The crosstalk between dying tumor cells and immune effectors within tumor microenvironment elicited by anti-cancer therapies dictates the therapeutic outcome

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UNIVERSITÉ PARIS-SUD 11

Faculté de Médecine Paris Sud

Année 2011 N° attribué par la bibliothèque

THESE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ PARIS-Sud 11

Champ Disciplinaire: Immunologie

Ecole Doctorale de rattachement: Cancérologie

Présentée et soutenue publiquement

Par

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Le 28 Juin 2011

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Acknowledgement

I would like to thank my mentor Pr Laurence ZITVOGEL for all her guidance and inspiring discussions during my thesis. I appreciate her encouragement to “look beyond what I see” and her help to explore my potential in science. I benefited a lot from our nice collaboration with Pr Guido KROEMER. His innovation, efficiency, devotion to science and elegance of reasoning set an example for me to continue my scientific research.

I appreciate Pr Mark J SMYTH’s passionate support and encouragement at the beginning of my scientific career. I would like to thank Pr Pablo PEREIRA, Pr Goro MATSUZAKI and Pr Koichi IKUTA who helped me get started with γδ T17 project.

I would like to acknowledge Pr Pierre GALANAUD, Pr Olivier LANTZ, Pr Jean-Jacques FOURNIÉ, Pr Marc BONNEVILLE and Pr Francine JOTEREAU who devoted their precious time reviewing my thesis, attending my thesis defense and providing valuable suggestions.

I would take the chance to thank China Scholarship Council for their financial support during my French PhD. I wish to express my gratitude to Pr Feili GONG, my previous mentor in China, who brought me into the field of medical research and inspired me in both science and life.

I enjoyed the pleasant collaboration with Noelia CASARES, Oliver KEPP, Ana ROMERO, Takahiro YAMAZAKI, Stephen MATTAROLLO, Daniel M ANDREWS, Rosa CONFORTI, Antonella SISTIGU, Dalil HANNANI, Isabelle MARTINE and Mickael MICHAUD. I would thank all my colleagues in INSERM U1015 for their support and accompany. I appreciate the invaluable help from Dr Patrick GONIN, Karine SER-LE ROUX, Melanie POLROT, Benoit PETIT, Justine DENIAU in the animal facility of Institut Gustave Roussy during my thesis.

Last but not the least, I wish to thank my parents Fangping MA and Laxia XU for their best gift of life, their whole-hearted love and unconditional support since I was born. It is my greatest joy to share all my honors and achievements with them!

This manuscript is dedicated to my beloved husband and soulmate Dr Heng YANG for his ten years of accompany, adoration, understanding, comfort and help!

Résumé

En dehors des effets cytostatiques ou cytotoxiques sur les cellules tumorales, certaines thérapies anti-cancéreuses (anthracyclines, l'oxaliplatine, rayons X) peuvent déclencher la mort cellulaire immunogénique, libérant ainsi les signaux de danger pour alerter le système immunitaire. Nous avons montré que les cellules T $CD8^+$ T (Tc1) productrices d'IFN-γ et spécifiques de la tumeur sont nécessaires pour le succès de la chimiothérapie et la diminution de la croissance tumorale. L'amorçage d'une réponse bénéfique Tc1 dépend de la sécrétion d'IL-1β par les cellules dendritiques confrontées à des cellules tumorales traitées avec de l'anthracycline libérant de l'ATP. Afin d'identifier les composants inflammatoires qui lient les réponses immunitaires innées et adaptatives, nous avons analysé l'influence de la chimiothérapie immunogène sur le microenvironnement de la tumeur. Nous avons identifié une up-régulation de gènes associés à la réponse Th1 et Th17 dans un modèle de tumeur répondant au traitement par les anthracyclines par un retard de croissance. En interférant avec les voies IFN-γ ou l'IL-17A, l'effet thérapeutique de la doxorubicine et l'oxaliplatine a été aboli dans ce modèle et le vaccin à base de cellules tumorales mortes a perdu de son efficacité à protéger les souris de la réintroduction de cellules tumorales vivantes. Nous avons également découvert que des sous-populations distinctes de lymphocytes T $\gamma\delta$ ($V\gamma4^+$ et $V\gamma6^+$) colonisent des tumeurs peu de temps après la chimiothérapie, où ils ont proliféré et sont devenus producteurs majeurs de l'IL-17 au sein de la tumeur. Nous avons constaté une forte corrélation entre la présence de lymphocytes T $\gamma\delta$ producteurs d'IL-17 ($\gamma\delta$ T17) et de TIL $CD8^+$ (Tc1) dans trois modèles différents de tumeurs traitées par la chimiothérapie ou la radiothérapie. IL-17A agit sur la signalisation en amont de l'IFN-γ puisqu'un défaut d'expression d'IL-17RA conduit à la perte complète de la production des Tc1 spécifiques de l'antigène. La contribution des cellules $\gamma\delta$ T17 ($V\gamma4^+$ et $V\gamma6^+$) dans l'effet bénéfique de la chimiothérapie est essentielle puisque les souris $V\gamma4/6^{-/-}$ ont montré une réduction de leur sensibilité à la chimiothérapie et la vaccination

anti-tumorale et l'infiltration tumorale par les cellules γδ T17 et Tc1 a été réduite au niveau basal chez ces souris. L'axe IL-1β/IL-1R, mais pas IL-23/IL-23R, est essentielle pour la production d'IL-17 par les cellules T et l'effet bénéfique de la chimiothérapie. Le transfert adoptif de lymphocytes γδ T peut rétablir l'efficacité de la chimiothérapie dans le modèle de souris IL-17A$^{-/-}$ et peut améliorer l'effet de la chimiothérapie chez la souris wt, à condition qu'ils conservent l'expression de l'IL-1R et de l'IL-17A. Nos résultats suggèrent fortement l'existence d'un axe fonctionnel: DC (IL-1β) → cellules T (IL-17) → Tc1 (IFN-γ), déclenché par la chimiothérapie induisant la mort des cellules tumorales, un phénomène essentiel pour une réponse thérapeutique favorable.

Pour renforcer la réponse immunitaire, nous essayons aussi de combiner la chimiothérapie « immunogène » avec le vaccin anti-tumoral en présence d'adjuvants (poly (A:U), l'agoniste de TLR3). Ce type de thérapie séquentielle combinée, que nous avons appelé VCT, pourrait retarder considérablement la croissance des tumeurs, voire éradiquer complètement la tumeur et établir une protection à long terme spécifique de la tumeur. Pour décortiquer l'effet de la poly (A:U) sur le système immunitaire et sur les cellules tumorales exprimant le TLR3, nous avons effectué un traitement VCT chez la souris nude, TRIF$^{-/-}$ et les souris présentant une diminution de l'expression de TRIF au niveau des cellules tumorales. Nos résultats montrent que l'effet anti-tumoral de VCT requiert les lymphocytes T et la voie de signalisation TRIF intacte au niveau de l'hôte et des cellules tumorales. Le traitement poly (A:U) peut induire un niveau élevé de production de certaines chimiokines associées à la réponse de type Th1 (CCL5 et CXCL10 notamment) par les cellules tumorales in vitro et in vivo, ce qui peut influencer négativement et positivement les résultats thérapeutiques. Le découplage de l'action de CCL5 et de CXCL10, pourrait améliorer le traitement par la VCT. En résumé, notre étude souligne le rôle des facteurs inflammatoires dérivés de la tumeur et de l'hôte dans la régulation de la réponse immunitaire anti-tumorale. Nous résultats suggèrent également que les applications thérapeutiques des agonistes TLR peuvent être optimisées grâce à la régulation du profil de chimiokines associées à la réponse de type Th1 produites in situ.

Mots-clés:

Tumeur, mort cellulaire, chimiothérapie, réponse immunitaire, leucocytes infiltrant les tumeurs, γδ T, IL-17A, IL-1β, poly (A: U), CCL5, CXCL10

Summary

Besides exerting cytostatic or cytotoxic effects on tumor cells, some anti-cancer therapies (anthracyclines, oxaliplatin, X-Rays) could trigger an immunogenic cell death modality, releasing danger signals to alert immune system. We have shown that tumor-specific IFN-γ producing $CD8^+$ T cells (Tc1) are mandatory for the success of chemotherapy to prevent tumor outgrowth. Priming of Tc1 response depends on IL-1β secretion by DC confronted with anthracycline-treated tumor cells releasing ATP. To identify the inflammatory components which link innate and cognate immune responses, we analyzed the influence of immunogenic chemotherapy on tumor microenvironment. We found an upregulated Th1- and Th17-related gene expression pattern in growth-retarded tumor after anthracycline treatment. By interfering with IFN-γ or IL-17A pathways, therapeutic effect of doxorubicin and oxaliplatin was abolished and dying tumor cell-based vaccine lost its efficacy to protect mice from live tumor cell rechallenge. Interestingly, we discovered that distinct subsets of γδ T lymphocytes ($V\gamma4^+$ and $V\gamma6^+$) colonized tumors shortly after chemotherapy, where they proliferated and became the dominant IL-17 producers within tumor beds. In three tumor models treated with chemotherapy or radiotherapy, a strong correlation between the presence of IL-17-producing γδ T (γδ T17) and IFN-γ-producing $CD8^+$ TIL (Tc1) was discovered. IL-17A signaling acts as upstream of IFN-γ since defect in IL-17RA led to complete loss of antigen specific Tc1 priming. The contribution of γδ T17 cells ($V\gamma4^+$ and $V\gamma6^+$) to chemotherapy is critical as $V\gamma4/6^{-/-}$ mice showed reduced sensitivity to chemotherapy and vaccination. Also, tumor infiltrating γδ T17 and Tc1 cells were reduced to basal level in this strain. IL-1β/IL-1R, but not IL-23/IL-23R, is pivotal for IL-17 production by γδ T cells and the success of chemotherapy. Importantly, adoptive transfer of γδ T cells could restore the efficacy of chemotherapy in $IL\text{-}17A^{-/-}$ mice and ameliorate the effect of chemotherapy in wild type host, provided that they retain the expression of IL-1R and IL-17A. Our research suggest a DC (IL-1β) → γδ T cells (IL-17) → Tc1 (IFN-γ) immune axis triggered by chemotherapy-induced dying tumor cells, which is critical for the favorable therapeutic response.

To boost the immune system, we try to combine immunogenic chemotherapy with tumor vaccine in the presence of TLR3 agonist Poly (A:U). This sequential combined therapy, which we named VCT, could significantly retard tumor growth or even completely eradicate tumor and establish long-term protection against rechallenge in highly tumorigenic models. To dissect the effect of Poly (A:U) on immune system and that on TLR3 expressing-tumor cells, we performed VCT treatment in nude mice, $TRIF^{-/-}$ mice and with TRIF-silencing tumors. Interestingly, our results suggested that anti-tumor effect of VCT required T cells and intact TRIF signaling pathway at the level of the host and that of tumor cells. Poly (A:U) treatment could induce high level of CCL5 and CXCL10 production from tumor cells both in vitro and in vivo, which could negatively and positively influence the therapeutic outcome. By uncoupling the effect of CCL5 from that of CXCL10, the VCT treatment can be ameliorated. Our study emphasizes that both tumor and host derived inflammatory factors participate in regulating anti-tumor response. We also highlight that therapeutic application of TLR agonists can be optimized through regulating the profile of chemokines and their downstream signaling events.

Keywords:

Tumor, cell death, chemotherapy, immune response, tumor infiltrating leukocytes, γδ T, IL-17A, IL-1β, Poly (A:U), CCL5, CXCL10

Abbreviations

ADCC: antibody-dependent cellular cytotoxicity

AhR: aryl hydrocarbon receptor

APC: antigen presenting cells

BAL: bronchoalveolar lavage

BST2: Bone marrow stromal protein 2

Bv8: bombina variagata peptide 8

cDC: conventional DC

CRT: calreticulin

CTL: cytotoxic T lymphocyte

DC: dendritic cells

DETC: dendritic epidermal $\gamma\delta$ T cells

DX: doxorubicin

ER: endoplasmic reticulum

FOXP3: forkhead box P3

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte macrophage colony-stimulating factor

HIF-1α: hypoxia-inducible factor-1α

HMGB1: High mobility group box 1 protein

HSP: Heat shock protein

IDO: indoleamine 2,3-deoxygenase

ILC: innate lymphoid cells

ILT7: immunoglobulin-like transcript 7

IPP: isopentenyl pyrophosphate

IRE1α: inositol-requiring enzyme 1 alpha

LTi: lymphoid tissue inducer cells

LOX-1: lectin-type oxidized LDL receptor 1

LXR: liver X receptor

MAIT: mucosal-associated invariant T cells

MAPK: mitogen-activated protein kinase

MDA5: melanoma differentiation-associated Gene 5

MDR: multidrug resistance

MDSC: myeloid-derived Suppressor Cells

MICA: MHC class I chain–related molecules A

MICB: MHC class I chain–related molecules B

Mo-DCs: monocyte-derived DCs

MMP: matrix metalloproteinase

MSR1: macrophage scavenger receptor 1

MTX: mitoxantrone

MyD88: myeloid differentiation primary response gene (88)

NALP3: NACHT, LRR and PYD domains-containing protein 3

N-BPs: aminobisphosphonates

NTPDase1: ecto-apyrase

NTPDase2: ecto-ATPase

OS: overall survival

OX: oxaliplatine

P2RX7: P2X purinoceptor 7

P2Y2: P2Y purinoceptor 2

PAMP: Pathogen associated molecular pattern

PD-1: programmed Death 1

pDC: plasmacytoid DC

PFS: progression-free survival

PGE2: prostaglandin E2

PI3K: phosphotidylinositol 3-kinase

PLG: poly lactide-co-glycolide

PRRs: pattern recognition receptors

Poly (A:U): Polyadenylic:polyuridylic acid

Poly (I:C): polyinosinic:polycytidylic acid

RIG-I: retinoic acid-inducible gene-I

Rorc: RAR-related orphan receptor C

ROS: reactive oxygen species

SNPs: single nucleotide polymorphisms

STAT3: signal transducer and activator of transcription 3

TAM: tumor-associated Macrophage

Th17: T helper 17 cells

TILs: tumor infiltrating leukocytes

Tim-3: T cell immunoglobulin mucin-3

TLR: Toll like receptor

TSLP: thymic stromal lymphopoietin

TNF-α: tumor necrosis factor-alpha

Treg: regulatory T cells

ULBP: UL-16–binding proteins

VEGF: vascular endothelial growth factor

XBP1: X-box binding protein 1

Introduction

Cancer, a leading lethal disease, could account for around one out of four deaths worldwide. In Europe alone, one in three people will be affected by cancer in their lifetime and approximately half of them will receive chemotherapy. Thus, it is of the utmost importance to understand how anticancer therapies occasionally work and elicit the host immune system for long-term tumor protection, and how to improve and predict the therapeutic effect shortly after chemotherapy.

