients with solid tumors other than EOC [140], partially due to forkhead box P3 (FOXP3) downregulation [141], and correlates partly with T and NK cell functional recovery [140]. Indeed, cyclophosphamide synergized with TNF receptor superfamily member 18 (TNFRSF18, also known as GITR) agonists (which also deplete T_{REG} cells) in preclinical immunocompetent models of melanoma and plasmacytoma [142]. Finally, cyclophosphamide synergized with CTLA4 blockers, despite systemic MDSC accumulation in immunocompetent mouse models of colorectal and renal carcinoma [143]. Whether any of these effects also occur in EOC models remains to be tested. Moreover, no T_{REG} cell depletion could be documented in a Phase II clinical study combining metronomic cyclophosphamide with a tumor-targeting vaccine in patients with EOC [144]. Thus, the immunostimulatory effects of metronomic cyclophosphamide in the EOC setting remain largely unexplored.

Gemcitabine can cooperate with other agents to induce ICD in preclinical models of pancreatic carcinoma [145,146]. Moreover, gemcitabine appears to deplete circulating MDSCs while promoting tumor infiltration by mature DCs and $CD8^+$ CTLs in preclinical models of lung carcinoma and mesothelioma [147]. However, whether these effects are conserved in the EOC settings has not yet been investigated. Conversely, gemcitabine promoted the upregulation of MHC Class I and PD-L1 on cultured EOC cells via an NF-κB-dependent mechanism, correlating with increased infiltration of mouse EOC cells by $CD8^+$ CTLs [100], and decreased abundance of intratumoral T_{REG} cells [104]. However, gemcitabine also appears to deplete M1-like TAMs [104], which could limit its therapeutic activity. Along similar lines, combining carboplatin with gemcitabine in preclinical EOC models appears to compromise the immunostimulatory effects of the former and establish an immunosuppressive TME that is characterized by abundant T_{REG} cell infiltration coupled with systemically increased levels of CCL4 and IL6 [104]. These findings suggest that, while gemcitabine mediates some immunostimulatory effects *per se* and, hence, may represent a promising partner for ICI-based immunotherapy (as demonstrated in preclinical models of tumors other than EOC) [148,149], the same may not hold true for carboplatin and gemcitabine combinations.

Taken together, these observations exemplify the ability of various chemotherapeutics commonly used for clinical EOC management to mediate immunostimulatory or immunosuppressive effects that offer a rationale for the development of ICI-based combinatorial regimens (Table 1).

Targeted anticancer agents

PARP inhibitors

PARP inhibition mediates robust anticancer effects against HR-defective EOC [14,150], resulting in the approval of at least three distinct PARP inhibitors (i.e., niraparib, olaparib, and rucaparib) as maintenance monotherapies for patients with platinum-sensitive EOC with *BRCA1* or *BRCA2* mutations [18,19,151]. Given accumulating DNA damage [38], PARP inhibitors also promote multiple immunostimulatory effects. PARP inhibitors promote the accumulation of nuclear DNA species in the cytosol of EOC cells [152–154] and other cancer cell types [155], culminating in cyclic GMP-AMP synthase (CGAS) activation coupled with stimulator of interferon response cGAMP interactor 1 (STING1)-dependent type I IFN secretion, and consequent tumor infiltration by $CD4^+$ and $CD8^+$ T cells and IFNγ signaling [154,156]. As such, PARP inhibitors synergize with different ICIs in various immunocompetent models of EOC [152–154,157], and in patient-derived EOC spheroids bearing immunological competence [158]. Moreover, PARP inhibitors appear to drive the upregulation of FAS and TNF receptor superfamily member 10b (TNFRSF10B; also known as DR5) on the surface of EOC cells, possibly increasing their sensitivity to GZMB-mediated lysis [159]. That said, both olaparib and talazoparib (a previously experimental PARP inhibitor) promoted PD-L1 upregulation on the surface of human breast cancer cells [160],

Table 1. Immunomodulation by cytotoxic chemotherapeutics commonly used in clinical EOC management

Setting	Model	Immunomodulatory effect	Note(s)	Refs
Carboplatin				
Human; *in vitro*	36M2 cells	PD-L1 overexpression	Gene expression microarray data set (GSE13525)	[100]
	A2780 cells; CAOV-3 cells; COV413B cells; OVCAR-3 cells; SK-OV-3 cells	IL6 secretion; prostaglandin E2 (PGE_2) secretion	Supporting M2-like TAM expansion	[110]
Human; *in vivo*	MA-148 cells	CCL2 overexpression; TAM recruitment	In combination with paclitaxel	[123]
Human; *in vitro*	MA-148 cells; SK-OV-3 cells	CCL2 overexpression	Supporting TAM migration	[123]
	SK-OV-3 cells	M6PR exposure	Supporting sensitivity to CAR-T cell-dependent lysis	[122]
		PD-L1 overexpression	Accompanied by PD-1 overexpression on co-cultured CAR T cells	[122]
Mouse; *in vitro*	ID8 cells; OV2944-HM-1 cells	MHC class II overexpression; p65 overexpression; PD-L1 overexpression	Dose-dependent effect	[100]
Mouse (C57BL/6); *in vivo*	ID8 cells	$CD4^+$ and $CD8^+$ T cell recruitment; decreased T_{REG} cell infiltration; decreased MDSC infiltration	Similar effects when carboplatin was combined with PD-L1 blocker	[103]
		Increased circulating IFNγ levels; M2-like TAM repolarization; reduced M1-like/M2-like TAM ratio	Significantly altered in combination with gemcitabine or paclitaxel	[104]
		CCL4 secretion; T_{REG} cell recruitment	In combination with gemcitabine	[104]
		No significant changes in TME	In combination with paclitaxel	[104]
	ID8-Vegf-Defb29 cells	$ARG1^+IL10^+$ myeloid cell recruitment	In combination with paclitaxel	[109]
Human patients	EOC specimens	ARG1 downregulation; suppressed IL10, TGFβ, and VEGFA secretion	In combination with paclitaxel	[128]
		CD4 and CD8A upregulation	Gene expression microarray data set (GSE15622)	[100]
		Stromal TIL recruitment; PD-L1 overexpression	Most patients also received paclitaxel	[106]
		T_H1-polarized $CD4^+$ T cell recruitment; $CD45RO^+$ and $IFNγ^+$ $CD8^+$ CTL, and NKT cell recruitment	Most patients also received paclitaxel	[127]
		T_H1-associated gene upregulation; granzyme A (GZMA), perforin 1 (PRF1), and PD-L1 overexpression; $CD4^+$ T cell activation; suppressed IL6, IL8, and TNF secretion; IFNγ secretion; decreased T_{REG} cell infiltration	Most patients also received paclitaxel	[101]
		MHC class II overexpression; $CD3^+$, $CD8^+$, and $CD20^+$ B cell recruitment	Most patients also received paclitaxel	[125]
Cisplatin				
Human; *in vitro*	A2780 cells; CAOV-3 cells; COV413B cells; OVCAR-3 cells; SK-OV-3 cells	IL6 secretion; PGE_2 secretion	Supporting M2-like TAM expansion	[110]
	IGROV1 cells; OVCAR-3 cells; SK-OV-3 cells	PD-L1 overexpression	Dose-dependent effect	[106]
	OVCA420 cells; OVCA432 cells	PD-L1 overexpression	Dose-dependent effect	[98]
Mouse; *in vitro*	2F8 cells	MHC Class I overexpression; PD-L1 overexpression	Increased in context of cisplatin resistance	[98]

