Table S1. Cont.

Cells	Primer
CSF3R	
long16F (PCR)	5′-CCCTGTGATATGGAAAAACTGACC-3′
long17R (PCR)	5′-CTGAGAAGTTTCCCCCTGACTGC-3′
FW (sequence)	5′-CACTATCTCCGCTGTGACTC-3′
RV (sequence)	5′-TAGTCATGGGCTTATGGAC-3′
XBP-1 splicing assay (semiquantitative RT-PCR)	
XBP-1	
XBP1 FW	5′-AAACAGAGTAGCAGCTCAGACTGC-3′
XBP1 RV	5′-TCCTTCTGGGTAGACCTCTGGGAG-3′

FW, forward; RV, reverse.

Cell Death and Differentiation (2014) 21, 39–49

www.nature.com/cdd

Review

Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments

H Inoue[1,2,3] and K Tani*[,1,3]

Apoptotic cell death generally characterized by a morphologically homogenous entity has been considered to be essentially non-immunogenic. However, apoptotic cancer cell death, also known as type 1 programmed cell death (PCD), was recently found to be immunogenic after treatment with several chemotherapeutic agents and oncolytic viruses through the emission of various danger-associated molecular patterns (DAMPs). Extensive studies have revealed that two different types of immunogenic cell death (ICD) inducers, recently classified by their distinct actions in endoplasmic reticulum (ER) stress, can reinitiate immune responses suppressed by the tumor microenvironment. Indeed, recent clinical studies have shown that several immunotherapeutic modalities including therapeutic cancer vaccines and oncolytic viruses, but not conventional chemotherapies, culminate in beneficial outcomes, probably because of their different mechanisms of ICD induction. Furthermore, interests in PCD of cancer cells have shifted from its classical form to novel forms involving autophagic cell death (ACD), programmed necrotic cell death (necroptosis), and pyroptosis, some of which entail immunogenicity after anticancer treatments. In this review, we provide a brief outline of the well-characterized DAMPs such as calreticulin (CRT) exposure, high-mobility group protein B1 (HMGB1), and adenosine triphosphate (ATP) release, which are induced by the morphologically distinct types of cell death. In the latter part, our review focuses on how emerging oncolytic viruses induce different forms of cell death and the combinations of oncolytic virotherapies with further immunomodulation by cyclophosphamide and other immunotherapeutic modalities foster dendritic cell (DC)-mediated induction of antitumor immunity. Accordingly, it is increasingly important to fully understand how and which ICD inducers cause multimodal ICD, which should aid the design of reasonably multifaceted anticancer modalities to maximize ICD-triggered antitumor immunity and eliminate residual or metastasized tumors while sparing autoimmune diseases.
Cell Death and Differentiation (2014) **21**, 39–49; doi:10.1038/cdd.2013.84; published online 5 July 2013

Facts

- Accelerated progresses and discoveries in the field of oncology, immunology, and virology have made it possible to translate several emerging immunostimulatory strategies to treat malignant cancers towards promising clinical benefits.
- Profound understanding of the process of immunogenic cell death (ICD) induction by different ICD inducers such as certain chemotherapeutic agents and oncolytic viruses has highlighted the importance of immunological antitumor effects and proposed novel anticancer therapies.
- The execution of different types of programmed cell death (PCD), including apoptosis, autophagy, necroptosis, and pyroptosis, which are driven by a plethora of stimuli, was recently found to be regulated by orchestrated interactions among them, and importantly, some of these types of PCD exhibit an ICD property.
- Tumors and cancer cells treated with certain chemotherapeutic agents and oncolytic viruses can undergo ICD and release tumor-associated antigens (TAAs) accompanied by diverse danger-associated molecular patterns (DAMPs) and inflammatory cytokines to restore the tumor microenvironment and incite TAA-specific antitumor immunity.

Open Questions

- What are the recent advances in the development of anticancer immunotherapeutic modalities in clinical settings?

[1]Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; [2]Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and [3]Department of Advanced Molecular and Cell Therapy, Kyushu University Hospital,Kyushu University, Fukuoka, Japan
*Corresponding author: K Tani, Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 92 642 6449; Fax: +81 92 642 6444; E-mail: taniken@bioreg.kyushu-u.ac.jp
Keywords: ICD; DAMPs; apoptosis; necroptosis; immunotherapeutic anticancer agents; oncolytic virotherapy
Abbreviations: ICD, immunogenic cell death; PCD, programmed cell death; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ecto-CRT, calreticulin exposure; HMGB1, high-mobility group protein B1; ATP, adenosine triphosphate; PS, phosphatidylserine; Hsp, heat-shock protein; ACD, autophagic cell death; ER, endoplasmic reticulum; ROS, reactive oxygen species; APCs, antigen-presenting cells; DCs, dendritic cells; TAAs, tumor-associated antigens; GM-CSF, granulocyte–macrophage colony-stimulating factor; PDT, photodynamic therapy; CVB3, coxsackievirus B3; CSCs, cancer stem cells
Received 11.3.13; revised 06.5.13; accepted 14.5.13; Edited by M Piacentini; published online 05.7.13

- In response to diversified ICD inducers, how are DAMPs such as CRT, high-mobility group protein B1 (HMGB1), and ATP expressed by or released from the dying cancer cells?
- How do the diverse types of PCD differentially induce ICD to mount an efficient antitumor immunity?
- What are the prerequisites for an ideal ICD inducer to obtain an optimum level of ICD for long-lasting antitumor effects?
- It is vital to understand the molecular mechanisms of how ICD inducers, for example, infection with oncolytic viruses and resultant DAMPs, affect the host immune system. Can manipulation of ICD induction and/or combined strategies synergize with current or emerging oncolytic virotherapies?

The concept of immunogenic cell death (ICD) has recently been introduced to describe dying cancer cells that release endogenous danger molecules, the so-called damage-associated molecular patterns (DAMPs), after the exposure to certain cytotoxic agents to be recognized by antigen-presenting cells (APCs) such as dendritic cells (DCs) followed by formation of T-cell-mediated adaptive immunity.[1] Although it has long been considered that apoptotic cell death is tolerogenic, DAMPs have also been found to be released from cells undergoing apoptosis, providing a promising anticancer efficacy.[2–4] Therefore, comprehension of ICD induction gradually increases its significance, particularly in the field of cancer immunotherapy.

Overall prognosis of advanced cancer patients still remains dismal, thus making it imminent for oncologists to develop novel anticancer strategies. Recently, sipuleucel-T (Provenge, Dendreon, Seattle, WA, USA), indicated for patients with metastatic castration-resistant prostate cancer, received FDA's approval as the first therapeutic cancer vaccine.[5] In addition, extensive Phase II clinical trials have demonstrated that the oncolytic herpes simplex virus talimogene laherparepvec (T-Vec, Amgen Inc., Thousand Oaks, CA, USA)[6] and vaccinia virus JX-547 (Pexa-Vec, Jennerex Biotherapeutics, Inc., San Francisco, CA, USA),[7] both of which carry the gene encoding the immunostimulatory cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF), hold great promise for the treatment of advanced cancer patients. Furthermore, cytotoxic T-cell responses directed against oncolytic virus-infected cancer cells have been identified as an essential factor in the process of destruction of cancer.[8] Moreover, proinflammatory cytokines generated in the virus-infected cancer cells can restore the immunosuppressive tumor microenvironment.[9–11] Thus, oncolytic viruses are recently viewed as anticancer immunotherapeutic agents. These backgrounds make it imperative to update the molecular pathways and/or cellular constituents that regulate ICD.

Here, we review the progress of research on ICD, emphasizing how apoptotic, autophagic, and necroptotic cell death, called type 1, 2, and 3 PCD, respectively, are induced by various ICD inducers to achieve successful antitumor immunity. These multiple modes can be categorized by describing initiating events, intermediated changes, terminal cellular events, and their immunological responses, which are summarized in Table 1. In the later section, we outline the characteristics of anticancer agents and oncolytic viruses and how they induce diversified forms of cell death and interact with host's immune system.

Apoptotic Cell Death as ICD

From ten million to billions of cells die per day as a consequence of normal tissue turnover,[12] which are vital for organisms to retain homeostasis.[13,14] Therefore, the existence of multiple modes of cell death in nature is not surprising. Apoptosis, type 1 PCD, is a specialized form of cell death, characterized by typical morphological changes, including chromatin condensation, nuclear fragmentation, and membrane blebbing (Table 1).[15] Apoptosis occurs ubiquitously in normal tissues and causes 'quiet' cell death that uses phosphatidylserine (PS) as an 'eat-me' signal to be quickly recognized by peripheral APCs. Although apoptotic cell death has been historically considered to be non-immunogenic,[16] recent studies unraveled that several anti-neoplastic agents, including doxorubicin,[1,17] oxaliplatin,[18,19] cisplatin,[20] and irradiation,[21,22,23] can trigger immunogenic apoptosis.[2] Mechanistically, the immunogenic apoptotic bodies induced by exposure to doxorubicin are sensed by APCs through their TLR-2/TLR-9-MyD88 signaling pathways.[17]

DAMPs: as Effectors in ICD

The primary conceptual theory of the pattern recognition of pathogen-associated molecular patterns (PAMPs), such as viral or bacterial components, has failed to fully explain the consequence of immunogenicity. Thus, the secondary concept of DAMPs has been proposed, which could provoke an immune response.[24] Released DAMPs as hallmarks of ICD consisted of adenosine triphosphate (ATP), high-mobility group protein B1 (HMBG1), and exposed molecules on the outer membrane of dying cells such as CRT (ecto-CRT), heat-shock proteins (Hsp90 and Hsp70), and endoplasmic reticulum (ER) sessile proteins.[25,26,27] The excretion of DAMPs was considered to occur during necrosis under inflammatory and/or pathological conditions. However, DAMPs have recently been reported to be produced from apoptotic cancer cells treated with chemotherapy[1,18] or radiotherapy.[21]

ICD Inducers

ICD inducers include multiple anticancer therapeutic modalities. It has been recently proposed that they can be classified into two categories, type I or II ICD inducers, based on their distinct actions to induce ER stress leading to apoptotic cell death (Tables 2 and 3).[27,28] The majority of ICD inducers such as chemotherapeutic agents (mitoxantrone,[29] anthracyclines,[2,30,31] oxaliplatin,[18,19] and cyclophosphamide[32]), shikonin,[33,34] the proteasome inhibitor bortezomib,[35] and 7A7 (an epidermal growth factor receptor-specific antibody),[36] cardiac glycosides,[37] and the histone deacetylase inhibitor (vorinostat)[38] are categorized as type I ICD inducers that primarily target cytosolic proteins, plasma membranes, or nucleic proteins. They also induce ER stress via collateral effects. Bortezomib, cardiac glycosides,

Table 1 Comparison of multiple forms of programmed cell death and necrosis

	Apoptosis (type 1 PCD)	Autophagic cell death (type 2 PCD)	Necroptosis (type 3 PCD)	Pyroptosis	Necrosis
Mode of cell death	Programmed	Programmed	Programmed	Programmed	Accidental
Initiators	TNF-α, FasL, or TRAIL, infectious pathogens	Nutrient deprivation, HDAC inhibitors, hypoxia, infectious pathogens	TNF-α, FasL, or TRAIL, microbial infections Ischemic injury	DAMPs, microbial infections	Toxins, infections, inflammation, trauma
Intermediate signalings	Mitochondrial pathway Caspase-3, -6, -7-dependent	Caspase-independent autophagosome formation Lysosomal protease	TNF receptor signaling JNK activation Caspase-independent RIP1/RIP3 necrosome	Nod-like receptors Caspase 1-dependent pyroptosome Inflammasome	-
Terminal cellular events	Non-lytic cell shrinkage DNA fragmentation apoptotic bodies	Non-lytic autophagic bodies	Non-lytic, loss of plasma membrane, swollen cellular organelles	Lytic, rapid loss of plasma membrane, cell swelling, pore formation	Lytic, plasma membrane rupture, leak of content
Inflammation Immunogenicity	Non-inflammatory +	Non-inflammatory +	Proinflammatory + +	Proinflammatory + +	Proinflammatory + + +
DAMPs released	Ecto-CRT HMGB1 and ATP release	HMGB1 and ATP release	Long genomic DNA IL-6	HMGB1 and ATP release IL-1α, IL-1β, IL-6, IL-18, and TNF-α chemokines	HMGB1 and ATP release IL-1α, IL-33 mRNA, and genomic DNA
Eat-me signals	Ecto-CRT	LPC secretion PS exposure	LPC secretion PS exposure	PS exposure	PS exposure