Immunosurveillance: friend or foe for tumor control?

Immunosurveillance versus immunediting

Immunosurveillance functions as an effective extrinsic tumor-suppressor system which influences the incidence and the clinical outcome of tumors [1]. In Japan, a 11-year follow-up of 3625 healthy people showed that reduced occurrence of common cancers was associated with medium and high cytotoxic activity of peripheral-blood lymphocytes [2]. Cancer may arise under conditions of reduced immune capacity. Immunosuppressive treatments applied in transplant recipients could increase the risk of malignancy and enable accelerated tumor growth by supporting oncogenesis caused by viruses or impaired immune surveillance [3,4]. In a cohort of 23729 female first cadaver kidney recipients who received therapeutic immunosuppression, the observed incidence of 11 major cancer types (except breast cancer) was significantly higher than expected [5]. In a retrospective cohort of 4178 patients undergoing renal replacement therapy, of whom 3592 were exclusively on dialysis and 1821 later had transplants, Birkeland *et al* observed significant excess cancer risks occurred after transplantation but not during dialysis [6]. Recently, it was reported that certain Immunosuppressive treatments display higher risk for post-transplant cutaneous squamous cell carcinoma [7].

Compelling evidences from mouse studies prove that the immune system can indeed protect mice from the outgrowth of various types of primary and transplanted tumors. The

incidence of spontaneous neoplasia upon aging and the susceptibility to MCA carcinogen-induced sarcomas are much more prominent in mice lacking IFN-γ [8-10] or type I IFN responsiveness [11,12], or in immunodeficient mice lacking T cell [13], T and B cell [10,14,15], NK [15,16], NKT [15,17] and γδ T cells [18,19]. The Link between susceptibility to tumorogenesis and defined immunodeficiencies has been comprehensively reviewed in mouse models [20].

The encounter between the immune system and nascent tumors initiates a process termed “cancer immunoediting” which puts functional imprint onto the emerging tumor repertoire. This event brings about three outcomes or stages of tumorigenesis: tumor elimination, tumor equilibrium and tumor escape [21-23]. Host immune system could eliminate some tumor cells but the this process is not always complete, thus surviving tumor variants may enter into the “equilibrium” phase, where tumor cells can become functionally dormant or even remain clinically unapparent throughout the life of the host [22-24]. During this phase, adaptive immune cells (eg. $CD4^+$, $CD8^+$ T cells) and effector molecules (eg. IFN-γ, IL-12) are responsible for preventing tumor outgrowth [23]. Under these selective pressures, some tumor cells eventually acquire further mutations, become more resistant to the immune destruction and therefore escape the immunosurveillance (Figure 1.1). Indeed, a significant portion (40%) of MCA sarcomas derived from immunodeficient $Rag2^{-/-}$ mice were spontaneously rejected when transplanted into naive syngeneic wild type mice, whereas 100% of MCA sarcomas derived from immunocompetent wild type mice grew progressively [10]. In this sense, immune system could facilitate tumor growth through “editing” or selecting tumor cells that are more capable of escaping immune detection, or by establishing the pro-tumoral microenvironment which favors the eventual tumor outgrowth [20,25]. Taken together, these findings suggest the dual host-protective and tumor-sculpting actions of immunity on developing tumors.

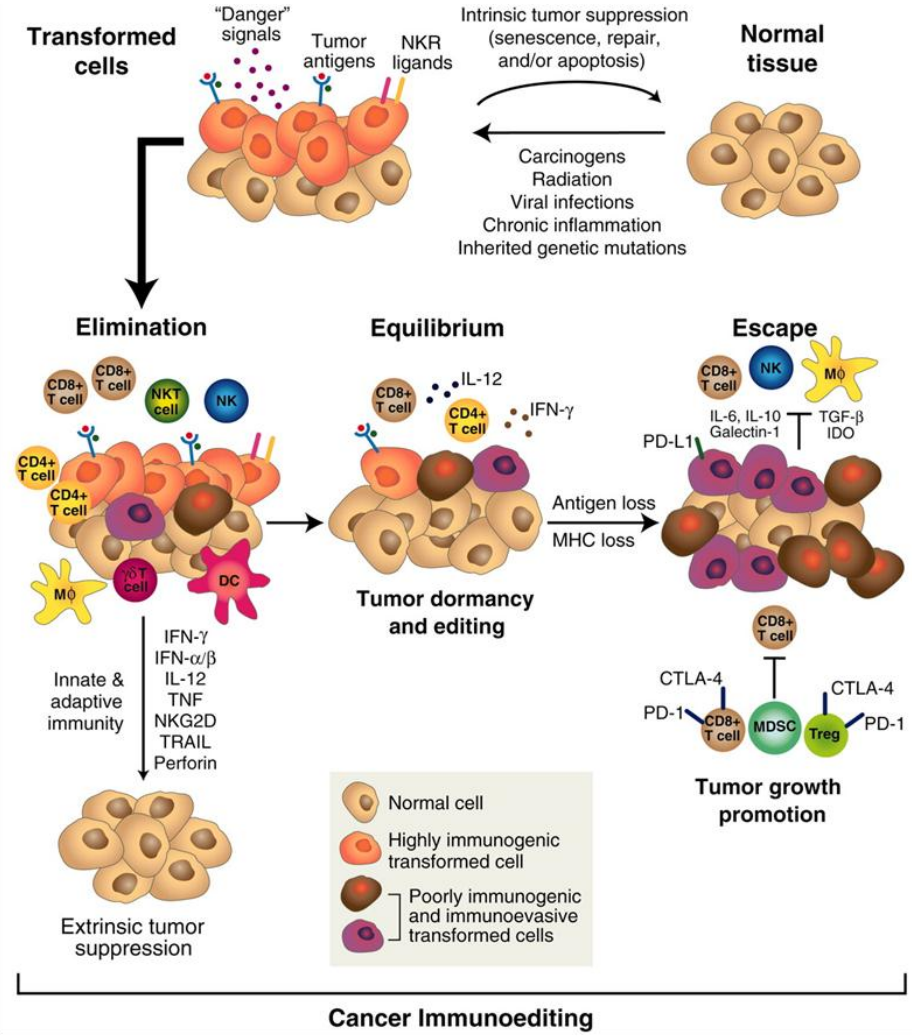

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Figure 1.1 ***Cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape.*** Immune molecules and cells could recognize and eliminate transformed cells to prevent tumor outgrowth. While some rare cancer cell variants are not completely destroyed in the elimination phase. They may then enter the equilibrium phase, accumulating mutations and acquire the ability to avoid immune eradication under the selection pressure of immune system. (figure from [25]).

Role of inflammation in oncogenesis and tumor escape

Some stimuli, such as tobacco smoke, contaminated or unhealthy food, infections, obesity, radiation and environmental pollutants, can induce and promote cancer development [26,27]. One common process induced by all these risk factors is inflammation. Inducers

of inflammation can be exogenous (microbial inducer, allergens, irritants, foreign bodies or toxic compounds) or endogenous (stressed, damaged cells or malfunctioning tissues) [28]. Chronic inflammation have a broad impact on tumor initiation by generating genotoxic stress, on tumor promotion by inducing cellular proliferation, and on tumor progression by enhancing angiogenesis and tissue invasion [26]. Thus, immune system does sometimes inadvertently provide a tumor-prone microenvironment and the causative connection between inflammation and tumors has been mechanistically established [26,29,30].

Up to 20% of all cancers arise in association with persistent and unresolving inflammation and most, if not all, solid tumors contain inflammatory infiltrates [31]. Inflammation, cytokines, and signal transducer and activator of transcription 3 (STAT3) have been classified as the “Unholy Trinity” that shapes the pro-tumorigenic microenvironment [32]. In ovarian, lung and gastro-oesophageal cancer patients, elevated IL-1β, IL-6 and IL-8 have been detected in ascites, bronchoalveolar lavage (BAL) fluid, serum and tumor tissues [33-36], correlating with a poor prognosis [35-43]. Chemical carcinogenesis in MCA-treated mice requires the participation of pro-inflammatory cytokines/signaling, such as IL-1β, IL-23, IL-10 and myeloid differentiation primary response gene (88) (MyD88) [20]. In murine transplantable tumor models, IL-1β derived from the host and that originated from tumor cells is involved in promoting tumor growth, angiogenesis, metastasis, myeloid cells recruitment as well as supporting drug resistance [44-46]. Tumor necrosis factor-alpha (TNF-α), a major inflammatory cytokine that can induce rapid hemorrhagic necrosis and tumor destruction, also plays a paradoxical tumor-promoting role through enhancing oncogene activation, tumor cell invasion, angiogenesis and chemotherapy resistance [47,48]. Plasma TNF-α was increased in various cancer patients, especially those with poor prognosis [49-51]. STAT3 acts as a point of convergence for numerous oncogenic signaling pathways. It is constitutively activated both in immune cells and in tumor cells within the tumor microenvironment. Activation of STAT3 induces upregulation of several key genes involved in cell proliferation and survival (eg. c-Myc, survivin, cyclin D1/D2). It also promotes pro-oncogenic inflammation (IL-6, VEGF, MMP2, MMP9, HIF-1α) and immune suppression (IL-10, TGF-β) while opposing STAT1- and NF-κB-mediated anti-tumor Th1

response (IL-12, CXCL10, IFN-γ, IFN-β, MHC class II, CD80, CD86) [52-54]. Unexpectedly, various factors derived from the tumor microenvironment also contribute to systemic anti-inflammation besides desensitizing local inflammation. Thus, neoplastic disorders are often associated with a defective capacity to mount inflammatory reactions at sites other than the tumor [29].

Dynamic interconnection between pro- and anti-tumor inflammation

Taken together, the crosstalk between tumor and immune system constitutes a paradox in terms of inflammatory response. The nature, timing, intensity and sites of inflammation might explain this apparent contradiction. Chronic inflammation might expedite tumor formation while acute inflammation might well hamper the process [55]. The tumor-promoting and tumor-disruptive inflammation could coexist within the same tumor bearer, even at the same tissue site, and are dynamically interconnected [20,25,56] (Figure 1.2). What's more, the same inflammatory molecule may promote or prevent tumor formation depending on the biological context in the tumor microenvironment [20]. This phenomenon is a vivid proof of the fascinating Yin-Yang dialectics and further investigations should bring us more clues to the proper manipulation of inflammation against cancer.

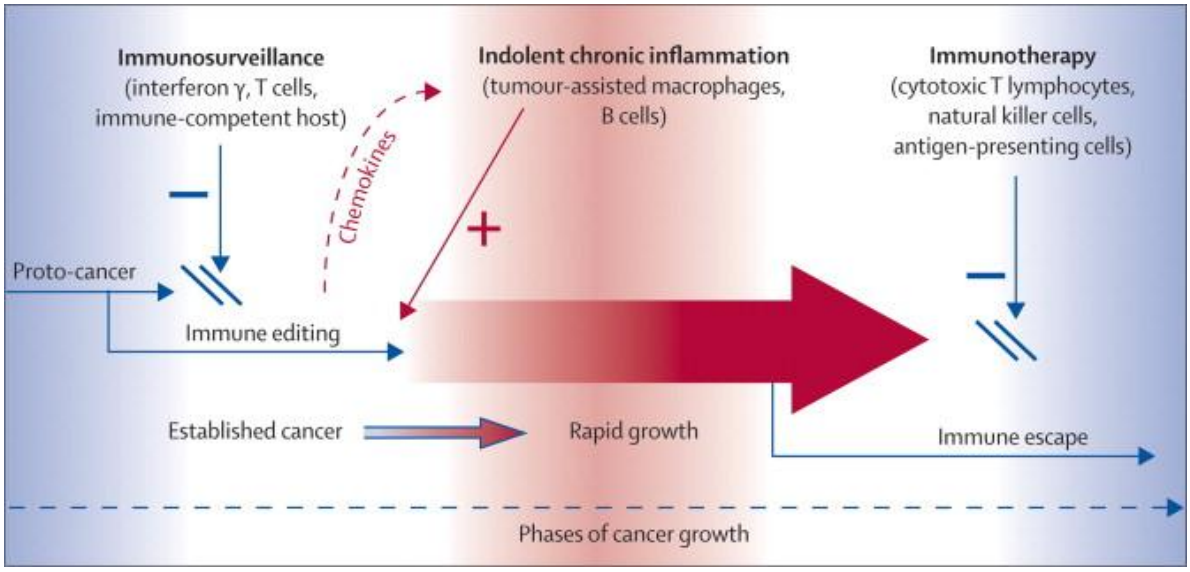

Figure 1.2 ***A schematic diagram of how inflammation participates in the different phases of tumor development.*** It shows how host immune system positively and negatively regulates tumor initiation and formation through immunosurveillance and immunoediting, how chronic inflammation assist rapid tumor progression and how acute inflammation induced by immunotherapy could retard tumor outgrowth [56].