(continued on next page)

Table 1. (continued)

Setting	Model	Immunomodulatory effect	Note(s)	Refs
Mouse; (*Muc1*$^{+/-}$ 129S1); *in vivo*	2F8 cells	$CD8^+$ CTL recruitment	Increased in context of cisplatin resistance	[98]
Doxorubicin				
Human; *in vitro*	OV90 cells	ICD-associated ER chaperone exposure	Supporting DC maturation and cross-priming	[133]
	Primary EOC cells	ICD-associated ER chaperone exposure	Supporting DC maturation and cross-priming	[133]
Mouse (C57BL/6); *in vivo*	MOSECs	IL12 secretion; induction of MOSEC-specific $CD4^+$ T cells	Therapeutic vaccine based on paclitaxel-resistant MOSECs treated with doxorubicin	[135]
	CTX-resistant ID8 cells	ICD induction	Further increased upon combination with oncolytic virus	[132]
Gemcitabine				
Human; *in vitro*	Ovary1847 cells; OVCAR8 cells; RMGII cells; SK-OV-3 cells	MHC class I overexpression; p65 overexpression; PD-L1 overexpression	Mechanistically dependent on NF-κB signaling	[100]
Mouse; *in vitro*	ID8 cells; OV2944-HM-1 cells	MHC class I overexpression; p65 overexpression; PD-L1 overexpression	Mechanistically dependent on NF-κB signaling	[100]
Mouse (C57BL/6); *in vivo*	ID8 cells	$CD4^+$ and $CD8^+$ T cell recruitment; decreased MDSC infiltration; p65 overexpression; PD-L1 overexpression	No therapeutic synergy with PD-L1 blocker	[100]
		Decreased T_{REG} cell infiltration; decreased M1-like TAM infiltration; reduced M1-like:M2-like TAM ratio	Significantly altered in combination with carboplatin	[104]
		CCL4 secretion; decreased M1-like TAM infiltration; T_{REG} cell recruitment	In combination with carboplatin	[104]
Paclitaxel				
Human; *in vitro*	MA-148 cells; SK-OV-3 cells	CCL2 overexpression	Supporting TAM migration	[123]
	Ovary1847 cells; OVCAR8 cells; RMGII cells; SK-OV-3 cells	MHC class I overexpression; p65 overexpression; PD-L1 overexpression	Mechanistically dependent on NF-κB signaling	[100]
	SK-OV-3 cells	M6PR exposure	Leads to synergistic cytotoxic killing in context of CAR-T cell therapy	[122]
		PD-L1 overexpression	Supporting sensitivity to CAR-T cell-dependent lysis	[122]
Mouse; *in vitro*	ID8 cells; OV2944-HM-1 cells	MHC class I overexpression; p65 overexpression; PD-L1 overexpression	Mechanistically dependent on NF-κB signaling	[100]
Mouse (C57BL/6); *in vivo*	ID8 cells	$CD4^+$ and $CD8^+$ T cell recruitment; p65 overexpression; PD-L1 overexpression	Therapeutic synergy with PD-L1 blocker	[100]
		Decreased M1-like TAM infiltration	Negligible changes in circulating cytokines	[104]
		No significant changes in TME	In combination with carboplatin	[104]
	ID8-Vegf-Defb29 cells	$ARG1^+IL10^+$ myeloid cell recruitment	In combination with carboplatin	[109]
	MA-148 cells	CCL2 overexpression; TAM recruitment	In combination with carboplatin	[123]
Human patients	EOC specimens	ARG1 downregulation; suppressed IL10, TGFβ, and VEGFA secretion	In combination with carboplatin	[128]
		Stromal TIL recruitment; PD-L1 overexpression	Most patients also received carboplatin	[106]
		$CD4^+$ and $CD8^+$ T cell recruitment; $GZMB^+$ T cell recruitment	Most patients also received carboplatin	[124]

Table 1. (continued)