Abbreviations: TNF-α, tumor necrosis factor- α; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; HDAC, histone deacetylase; IL-1α, interleukin-1α ; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-18, interleukin-18; IL-33, interleukin-33; ICD, immunogenic cell death; LPC, lysophosphatidylcholine; PS, phosphatidylserine; JNK, c-Jun N-terminal kinase.
The table gives a schematic overview of the multiple forms of cell death incuding apoptotic cell death (type 1 PCD), autophagic cell death (type 2 PCD), cell death induced by necroptosis (type 3 PCD), pyroptosis and necrosis. The extent of immunogenicity in each cell death subsection is scored as +, + +, and + + +, according to the expression levels of 'eat-me' signals and DAMPs emission. ICD in cancer can display different 'eat-me' signals, including ecto-CRT and LPC, on the cell membrane, as well as emission of DAMPs, ATP, and HMGB1. This peculiar ecto-CRT, which facilitates engulfment of TAAs from cancer cells by DCs, can only be found on cells that succumb to immunogenic apoptosis, whereas it is not present on cells dying in an immunologically silent manner. LPC secretion, PS exposure, and ATP release require autophagy induction. Numerous exquisite expression patterns shaped by the constituents of DAMPs and the interactive status of the immune system will predominantly determine the fate of subsequent immune responses, namely, immune tolerance or antitumor immunity

and shikonin effectively impede protumorigenic cytokine signaling.[27] Shikonin has been found to induce type 1 or 3 PCD, which is determined by caspase-8 activation as the 'decision-making switch'.[39] On the other hand, type II ICD inducers, which preferentially target the ER, include hypericin-based photodynamic therapy (PDT)[40,41,42] and oncolytic coxsackievirus B3 (CVB3).[9] Hypericin-based PDT is an anticancer therapy that utilizes hypericin to induce reactive oxygen species (ROS) in the vicinity of the ER.[43] Cancer cell infection with oncolytic viruses produce large amounts of viral proteins, which inevitably cause ER stress and ROS production to promote viral replication.[44,45] The quality and/or quantity of ER stress linking ROS triggered by ICD inducers may determine the ICD properties. Indeed, the previous finding that rigorous ROS-mediated ER stress augmented the release of DAMPs revealed an unrecognized role of RNA-dependent protein kinase (PKR)-like ER kinase (PERK) as a constituent of mitochondria-associated ER membranes to exert ROS-mediated mitochondrial apoptosis.[40,41,46] These observations indicate the superiority of type II ICD inducers with respect to immunological antitumor efficacies. However, further investigations to elucidate the precise interconnection between the ER stress and ROS production will be required to optimize antitumor immune responses.

Calreticulin Exposure

In response to specific chemotherapeutic agents, oncolytic viruses, and vorinostat, ecto-CRT has been found only on cells succumbing to immunogenic apoptosis.[2,9,38] This 'eat-me' signal promotes phagocytosis by DCs, thereby facilitating their tumor antigen presentation and incitement of TAA-specific cytotoxic T cells.[2,47] It has been shown that blockade of CRT inhibits phagocytosis of anthracycline-treated tumor cells by DCs and impairs their immunogenicity in mice.[2,47] In general, CRT exposure during ICD is an earlier process occurring within a few hours than PS externalization.[48] The ecto-CRT induction capacity of ICD inducers has been shown to depend on the properties of ER stress and ROS production.[2,37,49] Cancer cells can induce ecto-CRT followed by disturbance of the ER structure with GADD34 activation and PERK phosphorylation. It has been shown that depletion of PERK abolishes anthracycline-driven ecto-CRT and immunogenicity of cellular death (ER stress module),[19] and that caspase-8 acts upstream of apoptotic proteins Bax and Bak, and subsequent cleavage of its substrate Bap31 (apoptotic module) is indispensable for ecto-CRT induction.[19] Furthermore, a direct interaction between ecto-CRT and ERp57 was shown to be required for their cotranslocation to the cell surface (Figure 1).[29] Unlike the release of HMGB1 and ATP,

Table 2 Classification of type I ICD inducers determined by their major targets to provoke antitumor responses

Anticancer agents	Type of cell death induced	DAMPs	Major targets by ICD inducers	Preclinical observations for inciting antitumor immunity
Cytotoxic agents (mitoxantrone, oxaliplatin, anthracyclines)	Apoptosis, autophagic cell death, necroptosis	Ecto-CRT, ERp57, HMGB1, and ATP release	Nucleus (DNA or DNA-related proteins for cell mitosis)	*In vivo* antitumor effect is mitigated by depletion of $CD8^-$ T cells. Immunogenicity requires ecto-CRT in prophylactic tumor vaccination mouse models.
Cyclophosphamide (CTX)	Apoptosis	Ecto-CRT, HMGB1 release	Nucleus (DNA)	Metronomic doses of CTX deplete Treg from bed and tumors, CTX modulates DCs to produce IL-12
Shikonin	Apoptosis, necroptosis	Ecto-CRT, ecto-Hsp70	Cytosol (pyruvate kinase-M2 protein)	DCs incubated with shikonin increase Th1 cells but decrease Treg cells
Bortezomib	Apoptosis, autophagic cell death	Ecto-Hsp90	Cytosol (26S proteasome)	Cytotoxicity of NK cells against bortezomib-treated cells increased
7A7 (EGFR-specific antibody)	Apoptosis	Ecto-CRT, ERp57, ecto-Hsp70, ectp-Hsp90	Cell surface receptor (EGFR)	Contribution of $CD4^+$ T and $CD8^+$ T to 7A7-triggered suppression of metastasis in mice model
Cardiac glycosides	Apoptosis	Ecto-CRT HMGB1 and ATP release	Cell surface (Na^+/K^+-ATPase, enzyme)	Prophylactic antitumor immunity is partially dependent on $CD8^+$ T cells accompanied with Th17 cells
UVC irradiation	Apoptosis, necroptosis, necrosis	Ecto-CRT and ERp57, HMGB1 and ATP release	Nucleus (DNA)	UVC-treated cells increase susceptibility to attack by NK cells and total splenocytes
Vorinostat (HDAC inhibitor)	Apoptosis Autophagic cell death	Ecto-CRT	Nucleus (chromatin structure)	Promote the differentiation of $CD8^+$ T cells to memory cells

Abbreviations: Ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1; high-mobility group protein B1; Hsp, heat-shock protein; Treg, regulatory T cells; DCs, dendritic cells; IL-12, interleukin-12; NK, natural killer; EGFR, epidermal growth factor receptor; ATP, adenosine triphosphate; UVC, ultraviolet C

Table 3 Classification of type II ICD inducers determined by their major targets to provoke antitumor responses

Anticancer agents	Type of cell death induced	DAMPs	Major targets by ICD inducers	Preclinical observations for inciting antitumor immunity
PDT with hypericin	Apoptosis, autophagic cell death dependent on Bax/Bak, necroptosis	Ecto-CRT, ecto-Hsp70, ectp-Hsp90, HMGB1, and ATP release	ER (ROS generation)	PDT -hypericin therapy provokes antitumor immunity in both prophylactic and therapeutic murine tumor models. Same therapy-treated tumor cells result in phenotypic maturation of DCs and robust $CD4^+$T and $CD8^+$T cell expansion
CVB3	Apoptosis	Ecto-CRT, HMGB1 translocation, ATP release	ER (ROS generation)	Intratumoral CVB3 administration markedly recruited NK cells and granulocytes, both of which contribute to the antitumor effects as shown by depletion assays, macrophages, and mature DCs into tumor tissues
Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid, an hTERT promoter and human CD40L	Apoptosis	Ecto-CRT, HMGB1 release, ATP release	ER (ROS generation)	In two syngeneic mouse models, murine CD40L induced activation of APCs, leading to increased IL-12 production in splenocytes, associated with induction of the Th1 cytokines IFN-γ, RANTES, and TNF-α. Tumors treated with Ad5/3-CMV-mCD40L displayed an enhanced presence of macrophages and cytotoxic $CD8^-$ T cells
Edmonston strain MV	Apoptosis	IL-6 production, HMGB1 release	ER (ROS generation)	Coculture of MV-infected melanoma cells with human DCs led to both CD80 and CD86 upregulation on them. $CD8^+$ T cells cocultured with tumor cell-loaded and MV-infected DCs degranulated CD107a to target tumor cells with functional killing activity

Abbreviations: PDT, photodynamic therapy; CVB3, coxasackievirus B3; MV, measles virus; ROS, reactive oxygen species; ER, endoplasmic reticulum; hTERT, telomerase reverse transcriptase; hCD40L, human CD40 ligand; Th1, T helper type 1; RANTES, regulated and normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6

ecto-CRT could be one of the determinants that distinguishes between immunogenic and non-immunogenic cell death.[47]

HMGB1

HMGB1, one of the DAMPs, is a DNA-binding protein originally known as a nuclear non-histone chromatin-binding protein.[50] Although extracellular HMGB1 had been deemed to be released mainly from the nucleus during necrosis,[42] it was found to be excreted from cells undergoing late stage of apoptosis and autophagy.[30,51] HMGB1 inhibition in cancers undergoing immunogenic apoptosis impaired their ability to incite antitumor immunity in a prophylactic vaccination model.[30] HMGB1 initiates potent inflammation by stimulating the production of proinflammatory cytokines[52] from APCs via its binding to different surface receptors including receptor for advanced glycation end-products (RAGE), TLR2, TLR4, TLR9, and TIM3 (Figure 1).[53,54] Importantly, the binding of HMGB1 to TLR4 on APCs was required to suppress tumor development, which is consistent with clinical study showing that breast cancer patients harboring a single-nucleotide polymorphism (Asp299Gly) in the *TLR4* gene undergo an early relapse after anthracycline treatment.[30,55,56] In contrast, secreted HMGB1 could induce a protumor inflammation to facilitate tumor progression.[57] In addition, HMGB1 expression is significantly associated with overall survival of patients with bladder cancer.[58] As HMGB1 is an intrinsic sensor of oxidative stress,[59] the immunomodulatory properties of HMGB1 might be determined by its redox status.[60,61] Indeed, reduced HMGB1 production from dying cells was shown to trigger the immunogenic DCs, whereas oxidized HMGB1 during apoptosis fails.[51] As the extracellular space is usually oxidative under physiological conditions but is unpredictably variable under pathogenic conditions,[62] the unstable redox status of the tumor microenvironment might account for these inconsistent findings. However, the observation that the tumor microenvironment tends to be pro-oxidative[63] implies that a therapeutic approach using antioxidants to decrease ROS production would be favorable to stimulate antitumor immunity. Importantly, many anticancer agents, including chemotherapy,[30] radiation,[22] or oncolytic viruses,[9,64,65] have been shown to induce HMGB1 release from cancer cells, highlighting the significance of further addressing the mechanism of how these modalities affect the redox status of HMGB1.