Tumor microenvironment and tumor infiltrating immune cells

Solid tumors are not merely composed of heterogeneous clones of tumor cells. To some extent, tumor formation can resemble the process of tissue remodeling and organ development [57]. They contain multiple cell types (tumor cells, fibroblasts, endothelial cells, blood vessels, lymph vessels and immune cells), soluble factors (cytokines, chemokines and products of cellular metabolism) and extracellular matrix [1,57]. Immune cells infiltrate tumors and make up a significant component of the tumor microenvironment. The complicated cellular and molecular interactions between tumor cells and infiltrating immune cells influence the capacity of tumors to progress and to metastasize.

The impact of tumor infiltrating immune cells and their effector molecules on tumor progression

The type, density, activation status and location of tumors infiltrating lymphocytes could predict patient outcome. A high CD8 and low forkhead box P3 (FOXP3) cell infiltration pattern after neoadjuvant chemotherapy was significantly associated with improved progression-free survival (PFS) and overall survival (OS) [58]. A high infiltration of $CD3^+$, $CD8^+$, Granzyme B^+, and $CD45RO^+$ cells in the center and/or in the invasive margin of the tumors are of good prognosis and prolonged survival [59,60]. A coordinated expression of Th1 specific genes and cytotoxic markers (but not Th2-, immunosuppression-, inflammation- or angiogenesis-related genes) was associated with high density of $CD45RO^+$ memory cells. $CD8^+$ plus $CD45RO^+$ cells provide a useful immune criterion for

the prediction of low tumor recurrence and long-term survival in patients with early-stage colorectal cancer [61]. Furthermore, a significant positive correlations between markers of cytotoxic and effector memory T-cells, and that between markers of innate immune cells and early-activated T cells have been observed in colorectal cancer tumors with high densities of T cells, suggesting a coordinated immune reaction is needed to concentrate the most efficient effectors at the tumor site [62]. Based on the convincing evidence from colorectal tumors studies, Fridman and his colleagues proposed the primary tumor infiltration by memory T cells, particularly of the Th1 and cytotoxic types, as a strong prognostic factor in terms of DFS and OS. This immune scoring could help decision-making regarding the application of adjuvant therapies in early-stage human cancers [63,64]. Interestingly, the presence of tumor-associated lymphocytes in breast cancer is also an independent predictor of response to anthracycline/taxane neoadjuvant chemotherapy [65].

Even if, in general, a high lymphocytic infiltrate remains of good prognosis, tumors could also recruit various immune cells with “Janus face” [66]. NK cells are present in many tumors but elucidation of their role is still needed [67]. B cells infiltrating breast carcinoma show oligoclonal expansion and affinity maturation in situ, representing a tumor-specific humoral immune response [68,69]. In metastatic ovarian carcinoma, however, B cell and NK cell infiltration correlates with poor overall survival of patients [70]. In a mouse squamous carcinogenesis model, adoptive transfer of B cells or serum from K14-HPV16 transgenic mouse could reinstate necessary parameters that promote de novo carcinogenesis [71]. In this model, autoantibodies secreted by B cells are deposited in the tumor stromal and locally activate FcRγ-mediated signaling on resident and recruited myeloid cells to promote premalignant progression [72]. B cell derived IL-10 is also instrumental to induce M2 polarization of tumor-associated macrophages (TAM) [73]. Depending on the Th1- or Th2-like cytokine profile and co-stimulatory milieu, NKT cells could improve or suppress anti-tumor response [74-76]. Functionally distinct populations of NKT cells do exist in tumor models [76]. Whilst Vα14Jα18^{-} type II NKT cells may be responsible and sufficient for the negative regulation of antitumor immunity, Vα14Jα18^{+} type I NKT cells could provide protection against tumor [77,78].

In addition, tumors could hijack regulatory pathways of the host immune system and acquire resistance to immune attack. Tumor microenvironment can be dominated by regulatory T cells (Treg) which create a suppressive milieu [79,80]. High numbers of Treg cells can be detected in tumor bed [81-84], peripheral blood [85,86] and lymphoid aggregates [87], mitigating the immune response against cancer, negatively affecting the clinical disease course. Depleting Treg or inhibiting its immunosuppressive function could enhance anti-tumor response and improve therapeutic effect [88,89]. Myeloid derived suppressor cells (MDSC) are also present in most cancer patients and experimental animals bearing tumors [90], acting as regulators of immune system to facilitate tumor escape [91-93].

Tumor-derived factors impair the function of immune cells

Cancer cells can also instruct immune cells to undergo changes that promote malignancy. Tumor cells can block function of T and NK cells through secretion of soluble ligands of NKG2D [94]. TGF-β production by tumor cells can convert effector T cells into Treg which, in turn, suppress other tumor infiltrating effector T cells [95]. Unknown tumor-derived factors induce upregulation of macrophage scavenger receptor 1 (MSR1) on DCs, resulting in excessive uptake of extracellular lipids which reduces their capacity to process antigens [96]. Human breast cancer cells-derived thymic stromal lymphopoietin (TSLP) could drive the development of IL-13- and TNF-α-producing inflammatory Th2 cells, which are conducive to breast tumor development through inducing OX40 ligand expression on DC [97]. In tumor bearing mice, tumor derived granulocyte colony-stimulating factor (G-CSF) could promote bombina variagata peptide 8 (Bv8) expression in bone marrow $CD11b^+$ $Gr1^+$ cells, facilitating myeloid cell mobilization (especially neutrophils), tumor angiogenesis and promote tumor growth [98]. Bone marrow stromal protein 2 (BST2) released from tumor cells can subvert plasmacytoid DC (pDC) through immunoglobulin-like transcript 7 (ILT7) signaling and make pDC fail to respond to danger signals for type 1 IFN production. Pretreatment with IFN-γ and TNF-α significantly

increased BST2 secretion, suggesting that the inflammatory status of tumor microenvironment support this immunoregulatory pathway [99]. Human and mouse tumors releasing cholesterol metabolites could dampen the expression of CCR7 on maturing DC by triggering liver X receptor (LXR), thereby impairing DC migration and allowing tumor escape [100]. Co-expression of T cell immunoglobulin mucin-3 (Tim-3) and programmed Death 1 (PD-1) on CD8+T cells indicates an exhausting state. While PD-1 ligand (PDL1) and Tim-3 ligand (galectin-9) expression on tumor cells are involved in strengthen $CD8^+$ T cell exhaustion and increase the lethality of advanced acute myelogenous leukemia [101].

Compared with normal tissue, tumor microenvironment possesses several unique physiological features, such as hypoxia, low extracellular pH and high glycolysis. These factors could regulate global gene expression profiles [102] to facilitate metabolic adaptation, resistance to cell death, drug tolerance, angiogenesis and metastasis [103-108], as well as regulating the biological functions of tumor-infiltrating immune cells, such as neutrophils [109], dendritic cells (DC) [110], macrophages, Treg [111] and cytotoxic T lymphocytes (CTLs) [102,112].

Investigations on cell populations, distribution, activation states and functional polarization of immune infiltrates, as well as the tumor-stromal interactions in local microenvironment could provide targets for novel therapeutic approaches.

Immunogenicity of chemotherapy and radiotherapy induced cell death

Chemotherapy and radiotherapy could trigger immune response

Tumor could evolve into an immune privileged site due to its autologous origin, the process of immunoediting and its unique microenvironment [113]. Physiological cell death, which occurs as a continuous byproduct of cellular turnover, is non-immunogenic or even tolerogenic. While massive cell death caused by chemotherapy or radiotherapy might saturate the local capacity of silent corps removal, trigger acute inflammation and anti-tumor immunity. Vaccination with irradiated tumor cells engineered to secret GM-CSF

could recruit DC, plasma cells, invariant NKT cells and tumor reactive $CD4^+$ and $CD8^+$ T cells, both in mice and in metastatic cancer patients, and evoke potent, specific and long-lasting anti-tumor immunity [114]. Neoadjuvant irradiation of cutaneous melanoma tumors prior to surgical resection reduces lung metastases greater than 20-fold [115]. In the absence of any adjuvant, subcutaneously inoculation of dying tumor cells pretreated with doxorubicin or mitoxantrone could prevent tumor growth upon syngeneic live cell rechallenge in immunocompetent mice [116,117]. Local irradiation or chemotherapeutic drug gemcitabine treatment could induce sufficient tumor antigen cross-presentation by tumor stroma which leads to eradication of established cancer [118].

Checkpoints for generating immunogenic cell death and anti-tumor response

In addition to trigger protective antineoplastic immunity, cancer therapy can also trigger an inflammatory response by causing trauma, necrosis, and tissue injury that stimulate tumor re-emergence and resistance to therapy [26]. Whether or not a beneficial immune response can be triggered by chemotherapy is determined by at least four checkpoints [119], including: the intrinsic properties of drugs, the chemo-responsiveness and immunogenicity of tumors, the capability of the host to sense and to react to tumor cell death, and the coordinated infiltration of immune effectors into tumor microenvironment.

The property of the drugs

A striking diversity does exist among anticancer drugs concerning the ability to regulate immune response directly or indirectly. A unbiased functional screen by Tanaka et al. unveiled that most topoisomerase inhibitors and antimicrotubule agents, but not alkylating agents, antimetabolites, platinum-based compounds or hormonal agents, could promote DC maturation [120]. Some 20 different apoptosis-inducing agents that operate through distinct mechanisms were tested for their ability to induce protective immune response. Most of the compounds, including agents that target the endoplasmic reticulum (ER),

mitochondria or DNA did not induce immunogenic cell death. In sharp contrast, anthracyclines (doxorubicin, daunorubicin, idarubicin and mitoxanthrone) were the most potent inducers of immunogenic cell death in several mice tumor models [116,121]. Noticeably, chemotherapeutic drugs could regulate the function of immune effectors or sensitize tumors for the immune eradication [122]. Cytotoxic drugs could stress tumor cells through various signaling pathways, triggering differed death modalities and releasing diverse cell death derived danger signals. Thus, high-throughput screening the immunogenic property of drugs, both chemotherapeutic and other clinical approved ones, is of utmost importance for improving their clinical application [123].

Tumor derived factors and their perception

Whether tumor cells die in response to the therapeutic insult or the cell death occurs in an immunogenic manner [124] is another critical decision-making point. Multidrug resistance (MDR) gene products and genetic changes (eg, Bcl-2, Bcl-xL, Mcl-1 and p53) contributing to the anti-apoptotic pathways could account for the chemoresistance [122].

In the context that robust chemotherapy or radiotherapy could induce tumor cell death, cell death associated “danger signals” are emanated and they could trigger sterile inflammation. The release of positive and negative chemotactic signals and the ensemble of changing cell surface structures influence the choice of the host phagocytes to engulf, activate, differentiate and subsequently to prime the adaptive immunity [125]. The particular assembly of certain cell death-associated molecules originated from dying or dead cells could act like a combinatorial code to unlock antineoplastic inflammatory and immune responses. This “key-lock” paradigm is being progressively unraveled and corresponding counter-acting factors are also being demonstrated, explaining why current chemotherapy only works moderately.

Anthracyclines, oxaliplatin, UVC and γ-radiation could induce pre-apoptotic translocation of calreticulin (CRT) and the disulfide isomerase ERp57 to the tumor plasma membrane which became receptive for engulfment by dendritic cells (DC). Both CRT and

ERp57 exposure have been proved to determine the immunogenicity of cell death [121,126,127]. CD47 interacting with its receptor SIRP-α on macrophages could negatively regulate phagocytosis. It has been shown that pro-phagocytic effects of plasma membrane-exposed CRT are counteracted by the expression of CD47 on the same cell [128].

High mobility group box 1 protein (HMGB1), an abundant nuclear protein and transcription factor, are extruded from apoptotic cells into the extracellular milieu. HMGB1 could alert and recruit inflammatory cells, such as monocyte derived immature DC [129] and neutrophil [130]. Binding of HMGB1 to TLR4 could facilitate tumor antigen cross-presentation by DC [131], and negatively regulate the immunosuppressive function of Treg [132]. The immunostimulatory role of HMGB1 can be modified at the post-transcriptional level. Activation of caspases during apoptosis could induce ROS production which oxidizes HMGB1 and induce immune tolerance [133], albeit oxidized HMGB1 increases the cytotoxicity of chemotherapeutic agents or ionizing radiation and induces cancer cell apoptosis via the mitochondrial pathway [134].