Setting	Model	Immunomodulatory effect	Note(s)	Refs
		T_H1-polarized $CD4^+$ T cell recruitment $CD45RO^+$ and $IFN\gamma^+$ $CD8^+$ CTL, and NKT cell recruitment	Most patients also received carboplatin	[127]
		MHC Class II overexpression; $CD3^+$, $CD8^+$, and $CD20^+$ B cell recruitment	Most patients also received carboplatin	[125]
		CD4 and CD8A upregulation	Gene expression microarray data set (GSE15622)	[100]
		T_H1-associated genes upregulation; GZMA, PRF1, and PD-L1 overexpression; $CD4^+$ T cell activation; suppressed IL6, IL8, and TNF secretion; IFNγ secretion; decreased T_{REG} cell infiltration	Most patients also received carboplatin	[101]
PLD				
Mouse; (FVB); *in vivo*	$Myc^{TG}Akt1^{TG}Trp53^{-/-}$; EOCs	$CD4^+$, $CD8^+$, and T_{REG} cell recruitment	T cell recruitment dominated by $CD4^+$ T cells	[134]
Mouse; *in vitro*		MHC class I overexpression; FAS overexpression	Increased effect in *Brca1*$^{-/-}$ cells	[134]
	ID8 cells	MHC class I overexpression; FAS overexpression	Increased effect in ID8 cells resisting PLD	[136]
Mouse (C57BL/6); *in vivo*	ID8 cells	Decreased T_{REG} cell infiltration; decreased M1-like TAM infiltration; reduced M1-like:M2-like TAM ratio	Not tested in combination with other agents	[104]

while AG14361 (a PARP inhibitor that has not yet been approved for use in humans) drove systemic T_{REG} cell expansion, thereby delaying acute failure in a model of allograft rejection [161]. Whether any of these immunosuppressive effects apply to the EOC setting remains unclear.

Bevacizumab

Bevacizumab, a humanized VEGFA-neutralizing antibody that mediates potent antiangiogenic effects [162], is commonly used in combination with platinum/taxane doublets as first-line adjuvant therapy for Stage III–IV EOC [163,164]. Similar to other targeted anticancer agents [44], bevacizumab also exerts multipronged immunostimulatory effects [165,166]. It depletes T_{REG} cells in multiple preclinical models of neoplasms other than EOC [167,168], and in post-treatment samples from patients with various tumors, including EOC [169,170]. Moreover, patients with EOC responding to cytotoxic chemotherapy plus bevacizumab exhibit reduced circulating T_{REG} cell and IL10 levels compared with nonresponding patients, coupled with a preservation of originally elevated effector T (T_{EFF}) cells [169]. Similarly, patients with tumors other than EOC receiving bevacizumab exhibit decreased circulating levels of MDSCs coupled with a modest increase in blood-borne mature DCs [171]. Finally, resistance to VEGFA-targeting agents has been associated with the expansion of MDSCs in preclinical EOC models [172], while treatment efficacy reportedly involves accrued T cell recruitment to the tumor bed as a consequence of FAS downregulation on endothelial cells [173], as well as the restoration of VEGFA-suppressed NKT cell responses [174]. Indeed, VEGFA blockers potently synergize with PARP inhibitors and ICI-based immunotherapy in preclinical models of *BRCA1*-deficient EOC [175]. Therefore, both conventional chemotherapeutics and targeted anticancer agents that are commonly used for clinical EOC management represent promising agents to inflame the EOC microenvironment and improve ICI sensitivity (Table 2).

Clinical strategies to restore ICI sensitivity in patients with EOC

Patients with melanoma and NSCLC responding to ICIs often bear neoplasms that are abundantly infiltrated by immune effector cells at baseline [30]. Since a considerable fraction of

Table 2. Immunomodulation by targeted anticancer agents commonly employed in clinical EOC management[a]

Setting	Model	Immunomodulatory effect(s)	Note(s)	Refs
Bevacizumab				
Human; *in vitro*	OVCAR-3 cells	IL2 secretion; restored NKT cell function and CD1d-mediated antigen presentation	Blockade of VEGFA-induced NKT cell suppression	[174]
Human patients	PBMCs	$CD4^+$ T cell recruitment; decreased levels of activated and resting T_{REG} cells; IL10 production	Attenuation of effect after four treatment cycles	[169]
	EOC specimens	Decreased $CD8^+$ T cell infiltration; GM-CSF and p65 overexpression; MDSC recruitment	In settings of clinical resistance to bevacizumab	[172]
Olaparib				
Human; *in vitro*	UWB1.289 cells	Type I IFN overexpression in DCs; increased phosphorylation of IRF3 and TBK1	*BRCA1*$^{-/-}$ EOC cell line co-cultured with human DCs	[152]
Mouse; (FVB); *in vivo*	PBM tumors	MHC class II overexpression; $CD103^+$ DC recruitment; *Ifnb1* and *Cxcl10* overexpression; type I IFN secretion; $CD4^+$ and $CD8^+$ T cell recruitment and activation; IFNγ and TNF secretion; reduced LAG3, PD-1 and TIM-3 expression; decreased MDSC infiltration; PD-L1 overexpression	Restricted to *Brca1*-deficient settings	[152]
Mouse; *in vitro*	ID8 cells	PD-L1 overexpression	Restricted to *Brca1*-deficient settings	[175]
	PBM cells	*Ifnb1* and *Cxcl10* overexpression by BMDCs; Type I IFN secretion; increased phosphorylation of IRF3 and TBK1	*Brca1*-deficient PBM cells co-cultured with murine BMDCs	[152]
Mouse (C57BL/6); *in vivo*	ID8 cells	*Ccl5*, *Gzmb*, and *Ifng* overexpression; $CD3^+$, $CD4^+$, and $CD8^+$ T cell recruitment	Dual ICIs plus VEGFA blocker in *Brca1*-defective tumors	[175]
Talazoparib				
Human; *in vitro*	HOC1 cells; UPN251 cells	*CCL5* and *CXCL10* overexpression; CXCL10 secretion; increased phosphorylation of IRF3 and TBK1	Marked activation of STING signaling pathway	[153]
Mouse (C57BL/6); *in vivo*	ID8 cells	CCL5 secretion; $CD8^+$ T cell recruitment; PD-L1 overexpression	Therapeutic synergy with PD-L1 blocker	[153]
Mouse (FVB); *in vivo*	BR5FVB1-Akt cells	B, NK, and T_{REG} cell recruitment; *Ccl2* and *Ccl5* overexpression; IFNγ and TNF secretion ; $CD8^+$ T cell recruitment; decreased MDSC infiltration	Immunostimulatory effects restricted to NK and T cells, not macrophages	[156]
VEGFA blocker				
Mouse (B6C3F1); *in vivo*	OV2944-HM-1 cells	Decreased $CD8^+$ T cell infiltration; GM-CSF secretion; MDSC recruitment; accrued NF-κB signaling	Immunosuppression linked to therapeutic resistance upon treatment-driven hypoxia	[172]
Mouse (C57BL/6); *in vivo*	ID8 cells	*Ccl5*, *Gzmb*, and *Ifng* overexpression; $CD3^+$, $CD4^+$, and $CD8^+$ T cell recruitment	Dual ICIs plus olaparib in *Brca1*-defective tumors	[175]
Veliparib				
Mouse (FVB); *in vivo*	BR5FVB1-Akt cells	$CD8^+$ T cell recruitment; IFNγ and TNF secretion	Restricted to combination of veliparib plus CTLA4 blocker in *Brca1*-defective tumors	[157]

[a]Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell.