Adenosine Triphosphate

Extracellular ATP released from apoptotic cells is another important factor in ICD induction. ATP signaling recognized by P2Y2 receptors on phagocytes as a 'find-me' signal enables

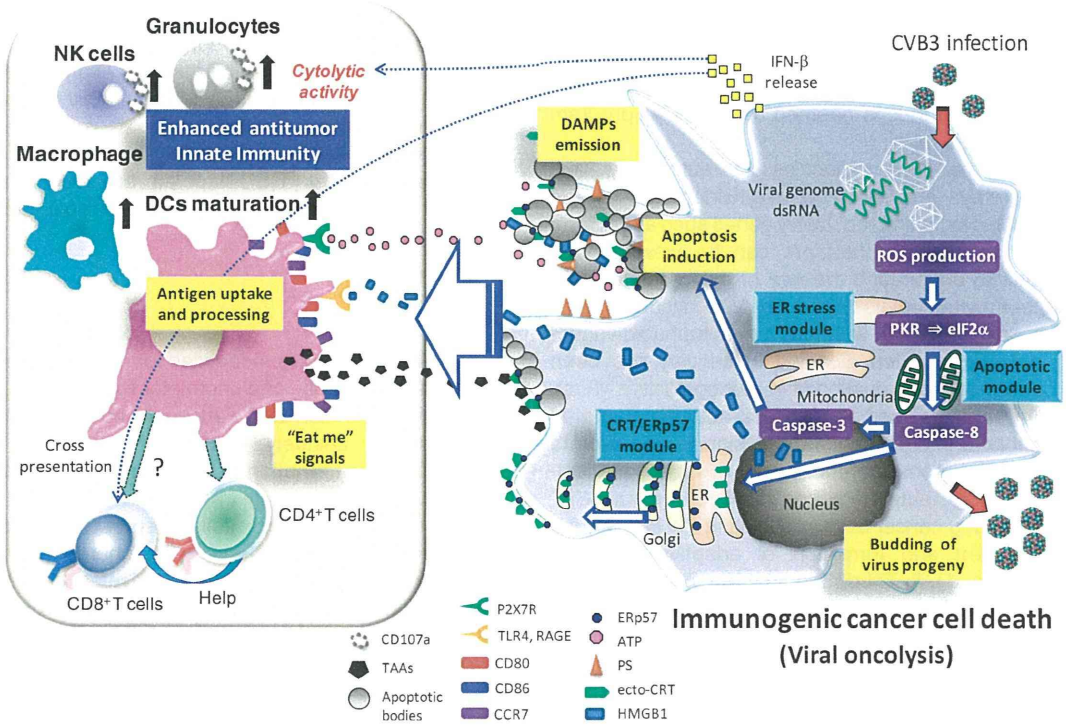

Figure 1 Oncolytic virus (CVB3) infection-triggered cancer cell death induces innate immune cell-mediated antitumor immunity. Intratumoral CVB3 infection-activated natural killer (NK) cells and granulocytes with enhanced expression of CD107a, a cytolytic degranulation marker, have been found to contribute to substantial antitumor effects as evidenced by NK cell and granulocyte depletion assays. Upon CVB3 infection, tumor cells can partially induce ecto-CRT on human tumor cells during early apoptosis, whereas majority of other viruses subvert ICD by circumventing ecto-CRT induction, and followed by robust release of DAMPs, including ATP and HMGB1, during later stages of cell death, which facilitates maturation of DCs via binding to Toll-like receptor 4 (TLR4)/RAGE and $P_2 \times _7R$, respectively. Viral genomes and/or viral progenies also stimulate DCs for their activation. Mature DCs may then efficiently phagocytose TAAs simultaneously released from dying cells and ultimately cross-present them to $CD8^-$ T cells with the support with $CD4^-$ T cells to elicit substantial antitumor immunity. Although ATP secretion relies on autophagic machinery, the other forms of cancer cell death, such as autophagic cell death and necroptosis, triggered by CVB3 infection have not yet been fully investigated

them to migrate into inflamed sites.[66] Indeed, ATP released from cancer cells treated with chemotherapeutic agents is essential for effective antitumor immune responses.[67] In addition, small interfering RNA-mediated inhibition of autophagic machinery abolishes ATP release from chemotherapy-treated tumor cells and mitigates the antitumor response.[68] Radiotherapy triggers ATP release from dying tumor cells through its interaction with the P2 × 7 purinergic receptor,[69] possibly resulting in the activation of the NLRP3–ASC–inflammasome axis and subsequent secretion of IL-1β.[70]

We and others recently showed that oncolytic viruses induce secretion of extracellular ATP from human cancer cells (Figure 1).[9,65] Unlike ecto-CRT induction, the release of ATP and HMGB1 is triggered by a range of death-inducing stimuli, and is not restricted to induction in apoptotic cell death.[47] Although ATP production is required for efficient vaccinia virus production[71] and facilitates HIV infection through its interaction with P2Y2 receptors,[72] there is little knowledge of how oncolytic viruses provoke ATP release.

Autophagic Cell Death

Autophagy physiologically has catabolic roles, particularly in cell survival.[73] However, persistent autophagy causes a caspase-independent form of cell death that is, morphologically defined as autophagic cell death (ACD), termed as type 2 PCD, through lysosomal proteinase-regulated elimination of cellular organelles.[74,75] Autophagy sometimes directs itself to cellular death, either in cooperation with apoptosis or as a back-up system, and thus is deemed as a cellular program with a 'double-faced' role.

Interestingly, the key molecules of autophagy and apoptosis pathways are intricately intertwined with shared several molecules including regulatory genes such as *p53* and *p19ARF*.[76] This crosstalk therefore can be viewed as a significant clue to understand the fate of dying cancer cells from therapeutic view points. Although ACD occurs without chromatin condensation but with massive autophagic vacuolization,[77] autophagy, often disabled in cancer, has been shown to be required for induction of immunogenicity.[68] First, dying cells in embryoid bodies that lack autophagy-related gene are unable to express the 'eat-me' signals and secrete lower levels of the 'come-get-me' signal, lysophosphatidylcholine.[78] Second, autophagy deficiency hinders ATP secretion from dying cancer cells, resulting in the impairment of DC recruitment and formation of adaptive immunity responses (Table 1).[68] Third, the inability of autophagy-deficient cancer cells to provoke antitumor immunity after chemotherapy can be reverted by suppression of extracellular ATP-degrading enzymes.[68,79] Therefore, immunogenicity of ACD could be mediated by subtle spatiotemporal alterations in the treated cancer cells.

Novel strategy of autophagy inhibition is therapeutically effective for eliminating apoptosis-resistant cancer cells based on the rationale that growing tumors may harness autophagy as an adaptation to resist therapeutic stresses.[80,81,82] Hence, more efforts should be made to elucidate the intricate interaction between autophagy inhibition and resulting effects on the immunogenicity.

Necrotic Cell Death and Necroptosis

Necrotic cell death is induced by external factors such as toxins, cancer, infections, and trauma, and is morphologically characterized by cellular swelling, rupture of the plasma membrane, and loss of cytoplasmic contents.[83] Understanding the immunogenicity of necrotic cell death is becoming important because it frequently induces robust inflammatory reactions to mount protective immune responses (Table 1).[84,85,86] Although necrosis has long been viewed as non-PCD, its execution was shown to be controlled by specific signal-transduction pathways and catabolic mechanism.[87,88,89] This alternative form of necrotic PCD, aptly termed necroptosis (type 3 PCD), is induced by tumor necrosis factor (TNF) receptor signaling that involves activation of the receptor-interacting protein (RIP) family. Upon inhibition of apoptotic pathway by the caspase inhibitor, activation of RIP1 and RIP3 kinase leads to mitochondrial instability and cell death.[90,91] Phosphorylated RIP1 and RIP3 generate a molecular complex called the necrosome, which initiates necroptosis. ROS production under necroptosis has been shown to facilitate TNF-α-induced cell death by sustaining c-Jun N-terminal kinase activation.[92] Intriguingly, necroptosis can also be executed via stimulation by apoptosis-inducible ligands such as TNF-α, FasL, or TRAIL (Table 1). Notably, cytotoxic agents are shown to induce necrotic cell death in apoptosis-defective cancer cells,[93] probably because necroptosis is principally induced when a cell cannot die via apoptotic pathways.[94] On the other hand, conventional therapy-resistant cancer stem cells (CSCs) have a higher antiapoptotic activity than that of their counterparts.[95,96] Therefore, it would be vital to clarify the key machinery of not only the necroptosis induction in cancer cells for CSC-directed therapeutic application but also the resultant immunogenicity to modulate antitumor immunity.

Pyroptosis

Pyroptosis is a recently indentified form of PCD stimulated by microbial infections and non-infectious stimuli such as myocardial infarction and cancer. In contrast to apoptosis, pyroptosis is uniquely mediated by caspase-1 activity triggered by the formation of a cytosolic complex termed the 'inflammasome', resulting in highly inflammatory outcomes (Table 1). Pyroptotic cells represent morphological characteristics, some of which are shared with apoptosis and necrosis.[97] The function of activated caspase-1 is to cleave proteolytically the proforms of the proinflammatory cytokines, IL-1β and IL-18, to their active forms.[97] Although pyroptosis has been intensively studied in the context of bacteria-infected macrophages,[98] it can also be triggered in human cancer cells infected with recombinant herpes simplex virus 2 (HSV-2) (Table 4).[99] Pyroptotic cancer cells induced by microbial infection have been recently shown to facilitate phagocytosis by macrophages, presumably through their PS exposure and ATP release.[100] Accordingly, the caspase-1-dependent generation of proinflammmatory cytokines and other DAMPs could be essential factors to provide a suitable inflammation for ICD induction.

Table 4 DNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

Oncolytic viruses	Type of cancer cells	Type of cell death induced	DAMPs	Possible mechanism of antitumor immunity
hTERT-Ad: CRAds regulated by human hTERT promoter	Human glioma, cervical and prostate cancer	Autophagy	NA	NA
hTERT-Ad	Human lung cancer	Autophagy via E2F1-miR-7-EGFR	NA	NA
OBP-702: p53-armed hTERT-Ad	Human osteosarcoma	Apoptosis Autophagy	NA	NA
CRAd-S-RGD: Ad5 carrying the RGD motif and survivin promoter	Human glioma cells	Autophagy	NA	NA
Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid with hTERT promoter	Murine urothelial carcinoma, melanoma	Apoptosis	Ecto-CRT, ATP and HMGB1	Enhanced recruitment of macrophages and $CD8^-$ T cells
ZD55-IFN-β: Oncolytic adenovirus carrying IFN-β	Human hepatoma, breast cancer	Apoptosis Necroptosis	NA	NA
Vaccinia virus	Human colon, breast, ovarian cancer	Not apoptosis Possibly necrosis	HMGB1 release	NA
vSP: antiapoptosis genes, SPI-1- and SPI-2-deleted vaccinia virus	Murine colon adenocarcinoma	Apoptosis Necrosis	HMGB1 release	NA
HSV2: Human simplex virus 2	Human endometrial cancer	Apoptosis Necrosis	HMGB1 release	NA
HSV-1716: a replication-restricted mutant herpes simplex virus	Murine ovarian cancer	NA	NA	Intratumoral injection induced IFN-γ, CXCL9 and CXCL10 with increase in NK and $CD8^-$ T cells
HSV-2 mutant ΔPK: ICP10PK-deleted HSV-2 virus	Human melanoma cells	Apoptosis Pyroptosis	NA	Dominant induction of $CD4^-$ Th1 cells

Abbreviations: hTERT, telomerase reverse transcriptase; CRAds, conditionally replicative adenoviruses; miR-7, microRNA-7, EGFR, epidermal growth factor receptor, ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1; high-mobility group protein B1;ATP, adenosine triphosphate; IFN-β, interferon-β; HSV, herpes simplex virus; IFN-γ, interferon-γ; CXCL9, chemokine (C–X–C motif) ligand 9; CXCL10, chemokine (C–X–C motif) ligand 10; NK, natural killer cells; NA, not assessed; Th1, T helper type 1

DAMPs Induced by Infection with Oncolytic Viruses

Because oncolytic viral infection can produce abundant PAMPs, including viral proteins and nucleic acids, followed by the release of DAMPs and the entire repertoire of TAAs from treated tumors,[101] oncolytic virus-triggered ICD may be more effective for induction of antitumor immunity. As viruses have developed sophisticated machineries to evade apoptotic cell death and interfere with ER stress and autophagy responses for their survival,[102,103] ICD may have played an essential role in the everlasting war between viruses and their hosts. We and other groups have found that many oncolytic viruses can induce apoptotic cell death and/or necrosis in cancer cells,[9,104,105,106] supporting their immunostimulatory potential to augment antitumor efficacy (Tables 4 and 5).[107,108] CVB3 infection induces multiple DAMPs including ecto-CRT, HMGB1 translocation from nuclei, and ATP release from human lung cancer cells. Importantly, intratumoral CVB3 administration can prominently recruit cytolytic degranulation marker CD107a-mobilized NK cells and granulocytes, and mature DCs into the tumor bed (Figure 1).[9,27] As pathogenic viruses have developed their strategies to subvert ecto-CRT and circumvent ICD induction,[109] it is noteworthy that CVB3 infection can induce ecto-CRT accompanied by other DAMPs.[9] Furthermore, we demonstrated that both NK cells and granulocytes substantially contributed to the CVB3-mediated antitumor efficacy as evidenced by *in vivo* depletion assays.[9]

Upon intratumoral replication of oncolytic viruses, resultant alterations in tumor microenvironment may restore the compromised antitumor immunity, presumably through induction of IFNs and/or cytokines that activate NK cells and APCs.[110,111] Although tumor-infiltrating DCs were impaired at maturation by immunosuppressive IL-10, PGE_2, and transforming growth factor β produced from tumor cells,[112] unidentified components in the culture media from reovirus-infected cancer cells facilitated maturation of DCs.[113]

Recent studies delineated that oncolytic viruses such as vaccinia, measles, HSV-2, and adenovirus cause the release of HMGB1.[64,65,114,115,116,117] Although HMGB1 interacts with viral components and may modulate viral replication,[117] the molecular mechanisms of how each oncolytic virus differentially produces these DAMPs remain largely elusive.