Heat shock protein (HSP) is commonly overexpressed in tumor, probably due to the stressful tumor microenvironment. It could inhibit apoptosis and exhibit cytoprotective activity. Binding of HSP72 to inositol-requiring enzyme 1 alpha (IRE1α) enhances IRE1α/XBP1 (X-box binding protein 1) signaling at the ER and inhibits ER stress-induced apoptosis [135]. HSP72 associated with tumor-derived exosomes could restrain tumor immune surveillance through promoting the suppressive function of MDSC by triggering STAT3 activation in the TLR2/MyD88-dependent manner [136]. Some HSP, such as HSP27, HSP70 and HSP90, are strongly induced upon anticancer drugs treatment, oxidative stress and irradiation. The membrane expression of HSP on stressed or dying tumor cells could be potently immunostimulatory, due to their ability to interact with certain receptors on antigen presenting cells (APC), such as CD91, lectin-type oxidized LDL receptor 1 (LOX-1) and CD40, which facilitate DC maturation, tumor antigens presentation [137-141]. Further, HSP could activate NK cells [142] and act as the immunoadjuvant.

Under physiological conditions, a 10^6-fold gradient between cytosolic and extracellular ATP is maintained as a result of the activities of extracellular ecto-apyrase (NTPDase1)

and ecto-ATPase (NTPDase2), which metabolize ATP into ADP, AMP and adenosine. The release and extracellular accumulation of ATP could promote immune cell activation and pro-inflammatory responses [143,144]. In response to chemoattractants, neutrophils release ATP from the leading edge of the cell surface to amplify chemotactic signals and promote orientated cell migration, in an autocrine feedback manner through P2Y2 and A3 receptors [145]. ATP could also provide a costimulatory signal to T cells during their brief encounter with APCs [146]. It is also involved in driving the differentiation of intestinal T helper 17 cells (Th17) [147]. Importantly, in the setting of chemotherapy, ATP released from dying tumor cells could trigger the activation of NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome through binding P2X purinoceptor 7 (P2RX7) on DC, cumulating in caspase-1 activation and IL-1β secretion which is indispensible for the anti-tumor CTL priming [117]. However, NTPDase1 and NTPDase2, expressed on tumor cells [148], Treg [149] and endothelial cells [150] may quench this potent immunostimulatory factors. In addition, ATP can also have anti-inflammatory effects, especially when extracellular ATP is generated chronically and at low concentrations [143].

Cell death derived danger molecules could indeed promote inflammation and their contribution to the success of chemotherapy is determined by their proper perception by the host immune system. These findings might ultimately lead to an algorithm which could predict anticancer immune responses elicited by chemotherapy or radiotherapy and provide instructions to induce the “desirable” cell death.

Hereditary factors from the host

The host intrinsic factors also present a checkpoint for chemotherapy to eradicate tumors. Several single nucleotide polymorphisms (SNPs) have been associated with the sensibility of tumor to chemotherapy. A sequence polymorphism in Tlr4 (896A/G, Asp299Gly, rs4986790) affecting the extracellular domain of TLR4 resulted in the impaired capacity of monocyte-derived DCs (Mo-DCs) to cross-present melanoma

antigens to CTLs. In breast cancer patients (n = 280) treated with local surgery, local radiotherapy and systemic anthracycline injections (FEC protocol: (Fluorouracil (5FU), epirubicin and cyclophosphamide), those who bear this loss-of-function allele of TLR4 developed metastasis more rapidly. And metastatic colorectal cancer patients (n = 338) bearing this SNPs undergoing an oxaliplatin-based regimen manifested a reduced PFS and OS [131]. A loss-of-function polymorphism that affects P2RX7 (Glu496Ala, rs3751143), lowering its affinity for ATP and thus IL-1β release in human monocytes, decrease the beneficial effect of anthracyclines to control metastasis in breast cancer [117]. The prognostic value of the SNPs in MMP genes is analyzed with 349 primary lung cancer patients. Tumor stage IIIB carrying MMP2 C-735T variant allele showed a significantly worse response. While the PFS and OS was significantly prolonged in MMP1 G-1607GG variant allele carriers and MMP12 A-82G variant allele bearers respectively in small cell lung cancer patients [151]. These studies strongly suggest the ability of host to sense tumor cell death and to create a beneficial environment for launching anti-tumor immunity is of vital importance for the therapeutic success. Verified results from these studies should be considered as criteria for personalized treatment in the future.

Coordinated function of immune cells and effector molecules

Immunogenic cell death and its perception modulate the immune contexture of tumor microenvironment, which also acts as a critical checkpoint for the therapeutic success. A series of immunosuppressive factors, such as S100A9 associated with MDSC, prostaglandin E2 (PGE2), IL-6, TGF-β, CXCL8, lL-10, gangliosides, reactive oxygen species (ROS), indoleamine 2,3-deoxygenase (IDO) and extracellular adenosine, have been described to interrupt the tumor→DC→T cell cascade, with STAT3 acting as the “evil core” [152]. This immunsuppresive host-tumor equilibrium is reversed during the course of successful chemotherapies (Figure 1.3).

Innate and cognate immune responses elicited by anti-cancer agents are required for an optimal therapeutic outcome. Indeed, tumoricidal activity of oxaliplatin or 10-Gy

irradiation against transplanted tumors was completely abolished in mice deficient for the recombination activating protein 2 (Rag2, which lack T, B and NKT cells), in athymic nu/nu mice (which lack T cells), and in wild type mice depleted of $CD8^+$ lymphocytes [117]. Using CD11c-DTR transgenic mice in which conventional DC could be depleted by diptheria toxin, it was found that DC mobilized by doxorubicin-treated dying tumor cells were indispensable to elicit specific anti-tumor immunity [116]. DC derived IL-1β was shown to be a key cytokine which gears the Tc1 polarization of TCR-triggered $CD8^+$ T cells and IFN-γ is indispensible for the tumor control by chemotherapy [117].

Immune effectors need to work in a coordinated fashion to achieve long-term protection by tumor vaccination, as well as to maximize tumor eradication. The cooperative functional pathways exist between various DC subsets [153]. Macroporous poly lactide-co-glycolide (PLG) matrices co-delivering GM-CSF, CpG-ODN and tumor lysates could recuit pDC, $CD8^+$ DC and $CD11b^+$ DC and potently prime local and systemic $CD8^+$ cytotoxic T lymphocyte (CTL). This tumor vaccine could completely eliminate distant and established melanoma and both pDC and $CD8^+$ conventional DC (cDC) are necessary for generating this protective antitumor immunity [154]. $CD11b^+$ stromal cells could capture tumor antigen from surrounding cancer cells, thus eliminating bystander antigen-loss tumor variants [155,156], in the IFN-γ- and TNF-α-dependent manner [157]. During this process, the cooperation between $CD4^+$ and $CD8^+$ T cells is mandatory because $CD4^+$ T cells were needed not only for optimal CD8+ T cell activation but also at the effector stage within the tumor microenvironment [158]. The crosstalk between NKT, $\gamma\delta$ T, NK and DC could also deliver substantial help for DC maturation and T cell priming [152] (Figure 1.3).

It is generally assumed that antitumor immune responses are generated and controlled in the tumor draining lymph nodes. However, the tumor microenvironment may contain lymphoid-like structures that could regulate local adaptive immune responses. In CRC and lung cancer, adjacent tertiary lymphoid structures, composed of mature dendritic cells (T and B cells organized as germinal centers), have been postulated as the site of tumor-initiated immune reaction [64]. Tumor infiltrating-bronchus associated lymphoid tissues (Ti-BALTs), orchestrated as DC-$Lamp^+$/$CD4^+$ T cell clusters surrounded by $CD20^+$ B-cell follicles, have been recently described in non-small cell lung cancers endowed with

favorable clinical outcome [159]. Lymphotoxin α, β and their receptor (LTβR), lymphoid tissue inducer (LTi) cells, dendritic cells, B and NK22 cells have been reported as contributive to tertiary lymphoid structures formation [160-164]. It remains to be investigated which cells are involved in tumor antigen uptaken and the priming of anti-tumoral adaptive immunity in tumor microenvironment.

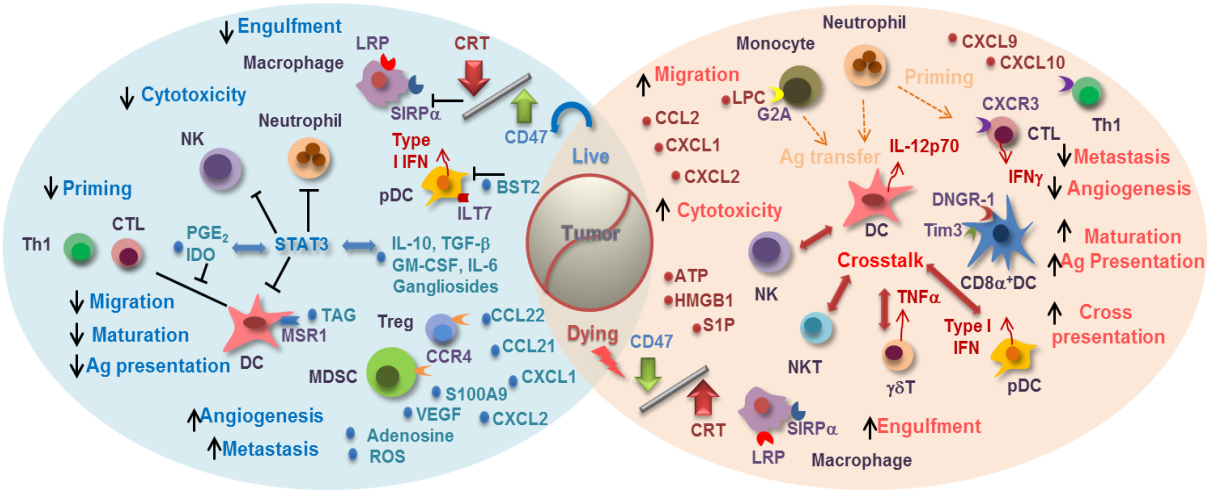

Figure 1.3 ***The Yin-Yang dialogue between tumor cells and immune cells.*** Live tumor cells produce or express a variety of metabolites or proteins that subvert the capacity of bona fide APC to initiate tumor-specific T cell responses (at all levels: engulfment, recruitment, differentiation, migration, activation, cross-presentation), contribute to activate MDSC or Treg competing against effector T cells and directly promoting angiogenesis or metastases (blue circle). In contrast, tumor cell death (intrinsically or extrinsically triggered) might either reenforce tumor-induced tolerance (via engulfment by inflammatory phagocytes and/or tolerogenic molecular pathways) or instead, reset immune responses by exposing appropriate 'cell death-associated molecular patterns' which recruite key innate effectors, reboot APC functions and T cell polarization (red circle).

Objectives of research

In this study, we are intended to investigate the immune-relevant genes expression profile and the dynamic changes of the frequency, composition, activation status and repertoire

of tumor infiltrating leukocytes (TILs). We also assessed the possibility to use certain immune signatures to predict therapeutic outcome of chemotherapy, and to develop strategies for compensating insufficient immune stimulation and augmenting therapeutic effect of conventional chemotherapy.

We are attempted to address these questions:

- ✧ What are the key mediators (immune cells and inflammatory molecules) during immunogenic chemotherapy?
- ✧ How does immunogenic chemotherapy modify the tumor microenvironment?
- ✧ What are the links and crosstalk among immunogenic cell death, innate and adaptive immunity triggered by chemotherapy?
- ✧ What are the factors to activate tumor infiltrating leukocytes? Are they critical or sufficient?
- ✧ The possible strategies to restore or potentiate anti-tumor immunity.

Materials and Methods

Mice. Wild type C57BLl/6 (H-2^b) and BALB/c (H-2^d) mice aged between 7-12 weeks were purchased from Harlan (Gannat, France). Nude mice were bred in the animal facility of IGR or obtained from the Centre d'élevage Janvier, the Mollegaard Breeding and Research Centre. TCR $\delta^{-/-}$, IL-1R1$^{-/-}$ and IL-17RA$^{-/-}$(H-2^b) mice were bred at CDTA, Orléans, France through BR and PP (as for TCR $\delta^{-/-}$). IL-23p19$^{-/-}$ and IL-17A$^{-/-}$ (H-2^b) were kindly provided by MJS. Vγ4γ6$^{-/-}$ mice (H-2^b) were kindly provided by GM and KI. CD1d$^{-/-}$ and CCR6$^{-/-}$ (H-2^b) were bred at St Vincent de Paul Hospital AP-HP, Paris, France and provided by KB. TRIF$^{-/-}$, CXCR3$^{-/-}$,CCR5$^{-/-}$ mice were bred at CNRS IEM 2815, Orléans, France and INSERM U543, Paris, France. Experimental protocols were approved by the Ethics Committee in the animal facility of Institut Gustave Roussy.

Genotyping. Mouse tail DNA was extracted with Maxwell® 16 Instrument. PCR was performed with GoTaq® Green Master Mix (Promega). For IL-17A KO genotyping, a 195bp PCR product can be obtained from WT or HE mice with the primers (forward) 5'-TCTCTGATGCTGTTGCTGCT-3' and (reverse) 5'-CGTGGAACGGTTGAGGTAGT-3'.

Cell lines. CT26 (H-2^d) colon cancer, MCA205 (H-2^b) and MCA2 (H-2^d) sarcoma, TS/A mammalian cancer (H-2^d) and EG7 thymoma (H-2^b), B16-OVA melanoma (H-2^b) cells were cultured in RPMI1640 containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES at 37°C, 5% CO_2. Murine GL26 glioma cells (H-2b) were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 10 mM HEPES and 50 µM beta-mercaptoethanol. Human breast cancer primary cultures were established at Institut Gustave Roussy from metastatic patients suffering from ascitis after informed consent. Cells were used after three passages propagation in AIM-V culture medium. All media were purchased from GIBCO, France.