EOCs also exhibits high TIL levels at baseline, one would expect such neoplasms to also respond to ICI-based immunotherapy. However, ICIs are poorly active in patients with EOC, with an overall response rate (ORR) of 8–9% and infrequent durable responses. Specifically, administration of the PD-1 blocker nivolumab to individuals with recurrent EOC was associated with 15% ORR and 10% durable responses in the first Phase II clinical trial testing this immunotherapeutic paradigm [176]. Similar results (ORR 9%) have also been documented with pembrolizumab (another PD-1 antagonist) in a cohort of more than 200 patients with recurrent EOC, correlating with frequent PD-L1 upregulation in the TME [24]. Thus, clinically viable strategies to inflame EOCs in support of restored ICI sensitivity are urgently needed.

The poor activity of ICIs used as standalone immunotherapeutics in patients with EOC partly reflects the dysfunctional state of T cells infiltrating the EOC microenvironment, which often express multiple co-inhibitory receptors, including PD-1, CTLA4, and TIM-3 [83–85]. In preclinical EOC models, single-agent ICI-based immunotherapy causes the compensatory upregulation of co-inhibitory receptors that are not directly targeted by the ICI [177]. While the validity of these latter findings has not been verified in post-treatment EOC samples, it is tempting to hypothesize that co-targeting nonredundant co-inhibitory pathways represents a valid strategy to expand the clinical benefit of ICIs in patients with EOC. As such, a Phase II trial testing nivolumab in combination with the CTLA4 blocker ipilimumab in patients with recurrent or persistent EOC revealed an ORR of 31.4% (compared with 12.2% for nivolumab alone) [23]. To the best of our knowledge, however, no Phase III clinical studies investigating this approach are active (source www.clinicaltrials.gov).

Combinatorial regimens involving cytotoxic chemotherapeutics and ICIs are emerging as an effective strategy for the management of multiple solid tumors, and considerable preclinical and clinical efforts are ongoing to expand the number of oncological indications benefitting from this approach [178]. Doxorubicin synergizes with ICIs in multiple preclinical models of neoplasms other than EOC [179,180]. Moreover, doxorubicin potently induces ICD coupled to the activation of tumor-targeting immune responses in several experimental settings, including immunocompetent EOC models [132,133], standing out as a promising partner for ICI-based immunotherapy in patients with EOC. Consistent with this possibility, PLD combined with pembrolizumab or the PD-L1 blocker durvalumab demonstrated good safety and a favorable therapeutic profile compared with standard-of-care chemotherapy in two Phase II clinical trials enrolling subjects with recurrent EOC [181,182]. However, data from a recent randomized Phase III clinical trial testing PLD plus avelumab (another PD-L1 antagonist) in this patient population failed to reveal any superiority over PLD alone [183]. Similarly, various PD-L1 antagonists failed to improve the therapeutic activity of platinum-based chemotherapy plus bevacizumab or a platinum/taxane doublet in recent Phase III clinical studies on patients with newly diagnosed stage III and IV EOC [27,28]. While various clinical studies testing chemotherapy plus ICIs in patients with EOC remain open, including several Phase III trials (Table 3), these findings suggest that chemotherapy is insufficient to restore ICI sensitivity in patients with EOC.

Early-phase clinical studies testing PARP inhibitors plus ICI-based immunotherapy in patients with recurrent EOC demonstrated good tolerability and at least some degree of clinical activity [26,184–186], spurring considerable interest in the initiation of randomized Phase III studies (Table 3) [187]. While the results of these trials will clarify the therapeutic potential of PARP inhibitors plus ICI-based immunotherapy, efforts are also being devoted to the identification of biomarkers that may predict the likelihood of patients with EOC to respond to this therapeutic paradigm. In this setting, it was recently reported that a mutational signature of HR deficiency and/or signs of an IFN-primed exhausted $CD8^+$ CTL response correlates with improved disease outcome in patients with EOC receiving niraparib and pembrolizumab [188]. Although additional studies are required to elucidate the actual predictive power of these signatures, identifying reliable predictive biomarkers remains of vital to prospectively identify patients who are likely to benefit from ICI-based immunotherapeutic regiments.

Data from a Phase II clinical study suggest that bevacizumab combined with nivolumab exerts promising clinical activity in subjects with EOC, especially in the platinum-sensitive population [189]. Similar results have been obtained in patients with EOC receiving olaparib, durvalumab, and cediranib (an anti-angiogenic drug) [186]. However, atezolizumab did not improve disease outcome in patients with stage III/IV EOC receiving platinum-based chemotherapy plus

Table 3. Active Phase III clinical trials testing strategies to restore ICI sensitivity in patients with EOC[a]