Multimodal PCD Induced by Oncolytic Viruses

We showed that approximately 20% of CVB3-mediated cytotoxicity of A549 cells resulted from apoptotic cell death.[9]

Table 5 RNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

Oncolytic viruses	Type of cancer cells	Type of cell death induced	DAMPs	Possible mechanism of antitumor immunity
Edmonston vaccine strain of MV	Human melanoma	NA	IL-6 HMGB1 release	Human DC maturation Priming an adaptive T-cell response
MV-NPL: genetically engineered MV	Human renal cell carcinoma	Apoptosis	NA	NA
MV-CEA:Edmonston vaccine MV genetically engineered to produce CEA antigen	Human breast cancer	Apoptosis	NA	NA
CVB3	Human non-small-cell lung cancer	Apoptosis	Preapoptotic ecto-CRT, HMGB1 translocation, ATP release	Phenotypic activation of immature DCs and lytic NK cells in tumors. Deletion of NK and granulocytes abrogated the CVB3-induced *in vivo* antitumor immunity
NDV	Human glioma	Autophagy	NA	NA
Reovirus	Human multiple myeloma	Apoptosis Autophagy	NA	NA
Live-attenuated poliovirus	Human neuroblastoma	Apoptosis	NA	NA
M51R: M protein mutant VSV	Human glioblastoma multiforme	Apoptosis	NA	NA
Interferon-sensitive VSV (AV3 strain)	Human prostate cancer	Apoptosis	NA	NA

Abbreviations: MV, measles virus; CVB3, coxasackievirus B3; NDV, New castle disease virus; CEA, carcinoembryonic antigen; VSV, vesicular stomatitis virus

This induction was presumably due to the capacity of CVB3 infection to induce PKR-mediated phosphorylation of eIF2 and caspase-8-mediated activation of proapoptotic mediator, caspase-3 (Figure 1).[118,119] Other DNA and RNA oncolytic viruses have been reported to induce apoptotic cancer cell death (Tables 4 and 5). However, there are only two reports showing that virus-induced ecto-CRT was correlated with enhanced intratumoral infiltrations of immune subpopulations, which accounted for the '*in vivo*' remarkable antitumor immunity.[9,65]

Several studies showed that recombinant oncolytic adenoviruses induced ACD in human malignant glioma cells,[120] brain tumor stem cells,[121] osteosarcoma cells,[105] and lung cancer cells.[122] Newcastle disease virus also triggered autophagy in glioma cells to promote its viral replication.[123] Reovirus-mediated oncolysis of multiple myeloma was reported to be orchestrated via upregulation of autophagy.[124] Because cancer cells are largely refractory to apoptotic inducers but vulnerable to necroptosis,[39] overcoming anticancer drug resistance may be achieved by activation of necroptotic rather than apoptotic pathways, where the former might be the intrinsic 'Achilles heel' of cancers.[125] So far only recombinant adenovirus has been shown to facilitate both necroptotic and apoptotic cell death with a synergistic effect on cancer cells when combined with doxorubicin (Tables 4 and 5).[126] In addition, most oncolytic viruses may induce pyroptotic cancer cell death accompanied by abundant proinflammatory cytokines and DAMPs. Accordingly, some oncolytic viruses may induce multimodal ICD, allowing them to be a plausible modality as promising agents of immunotherapy.

Strategies to Enhance the Potentials of ICD Induced by Oncolytic Viruses

Besides DAMPs, massive production of type I IFNs (IFN-α/β) upon oncolytic viral infection can be a potent immunomodulator through their indirect immunostimulatory effects on neutrophils and T cells,[127,128] as well as through their direct antiproliferative effects.[129] Despite a creation of multimodal ICD by oncolytic viruses to facilitate antitumor immunity, much attention should be paid to the preferential antiviral immunity that might impede direct viral oncolysis-mediated tumor destruction. To avoid this, cyclophosphamide is shown to retard immune removal of oncolytic viruses, enhancing the persistence of viral infection.[130] Another promising strategy to overcome antiviral immunity could be potentiating immune responses by gene modification of oncolytic viruses to arm them with immunostimulatory cytokines, such as GM-CSF, IL-2, IL-12, and IL-15. Indeed, the results of clinical trials of the GM-CSF gene-harboring oncolytic vaccinia virus JX-594 and the GM-CSF gene-harboring oncolytic herpes virus talimogene laherparepvec demonstrated that a clinical benefit can be accomplished by combined respective oncolytic activity with the recruitment of immune cells.[6,7,131] The combination of adoptive T-cell therapy with oncolytic viruses is shown to elicit an increased antitumor effect.[131,132] Collectively, the design of combinatorial therapies of oncolytic viruses with immunotherapeutic modalities may

hold the key to mount maximally a multifaceted attack against cancers.

Conclusions

Although mechanism of ICD induction is a very complicated process, we need to elucidate how dying cells become much more stimulatory in shaping antitumor immune responses than was ever expected. Notably, intermediate death processes, including caspase activation, mitochondrial degradation by autophagy, ROS production, and oxidative modification of DAMPs, have been found to fine-tune the balance between antitumor tolerance and immunity, providing implications in manipulation of ICD.

Four forms of PCD, apoptosis, autophagy, necroptosis, and pyroptosis, may jointly decide the fate of cells of malignant cells. However, in terms of immunogenicity, investigations of only apoptotic cell death in cancer cells have just begun. Therefore, further elucidation of determinants of respective PCD-inducing pathway and characterization of resultant ICD should aid to develop novel anticancer strategies. A recent review advocates a list of characteristics for an ideal ICD inducer,[27] as follows: (1) efficiently activates apoptosis or necrosis leading to emission of multiple DAMPs and TLR agonists;[133,134] (2) irrelevant in drug-efflux pathways;[135] (3) can induce ER stress;[134] (4) has negligible suppressive or inhibitory effects on immune cells;[136] (5) counteracts immunosuppressive responses;[136,137] and (6) directly targets not only the primary tumor but also metastases.[138] No ideal ICD inducer exists, but it is important to seek for ideal combinatorial therapies that could achieve these properties. Of the currently known relevant ICD inducers, those that meet most of these properties include mitoxantrone, hypericin-PDT, and shikonin. However, diverse oncolytic viruses could be the promising ICD inducer as we gain more knowledge about the properties yet to be investigated. Evidently, they can destroy conventional therapy-resistant CSCs,[139] possibly through their ability to induce distinctive PCD and/or modification to express genes that target CSC-specific signaling pathways underpinning their cell survival.[140]

Gaining more detailed insights into the mechanisms of ICD induction, to be perceived by the immune system, will not only ameliorate the development of promising anticancer agents or combinatorial therapies but also offer useful knowledge in various life science fields including virology, immunology, and clinical medicine.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (17016053 and 23240133), and the Ministry of Health Labour and Welfare (23080101), Japan.

1. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N *et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* 2005; **202**: 1691–1701.
2. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL *et al.* Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007; **13**: 54–61.
3. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**: 1456–1462.
4. Garg AD, Krysko DV, Verfaillie T, Kaczmarek A, Ferreira GB, Marysael T *et al.* A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *EMBO J* 2012; **31**: 1062–1079.
5. Tanimoto T, Hori A, Kami M. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010; **363**: 1967–1968; 1966; author reply.
6. Senzer NN, Kaufman HL, Amatruda T, Nemunaitis M, Reid T, Daniels G *et al.* Phase II clinical trial of a granulocyte–macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol* 2009; **27**: 5763–5771.
7. Heo J, Reid T, Ruo L, Breitbach CJ, Rose S, Bloomston M *et al.* Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med* 2013; **19**: 329–336.
8. Sobol PT, Boudreau JE, Stephenson K, Wan Y, Lichty BD, Mossman KL. Adaptive antiviral immunity is a determinant of the therapeutic success of oncolytic virotherapy. *Mol Ther* 2011; **19**: 335–344.
9. Miyamoto S, Inoue H, Nakamura T, Yamada M, Sakamoto C, Urata Y *et al.* Coxsackievirus B3 is an oncolytic virus with immunostimulatory properties that is active against lung adenocarcinoma. *Cancer Res* 2012; **72**: 2609–2621.
10. Contag CH, Sikorski R, Negrin RS, Schmidt T, Fan AC, Bachireddy P *et al.* Definition of an enhanced immune cell therapy in mice that can target stem-like lymphoma cells. *Cancer Res* 2010; **70**: 9837–9845.
11. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol* 2012; **30**: 658–670.
12. Curtin JF, Cotter TG. Apoptosis: historical perspectives. *Essays Biochem* 2003; **39**: 1–10.
13. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV *et al.* Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ 2012* **19**: 107–120.
14. Brenner C, Kroemer G. Apoptosis. Mitochondria–the death signal integrators. *Science* 2000; **289**: 1150–1151.
15. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; **35**: 495–516.
16. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 2001; **14**: 715–725.
17. Krysko DV, Kaczmarek A, Krysko O, Heyndrickx L, Woznicki J, Bogaert P *et al.* TLR-2 and TLR-9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation. *Cell Death Differ* 2011; **18**: 1316–1325.
18. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F *et al.* Immunogenic death of colon cancer cells treated with oxaliplatin. *Oncogene* 2010; **29**: 482–491.
19. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund AC, Chapman DC *et al.* Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J* 2009; **28**: 578–590.
20. Merritt RE, Mahtabifard A, Yamada RE, Crystal RG, Korst RJ. Cisplatin augments cytotoxic T-lymphocyte-mediated antitumor immunity in poorly immunogenic murine lung cancer. *J Thorac Cardiovasc Surg* 2003; **126**: 1609–1617.
21. Obeid M, Panaretakis T, Joza N, Tufi R, Tesniere A, van Endert P *et al.* Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. *Cell Death Differ* 2007; **14**: 1848–1850.
22. Suzuki Y, Mimura K, Yoshimoto Y, Watanabe M, Ohkubo Y, Izawa S *et al.* Immunogenic tumor cell death induced by chemoradiotherapy in patients with esophageal squamous cell carcinoma. *Cancer Res* 2012; **72**: 3967–3976.
23. Rubner Y, Wunderlich R, Ruhle PF, Kulzer L, Werthmoller N, Frey B *et al.* How does ionizing irradiation contribute to the induction of anti-tumor immunity? *Front Oncol* 2012; **2**: 75.
24. Matzinger P. The danger model: a renewed sense of self. *Science* 2002; **296**: 301–305.
25. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994; **12**: 991–1045.
26. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 2012; **31**: 51–72.
27. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* 2012; **12**: 860–875.
28. Dudek AM, Garg AD, Krysko DV, De Ruysscher D, Agostinis P. Inducers of immunogenic cancer cell death. *Cytokine Growth Factor Rev* 2013.
29. Panaretakis T, Joza N, Modjtahedi N, Tesniere A, Vitale I, Durchschlag M *et al.* The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death Differ* 2008; **15**: 1499–1509.
30. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A *et al.* Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007; **13**: 1050–1059.
31. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C *et al.* Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 2009; **15**: 1170–1178.

32. Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F *et al.* Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Res* 2011; **71**: 768–778.
33. Chen J, Xie J, Jiang Z, Wang B, Wang Y, Hu X. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene* 2011; **30**: 4297–4306.
34. Yang H, Zhou P, Huang H, Chen D, Ma N, Cui QC *et al.* Shikonin exerts antitumor activity via proteasome inhibition and cell death induction *in vitro* and *in vivo*. *Int J Cancer* 2009; **124**: 2450–2459.
35. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. *Blood* 2007; **109**: 4839–4845.
36. Garrido G, Rabasa A, Sanchez B, Lopez MV, Blanco R, Lopez A *et al.* Induction of immunogenic apoptosis by blockade of epidermal growth factor receptor activation with a specific antibody. *J Immunol* 2011; **187**: 4954–4966.
37. Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S *et al.* Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. *Sci Transl Med* 2012; **4**: 143ra199.
38. Sonnemann J, Gressmann S, Becker S, Wittig S, Schmudde M, Beck JF. The histone deacetylase inhibitor vorinostat induces calreticulin exposure in childhood brain tumour cells *in vitro*. *Cancer Chemother Pharmacol* 2010; **66**: 611–616.
39. Han W, Li L, Qiu S, Lu Q, Pan Q, Gu Y *et al.* Shikonin circumvents cancer drug resistance by induction of a necroptotic death. *Mol Cancer Ther* 2007; **6**: 1641–1649.
40. Galluzzi L, Kepp O, Kroemer G. Enlightening the impact of immunogenic cell death in photodynamic cancer therapy. *EMBO J* 2012; **31**: 1055–1057.
41. Verfaillie T, Rubio N, Garg AD, Bultynck G, Rizzuto R, Decuypere JP *et al.* PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. *Cell Death Differ* 2012; **19**: 1880–1891.
42. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; **418**: 191–195.
43. Karioti A, Bilia AR. Hypericins as potential leads for new therapeutics. *Int J Mol Sci* 2010; **11**: 562–594.
44. Zhang L, Wang A. Virus-induced ER stress and the unfolded protein response. *Front Plant Sci* 2012; **3**: 293.
45. Schwarz KB. Oxidative stress during viral infection: a review. *Free Radic Biol Med* 1996; **21**: 641–649.
46. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. *Cancer Immunol Immunother* 2012; **61**: 215–221.
47. Obeid M, Tesniere A, Panaretakis T, Tufi R, Joza N, van Endert P *et al.* Ecto-calreticulin in immunogenic chemotherapy. *Immunol Rev* 2007; **220**: 22–34.
48. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; **182**: 1545–1556.
49. Martins I, Kepp O, Schlemmer F, Adjemian S, Tailler M, Shen S *et al.* Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. *Oncogene* 2011; **30**: 1147–1158.
50. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta* 2010; **1805**: 53–71.
51. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 2008; **29**: 21–32.
52. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H *et al.* High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 2000; **192**: 565–570.
53. Tesniere A, Apetoh L, Ghiringhelli F, Joza N, Panaretakis T, Kepp O *et al.* Immunogenic cancer cell death: a key–lock paradigm. *Curr Opin Immunol* 2008; **20**: 504–511.
54. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H *et al.* Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol* 2012; **13**: 832–842.
55. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R *et al.* The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol Rev* 2007; **220**: 47–59.
56. Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J *et al.* A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci USA* 2010; **107**: 11942–11947.
57. Jube S, Rivera ZS, Bianchi ME, Powers A, Wang E, Pagano I *et al.* Cancer cell secretion of the DAMP protein HMGB1 supports progression in malignant mesothelioma. *Cancer Res* 2012; **72**: 3290–3301.
58. Yang GL, Zhang LH, Bo JJ, Huo XJ, Chen HG, Cao M *et al.* Increased expression of HMGB1 is associated with poor prognosis in human bladder cancer. *J Surg Oncol* 2012; **106**: 57–61.
59. Hoppe G, Talcott KE, Bhattacharya SK, Crabb JW, Sears JE. Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. *Exp Cell Res* 2006; **312**: 3526–3538.
60. Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F *et al.* Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *J Exp Med* 2012; **209**: 1519–1528.
61. Yang H, Lundback P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME *et al.* Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). *Mol Med* 2012; **18**: 250–259.
62. Chaiswing L, Oberley TD. Extracellular/microenvironmental redox state. *Antioxid Redox Signal* 2010; **13**: 449–465.
63. Policastro LL, Ibanez IL, Notcovich C, Duran HA, Podhajcer OL. The tumor microenvironment: characterization, redox considerations, and novel approaches for reactive oxygen species-targeted gene therapy. *Antioxid Redox Signal* 2012.
64. Guo ZS, Naik A, O'Malley ME, Popovic P, Demarco R, Hu Y *et al.* The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2. *Cancer Res* 2005; **65**: 9991–9998.
65. Diaconu I, Cerullo V, Hirvinen ML, Escutenaire S, Ugolini M, Pesonen SK *et al.* Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. *Cancer Res* 2012; **72**: 2327–2338.
66. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF *et al.* Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 2009; **461**: 282–286.
67. Martins I, Tesniere A, Kepp O, Michaud M, Schlemmer F, Senovilla L *et al.* Chemotherapy induces ATP release from tumor cells. *Cell Cycle* 2009; **8**: 3723–3728.
68. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P *et al.* Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 2011; **334**: 1573–1577.
69. Ohshima Y, Tsukimoto M, Takenouchi T, Harada H, Suzuki A, Sato M *et al.* Gamma-irradiation induces P2X(7) receptor-dependent ATP release from B16 melanoma cells. *Biochim Biophys Acta* 2010; **1800**: 40–46.
70. Petrovski G, Ayna G, Majai G, Hodrea J, Benko S, Madi A *et al.* Phagocytosis of cells dying through autophagy induces inflammasome activation and IL-1beta release in human macrophages. *Autophagy* 2011; **7**: 321–330.
71. Chang CW, Li HC, Hsu CF, Chang CY, Lo SY. Increased ATP generation in the host cell is required for efficient vaccinia virus production. *J Biomed Sci* 2009; **16**: 80.
72. Seror C, Melki MT, Subra F, Raza SQ, Bras M, Saidi H *et al.* Extracellular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection. *J Exp Med* 2011; **208**: 1823–1834.
73. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2007; **8**: 741–752.
74. Amaravadi RK, Thompson CB. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res* 2007; **13**: 7271–7279.
75. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000; **290**: 1717–1721.
76. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ* 2009; **16**: 966–975.
77. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol* 2008; **9**: 1004–1010.
78. Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN *et al.* Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell* 2007; **128**: 931–946.
79. Martins I, Michaud M, Sukkurwala AQ, Adjemian S, Ma Y, Shen S *et al.* Premortem autophagy determines the immunogenicity of chemotherapy-induced cancer cell death. *Autophagy* 2012; **8**: 413–415.
80. Townsend KN, Hughson LR, Schlie K, Poon VI, Westerback A, Lum JJ. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. *Immunol Rev* 2012; **249**: 176–194.
81. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI *et al.* Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007; **117**: 326–336.
82. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther* 2011; **10**: 1533–1541.
83. Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: a specific form of programmed cell death? *Exp Cell Res* 2003; **283**: 1–16.
84. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239–257.
85. Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T. The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. *Sci Signal* 2010; **3**: re4.
86. Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; **68**: 251–306.
87. Van Herreweghe F, Festjens N, Declercq W, Vandenabeele P. Tumor necrosis factor-mediated cell death: to break or to burst, that's the question. *Cell Mol Life Sci* 2010; **67**: 1567–1579.
88. Festjens N, Vanden Berghe T, Vandenabeele P. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta* 2006; **1757**: 1371–1387.

89. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci* 2007; **32**: 37–43.
90. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC *et al.* RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009; **325**: 332–336.
91. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S *et al.* Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* 2000; **1**: 489–495.
92. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 2005; **120**: 649–661.
93. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 2004; **18**: 1272–1282.
94. Skulachev VP. Bioenergetic aspects of apoptosis, necrosis and mitoptosis. *Apoptosis* 2006; **11**: 473–485.
95. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F *et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007; **1**: 389–402.
96. Madjd Z, Mehrjerdi AZ, Sharifi AM, Molanaei S, Shahzadi SZ, Asadi-Lari M. CD44$^+$ cancer cells express higher levels of the anti-apoptotic protein Bcl-2 in breast tumours. *Cancer Immun* 2009; **9**: 4.
97. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 2009; **7**: 99–109.
98. Frantz S, Ducharme A, Sawyer D, Rohde LE, Kobzik L, Fukazawa R *et al.* Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction. *J Mol Cell Cardiol* 2003; **35**: 685–694.
99. Colunga AG, Laing JM, Aurelian L. The HSV-2 mutant DeltaPK induces melanoma oncolysis through nonredundant death programs and associated with autophagy and pyroptosis proteins. *Gene Ther* 2010; **17**: 315–327.
100. Wang Q, Imamura R, Motani K, Kushiyama H, Nagata S, Suda T. Pyroptotic cells externalize eat-me and release find-me signals and are efficiently engulfed by macrophages. *Int Immunol* 2013; **25**: 363–372.
101. Bridle BW, Stephenson KB, Boudreau JE, Koshy S, Kazdhan N, Pullenayegum E *et al.* Potentiating cancer immunotherapy using an oncolytic virus. *Mol Ther* 2010; **18**: 1430–1439.
102. Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of mitochondrial apoptosis. *PLoS Pathogen* 2008; **4**: e1000018.
103. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature* 2011; **469**: 323–335.
104. Meng X, Nakamura T, Okazaki T, Inoue H, Takahashi A, Miyamoto S *et al.* Enhanced antitumor effects of an engineered measles virus Edmonston strain expressing the wild-type N, P, L genes on human renal cell carcinoma. *Mol Ther* 2010; **18**: 544–551.
105. Hasei J, Sasaki T, Tazawa H, Osaki S, Yamakawa Y, Kunisada T *et al.* Dual programmed cell death pathways induced by p53 transactivation overcome resistance to oncolytic adenovirus in human osteosarcoma cells. *Mol Cancer Ther* 2013; **12**: 314–325.
106. Moussavi M, Fazli L, Tearle H, Guo Y, Cox M, Bell J *et al.* Oncolysis of prostate cancers induced by vesicular stomatitis virus in PTEN knockout mice. *Cancer Res* 2010; **70**: 1367–1376.
107. Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat Med* 2001; **7**: 781–787.
108. Prestwich RJ, Harrington KJ, Pandha HS, Vile RG, Melcher AA, Errington F. Oncolytic viruses: a novel form of immunotherapy. *Expert Rev Anticancer Ther* 2008; **8**: 1581–1588.
109. Kepp O, Senovilla L, Galluzzi L, Panaretakis T, Tesniere A, Schlemmer F *et al.* Viral subversion of immunogenic cell death. *Cell Cycle* 2009; **8**: 860–869.
110. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B *et al.* Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol Ther* 2001; **3**(Part 1): 697–707.
111. Benencia F, Courreges MC, Conejo-Garcia JR, Mohamed-Hadley A, Zhang L, Buckanovich RJ *et al.* HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. *Mol Ther* 2005; **12**: 789–802.
112. Vicari AP, Chiodoni C, Vaure C, Ait-Yahia S, Dercamp C, Matsos F *et al.* Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med* 2002; **196**: 541–549.
113. Errington F, Steele L, Prestwich R, Harrington KJ, Pandha HS, Vidal L *et al.* Reovirus activates human dendritic cells to promote innate antitumor immunity. *J Immunol* 2008; **180**: 6018–6026.
114. Donnelly OG, Errington-Mais F, Steele L, Hadac E, Jennings V, Scott K *et al.* Measles virus causes immunogenic cell death in human melanoma. *Gene Ther* 2013; **20**: 7–15.
115. Huang B, Sikorski R, Kirn DH, Thorne SH. Synergistic anti-tumor effects between oncolytic vaccinia virus and paclitaxel are mediated by the IFN response and HMGB1. *Gene Ther* 2011; **18**: 164–172.
116. Borde C, Barnay-Verdier S, Gaillard C, Hocini H, Marechal V, Gozlan J. Stepwise release of biologically active HMGB1 during HSV-2 infection. *PLoS One* 2011; **6**: e16145.
117. Moisy D, Avilov SV, Jacob Y, Laoide BM, Ge X, Baudin F *et al.* HMGB1 protein binds to influenza virus nucleoprotein and promotes viral replication. *J Virol* 2012; **86**: 9122–9133.
118. Zhang HM, Ye X, Su Y, Yuan J, Liu Z, Stein DA *et al.* Coxsackievirus B3 infection activates the unfolded protein response and induces apoptosis through downregulation of p58IPK and activation of CHOP and SREBP1. *J Virol* 2010; **84**: 8446–8459.
119. Chau DH, Yuan J, Zhang H, Cheung P, Lim T, Liu Z *et al.* Coxsackievirus B3 proteases 2A and 3C induce apoptotic cell death through mitochondrial injury and cleavage of eIF4GI but not DAP5/p97/NAT1. *Apoptosis* 2007; **12**: 513–524.
120. Ito H, Aoki H, Kuhnel F, Kondo Y, Kubicka S, Wirth T *et al.* Autophagic cell death of malignant glioma cells induced by a conditionally replicating adenovirus. *J Natl Cancer Inst* 2006; **98**: 625–636.
121. Jiang H, Gomez-Manzano C, Aoki H, Alonso MM, Kondo S, McCormick F *et al.* Examination of the therapeutic potential of Delta-24-RGD in brain tumor stem cells: role of autophagic cell death. *J Natl Cancer Inst* 2007; **99**: 1410–1414.
122. Tazawa H, Yano S, Yoshida R, Yamasaki Y, Sasaki T, Hashimoto Y *et al.* Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1–microRNA-7–epidermal growth factor receptor axis. *Int J Cancer* 2012; **131**: 2939–2950.
123. Meng C, Zhou Z, Jiang K, Yu S, Jia L, Wu Y *et al.* Newcastle disease virus triggers autophagy in U251 glioma cells to enhance virus replication. *Arch Virol* 2012; **157**: 1011–1018.
124. Thirukkumaran CM, Shi ZQ, Luider J, Kopciuk K, Gao H, Bahlis N *et al.* Reovirus modulates autophagy during oncolysis of multiple myeloma. *Autophagy* 2013; **9**: 413–414.
125. Hu X, Xuan Y. Bypassing cancer drug resistance by activating multiple death pathways–a proposal from the study of circumventing cancer drug resistance by induction of necroptosis. *Cancer Lett* 2008; **259**: 127–137.
126. Huang H, Xiao T, He L, Ji H, Liu XY. Interferon-beta-armed oncolytic adenovirus induces both apoptosis and necroptosis in cancer cells. *Acta Biochim Biophys Sin (Shanghai)* 2012; **44**: 737–745.
127. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest* 2010; **120**: 1151–1164.
128. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. *Science* 1996; **272**: 1947–1950.
129. Qin XQ, Beckham C, Brown JL, Lukashev M, Barsoum J. Human and mouse IFN-beta gene therapy exhibits different anti-tumor mechanisms in mouse models. *Mol Ther* 2001; **4**: 356–364.
130. Chiocca EA. The host response to cancer virotherapy. *Curr Opin Mol Ther* 2008; **10**: 38–45.
131. Melcher A, Parato K, Rooney CM, Bell JC. Thunder and lightning: immunotherapy and oncolytic viruses collide. *Mol Ther* 2011; **19**: 1008–1016.
132. Kottke T, Diaz RM, Kaluza K, Pulido J, Galivo F, Wongthida P *et al.* Use of biological therapy to enhance both virotherapy and adoptive T-cell therapy for cancer. *Mol Ther* 2008; **16**: 1910–1918.
133. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. DAMPs and PDT-mediated photo-oxidative stress: exploring the unknown. *Photochem Photobiol Sci* 2011; **10**: 670–680.
134. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. The emergence of phox-ER stress induced immunogenic apoptosis. *Oncoimmunology* 2012; **1**: 786–788.
135. Shen F, Chu S, Bence AK, Bailey B, Xue X, Erickson PA *et al.* Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J Pharmacol Exp Ther* 2008; **324**: 95–102.
136. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; **12**: 298–306.
137. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**: 883–899.
138. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
139. Kanai R, Wakimoto H, Martuza RL, Rabkin SD. A novel oncolytic herpes simplex virus that synergizes with phosphoinositide 3-kinase/Akt pathway inhibitors to target glioblastoma stem cells. *Clin Cancer Res* 2011; **17**: 3686–3696.
140. Cripe TP, Wang PY, Marcato P, Mahller YY, Lee PW. Targeting cancer-initiating cells with oncolytic viruses. *Mol Ther* 2009; **17**: 1677–1682.