Reagents. Recombinant mouse IL-1β, IL-23, IL-6, TGF-β and IL-18 BPd/Fc were from R&D system. AhR antagonist CH223191 was from Calbiochem. Doxorubicin hydrochloride (D1515), mitoxantrone dihydrochloride (M6545) and DiOC6(3) were from Sigma Aldrich. Mouse IL-17A, IL-1β, IL-23p19 ELISA kits were purchased from eBioscience. Mouse ELISA kits and neutralizing antibody for IL-22 (AF582) (AB108C as isotype control) were purchased from R&D system. Antibodies for CD45.2 (104), CD3ε (145-2C11 or 17A2), CD4 (GK1.5), CD8α (53-6.7), TCR δ (GL-3), CD69 (H1.2F3), IL-17A (TC11-18H10) or IFN-γ (XMG1.2) were from BD bioscience or eBioscience. Anti-SCART2 polyclonal serum was provided by Dr Jan Kisielow, Swiss Federal Institute of Technology (ETH), Switzerland. Neutralizing antibodies for IL-17A (MAB421), IFN-γ (XMG1.2), CCL20 (MAB760), IL-23 (AF1619), IL-23R (MAB1686) and IL-6 (MAB406) were from R&D system. CpG oligodeoxynucleotide (ODN) 1668 was from MWG Biotech AG. Anti-TGF-β peptide P17 and control peptide were from JJL [165].

Poly(A:U) was obtained from Innate Pharma (Marseille, France). The murine type I interferon was produced by M.F (Istituto Superiore di Sanità, Rome, Italy). Human interferon α2b and ELISA kits for CCL5 and CXCL10 were from R&D Systems, Europe (Lille, France). Ovalbumin holoproteins were from Calbiochem (France Biochem, Meudon, France). CpG oligodeoxynucleotide (ODN) 1668 was purchased from MWG Biotech AG (Ebersberg, Germany). Culture medium, fetal bovine serum and antibiotics were obtained from GIBCO (Invitrogen, France) or Sigma Chemicals (St. Louis, MO, USA). MetRantes was kindly provided by A.P (Merck Serono Geneva Research Center, Switzerland).

Tumor models and chemo/radiotherapy. 8×10^5 MCA205, EG7, CT26, TS/A or MCA2 tumor cells were inoculated s.c. near the thigh into syngeneic mice. Chemotherapy was performed in MCA205 and CT26 models by intratumoral injection of DX (2 mM, 50 μl, i.t) or OX (5 mg/kg body weight, i.p) when tumors reached the size of 25-45mm^2. Radiotherapy was performed by local X-ray irradiation (10 Gy, RT250, Phillips) at the unshielded tumor area when TS/A tumor reached a size 40-60 mm^2.

3×10^5 B16-OVA or 6×10^5 GL26 cells were inoculated s.c. into the left flank of C57BL/6 mice. Vaccines were composed either of CpG ODN 1668 (5 μg/mouse) plus Ovalbumin (1

mg/mouse) or cells (10^6 B16-OVA or GL26) pretreated with type I IFN (1000 IU/ml) for 18hrs and then 20μM of doxorubicin for 24 hrs. Vaccines were injected into the right footpad (for CpG ODN) or right flank (for cell-based vaccines). Chemotherapy (oxaliplatin) was applied i.p. at 5mg/kg. Poly(A:U) was injected i.p. at 100 μg/mouse in B16-OVA model and at 500 μg/mouse in the GL26 model. MetRantes (10 μg/mouse) was injected i.p. daily for 3 weeks to block CCL5. Necrotic cells (F/T) were obtained following 2 consecutive cycles of freezing (in liquid nitrogen) and thawing (at 37°C). For pre-immunization, CpG ODN 1668 (5 μg/mouse) plus Ovalbumin (1 mg/mouse) were injected into the right footpad 7 days before inoculation of tumor cells. To neutralize CXCR3, Anti-CXCR3-173 neutralizing monoclonal Ab and the control monoclonal Ab (PIP) were injected i.p. at 200 μg/mouse every other day for 12 days starting from 5 days before challenging with live tumor cells.

***Gene Expression Assays*.** Whole RNA was extracted using RNeasy Mini Kit, QIAGEN from tumor homogenates. 5 μg of RNA from each sample were reverse-transcribed using Quantitect Reverse Transcription Kit (QIAGEN). Gene expression assays were performed with custom TaqMan® Low Density Arrays using StepOnePlus™ Real-Time PCR System. PPIA was chosen as the endogenous control to perform normalization between different samples.

Tumor dissection and FACS analysis. Tumor burdens were carefully removed, cut into small pieces and digested in 400 U/ml Collagenase IV and 150 U/ml DNase I for 30 min at 37°C. Single cell suspension was obtained by grinding the digested tissue and filtering through 70 μM cell strainer. Cells were blocked with 10 μg/ml anti-CD16/CD32 (eBioscience) before surface staining (2.5 μg/ml of each antibody). LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was used to distinguish live and dead cells. For intracellular staining, freshly isolated cells were treated with 50 ng/ml PMA, 1 μg/ml ionomycin and Golgi-stop (BD Pharmingen) for 4hrs, at 37°C in RPMI containing 2% mouse serum (Janvier, France). Cells were then stainied with anti-IFN-γ and anti-IL-17A using BD Cytofix/Cytoperm™ Kit.

Protein extraction. Tumors were mechanically dissociated with electronic homogenizer in lysis buffer (T-PER Tissue Protein Extraction Reagent, PIERCE) containing protease inhibitor (complete Mini EDTA-free, Roche). Tumor lysate was then centrifuged at 10000×g, 5min, 4°C to obtain supernatant. The supernatant were either tested freshly or aliquoted and stored at -80°C.

Purification and adoptive transfer of γδ T cells. The skin-draining LNs (inguinal, popliteal, superficial cervical, axillary and brachial LNs) were harvested from naïve mice (8-12 weeks). Dead cells were removed from single cell suspension (Dead Cell Removal Kit) before γδ T cells purification (TCRγ/δ$^+$ T Cell Isolation Kit) using AutoMACS™ Separator (Miltenyi Biotec) with recommended programs. Purity of this isolation normally reached above 95%. The TCR δ$^-$ CD3$^+$ cell fraction was also collected and used as 'non γδ T cells for some experiments. Day 2 after chemotherapy, 2.5×10^5 cells were injected directly into the tumor with insulin syringes for the adoptive transfer setting.

T cell priming and tumor vaccination. EG7 cells pretreated with 5 μg/ml OX overnight or left untreated were washed thoroughly and injected at 1 million/50 μl into the foodpad of naïve syngeneic mice. CpG/OVA (5 μg CpG+1 mg OVA/mouse) and PBS injection were used as positive and negative controls. In some setting, neutralizing antibody (200 μg/mouse) for IL-17A or CIg was injected i.p. 5 days later, the popliteal lymph node cells were harvested, seeded in a 96 well plate at 3×10^5/well and restimulated with 1 mg/ml OVA protein. IFN-γ secretion was measured by OptEIA™ Mouse IFN-γ ELISA kit (BD Bioscience). MCA205 cells were treated with 2 μM MTX overnight, washed thoroughly and injected into left flank s.c. at 0.3 million/mouse. PBS was used as control. Mice were rechallenged with 5×10^4 live MCA205 cells in the right flank 7 days later. Tumor growth was monitored every 2-3 days.

DC-tumor mixed lymphocyte cultures. DC were propagated in Iscoves's medium (Sigma Aldrich) with J558 supernatant (containing 40ng/ml GM-CSF), 10% FCS, 100

IU/ml penicillin/streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (Sigma) and used between day 8 and 12 when the proportion of CD11c/MHC class II+ cells was > 80%. In mixed cocultures, DC were seeded at 10^5/100 μl/well in U bottom 96 well plates. Tumor cells were treated overnight with 25μM DX or left untreated, washed and used at 7.5×10^4/100 μl/well. 2×10^4/50 μl γδ T cells were added 12 hrs later. Supernatant was collected 36 hrs later.

***In vitro tumor stimulation with type I IFN and TLR3L assays*.** 5×10^4 B16-OVA or GL26 and 2×10^5 primary human breast cancer cells were seeded in 24 well plates, treated with 1000 IU/ml of type I interferon for 18hrs, then washed and cultured with fresh medium or medium containing poly(A:U) for 48hrs. Supernatants were collected to dose production of CCL5 and CXCL10 by ELISA.

Lentivirus based shRNA construction. The lentivirus construction and viral particules were designed and produced by Vectalys SA (Labège, Toulouse, France). As for the lentivirus carrying the shRNA knocking down RANTES, the forward primer 5'-CGC GAC GTC AAG GAG TAT TTC TAT TCA AGA GAT AGA AAT ACT CCT TGA CGT TTT TTT GCA-3' and the reverse primer 3'- TGC AGT TCC TCA TAA AGA TAA GTT CTC TAT CTT TAT GAG GAA CTG CAA AAA A-5' were annealed and ligated into lentiviral vector (pLV-H1-EF1-PURO-IRES-GFP (pV2.3.127)) containing a RNA polymerase III promoter, by cohesive MluI/NsiI ligation to generate pLV-H1-shRANTES-EF1-PURO-IRES-GFP vector. A similar technical approach was used to generate control pLV-H1-shLaminA/C-EF1-PURO-IRES-GFP designed to knock down Lamin A/C expression (forward primer: 5'-CGC GGA AGG AGG GTG ACC TGA TAT TCA AGA GAT ATC AGG TCA CCC TCC TTC TTT TTT GCA-3'; reverse primer: 5'-AAA AAG AAG GAG GGT GAC CTG ATA TCT CTT GAA TAT CAG GTC ACC CTC CTT C-3').

Statistical analyses of experimental data. All results are expressed as mean ± SEM or as ranges when appropriate. For two groups, normal distributions were compared by

unpaired t test. Non-normal samplings were compared using the Mann-Whitney's test or Wilcoxon matched paired test when appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curve. Statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA). P values of <0.05 were considered significant.

Results and Discussion

Part I. The contribution of IL-17 producing γδ T cells during chemotherapy and radiotherapy

This works has been published in The Journal of Experimental Medicine (2011 Mar 14;208(3):491-503. PMID: 21383056) as attached in annex 3.

Chemotherapy induced the expression of immune genes in tumor bed

Doxorubicin (DX) has been identified as one of the most potent chemotherapeutic drugs to induce immunogenic cell death according to our previous work. And it could significantly control the outgrowth of MCA205 sarcoma. To identify the profile of triggered immune response in tumor bed, we first selected 40 immune genes (including transcription factors, chemokines, cytokines and cytokine receptors) and compare gene expression in DX versus PBS treated tumor with quantitive RT-PCR using Custom TaqMan® Array 96-Well Plates. Interestingly, we noticed from the unsupervised hierarchical clustering analysis that Th1 response related genes expression (Eomes, Tbx21, IFN-γ, Ltxb, Ccl5, Cxcl10, Cxcl9, and Tnf) were significantly upregulated. Another set of genes encoded IL-7R, IL-21, AhR, CXCL2, and Foxp3 were also overexpressed, correlating with Th17 response. The expression of some IL-1 family members (IL-1α, membrane-bond and soluble form of IL1RL1) or immunosuppressive factors (IL-10, Foxp3, IL-27) were also enhanced, suggesting a new balance between inflammation and anti-inflammation factors has been triggered by chemotherapy. While Ccl2, Il6, RAR-related orphan receptor C (Rorc), Ccl25, Cxcl1 expression were not changed compared with PBS group (Figure a). To confirm this finding at the protein level, we dosed IFN-γ and IL-17A, representative cytokines for Th1 and Th17 response, within tumor lysate by ELISA, or with single cell suspension dissociated from tumor by FACS. Indeed,

IFN-γ and IL-17A secretion was enormously increased 8 days after DX treatment as shown by both ELISA and intracellular staining. Interestingly, more IL-17A accumulated in tumor bed only 3 days after chemotherapy, indicating that IL-17A and IFN-γ related response initiated at different phases and could be originated from separate sources (shown in published Figure 1A-E).

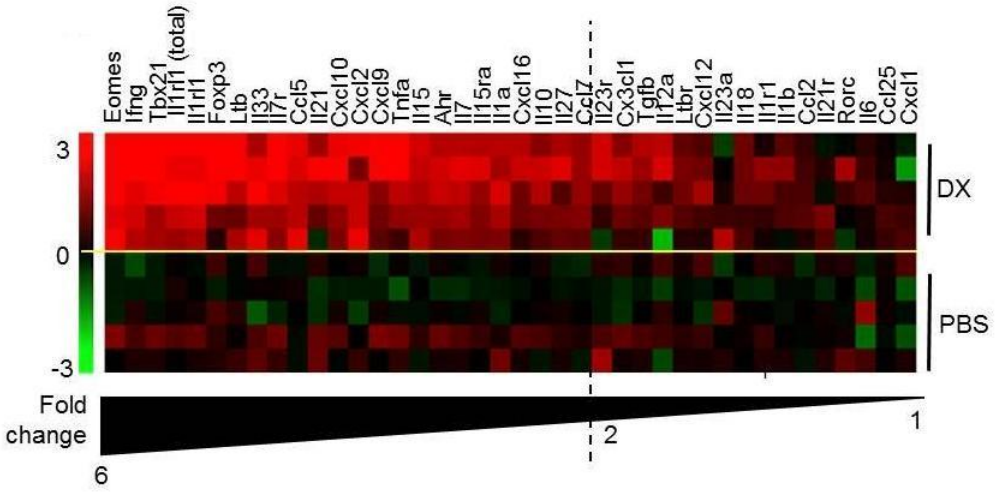

Figure a. ***Gene expression pattern in MCA205 tumor bed 8 days after DX versus PBS treatment.*** Relative gene expression normalized with Ppia is shown as a heat map and the fold change above 2 is used as the threshold of significance.