ICI(s)	Status	Notes	Clinicaltrials.gov ID
Bevacizumab			
Atezolizumab	Active, not recruiting	Patients also receive carboplatin and paclitaxel	NCT03038100
		Patients also receive carboplatin and investigator's choice of paclitaxel, gemcitabine, or PLD	NCT02891824
		Patients also receive PLD	NCT02839707
	Recruiting	Patients also receive paclitaxel or PLD	NCT03353831
Dostarlimab	Active, not recruiting	Patients also receive carboplatin, niraparib, and paclitaxel	NCT03602859
Durvalumab	Recruiting	Patients also receive carboplatin, olaparib, and paclitaxel	NCT03737643
Pembrolizumab	Not yet recruiting	Patients also receive paclitaxel (or docetaxel in case of hypersensitivity to paclitaxel)	NCT05116189
Carboplatin			
Atezolizumab	Active, not recruiting	Patients also receive bevacizumab and paclitaxel	NCT03038100
		Patients also receive bevacizumab and investigator's choice of paclitaxel, gemcitabine, or PLD	NCT02891824
	Recruiting	Patients also receive investigator's choice of niraparib and paclitaxel, gemcitabine, or PLD	NCT03598270
Avelumab	Active, not recruiting	Patients also receive paclitaxel and talazoparib	NCT03642132
Dostarlimab	Active, not recruiting	Patients also receive bevacizumab, niraparib, and paclitaxel	NCT03602859
Durvalumab	Recruiting	Patients also receive bevacizumab, olaparib, and paclitaxel	NCT03737643
Pembrolizumab	Active, not recruiting	Patients also receive olaparib and paclitaxel (or docetaxel in case of hypersensitivity to paclitaxel)	NCT03740165
	Recruiting	Patients also receive paclitaxel	NCT03914612
Gemcitabine			
Atezolizumab	Active, not recruiting	Patients also receive bevacizumab and carboplatin	NCT02891824
	Recruiting	Patients also receive carboplatin and niraparib	NCT03598270
Niraparib			
Atezolizumab	Recruiting	Patients also receive investigator's choice of carboplatin and paclitaxel, gemcitabine, or PLD	NCT03598270
Dostarlimab	Active, not recruiting	Patients also receive bevacizumab, carboplatin, and paclitaxel	NCT03602859
	Recruiting	No other therapeutic agents	NCT04679064
			NCT03651206
Olaparib			
Durvalumab	Recruiting	Patients also receive bevacizumab, carboplatin, and paclitaxel	NCT03737643
Pembrolizumab	Active, not recruiting	Patients also receive carboplatin and paclitaxel (or docetaxel in case of hypersensitivity to paclitaxel)	NCT03740165
Paclitaxel			
Atezolizumab	Active, not recruiting	Patients also receive bevacizumab and carboplatin	NCT03038100
			NCT02891824
	Recruiting	Patients also receive carboplatin and niraparib	NCT03598270
		Patients also receive bevacizumab	NCT03353831
Avelumab	Active, not recruiting	Patients also receive carboplatin and talazoparib	NCT03642132
Dostarlimab	Active, not recruiting	Patients also receive bevacizumab, carboplatin, and niraparib	NCT03602859
Durvalumab	Recruiting	Patients also receive bevacizumab, carboplatin, and olaparib	NCT03737643
Pembrolizumab	Active, not recruiting	Patients also receive carboplatin and olaparib (paclitaxel is replaced with docetaxel in case of hypersensitivity)	NCT03740165
	Not yet recruiting	Patients also receive bevacizumab (paclitaxel is replaced with docetaxel in case of hypersensitivity)	NCT05116189
	Recruiting	Patients also receive carboplatin	NCT03914612

Table 3. (continued)

ICI(s)	Status	Notes	Clinicaltrials.gov ID
PLD			
Atezolizumab	Active, not recruiting	Patients also receive bevacizumab and carboplatin	NCT02891824
		Patients also receive bevacizumab	NCT02839707
	Recruiting	Patients also receive carboplatin and niraparib	NCT03598270
		Patients also receive bevacizumab	NCT03353831
Avelumab	Active, not recruiting	No other therapeutic combination	NCT02580058
Rucaparib			
Nivolumab	Active, not recruiting	No other therapeutic combination	NCT03522246
Talazoparib			
Avelumab	Active, not recruiting	Patients also receive carboplatin and paclitaxel	NCT03642132
	Recruiting	No other therapeutic combination	NCT05059522

[a]Limited to recruitment status of 'not yet recruiting', 'recruiting', 'enrolling by invitation' and 'active, not recruiting', as of November 22, 2021.

bevacizumab [27]. Nonetheless, various other Phase III clinical trials investigating the therapeutic profile of bevacizumab combined with ICI-based immunotherapy in patients with recurrent EOC are ongoing (Table 3). The results of these studies are urgently awaited.

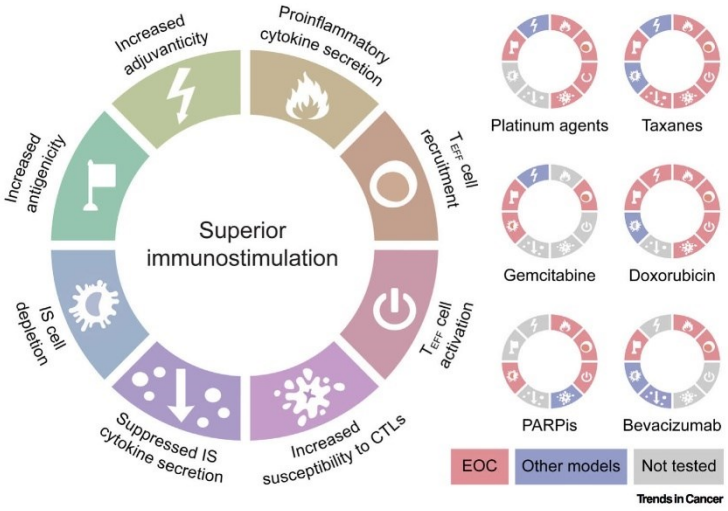

Figure 2. Immunostimulation by therapeutics commonly used for clinical epithelial ovarian cancer (EOC) management. A variety of agents routinely used in the clinical management of patients with EOC mediate robust immunostimulatory effects that may be harnessed to inflame the tumor microenvironment in support of restored immune checkpoint inhibitor (ICI) sensitivity. These effects encompass increased antigenicity or adjuvanticity, proinflammatory cytokine secretion, recruitment of effector T (T_{EFF}) cells and their activation, increased cancer cell susceptibility to lysis by cytotoxic T lymphocytes (CTLs), as well as reduced production of immunosuppressive (IS) cytokines and depletion of IS cells. However, while some of these activities have been documented in preclinical models of EOC, others have only been verified in models of other tumors, or not tested altogether yet. Abbreviation: PARPi, poly(ADP-ribose) polymerase inhibitor.