Int J Hematol (2013) 98:355–360
DOI 10.1007/s12185-013-1408-7

ORIGINAL ARTICLE

B-cell function after unrelated umbilical cord blood transplantation using a minimal-intensity conditioning regimen in patients with X-SCID

Satoru Kumaki · Yoji Sasahara · Yoshiro Kamachi · Hideki Muramatsu · Tomohiro Morio · Kumiko Goi · Kanji Sugita · Tomonari Urabe · Hidetoshi Takada · Seiji Kojima · Shigeru Tsuchiya · Toshirou Hara

Received: 10 January 2013 / Revised: 30 July 2013 / Accepted: 6 August 2013 / Published online: 17 August 2013

Abstract Patients with X-linked severe combined immunodeficiency (X-SCID) suffer from severe and persistent infections, and usually die early in life unless treated by hematopoietic stem cell transplantation. If a patient has an HLA-identical sibling donor, preparative conditioning is not necessary for T-cell engraftment and B-cell function. However, in the absence of such a donor, long-term reconstitution of full B-cell function is often problematic, leading in many cases to a lifetime requirement for immunoglobulin replacement therapy. Preparative myeloablative conditioning has been shown to improve long-term B-cell function, but may aggravate pre-existing infection and transplant-related toxicity. It is thus important to determine the minimum intensity of conditioning that assures immunoglobulin production. In the present study, we performed reduced-intensity conditioning (RIC), consisting of fludarabine 125 mg/m^2 and melphalan 80 mg/m^2, prior to unrelated umbilical cord blood transplantation (UCBT) for five patients with X-SCID, none of them had an HLA-identical donor. Four patients survived more than 4 years without sequelae, and none required long-term immunoglobulin replacement therapy. One patient succumbed to sepsis in conjunction with severe GVHD. Our result demonstrates that the RIC regimen described above in combination with UCBT is an effective and less toxic conditioning to correct B-cell function in patients with X-SCID.

Keywords X-SCID · Reduced-intensity conditioning · Umbilical cord blood transplantation · Fludarabine/melphalan

S. Kumaki (✉) · Y. Sasahara · S. Tsuchiya
Department of Pediatrics, Tohoku University Graduate School of Medicine, 1-1 Seiryo Machi, Aoba-ku, Sendai 980-8574, Japan
e-mail: kumaki@med.tohoku.ac.jp

Y. Kamachi · H. Muramatsu · S. Kojima
Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan

T. Morio
Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan

K. Goi · K. Sugita
Department of Pediatrics, School of Medicine, University of Yamanashi, Yamanashi, Japan

T. Urabe
Department of Pediatrics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

H. Takada · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Introduction

X-linked severe combined immunodeficiency (X-SCID), which accounts for approximately half the cases of SCID, is caused by mutations of the γc chain. Immunological characteristics of this disease include profound impairment of both cellular and humoral immunity due to the absence or diminished numbers of T cells and natural killer (NK) cells, and abnormal B-cell function in spite of normal or elevated numbers of B cells. Therefore, patients with X-SCID suffer from severe and persistent infections, including opportunistic pathogens, and usually die early in life unless treated by hematopoietic stem cell transplantation (HSCT) or gene therapy [1]. Previous reports demonstrated excellent results of HLA-identical BMT with

survival rate over 90 %, and full restoration of T- and B-cell function [2, 3]. Since most patients do not have an HLA-identical sibling donor, HLA-haploidentical bone marrow transplantation (BMT) was developed in the early 1980s, when better T-cell depletion methods became available. However, the survival rate was lower, at around 60–80 %, and about half of the patients required life-long immunoglobulin replacement therapy despite normal T-cell immunity with or without pre-transplant conditioning [2–4]. These results suggested that T-cell-depleted, HLA-haploidentical bone marrow cells might not be a suitable source of HSCT for correcting B-cell function. Another strategy to treat this condition is to use unrelated donor HSCT with conventional myeloablative conditioning regimens, which leads to stable reconstitution of T- and B-cell function [5]. However, this approach has been associated with significant treatment-related toxicities and aggravation of pre-existing infections. To avoid these problems, reduced-intensity conditioning (RIC) regimens have been developed. Recently, Rao et al. [6] reported that a RIC regimen using a total dose of 150 mg/m^2 of fludarabine, 140 mg/m^2 of melphalan, and Campath 1H or ATG resulted in an improved survival and reduced transplantation-related mortality, compared with myeloablative conditioning, in children with primary immunodeficiency (PID) undergoing unrelated BMT. They used the same regimen for patients with X-SCID who would be expected to need less intensive conditioning because their immune system is already profoundly impaired. Based on their report, we performed unrelated umbilical cord blood transplantation (UCBT) with pre-transplant conditioning using a further reduced dosage of fludarabine and melphalan, in the absence of Campath 1H or ATG, to investigate whether a minimally intensive conditioning regimen could assure correction of B-cell function in X-SCID patients.

Patients and methods

Patients

Five patients with typical X-SCID received unrelated UCBT because they had no HLA-matched sibling donor. As shown in Table 1, mutations in the γc chain gene were detected in all patients. Patient 3 suffered from pneumonia caused by *Pneumocystis jiroveci* at the time of diagnosis of X-SCID. All patients except for patient 3 were diagnosed with X-SCID at birth because their brothers had the same disease. Immunoglobulin replacement therapy was initiated once hypogammaglobulinemia was confirmed, and IgG trough levels were maintained over 500 mg/dL. This study was performed with the approval of Institutional Review Board at each university and with the written informed consents of the parents.

Conditioning regimen and GVHD prophylaxis

Pre-transplant conditioning for all patients consisted of fludarabine (25 mg/m^2 per day) from day −7 to day −3

Table 1 Patient characteristics

Patient	1	2	3	4	5
Age at diagnosis (months)	0	0	4	0	0
Age at UCBT (months)	3	3	10	3	3
Mutations in the γc chain	868 G > A	691 G > A	c.735 741*	IVS4 + 2 T > A	568A > G
HLA identity	6/6	5/6	6/6	5/6	5/6
Nucleated cell dose (×10^7/kg)	7.1	10.0	5.0	9.5	11.2
CD34+ cell dose (×10^5/kg)	1.09	3.65	ND	3.50	1.68
Hematological recovery					
Nt > 500/μL	30	20	36	20	12
Plt > 5 × 10^4/μL	10	16	95	17	16
Ret > 1 %	18	16	38	20	15
Complications at UCBT	None	None	Pneumonia	None	None
Additional infections during UCBT	None	None	Sepsis	CMV	Sepsis
GVHD					
Prophylaxis	CyA	FK + sMTX	FK	FK + sMTX	CyA + PSL
Acute (grade)	0	0	II	II	III
Chronic	–	–	Extensive	–	Extensive
Therapy	–	–	FK + mPSL	FK + PSL	FK + MMF + PSL

*c.735 741** c.735_741delAGCCACC→insGGGAGCAATACTT, *ND* not determined, *Nt* neutrophils, *Plt* platelets, *Ret* reticulocytes, *sMTX* short-term methotrexate

(total dose 125 mg/m^2) and melphalan (40 mg/m^2 per day) from day −4 to day −3 (total dose 80 mg/m^2). Neither ATG nor Campath 1H was included in the conditioning regimen.

Prophylaxis for acute GVHD was performed with either cyclosporine A (CyA) with/without prednisolone or FK506 with/without short-term methotrexate as shown in Table 1.

Graft characteristics

As shown in Table 1, UCB units were either serologically full-matched or one locus mismatched at 6/6 (A, B, DR) HLA loci. Infused nucleated cell doses were 5.0 × 10^7/kg–11.2 × 10^7/kg (mean 8.6 × 10^7/kg), which contained CD34+ stem cells, ranging from 1.09 × 10^5/kg to 3.65 x 10^5/kg (mean 2.48 × 10^5/kg) except for patient 3, whose information on CD34+ cells was not available.