The source of IL-17A and IFN-γ in tumor bed triggered by chemotherapy

The role of IL-17 in generation of cytokine and chemokine responses, induction of antimicrobial proteins, recruitment for neutrophils and monocytes to the inflammatory site and triggering of adaptive immunity has emerged [166]. IL-17 and other Th17-related cytokines are involed in multiple pathological processes [167,168], such as autoimmune diseases (EAE/multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus) [169-171], inflammatory skin and bowel disease [172,173], transplant rejection [174-176], host defense against infection [177], AIDS pathogenesis [178] and cancer [179].

Both innate and adaptive immune cells could be the source of IL-17, activated through various signal pathways in several pathological processes. $CD4^+$T [167], Treg [180,181], $CD8^+$ T [182-184], $CD4^-$ NKT [185,186], γδ T [187,188], mucosal-associated invariant T

cells (MAIT) [189], NK [190], neutrophils [191], eosinophils [192] and several newly discovered innate lymphoid cells (ILC) including lymphoid tissue inducer cells (LTi) [193-195] and LTi-like cells [196] have been reported capable of secreting IL-17. A αβ-TCR^{+} CD4^{-}CD8^{-}T cell population can also produce IL-17 in both human and mouse [197,198].

By careful analysis with a combination of cell surface markers, we revealed that IL-17 was originated from CD3^{+} CD45^{+} TILs rather than from tumor cells. The majority of IL-17A-producing lymphocytes were CD3^{bright}, a phenotype indicating that they were most likely to be γδ T cells [199]. A reverse gating on all IL-17A^{+} cells confirmed that most of them (60-70%) do co-express CD3 and TCR δ, but not CD4, CD8 or Gr1. CD8^{+} T cells were the major contributor of IFN-γ. It is interesting that γδ T cells exclusively produce IL-17A but rarely IFNγ and we could not see IFN-γ and IL-17 co-expressing cells (shown in published Figure 1F-G). Since the remaining 20-30% of the IL-17 producing cells did not express CD8 and they are partially CD4^{+}, it is possible that Th17 and probably αβ-TCR^{+} CD4^{-}CD8^{-} T and/or CD4^{-} NKT cells constitute this fraction.

The contribution of IFN-γ and IL-17A during chemotherapy

Consistently, IFN-γ and IL-17 production by tumor-infiltrating CD8^{+}T and γδ T cells were substantially enhanced by DX (shown in published Figure 1F-G and S2A). These findings can also be extended to CT26 colon cancer and TS/A mammalian cancer models treated with DX and radiotherapy. In TS/A model, it should be noted that the accumulation of both Tc1 and γδ T17 lymphocytes occurred only in regressing tumors responding to radiotherapy but not in progressing tumors (shown in published Figure 3A-F). Thus, it remains possible that coordinated Tc1 and γδ T17 infiltration in tumor bed after chemotherapy or radiotherapy could be a beneficial prognostic factor for therapeutic response.

IFN-γ is widely recognized to be contributive for immunosurveillance and it plays a central role in coordinating anti-tumor immune responses [200,201]. IFN-γ could restrain

the outgrowth of chemically induced primary tumors and transplanted tumors. It could enhance MHC class I expression on tumors and promote their recognition by tumor-specific T cells. IFN-γ exerts anti-proliferative and pro-apoptotic effect on tumor cells and it could induce angiostasis [202]. While it could also upregulate classical and nonclassical MHC class I expression on tumor cells, which promote the activation of inhibitory NK receptors (such as CD94/NKG2A) on NK cells and CTLs and suspended their license to kill [203].

In the context of chemotherapy, genetic defects in IFN-γ or IFN-γR or IFN-γ neutralization with antibody totally abolished the efficacy of oxaliplatin and doxorubicin against EG-7/EL-4 thymoma and CT26 colon cancer [117]. By system administration of IFN-γ neutralizing antibody (clone XMG1.2, 200 μg per injection for total 3 injections, every other day since the day of DX treatment), we confirmed the critical role of IFN-γ during anthracycline DX-based treatment (shown in published Figure 4A). In the follow-up research (collaboration with Mark J Smyth's lab), we proved that deleting $CD8^+$ T cells (with CD8α- or CD8β- specific antibody), the cellular source of IFN-γ, also resulted in failure of chemotherapy. The IFN-γ/$CD8^+$T-dependency of chemoefficacy could be generalized to other transplanted tumor models, such as C57BL/6-derived AT3 and E0771 tumors and BALB/c-derived H2N100 mammary adenocarcinoma and MCA2 fibrosarcoma, as well as MCA induced primary tumors (Mattarollo SR, Cancer Res. 2011, accepted).

The function of IL-17A/IL-17R pathway in the context of cancer is quite controversial [204,205]. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators, including vascular endothelial growth factor (VEGF), IL-6 and PGE_2, which markedly promote inflammation and tumor angiogenesis [206]. IL-17-overexpressing human derived cancer cells showed greater ability to form tumors in immunocompromised mice [207,208]. Poor immunogenic MCA205 murine fibrosarcoma transfected with retroviral vector carrying mouse IL-17 showed enhanced tumorigenic growth in syngenic immunocompetent as well as irradiated mice, suggesting the critical role of non-hematopoietic derived factors. And the level of Th17 cells was positively correlated with microvessel density in tumors [209]. In contrast, IL-17-transduced Meth-A

fibrosarcoma cells did not show any differences in tumor growth when inoculated subcutaneously into BALB/c nude mice while they were rejected in conventional BALB/c mice, due to their augmented expression of MHC class I and class II antigens and induction of tumor-specific antitumor immunity [210].

Mast cells, MDSC-derived IL-17A and Treg-erived IL-9 acted as a positive feedback loop to strengthen immunosuppression in tumor microenvironment [211]. In B16 melanoma and MB49 bladder carcinoma where $CD4^+$ T cells were the source of IL-17A, this cytokine promoted IL-6 production by IL-17 receptor-bearing tumor cells and tumor-associated stromal cells, activating STAT3 which could up-regulate pro-survival and pro-angiogenic genes [212]. STAT3 masters the balance between IL-23 and IL-12 favoring the procarcinogenic immune responses [213]. However, still in B16 melanoma, in vitro polarized tumor-specific Th17 exhibited stronger therapeutic efficacy than Th1 cells upon adoptive transfer. These Th17 cells converted into effective IFN-γ producers after infusion [214]. In the tumor model which B16-F10 melanoma were injected intravenously, IL-17A deficient mice exhibited increased numbers of tumor foci and larger tumor size. Adoptively transferred Th17 cells retained their IL-17-producing signature and triggered the expansion, differentiation, and tumor-homing of tumor-specific $CD8^+$ T cells, exhibiting stronger therapeutic efficacy than Th1 cells [215]. IL-17 could induce Th1-type chemokines CXCL9 and CXCL10, recruiting effector T cells to the tumor microenvironment. And IL-17 in tumor ascites was reported as a significant predictor of patient survival [216]. Inhibiting pDC derived immunoregulatory enzyme IDO induced in situ conversion of Treg to the Th17 phenotype, which markedly enhanced the activation and antitumor efficacy of $CD8^+$ T cells [217]. IL-17-producing $CD8^+$ T cells could differentiate into long-lasting IFN-γ producers and reduce the volume of large established tumors [218]. In contrast, Kwong et al. described a tumor-promoting, IL-17-producing TCR $\alpha\beta^+CD8^+$ tumor infiltrating subset with regulatory potential and reduced cytotoxicity in the two-stage chemical cutaneous carcinogenesis model [219]. Therefore, the heterogeneous source, differed doses, varied target cells in the microenvironment and different stages of disease determine the biological function of IL-17, especially in cancer.

Whether conventional anticancer therapies could modulate IL-17 secretion and/or Th17

polarization, and whether IL-17 contribute to anti-tumor response remain to be explored [220]. In IL-17A deficient mice, or by neutralizing IL-17A or blocking IL-17R, we found that the therapeutic effect was completely abolished in MCA205, MCA2 treated with doxorubicin, and EG-7, CT26 treated with oxaliplatin (shown in published Figure 4A-E), suggesting IL-17 acted as an indispensible anti-tumor factor. Reinforcing this discovery, we confirmed that IL-17A/ IL-17RA signaling is also critical to control tumor outgrowth of AT3, H2N100 and E0771 mammary gland carcinoma in DX based chemotherapy. And this conclusion also held true in the treatment of MCA induced primary carcinoma (Mattarollo SR, Cancer Res. 2011, accepted). We noticed that IFN-γ, but not rIL-17, could significantly suppress tumor proliferation in vitro (data not shown), indicating that IL-17 exert its tumor-retarding effect indirectly through regulating the inflammatory tumor microenvironment. These data suggest that though IL-17 can be involved in pro-tumoral chronic inflammation and angiogenesis, it is a critical acute inflammatory factor for inducing protective anti-tumor immunity when massive tumor cell death occur upon the lethal hit of chemotherapy and radiotherapy.

The role of γδ T cells and associated cytokines during chemotherapy

γδ T cells are involved in both immunosurveillance and immunoregulation [221]. They are recognized as one of the most potent antitumor cytolytic mediators since they could kill a vast repertoire of tumors cell lines, primary tumor samples and tumor stem cells in a major histocompatibility complex-independent manner [222]. Both human and mouse γδ T cells could mediate antibody-dependent cellular cytotoxicity (ADCC) [223-225]. They produce cytokines like IFN-γ and IL-17 rapidly and promote inflammation, partly due to their inherent epigenetic and transcriptional programs [226]. Certain subsets of γδ T cells also possess the antigen-presenting ability, such as human $V\gamma9V\delta2^{+}$ T cells [227-230] and murine dendritic epidermal γδ T cells (DETC) [231,232]. Similar with other T cell populations, γδ T cells also show regulatory activities. They are involved in inflammation resolution [233-235]. Distinct subsets of γδ T cells have non-overlapping

functional potentials and the outcome depends on their functional balance [236]. Interestingly, γδ T cells were found as the dominant producers of IL-17 in both ischemic brain injury and bleomycin induced lung injury models while the “same” population exerted completely opposite effects by either promoting or controlling inflammation [235,237].

The contribution of γδ T cells in tumor immunosurveillance is still elusive [238]. In C57BL/6 mice, γδ T cells strongly protected the host against chemically induced cutaneous malignancy in a NKG2D-dependent manner [18,239]. γδ T cells were recruited quickly to the tumor sites and provided an early source of IFN-γ which was critical to induce tumor antigen-triggered αβ T cell response [19]. Tumor inifiltrating IL-17-producing γδ T mediated the therapeutic effect of BCG treatment against bladder cancer through recruiting neutrophils [240]. However, this population was supportive for tumor progression through promoting angiogenesis in fibrosarcoma- or skin carcinoma-bearing BALB/c mice while IL-23, IL-6, TGF-β, TCR and NKG2D contributed to the IL-17 production [241]. γδ T cells have been suggested as suppressor cells in early tumor formation. They secreted IL-10 and TGF-β, but not IL-4 or IFN-γ, which attenuated the activity of CTLs and NK cells [242,243]. In human, $V\delta1^+$ γδ T cells infiltrating breast tumors suppressed naive and effector T cell responses and blocked the maturation and function of dendritic cells [244]. On the contrary, they were associated with a reduced occurrence of cancers in transplanted patients bearing a CMV infection [245,246], and with long-term relapse-free survival after bone marrow transplantation [247]. $V\delta2^+$ γδ T cells can be activated by various synthetic ligands to produce Th1-like cytokines, exhibit cytotoxic functions against tumors [248] and mediate anti-tumor effects in patients [249,250]. In summary, the cell subsets, effector molecules and corresponding activating factors in the microenvironment, the stage of cancer and the genetic background of the host determined the anti- or pro-tumoral behaviour of γδ T cells.

γδ T17 cells have been reported to share most phenotypic markers with Th17 cells (expressing CCR6, RORγt, AhR, IL-23R, IL-17A, IL-22) [251]. They are unrestricted by Vγ usage (although they are mostly Vγ4 in the context of mycobacteria [251] and experimental autoimmune encephalitis [252]. Recent work suggests that thymic selection does little to constrain γδ T cell antigen specificities, but instead determines their effector

fate. When triggered through the T cell receptor, ligand-experienced cells make IFN-γ, strongly self-reactive cells make IL-4 whereas ligand-naïve γδ T cells produce IL-17 [188,253,254]. CD27 also regulates γδ T cells differentiation. $CD27^+$ γδ thymocytes expresse LTβR and genes associate with a Th1 phenotype, in contrast to $CD27^-$ γδ thymocytes which give rise to IL-17-producing cells [255]. It is reported that IL-17- and IFN-γ-producing γδ T cells express CD25 (IL-2Rα) and CD122 (IL-2Rβ) respectively. CD25 expression may be downregulated on γδ T cells that have experienced antigen in the thymus, making them less sensitive to endogenous IL-2 production [256]. IFN-γ-secreting γδ T cells in the periphery also express NK1.1, whereas IL-17-secreting γδ T cells express CCR6, which is also known to be expressed by Th17 cells [257]. Finally, the scavenger receptor SCART2 is highly expressed on IL-17 producing γδ T cells homing to the peripheral LN and dermis. $SCART2^{high}$ cells are enriched for Vγ4 but strong TCR stimulation leads to SCART2 down-regulation [258].