Concluding remarks

While ICIs have revolutionized the clinical management of multiple solid tumors [190], EOC remains largely resistant to ICI-based immunotherapy [191], a least in part reflecting robust immunosuppression at baseline [31,34]. Multiple cytotoxic chemotherapeutics and targeted anticancer agents routinely used for clinical EOC management stand out as promising agents to inflame such a cold TME and restore ICI sensitivity [192] (Figure 2). However, recent Phase III clinical trials investigating this combinatorial treatment paradigm in patients with recurrent EOC generally failed to demonstrate any superiority with respect to standard-of-care therapeutic approaches [27,28,183], pointing to the existence of several, hitherto unresolved challenges (see Outstanding questions). First, most combinatorial approaches involving clinically relevant approaches against EOC and ICIs have been developed harnessing mouse EOC cells grafted subcutaneously or intraperitoneally in immunocompetent syngeneic mice [193,194]. While this approach is advantageous because it provides a relatively homogenous model and an immunologically intact setting, unlike carcinogen-driven models, mouse EOC cell lines evade immune recognition and elimination in the original host from which they were isolated and expanded, thus failing to recapitulate natural oncogenesis in the context of failing immunosurveillance when reintroduced in a tumor-naïve recipient [65,193]. Second, insufficient emphasis is given to treatment schedule during preclinical development. Most combinatorial regimens are developed according to co-administration paradigms that may not necessarily be the most efficacious approach and/or implementable in the clinics [195,196]. Third, the immunosuppressive mechanisms in the EOC microenvironment may not be actionable with clinically approved ICIs, such as PD-1-, PD-L1-, and CTLA4-targeting agents [49,72,83,197]. Fourth, EOCs exhibit a considerable degree of intratumoral heterogeneity, encompassing both malignant and non-malignant components of the TME [198]. Therefore how the selective pressure imposed by common therapeutic agents shapes the EOC microenvironment with respect to immunotherapy sensitivity remains to be clarified [58]. Finally, while various immunological features of the TME at baseline have been attributed to robust predictive value for ICI sensitivity in other settings (e.g., melanoma and NSCLC) [199–201], the same does not hold true for EOC, as reflected in the limited ORR among unselected patients.

We surmise that: (i) the development of endogenous EOC models that fully recapitulate oncogenesis and disease progression in the context of failing immunosurveillance; (ii) the implementation of systematic preclinical studies comparatively assessing combinatorial treatments delivered according to various, clinically relevant, schedules; (iii) the in-depth characterization of the immunological EOC microenvironment (with a focus on spatial and temporal intratumoral heterogeneity) and the design of novel immunotherapeutic agents to action it; and (iv) the initiation of accurately designed clinical studies involving a diversified immune-monitoring program, will unlock the full therapeutic potential of modern ICI-based combinatorial regimens against EOC.

Outstanding questions

Can carcinogen-driven models of EOC be developed to recapitulate oncogenesis and tumor progression in the context of failing immunosurveillance?

What are the best administration schedules to combine conventional chemotherapeutic or targeted anticancer agents with ICI in the EOC setting?

What are the key mechanisms of immunosuppression in the EOC microenvironment?

How do conventional treatments shape the intratumoral heterogeneity of EOCs with respect to ICI sensitivity?

Can robust predictive biomarkers of responsiveness to ICI-based combinatorial regimens be identified?

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Author contributions

J.F. conceived the article and wrote the first version of the manuscript under the supervision of R.S. and L.G., with critical conceptual and writing input from all authors. J.F. and V.K. prepared display items under supervision of L.G. All authors approved the final version of the article.

Declaration of interests

J.F. and R.S. are full-time employees of Sotio Biotech. I.V. has received consulting/advisory honoraria from AstraZeneca, Clovis Oncology Inc., Carrick Therapeutics, Deciphera Pharmaceuticals, Elevar Therapeutics, Roche, Genmab, GSK, Immunogen, Jazzpharma, Mersana, Millennium Pharmaceuticals, MSD, Novocure, Octimet Oncology, Oncoinvent, Sotio, Verastem Oncology, and Zentalis; and has performed contracted research for Amgen, Genmab, Oncoinvent, and Genmab. E.G.M. has held research contracts with Roche, has received consulting/advisory honoraria from AstraZeneca and Clovis, as well as speaker honoraria from GSK, AstraZeneca, MSD, Clovis, and Roche. L.G. has held research contracts with Lytix Biopharma and Phosplatin, and has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, Sotio, The Longevity Labs, Inzen, and the Luke Heller TECPR2 Foundation. All other authors have no conflicts to declare.

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5 Discussion

The activation of anti-cancer immune responses is essential for efficient control of tumor cell growth. For this reason, cancer cells need to be perceived as a danger to the organism and elicit an immune response. The overall immunogenicity of neoplastic cells is mostly determined by a combination of their antigenicity and adjuvanticity, which is represented by the capacity to expose and release sufficient levels of DAMPs (Galluzzi et al., 2017). Although a living cancer cell is poorly immunogenic, tumor cells dying under specific stress conditions might release, secrete, or expose various DAMPs, thus rendering themselves immunogenic. To date, numerous studies have focused on ICD-mediated activation of effector adaptive immune responses and the associated clinical benefits. Although the impact of DAMPs and associated ICD-signaling on the development of adaptive anticancer immunity has been largely covered by the accumulated preclinical and clinical literature, little is known about the impact of DAMPs on activation of innate anticancer immunity. Thus, the first aim of this thesis was to further explore the impact of DAMPs and danger signaling on activation of innate anticancer immunity.