Chimerism studies

Hematological recovery was defined as achievement of absolute neutrophil count (ANC) >500/μL for 3 consecutive days and a platelet count >5.0 × 10^4/μL for 7 consecutive days without need for further transfusion. Chimerism was analyzed at Human Leukocyte Antigen Laboratory (Kyoto, Japan) as described previously [7]. Briefly, T cells, B cells and NK cells were separated by anti-CD3, anti-CD19 and anti-CD56 microbeads (Invitrogen Dyanl AS, Oslo, Norway), respectively. Donor- and recipient-specific polymorphic short tandem repeats (STR) were amplified by PCR, and subsequently analyzed by SDS-PAGE.

Immunological reconstitution studies

Immunological reconstitution status after transplantation was monitored by serum immunoglobulin levels (IgG, IgA, IgM and IgE), isohemagglutinin, and specific antibodies, and by flow cytometry analyses of peripheral mononuclear cells for CD3, CD4, CD8, CD19, CD16 and CD56.

Results

The age at transplantation was 3 months in four patients and 10 months in one patient (Table 1). All patients received UCBT using fludarabine (125 mg/m^2) and melphalan (80 mg/m^2) as a pre-transplant conditioning. They all achieved engraftment of ANC > 500 μL and platelets > 5.0 × 10^4/μL at a mean of 23.6 days (range 12–36 days) and 30.8 days (range 10–95 days), respectively. All but one survived more than 4 years without complication. One patient, patient 5, succumbed to sepsis in conjunction with severe GVHD.

Infections

Patient 3 suffered pneumonia due to *P. jiroveci* infection prior to admission and intravenous trimethoprim/sulfamethoxazole therapy was initiated. The pneumonia resolved with the engraftment of donor cells. He also experienced an episode of sepsis due to enterococci after UCBT, which was cured by appropriate antibiotics. Patients 1, 2, 4, and 5 were diagnosed with X-SCID at birth by sequencing of the γc chain because their brothers had the same disease. They had been protected in a clean environment soon after birth and they did not experience any infection until UCBT. Patient 5 developed sepsis due to a catheter infection, which was the cause of death at day 491 after UCBT.

Regimen-related toxicity and GVHD

Mild mucositis and myelosuppression were observed with this reduced-intensity conditioning, and no other regimen-related toxicity was noted.

Patient 3 developed acute GVHD grade II (skin stage 3) and extensive chronic GVHD, while patient 4 developed acute GVHD grade II (skin stage 3, liver stage 1 and gut stage 1). Symptoms in both cases resolved on prednisolone and FK506. Patient 5 developed acute GVHD grade III (skin grade 1, liver grade 3 and gut stage 3), followed by extensive chronic GVHD. He succumbed to sepsis in conjunction with uncontrolled GVHD, although he was treated with prednisolone, FK506 and mycophenolate mofetil (MMF).

Chimerism

Median follow-up was 68 months (range 48–73 months). As shown in Table 2, all survivors had complete donor T-cell chimerism. One survivor, patient 3, also had complete lymphocyte and granulocyte chimerism, which was confirmed by day 52. The others demonstrated mixed chimerism in these cell lineages. The percentage of the donor cells in each cell lineage had been stable since day 168 after UCBT in patient 1. In patients 2 and 4, detailed chimerism using fractionated cells was analyzed only the date indicated in Table 2. Donor cells of patient 5 constituted only 5 % of his peripheral blood nucleated cells at day 420 after UCBT, although T cells were 100 % of donor origin.

Immune reconstitution

Table 3 shows the results of immunologic evaluation at the most recent follow-up after UBCT in all survivors. Absolute numbers of lymphocytes were normal after

Table 2 Leukocyte chimerism

Patient	1		2		3		4		5
Days after UCBT[a]	168	1620	60	1098	52	2021	90	2078	420
T cell (donor %)	100	>95	90	94		100	100	92	100
B cell (donor %)	24	20	20	8		100	70	50	ND
NK cell (donor %)	55	69	15	33		100	90	84	ND
Granulocyte (donor %)	65	59	18	48	>95	100	20	13	ND
Lymphocyte (donor %)					>95				

ND not determined

[a] Days after UCBT when chimerism was determined

Table 3 Immune reconstitution

Patient	1	2	3	4	5 (at day 470)
WBC (/μL)	7700	7400	4710	9200	1000
Lymphocyte (/μL)	3700	4370	4120	4100	400
CD3 (%)	82.2	60.4	63.7	81.3	38.6
CD4 (%)	48.4	24.4	35.3	42.0	33.0
CD8 (%)	27.8	28.7	25.7	32.2	11.5
CD19 (%)	13.4	37.9	32.0	15.1	0.0
CD16/56 (%)	1.8	0.6	4.0	2.7	13.0
B-cell function					
IgG (mg/dL)	937	531	692	1157	660 (under i.v.Ig)
IgA (mg/dL)	58	32	55	101	89
IgM (mg/dL)	117	77	115	231	112
IgE (IU/mL)	37	<3	4.2	1	ND
Isohemagglutinin	+	+	–	+	–
Specific antibody	–	+	+	+	ND
T-cell function					
PHA stimulation (SI)	164.4	243.6	220.9	1213.4	1.1
ConA stimulation (SI)	897.7	322.1	225.5	713.1	1.1
NK activity (%)	15	4	10	19	ND

Normal values; PHA stimulation (SI) >100, ConA stimulation (SI) >75, NK activity 18–40 %

ND not determined, *SI* stimulation index

transplantation (Table 3). Numbers of CD3+, CD4+, CD8+ T cells and CD19+ B cells were within normal ranges, and T-cell function was normal by assessment with PHA and ConA stimulation. Immunoglobulin serum levels were within normal ranges of age-matched controls in all four patients, and none requires IgG substitution (Tables 3, 4). Also each patient had a positive antibody response. NK activity was lower than normal in all but patient 4.

Growth and psychomotor development

As shown in Table 4, all survivors have shown normal height, body mass index (BMI), psychomotor development and performance status to date.

Discussion

We report the outcome of unrelated UCBT in five patients with X-SCID using a RIC regimen. The most important result of this study is all four survivors are free from immunoglobulin replacement therapy.

Previous studies showed that about two-thirds of SCID patients required immunoglobulin replacement therapy after T-cell-depleted, HLA-haploidentical BMT from related donors without pre-transplant conditioning [2, 8]. In Europe, about half of SCID patients who received HLA-haploidentical related marrow cells were conditioned mostly with busulfan (8 mg/kg) and cyclophosphamide (200 mg/kg) [3]. However, the mortality rate for this type of conditioning was higher than that of patients without conditioning. Further, pre-transplant conditioning in combination with HLA-haploidentical related marrow cells did not always result in correction of B-cell function, and about one-third of the SCID patients continue to require immunoglobulin replacement therapy. In contrast, all surviving SCID patients, who had received bone marrow cells from unrelated donors after conventional conditioning with busulfan (16 mg/kg) and cyclophosphamide (200 mg/kg), did not require immunoglobulin replacement therapy [5, 6]. This conventional conditioning regimen, however, has been associated with a significant mortality rate due to treatment-related toxicities such as profound pancytopenia, severe organ toxicity, and exacerbation of pre-existing infections. In addition, children treated with myeloablative regimens often suffer from delayed effects such as infertility, hormonal dysfunction, growth failure and secondary malignancies [9]. Recently, Rao et al. [6] reported the outcome of 33 patients with primary immunodeficiency (PID) [SCID ($n = 6$) and non-SCID ($n = 27$)] who received unmodified unrelated donor marrow grafts following reduced-intensity conditioning consisting of fludarabine (150 mg/m^2), melphalan (140 mg/m^2), and alemtuzumab (Campath 1H) or anti-thymocyte globulin (ATG). All patients had primary engraftment, and most patients achieved normal immunoglobulin production and B-cell function, although it is not clear whether patients with SCID were on immunoglobulin replacement therapy or not. From these

Table 4 Current status

Patient	1	2	3	4	5
Clinical status	Alive	Alive	Alive	Alive	Dead (at 17 months)
Follow-up (months)	68	48	73	69	17 months
Last i.v.Ig (months)	44	32	8	3	17 months
i.v.Ig at present	Off	Off	Off	Off	NA
Height	−1.0 SD	+1.92 SD	−1.0 SD	−0.2 SD	Short stature
Body mass index	15.9	14.5	14.5	15.2	BW 6 kg
Mental status	Normal	Normal	Normal	Normal	Normal
Karnofsky performance status	100 %	100 %	100 %	100 %	30 %

i.v.Ig intravenous immunoglobulin, *NA* not applicable, *SD* standard deviation, *BW* body weight

results, we speculated that T-cell depletion might interfere with B-cell engraftment and function. In this context, it is interesting to note that patients in our study who had acute GVHD complications showed higher B-cell chimerism and early immunoglobulin production after UCBT. However, one of our patients succumbed to sepsis in conjunction with severe GVHD. Unlike patients with hematologic malignancies, who benefit from the graft-vs-leukemia effect of donor cells, there is no such benefit from GVHD in patients with PID [10]. Thus, it is inevitable to use immunosuppressive drugs to prevent GVHD, and modifications such as the addition of ATG to our protocol to reduce the risk of GVHD will need to be evaluated in a future study [11]. Of note, two of our patients who did not develop acute GVHD gradually corrected their B-cell function, and immunoglobulin replacement therapy could be discontinued 32 and 44 months after UCBT. These results suggest that the RIC regimen described here may provide a minimal-intensity conditioning regimen in combination with UCB, which can assure sufficient production of immunoglobulin.

Some reports have raised concern about cardiac toxicity associated with high-dose melphalan and fludarabine used in combination [12, 13]. However, patients with this adverse event had been suffering from advanced hematologic malignancies and had been heavily treated with cytotoxic drugs including anthracyclines prior to pre-transplantation conditioning, and the total dosage of fludarabine (150 mg/m^2) and melphalan (140 mg/m^2) used for conditioning was much higher than the present study. In addition, reduction of melphalan from 140 to 80 mg/m^2 is expected to result in a lower frequency of cardiac toxicity. We only observed mild myelosuppression and mucositis as adverse events of the RIC regimen. Engraftment of unrelated cord blood cells, which might not be achieved with lower concentration of melphalan, was observed in all patients in our study. To date, none of our patients has shown any delay in growth or mental development. Long-term follow-up is necessary to validate the efficacy and safety of this RIC regimen.

Regarding B-cell engraftment and function, T-cell depletion from related donor bone marrow cells may not be a suitable source of HSCT for PID patients who do not have an HLA-identical sibling donor as described above. Recently, it was reported that UCB from unrelated donors could be used successfully for patients with PID [14, 15]. As UCB contains T cells, faster emergence of donor T cells is expected even though the infused T cells are functionally naïve. UCB recipients were able to discontinue immunoglobulin replacement therapy sooner and more frequently compared with T-cell-depleted bone marrow recipients although the estimated 5-year over all survival rates were comparable when UCB recipients received a myeloablative conditioning regimen [15]. In addition, UCBT is more tolerant of HLA disparity because the incidence and severity of GVHD is lower than for unrelated BMT. These results together with ours support the application of UCBT for patients with X-SCID who do not have an HLA-identical sibling donor.

Another risk factor for a poor outcome using HSCT for SCID is a pre-existing infection [8]. In our patients, all but one were diagnosed with X-SCID at birth from their family histories, and they had been kept in a protective environment for 3 months until they received UCBT. There are two reasons why we performed UCBT at the age of 3 months. One is to minimize regimen-related toxicities because infants are more susceptible to cytotoxic drugs, and the other is to expect higher survival rate after transplantation in the first 3.5 months of life as described previously [2, 16]. Early diagnosis before any infectious episodes is necessary for safe HSCT in the patients with SCID. Recently, screening of newborns for SCID has been recommended [17], and the RIC regimen described above in combination with UCBT is an alternative to HLA-haploidentical BMT for such patients.

In conclusion, our regimen in combination with UCBT is well tolerated and resulted in normal immunoglobulin production and B-cell function in our patients. Future studies with a modification of GVHD prophylaxis for patients with X-SCID who do not have an HLA-matched

sibling donor will be needed to further improve the outcome.

Acknowledgments We thank Dr. David Cosman for critical reading of this manuscript. This study was supported by the Japanese Research Group on Primary Immunodeficiency Diseases, supported by the Ministry of Health, Labour and Welfare in Japan.