To analyse the functional relevance of IL-17-producing γδ T cells (that we termed "γδ T17") in cancer, we performed phenotype of these tumor infiltrating cells after chemotherapy. Most γδ T17 TILs showed an effector memory phenotype (preponderantly $CD69^+$ granzyme B^+ $CD44^+$ $CD62L^-$). They did not express CD27, CD122, CD24, c-kit or NKG2D and they were negative for Scart 2 and CCR6, perhaps due to activation-induced down regulation (Figure. b). Ectonucleotidase CD39 (ENTPDase1) and ecto-5'-nucleotidase CD73, which are responsible to generate the immunosuppressive and pro-angiogenic extracellular adenosine [259], were highly expressed on tumor infiltrating Treg while γδ T17 TILs were $CD39^{low}$ (Figure. c).

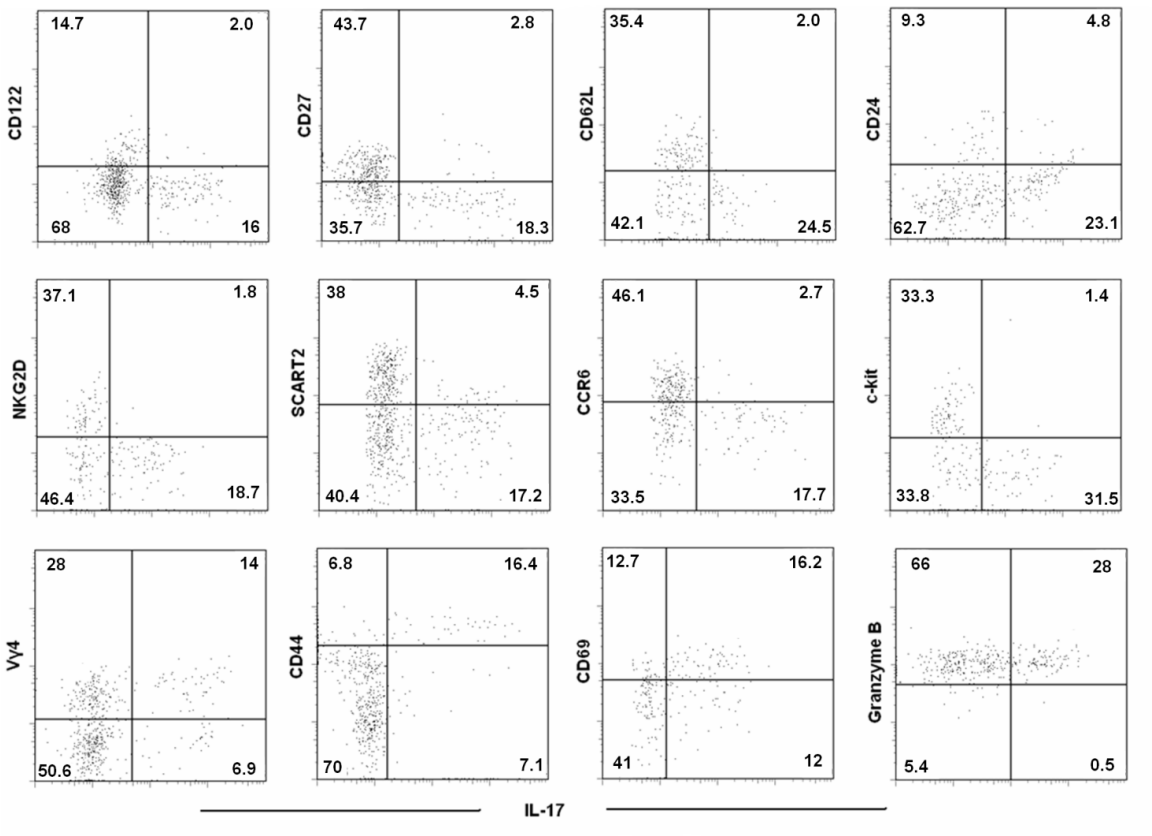

Figure b. ***Phenotype of tumor infiltrating γδ T cells after DX therapy.*** MCA205 tumors were analyzed 8 days post-DX. After gated on live $CD45.2^{+}CD3^{+}TCR\ \delta^{+}$ cells, IL-17 production versus certain molecule expression was analyzed. A typical dot plot is depicted. The experiment has been performed 3 times yielding similar results.

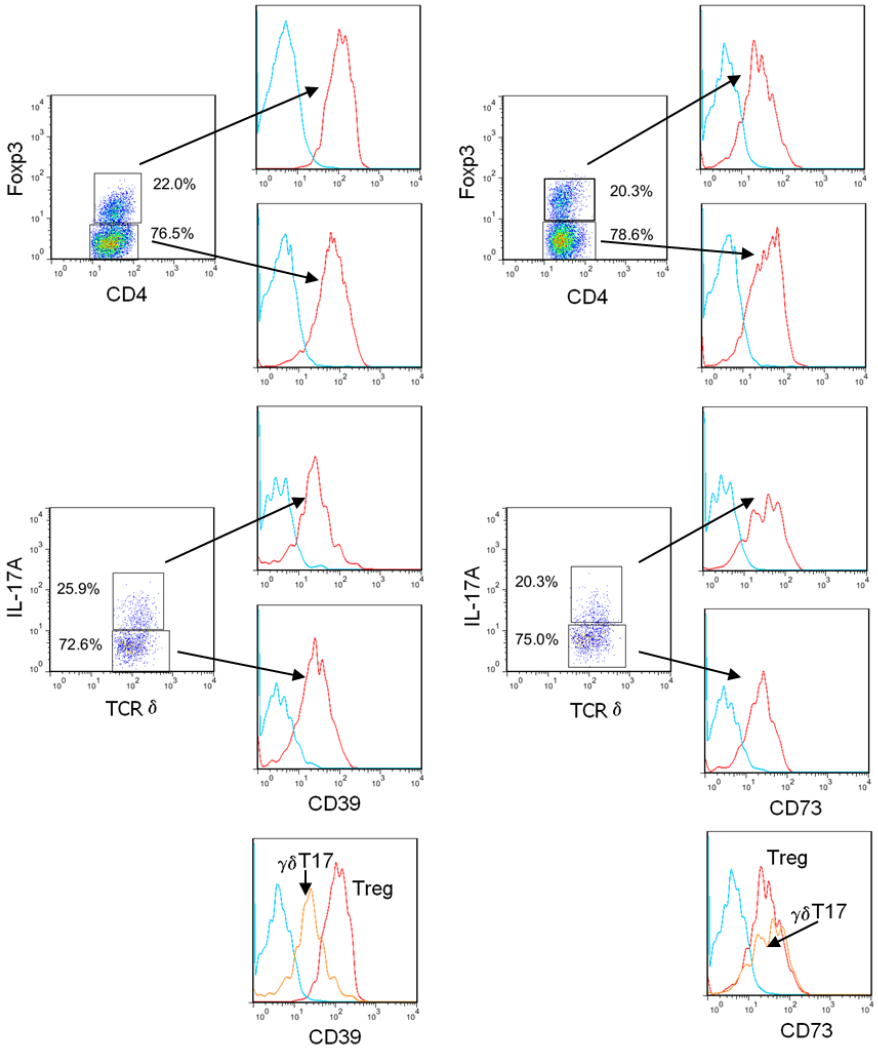

Figure c. CD39 and CD73 expression on tumor infiltrating Treg and γδ T17 after DX treatment. MFI of CD39 (left) and CD73 (right) staining on CD4+ T and γδ T cells were shown, comparing $Foxp3^+$ vs $Foxp3^-$, $IL\text{-}17A^+$ vs $IL\text{-}17^-$ fractions.

FACS indicated that around 60-75% of γδ T17 utilized Vγ4 chain according to Heilig and Tonegawa's nomenclature [260] (Figure. b and published Figure S2B) but expression of Vγ1 and Vγ7 chain was rarely found (published Figure S2B). We then sorted $Vγ1^-Vγ4^-Vγ7^-$ γδ T17 TILs and performed single-cell PCRs [261] to examine their Vγ chain usage. These experiments revealed that 21 out of 23 sorted single cells contained a functional Vγ6 rearrangement identical to the one found in fetal γδ T cells (shown in published Figure S2C-E), suggesting that most γδ T17 TILs express either Vγ4 or Vγ6. Indeed, γδ T cell subsets appear to be biased to carry out particular functions which are somehow

predetermined in the thymus [262]. $V\gamma4^+$ $\gamma\delta$ T cells are expanded and they potently produce IL-17A at the inflammatory sites in the murine collagen-induced arthritis (CIA) [263] and experimental autoimmune encephalomyelitis (EAE) models [252], exacerbating the disease process. The TCR invariant $V\gamma6V\delta1$ T cells functionally differentiate to produce IL-17 within thymus and thereafter express CD25 to be maintained in the periphery [256]. They form a small population at steady-state while expanding rapidly only when their functions are required. During *E. coli* and *Listeria* infection, $V\gamma6V\delta1$ cells become responsible for most of the IL-17 production within hours. They present in expanded numbers and continue to produce IL-17 for several days [264,265].

DX based chemotherapy could efficiently inhibit tumor growth in immunocompetent wild type mice while its efficacy was greatly dampened when the bulk of $\gamma\delta$ T cells were absent or the $V\gamma4^+$ and $V\gamma6^+$ cells were deficient (shown in published Figure 6A). Consistently, IL-17A production by tumor infiltrating cells was abolished in $V\gamma4/6^{-/-}$ mice (shown in published Figure 6B). Importantly, $\gamma\delta$ T cells are critical for the tumor-retarding effect of DX in both MCA205 sarcoma and AT3 breast cancer while NKT cells, especially the anti-tumor $J\alpha18^+$ NKT1, are dispensable (Mattarollo SR, Cancer Res. 2011, accepted). Mitoxantrone treated dying MCA205 tumor cells could vaccinate wild type mice against syngeneic tumor cell rechallenge. This vaccine could not protect athymic nude mice, suggesting the critical contribution of T, B and NKT cells. Importantly, the percentages of tumor free mice were significantly reduced in $V\gamma4/6^{-/-}$ mice but not in NKT cell deficient $CD1d^{-/-}$ mice (shown in published Figure 5B), indicating that the indispensible role of IL-17A/IL-17R pathway and $V\gamma4^+$ and $V\gamma6^+$ cells in triggering protective adaptive immunity against tumor.

The biological rationale for using adoptively transfer ex vivo-expanded autologous $\gamma\delta$ T cells in clinical trials of cancer treatment has been shown in mouse prostate tumor model. Tumor-bearing mice treated i.v. with supraphysiological numbers of syngeneic $\gamma\delta$ T cells developed measurably less disease and superior survival [266]. We further investigated the possibility to potentiate the tumor-retarding effect of chemotherapy by adoptive transferring $\gamma\delta$ T17 cells. We chose to purify $\gamma\delta$ T17 cells from peripheral LNs (inguinal, popliteal, superficial cervical, axillary and brachial LNs) rather than spleen or thymus

because IL-17$^+$ γδ T cells are much more enriched in LNs than that in the spleen or thymus (Figure d). Similarly, it has been reported by other group that IL-17$^+$ γδ T cells in the spleen were present at a low frequency (~3%) and they were absent in the mesenteric LN [267]. It is probably due to the fact that LN γδ T cells express higher IL-23R [268]. Consistently, by using IL-23 receptor GFP reporter mice, Awasthi and colleagues found that expression of IL-23R were detected mainly in the LNs but not in the spleen. The small proportion of IL-23R-expressing cells (~1.4%) were CD3$^+$ and the majority of them were γδ T cells [269]. It would be also interesting to know whether γδ T cells from different organs or various subsets of γδ T cells express IL-1R at different levels.

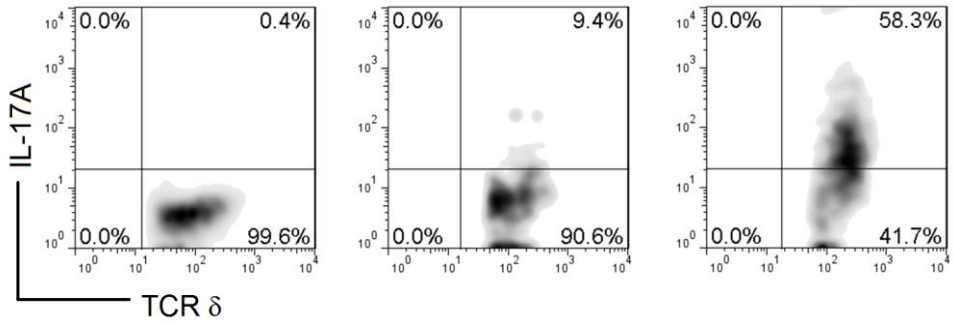

Figure d. ***IL-17 production by γδ T cells in spleen, thymus and peripheral LNs.*** Single cell suspension from spleen (left), thymus (middle) and peripheral LNs (right) were stimulated with PMA and ionomycin in the presence of GolgiStop for 4 hrs before intracellular staining for IL-17A.