Inspired by our previous study documenting the impact of ecto-CALR expressed by malignant blasts of AML patients on increased frequency of antigen specific $CD4^+$ and $CD8^+$ T cells and increased frequency of circulating NK cells (Fucikova, Truxova, et al., 2016), we hypothesized that ecto-CALR might also impact the development of the innate immune response. Since NK cells have a vital role in cancer immunosurveillance (Meza Guzman, Keating, & Nicholson, 2020), we decided to extend our previous knowledge about the connection between ecto-CALR and NK cells in AML patients. Indeed, the NK cells from patients with high levels of ecto-CALR on their malignant cells exhibited superior secretory cytotoxic and cytolytic activity compared with those from patients with low ecto-CALR (Truxova et al., 2020). In line with a preclinical study performed by Chen et al (X. Chen et al., 2017), we demonstrated that the NK cell cytotoxicity was indirectly activated by DCs, which undergo maturation and activation upon exposure to ecto-CALR. In this process, DCs secrete pro-inflammatory cytokines, including type I IFNs, and express high levels of costimulatory molecules such as CD86, HLA-DR, and IL15Rα, which stimulate the NK cells. Although we did not observe a direct effect of soluble CALR on NK cell activity *in vitro*, it was recently reported that ecto-CALR on cancer cells is generally directly recognized by NK cells via the NKp46 receptor, which culminates in increased lysis of the

target cells (Sen Santara et al., 2023). Both of these mechanisms clearly demonstrate that ICD-induced ecto-CALR plays an important role in the development of innate and adaptive anticancer immunity (Guilbaud, Kroemer, & Galluzzi, 2023).

Type I IFNs play a crucial role as a first-line defense system against viral pathogens. In addition, accumulating evidence has demonstrated that the production and signaling of type I IFNs in the TME also contribute to cancer immunosurveillance and impact the response to therapy, including ICD inducers (Anguille et al., 2011). The second aim of my thesis was to determine the role of type I IFNs and danger signaling in AML patients. Type I IFNs are released from the malignant blasts of AML patients in a TLR3-dependent fashion that most likely originates from the RNA species of dying malignant cells (Bernard et al., 2012; Sistigu et al., 2014). This extends previous findings describing the important role of TLR3 signaling in type I IFN production and release in experimental mice models and breast cancer patients (Borden, 2019; Sistigu et al., 2014). Despite our expectations, we did not observe correlations between high levels of type I IFNs and activation of the adaptive and innate immune systems. In line with abundant literature documenting the potent immunosuppressive capacity of malignant blasts, we found that cancer cell-mediated immunosuppression blunts the immunostimulatory capacity of type I IFNs onto both $CD8^+$ T cells and NK cells, via several potential mechanisms, including IL-10 release, TNFSF18 signaling, and direct inhibition via CD200 (Baessler et al., 2010; Baessler et al., 2009; Coles et al., 2011). Therefore, even though type I IFNs have a crucial role in promoting immunosurveillance, we failed to observe this effect in AML patients.

In addition, preclinical and clinical data provide strong evidence that the concept of ICD confers great potential for improving the existing and developing new anti-cancer treatments. Also, the capacity of TME to successfully activate ICD signaling accompanied with the release of DAMPs proved to be a good predictor of prognosis and response to anticancer therapy in cancer patients (Y. J. Wang, Fletcher, Yu, & Zhang, 2018). In line with these premises, we observed that type I IFN secretion by malignant blasts not only exert direct cytotoxicity against malignant cells, but also potentiate standard of care treatment in AML patients. Using immunodeficient AML xenograft mice model we demonstrated the synergy between type I IFN and standard of care, consisting of ARA-C and DNR, which significantly improves OS in mice model (Holicek et al., 2023). These data are consistent with a previous report using another immunodeficient murine AML xenograft model, in which continuous delivery of type I IFN by an adenoviral vector improved OS (Benjamin

et al., 2007). Similarly, a high level of type I IFNs was independently associated with superior OS and RFS in our cohort of 132 AML patients. Although type I IFN was previously shown to be correlated with improved disease outcomes in glioblastoma and breast carcinoma (Sistigu et al., 2014; Snijders et al., 2014; Zhu et al., 2019), independent studies of breast and colorectal carcinoma patients revealed a negative prognostic impact of type I IFN signaling (Musella et al., 2022; Rodriguez-Ruiz et al., 2019; Weichselbaum et al., 2008). This clear discrepancy could be attributed to the differential effect of acute versus chronic type I IFN signaling and the overall immunological contexture of the TME (Vanpouille-Box, Demaria, Formenti, & Galluzzi, 2018). Taken together, our findings support the accumulating preclinical and clinical data providing strong evidence that danger signaling offers great potential for improving the existing and for developing new anticancer treatments.

ICD might be induced by different stressors, such as chemotherapy, targeted anticancer agents, and physical modalities. In line with this notion, some novel approaches to drive ICD have been harnessed for the development of therapeutic DC-based vaccines, which are currently under clinical evaluation. DCs-based immunotherapy is a promising approach to boost clinically relevant anticancer immune responses. Despite various improvements in recent years, the overall response rate to DCs-based immunotherapies is only about 7–15% (Sprooten et al., 2019), therefore, further development and identification of predictive biomarkers is urgently needed. DCVAC is a cellular-based immunotherapy consisting of autologous DCs, which are primed and loaded with allogeneic tumor cells killed by an ICD inducer—high hydrostatic pressure (HHP)—which was previously identified by our group. DCVAC has been tested in several phase II and III randomized, multicenter clinical trials (SOV01, NCT02107937; SLU01, NCT02470468; SOV02, NCT02107950; SP005, NCT02111577) for the treatment of prostate (mCRPC), lung (NSCLC) and ovarian (OC) cancer patients. In more than 1400 cancer patients, DCVAC was well tolerated and significantly prolonged PFS and OS versus SoC in OC and NSCLC patients, but it failed to extend OS in mCRPC patients (Cibula et al., 2021; Rob et al., 2022; Vogelzang et al., 2022; Zemanova et al., 2021). Analyzing the peripheral blood of 1000 patients randomized within the individual studies, we demonstrated that the circulating immune-related gene signatures associated with adaptive immunity and T cell activation are linked to the good prognosis and enhanced response rate to DCVAC in mCRPC and NSCLC patients. Interestingly, in OC patients, the clinical benefits of DCVAC were associated with rather low expression of a

T_H2-like signature and immunosuppressive regulatory T cells (Tregs). The association of such dramatically different gene signatures with better response to the same immunotherapy could be explained by the observations made by us and others, that OC patients have significantly higher baseline levels of immunosuppressive like gene signatures (e.g. ARG1, IL6, IL13, TGFB1, TNFA, FOXP3, PDCD1 and TIGIT) compared with mCRPC and NSCLC patients (Coosemans et al., 2016; De Bruyn et al., 2021; Sprooten et al., 2021). This elevated baseline immunosuppression might eventually inhibit the DCVAC-driven cytotoxic T cell responses, which might be associated with poor responses to DCVAC therapy.