Conflict of interest The authors declare no conflict of interest.

References

1. Fischer A, Le Deist F, Hacein-Bey-Abina S, André-Schmutz I, Basile Gde S, de Villartay JP, et al. Severe combined immunodeficiency. A model disease for molecular immunology and therapy. Immunol Rev. 2005;203:98–109.
2. Railey MD, Lokhnygina Y, Buckley RH. Long-term clinical outcome of patients with severe combined immunodeficiency who received related donor bone marrow transplants without pretransplant chemotherapy or post-transplant GVHD prophylaxis. J Pediatr. 2009;155:834–40.
3. Antoine C, Müller S, Cant A, Cavazzana-Calvo M, Veys P, Vossen J, et al. Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968–99. Lancet. 2003;361:553–60.
4. Haddad E, Deist FL, Aucouturier P, Cavazzana-Calvo M, Blanche S, Basile GD, et al. Long-term chimerism and B-cell function after bone marrow transplantation in patients with severe combined immunodeficiency with B cells: a single-center study of 22 patients. Blood. 1999;94:2923–30.
5. Grunebaum E, Mazzolari E, Porta F, Dallera D, Atkinson A, Reid B, et al. Bone marrow transplantation for severe combined immune deficiency. JAMA. 2006;295:508–18.
6. Rao K, Amrolia PJ, Jones A, Cale CM, Naik P, King D, et al. Improved survival after unrelated donor bone marrow transplantation in children with primary immunodeficiency using a reduced-intensity conditioning regimen. Blood. 2005;105:879–85.
7. Grubwieser P, Zimmermann B, Niederstätter H, Pavlic M, Steinlechner M, Parson W. Evaluation of an extended set of 15 candidate STR loci for paternity and kinship analysis in an Austrian population sample. Int J Leg Med. 2007;121:85–9.
8. Buckley RH. B-cell function in severe combined immunodeficiency after stem cell or gene therapy: a review. J Allergy Clin Immunol. 2010;125:790–7.
9. Sanders JE, The Seattle Marrow Transplant Group. Effects of bone marrow transplantation on reproductive function. In: D'Angio GJ, Green DM, editors. Late effects of treatment for childhood cancer. New York: Wiley-Liss Publishing Ltd; 1992. p. 95–101.
10. Burroughs LM, Storb R, Leisenring WM, Pulsipher MA, Loken MR, Torgerson TR, et al. Intensive postgrafting immune suppression combined with nonmyeloablative conditioning for transplantation of HLA-identical hematopoietic cell grafts: results of a pilot study for treatment of primary immunodeficiency disorders. Bone Marrow Transpl. 2007;40:633–42.
11. Hatanaka K, Fuji S, Ikegame K, Kato R, Wake A, Hidaka M, et al. Low incidences of acute and chronic graft-versus-host disease after unrelated bone marrow transplantation with low-dose anti-T lymphocyte globulin. Int J Hematol. 2012;96:773–80.
12. Ritchie DS, Seymour JF, Roberts AW, Szer J, Grigg AP. Acute left ventricular failure following melphalan and fludarabine conditioning. Bone Marrow Transpl. 2001;28:101–3.
13. Morandi P, Ruffini PA, Benvenuto GM, Raimondi R, Fosser V. Cardiac toxicity of high-dose chemotherapy. Bone Marrow Transpl. 2005;35:323–34.
14. Morio T, Atsuta Y, Tomizawa D, Nagamura-Inoue T, Kato K, Ariga T, Japanese Cord Blood Bank Network, et al. Outcome of unrelated umbilical cord blood transplantation in 88 patients with primary immunodeficiency in Japan. Br J Haematol. 2011;154:363–72.
15. Fernandes JF, Rocha V, Labopin M, Neven B, Moshous D, Gennery AR, et al. Transplantation in patients with SCID: mismatched related stem cells or unrelated cord blood? Blood. 2012;119:2949–55.
16. Straathof KC, Rao K, Eyrich M, Hale G, Bird P, Berrie E, et al. Haemopoietic stem-cell transplantation with antibody-based minimal-intensity conditioning: a phase 1/2 study. Lancet. 2009;374:912–20.
17. Buckley RH. The long quest for neonatal screening for severe combined immunodeficiency. J Allergy Clin Immunol. 2012;129:597–604.

J Clin Immunol (2013) 33:1018–1026
DOI 10.1007/s10875-013-9897-y

ORIGINAL RESEARCH

A Non-invasive Diagnosis of Histiocytic Necrotizing Lymphadenitis by Means of Gene Expression Profile Analysis of Peripheral Blood Mononuclear Cells

Masataka Ishimura · Hiroyuki Yamamoto · Yumi Mizuno · Hidetoshi Takada · Motohiro Goto · Takehiko Doi · Takayuki Hoshina · Shouichi Ohga · Koichi Ohshima · Toshiro Hara

Received: 12 January 2013 / Accepted: 9 April 2013 / Published online: 23 April 2013

Abstract Histiocytic necrotizing lymphadenitis (HNL), also called Kikuchi-Fujimoto disease, is a benign, self-limiting inflammatory disease with fever and painful cervical lymphadenopathy of unknown etiology. A lymph node biopsy is required for the definitive diagnosis because of no specific symptoms or laboratory findings for HNL. To establish the rapid non-invasive diagnostic method for this disease, we investigated genes specifically expressed in the patients by analyzing whole transcriptome using microarray analysis of peripheral blood mononuclear cells (PBMC). The top five up-regulated genes (*IFI44L*, *CXCL10*, *GBP1*, *EPSTI1* and *IFI27*) in HNL were interferon-induced genes (ISGs). The expression levels of the up-regulated genes by microarray were verified by quantitative PCR. High levels of serum CXCL10 concentration were confirmed at the symptomatic phase of HNL patients. The expression levels of these 5 genes positively correlated with each other (r^2=0.28–0.60). The genes were also highly expressed in HNL lymph nodes. The discriminant analysis using the expression levels of these five genes distinguished HNL with 84 % accuracy. The combination of up-regulated ISGs in HNL seemed to be a specific response induced by viral infections or autoantigens. An analysis of the gene expression profile of PBMC may provide a rapid non-invasive diagnosis of HNL.

Keywords Histiocytic necrotizing lymphadenitis · Kikuchi-Fujimoto disease · interferon-stimulated genes · gene expression · discriminate analysis

Abbreviations

ACTB	Beta actin
ADV	Adenovirus
AUC	Area under the curve
cDNA	Complementary DNA
C_T	Threshold cycle
CXCL10	Chemokine (C-X-C motif) ligand 10
EPSTI1	Epithelial stromal interaction 1 (breast)
FluA	Influenza type A virus
GBP1	Guanylate binding protein 1 interferon-inducible
HNL	Histiocytic necrotizing lymphadenitis
IFN	Interferon
IFI27	Interferon alpha-inducible protein 27
IFI44L	Interferon-induced protein 44-like
IL	Interleukin
IM	Infectious mononucleosis

Masataka Ishimura and Hiroyuki Yamamoto contributed equally to this paper.

M. Ishimura (✉) · H. Yamamoto · H. Takada · M. Goto · T. Doi · T. Hoshina · S. Ohga · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: ischii@pediatr.med.kyushu-u.ac.jp

Y. Mizuno
Fukuoka Children's Hospital and Medical Center for Infectious Diseases, Fukuoka, Japan

S. Ohga
Department of Perinatal and Pediatric Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

K. Ohshima
Department of Pathology, School of Medicine, Kurume University, Kurume, Japan

ISG	Interferon-stimulated gene
KD	Kawasaki disease
LNitis	Purulent lymphadenitis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
SLE	Systemic lupus erythematosus
SoJIA	Systemic onset juvenile idiopathic arthritis

Introduction

Histiocytic necrotizing lymphadenitis (HNL), also called Kikuchi-Fujimoto disease, is a benign, self-limiting inflammatory disease with fever and tender cervical lymphadenopathy of unknown etiology [1, 2]. Severe HNL patients with hemophagocytic syndrome or prolonged fever need immunosuppressive therapy [3]. It is necessary to distinguish it from other febrile diseases with lymphadenopathy including leukemia, malignant lymphoma, infectious mononucleosis (IM), purulent/tuberculous lymphadenitis, Kawasaki disease (KD), systemic juvenile idiopathic arthritis (SoJIA), and systemic lupus erythematosus (SLE). Lymph node biopsies are required for the definitive diagnosis because of no specific symptoms or laboratory findings including imaging tests for HNL.

Histologic findings of involved lymph nodes include paracortical areas of coagulative necrosis with abundant karyorrhectic debris. Karyorrhectic foci consist of histiocytes, plasmacytoid dendritic cells, immunoblasts, and lymphocytes [1, 2]. Neutrophils and plasma cells are absent or scarce. Assessed by immunohistochemical analysis, histiocytes are positive for myeloperoxidase and CD68 antigen, and lymphocytes are predominantly $CD8^+$ cytotoxic T cells [1, 2, 4]. In the earlier stages, histiocytes and plasmacytoid dendritic cells (pDCs) are usually prominent in the lesions, suggesting that these cells have a close correlation with the pathogenesis of HNL [1, 2, 5]. By immunohistochemical analyses, it has been suggested that perforin and Fas pathways play important roles in the induction of apoptosis and necrotizing lesions [6, 7]. It was supported by the findings of microarray analysis which showed up-regulation of apoptosis- and cell cycle-associated genes in lymphnodes of HNL patients [8].

Some HNL cases were reported to occur in association with viral infections (e.g. Epstein-Barr virus, human herpes virus type 6, and human T-lymphotropic virus type 1) or autoimmune disease (SLE) [1, 9]. Increased serum concentrations of interferon (IFN)-γ, interleukin (IL)-6, and 2',5'-oligoadenylate synthetase as well as pyrexia and extranodal involvement of skin, bone marrow, and liver in the patients suggest the exaggerated systemic inflammatory response to viral pathogens or to autoantigens in the pathophysiology of HNL [10, 11].

To establish a rapid non-invasive diagnostic method for HNL, and to characterize the molecular pathophysiology of the disease, we investigated the gene expression profile of peripheral blood mononuclear cells (PBMC) by microarray analysis and performed discriminate analysis.

Materials and Methods

Patients

Twenty-four patients with HNL participated in this study. The clinical features of the patients are shown in Table I. The specimens were obtained from the patients before or without the treatment with steroids or immunosuppressive drugs etc. In addition, 93 disease controls and 34 healthy donors were included in this study (Table II). Cervical lymph node samples of patients with HNL (n=9) and reactive lymphadenopathy (n=4) were provided from Department of Pathology, Faculty of Medicine, Fukuoka University, Japan. For the analysis of serum concentrations of CXCL10, blood samples were obtained from 12 patients with HNL, 5 with KD, 4 with IM, and 5 with bacterial lymphadenitis. All patients were diagnosed by trained pediatricians and pathologists, according to the clinical manifestations, laboratory findings, and histological examinations. Informed consent was obtained from all participants for this study, according to the process approved by the Ethical Committee of Kyushu University, Fukuoka, Japan.

Total RNA Extraction and RNA Amplification

PBMC were separated from peripheral blood by density gradient centrifugation using Lymphocyte Separation Medium (MP Biomedicals LLC, CA, USA). Total RNA was extracted from PBMC or lymph nodes cells using an RNA extraction kit (Isogen) (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. An Amino Allyl MessageAmp aRNA Kit (Life Technologies, CA, USA) was used to amplify the total RNA.

Microarray Analysis

In order to characterize the gene expression profile of PBMC in HNL patients, microarray analysis of PBMC was performed using an AceGene Human Oligo Chip 30 K (Hitachi Solutions, Tokyo, Japan) that contains approximately 30,000 genes. The arrays were scanned by FLA-8000 (Fujifilm, Tokyo, Japan), and changed to the numerical values by ArrayVision Software (GE Healthcare, Buckinghamshire, UK). The numerical data were normalized using the LOWESS method, as described previously [12]. In the microarray analysis of PBMC, data from 2 patients with HNL, 5 with SoJIA, 3 with KD and a healthy donor were compared. Genes that were consistently up-regulated in PBMC of HNL patients, compared with patients with SoJIA or KD and a healthy donor, with more than