All of these findings clearly demonstrate robust systematic and intratumoral immunosuppression in OC, and are urge us to develop combinatorial treatment strategies (Fucikova et al., 2022; Garg, Coulie, Van den Eynde, & Agostinis, 2017; Kandalaft, Odunsi, & Coukos, 2019). Overcoming immunosuppression is critical for improving the response to immunotherapies, including DC-based immunotherapies. The accumulating preclinical and clinical evidence indicates that chemotherapy regimens and targeted anticancer agents used for clinical management of OC patients, can induce anticancer immunity through various mechanisms. To this end, we also summarized the available preclinical and clinical data regarding the immunomodulatory ability of chemotherapies and targeted anticancer agents, emphasizing pathways that may render OC particularly sensitive towards ICIs, especially pathways related to ICD. We also surmise that, to successfully achieve the therapeutical potential of combinatorial regimes in EOC, it is necessary to develop/modify our overall approach in testing by: (i) using preclinical models that more adequately reflect actual oncogenesis and progression; (ii) focusing more on dosing because the responses vary greatly among schedules; (iii) characterizing the OC microenvironment, especially spatial and temporal intertumoral heterogeneity; and (iv) design and initiate clinical studies, which include a diversified immune-monitoring program.

Despite rapid advances, the field of ICD application still has several obstacles to overcome before it yields its full clinical potential. So far, the majority of clinically utilized ICD inducers were tested in immunodeficient models, meaning we have a little understanding of how they affect the host's immune system (Buque & Galluzzi, 2018; Zitvogel, Pitt, Daillere, Smyth, & Kroemer, 2016). We also still have rather limited understanding of immunosuppressive DAMPs, such as phosphatidylserine, prostaglandin E2, and adenosine, that are released by cells, especially in the context of ICD (Bondanza et al., 2004; Cekic & Linden, 2016; Garg et al., 2015; Hangai et al., 2016) and how other physiological aspects

that commonly affect immune responses, such as the gut microbiota, central nervous system, overall host metabolism or TCR repertoire, impact ICD (Galluzzi et al., 2020).

6 Conclusion

The aim of this thesis was to investigate the biological and clinical relevance of ICD signaling and associated molecules in cancer therapy. We successfully managed to address all the outlined aims and hypothesis, finding that:

1. ICD-signaling and the emission of DAMPs such as ecto-CALR and type I IFNs by malignant blasts associate with improved OS in AML patients. Ecto-CALR not only mediate activation of adaptive immune response as previously described by our group (Fucikova, Truxova, et al., 2016), but also indirectly increases total NK cell numbers and cytolytic properties in peripheral blood of AML patients (Truxova et al., 2020). In addition, we observed that prominent immunostimulatory properties of type I IFNs in peripheral blood of AML patients might be abolished by immunosuppressive mechanism driven by malignant blasts (Holicek et al., 2023).

2. Type I IFNs release, via a TLR3 dependent mechanism by malignant blasts exert direct cytotoxic, cytostatic and chemosensitizing activity in AML patients, leukemic stem cells from AML patients and AML xenograft models (Holicek et al., 2023). Thus, our findings document a beneficial impact of cancer cell-autologous type I IFN signaling on AML treatment sensitivity. In similar line, chemotherapy and targeted anti-cancer therapy driven immunogenicity might well synergize with immunotherapy of solid cancer malignancies as comprehensively reviewed by our group (Fucikova et al., 2022).

3. We identified prognostic and predictive biomarkers for response rate to ICD-based immunotherapy DCVAC. Gene signatures reflecting adaptive immunity and T cell activation were associated with survival benefits in responses to DCVAC in patients with mCRPC and NSCLC. By contrast, reduced expression of T_H2-like signature and immunosuppressive genes associated with regulatory T cells (T_{reg}) were associated with clinical benefits to DCVAC therapy in OC patients (Hensler et al., 2022).

7 Summary

Chemotherapy, radiation therapy, physical modalities, and targeted anticancer agents can induce clinically relevant anticancer immune responses, which rely on the antigenicity of neoplastic cells and their capacity to mediate adjuvant signals. ICD is accompanied by the exposure and release of various DAMPs, which are associated with adjuvanticity of dying cancer cells, thus triggering activation of both innate and adaptive immunity. Thus, ecto-CARL, secreted ATP, ANXA1, type I IFN, and HMGB1 might support the migration, activation, and cross-presentation of tumor antigens by DCs to activate adaptive anticancer immunity in cancer patients, as comprehensively documented by our research group and others. Besides the impact of ecto-CALR on adaptive cellular and humoral immunity, it also positively impacts the frequency and cytotoxicity of NK cells in AML patients. Moreover, our findings support previous observations that danger signaling, and expression of DAMPs might support the response to standard of care therapy and immunotherapy in cancer patients and confer a potential clinical benefit, a possibility that is currently being evaluated in multiple ongoing clinical studies. Finally, ICD inducers might be well implemented in manufacturing of cellular-based immunotherapies, including DC-based vaccines, antibody–drug conjugates, and other targeted therapies with promising clinical outcomes in cancer patients. Supporting this notion, further identification of reliable biomarkers, which either reflect the development of anticancer immunity or intratumoral danger signaling, might support the development of personalized anticancer regimens, and the identification of optimal therapeutic combinations for the clinical management of cancer.

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