We purified γδ T cells with AutoMACS (purity above 90%, shown in Figure e) and adoptively transfer these cells directly into tumor bed 2 days after chemotherapy. γδ T cells transfer showed addictive effect to control tumor growth provided that chemotherapy (DX) was applied in advance (shown in published Figure 7A). IL-17$^{-/-}$ γδ T cells adoptive transfer could not improve the efficacy of chemotherapy but IL-17-sufficient wild type γδ T cells could restore the chemosensitivity of MCA205 sarcoma. These findings strongly suggest that IL-17 derived from γδ T cells is critical for the success of DX based chemotherapy (shown in published Figure 7B, 7D).

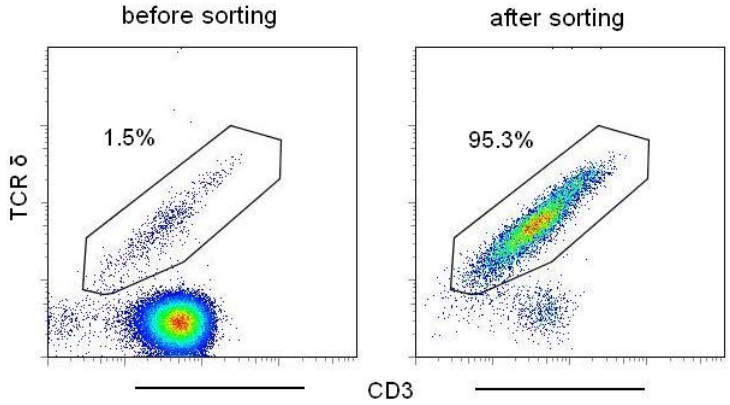

Figure e. ***The percentage of TCR δ+ CD3+ cells before and after AutoMACS sorting.***

The link between IL-17A/γδ T and IFN-γ/CD8$^+$T response

To analyze the kinetics of TILs accumulation and activation in MCA205 tumor, we sacrificed tumor-bearing mice 2 days before and 4, 6, 8 or 10.days after chemotherapy. γδ TILs were quickly switched on to proliferate and produce IL-17A within 4 days after DX, resulting in around 9 fold increase of γδ T17 cells above the basal level. Their activation sustained and peaked around day 8 after DX. In sharp contrast, the priming of Tc1 response act in the exponential manner. It took more than 6 days to "warm up" and become fully primed (as indicated in their IFN-γ$^+$ Ki-67$^+$ phenotype) within the following 2 days. PBS treatment could modify neither IFN-γ or IL-17A secretion throughout the chosen time points (shown in published Figure 2A, 2C). γδ T cells activated by IL-1β and IL-23 could promot IL-17 production by CD4$^+$ T cells and increase susceptibility to EAE [252]. We noticed that tumor infiltrating γδ T17 cells did not sustain long after Th1 response was primed. And we could not find significant increase of Th17 TILs, suggesting that albeit γδ T17 was the major source of IL-17A in tumor bed, they did not manage to amplify the adaptive Th17 response after chemotherapy. Instead, we noticed that a clear positive correlation existed between tumor invading γδ T17 and Tc1 cells in MCA205, CT26 and TS/A models as analyzed by linear regression (shown in published Figure 3G), implying that early IL-17 production may facilitate the priming of Tc1 adaptive immune response.

To test this hypothesis, we checked the role of IL-17A during Tc1 priming triggered by tumor cells undergoing immunogenic cell death. We have previously reported that IFN-γ production by OVA-specific T cells in the draining LNs could be triggered by footpad injection of oxaliplatine-treated EG7 cells. The absence of IL-17RA fully abolished antigen-specific T cell priming in response to dying cells, yet had no negative effect on T cell priming by OVA holoprotein admixed with CpG oligodeoxynucleotides. Consistently, a neutralizing anti-IL-17A antibody but not the isotype control immunoglobulin markedly impaired the OVA-specific T cell induction in the same setting (shown in published Figure 5A). And IL-17R$^{-/-}$ mice could not mount a protective immunity after dying tumor vaccine (shown in published Figure 5B). We also noticed that tumor infiltrating CD8^{+} T cells failed to produce IFN-γ in Vγ4/6$^{-/-}$ mice (shown in published Figure 6B).

IL-17 production by innate cells may function as a bridge between innate and adaptive immunity. The link between IL-17 and Th1 (IFN-γ) response has been reported in mouse models and clinical research. IL-17 is required in the induction of optimal Th1 response and protective immunity against *M. bovis BCG* infection [270]. We could not find enhanced proliferation of CD8^{+} T cells or upregulated CD121a (IL-1R1) expression on CD8^{+} T cells after incubation with IL-17A with or without CD3 and CD28 crosslinking (data not shown). Thus, it is unlikely that IL-17A could directly act on CD8^{+} T cells. IL-17R is ubiquitously expressed by a variety of target cells including fibroblasts, epithelial and endothelial cells, monocytes/macrophages and mast cells. We noticed that IL-17R are mainly expressed on CD3^{-} fractions in spleen and LNs, implying that the potential target of IL-17A were probably not T cells directly (data not shown). IL-17A could induce IL-12 production by dendritic cells and mediate Th1 responses against intracellular pathogen *F. tularensis* [271]. IL-17A signaling was suggested proximal to its downstream IL-12/IFN-γ signaling in a mouse kidney ischemia-reperfusion injury model [191]. Though IL-12 contributes to the chemotherapeutic effect, it doesn't seem to be the sole key factor since DX partially retained its efficacy in IL-12p35 deficient mice (Mattarollo SR, Cancer Res. 2011, accepted). IL-17A derived from γδ T cells is required for the generation of antigen-specific CD8^{+} CTL response against primary *Listeria monocytogenes* infection through enhancing DC cross presentation [272]. IL-23-activated γδ T cells produce IL-17

and they could render αβ effector T cells refractory to the suppressive activity of Treg cells and also prevent the conversion of conventional T cells into Foxp3$^+$ Treg cells in vivo [273]. What's more, IL-17 could synergize with IFN-γ to stimulate CXCL9 and CXCL10 production and recruit effector T cells to the tumor microenvironment [216]. Interestingly, in experimental lung metastasis B16 and 3LL tumor models, tumor microenvironment derived IL-1β promoted γδ T cells recruitment and IL-17 production. IL-1β deficiency resulted in reduced IFN-γ production by CD8$^+$ TILs while IL-17, which acted mainly downstream of IL-1β, did not contribute to DC maturation or Tc1 response and it suppressed IL-12 production by CD11c$^+$ cells in tumor bed [274]. Thus, coordinated IL-17 production by γδ T and IFN-γ secretion by CD8$^+$ T cells predict the beneficial anti-tumor response. The orchestration and interactions of γδ T cell with other immune cell in the tumor microenvironment may be critical to determine their host protective and immune regulatory function. Further studies are needed to dissect the cell subsets and contributive factors linking IL-17 and Tc1 response.

Contributive factors for γδ T17 activation in the context of chemotherapy induced tumor cell death

The differentiation factors (TGF-β plus IL-6 or IL-21), the growth and stabilization factor (IL-23) and the transcription factors (STAT3, RORγt, and RORα) are involved in the development of Th17 cells. Innate IL-17 producing cell populations share some common activating signals, as well as unique pathways [275]. Mouse γδ T, NKT, memory T cells and human MAIT cells express IL-23R and RORγt constitutively and they could rapidly produce IL-17 after TCR ligation and/or IL-23 stimulation. Pathogen associated molecular pattern (PAMP), stress-induced proteins, pathogen metabolites and glycolipids could either activate γδ T cells directly or through inducing IL-1β and/or IL-23 production from antigen presenting cells [251]. γδ T cells express pattern recognition receptors (PRRs), such as Dectin-1 and TLR1 and TLR2, which could be engaged by corresponding ligands to induce IL-17 production and IL-23 could amplify this process [251]. Soluble factors (IL-6,

IL-23 and TGF-β), engagement of NKG2D and TCR has been reported to activate γδ T produce IL-17 [241].

By in vitro experiment, we confirmed that γδ T from peripheral LNs potently produced IL-17A, and IL-22 to a lesser extent, in response to IL-1β+IL-23, CD3 crosslinking and IL-1β or/and IL-23 (Figure f). To mimic the microenvironment after chemotherapy, we cocultured γδ T with BMDC and dying/live tumor cells and tested IL-17A, IL-22 and IFN-γ in the supernatant. Although DX-treated MCA205 cells failed to directly induce IL-17 or IL-22 secretion by γδ T cells, they could do so indirectly through triggering IL-1β production by BMDC. It is noteworthy that these stimuli specifically activated IL-17A, but not IFN-γ production by γδ T cells. And none of these cytokins were induced by live tumor cells. We and others have described that peripheral LN γδ T cells express IL-18R and could produce IL-17 in response to IL-18 plus IL-23 without TCR ligation [276,277]. The DC/γδ T cell cross-talk in the presence of dying cells relied on IL-1β/IL-1R but not IL-18/IL-18R, as IL-1RA completely abrogated IL-17 production while IL-18BP did not interfere with it. IL-23/IL-23R signaling only moderatly regulated IL-17 production by γδ T cells. IL-22 production was completely abolished by blocking the IL-1β/IL-1R or IL-23/IL-23R pathways but not affected by IL-18R blockade (shown in published Figure 5C). Although IL-1β production could be readily detected in dying tumor/BMDC coculture, IL-23 production form this system was below the detection limit of our ELISA kits (from R&D and eBioscience). Interestingly, chemotherapy completely lost its anticancer activity in IL-1R1 deficient mice, yet maintained its efficacy in mice treated with IL-23p19 neutralizing antibodies or in IL-23p19$^{-/-}$ mice (shown in published Figure 5E-G). Adoptive trasfer of IL-1R$^{-/-}$ γδ T cells could not improve the efficacy of chemotherapy. These findings emphasize the pivotal contribution of IL-1β, but not IL-18 or IL-23, during chemotherapy.

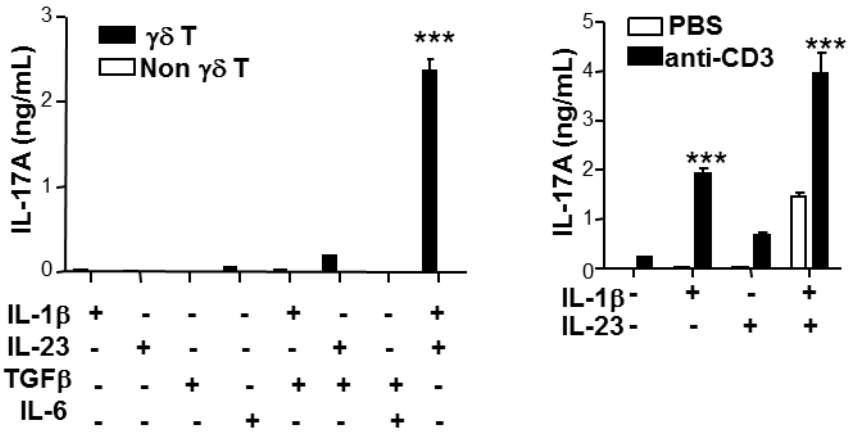

Figure f. ***IL-17 secretion by T cells purified from peripheral LNs in the presence of various cytokines and CD3 crosslinking.***

IL-22 is a member of the IL-10-related cytokine family which comprises IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29. It signals through a heterodimeric receptor that consists of the IL-10Rβ and the IL-22R chain. IL-22 signalling is mediated by JAK 1 and STAT1, 3 and 5 [278]. It can be secreted by Th17 [279], γδ T [251], NK22 [280,281], LTi and RORγt$^+$ NKR-LTi cells [282,283], involving in protection against infection and homostasis. Interestingly, IL-22R expression is restricted to cells of the non-haematopoietic lineage, therefore IL-22 does not serve the communication between immune cells but is a T cell mediator that directly promotes the innate, nonspecific immunity of tissues [284]. Innate IL-22-producing cells have a largely protective function probably through their instruction of epithelial cells to express antimicrobial and tissue-protective genes. In induced pulmonary fibrosis model, γδ T cells derived IL-17 [285] and IL-22 [286] act as the critical factor to in either tissue destruction or tissue protection through differed mechanisms, supporting the notion that their functional spectra are generally distinct [287]. IL-22 is tissue protective in the absence of IL-17A while it is proinflammatory in the presence of IL-17A. IL-17A inhibited IL-22 production by Th17 cells, suggesting the balance between IL-17 and IL-22 production is decisive for a disease-promoting or protective role of these cells [288]. In our experiments, IL-1β-activated γδ T cells produced IL-17 as well as IL-22 in vitro. However, we could not detect IL-22 expression at mRNA level in CD45$^+$ cells purified from tumor bed. Neither could we obtain postive intracellular staining of IL-22 in

TILs by FACS. We also proved that IL-22 did not play an essential role in the antitumor effects promoted by chemotherapy (shown in published Figure S3A).

It remains possible that TCR signaling facilitates γδ T17 activation in our setting. Possible ligands of γδ TCR have been summarized elsewhere [289,290]. Irradiation and chemotherapeutic drugs, especially anthracyclines, could generate high level of oxidative stress on tumor cells [291] which is known to induce cardiolipin remodeling. Cardiolipin moves from the inner to the outer mitochondrial membranes during apoptosis and this phospholipid provides a recognition site for Bcl2 proteins, notably t-Bid, to bind to mitochondria and promote the apoptotic process [292]. Cardiolipin is also expressed on the surface of apoptotic cells, showing a clustered distribution localized mostly on surface blebs before the incidence of DNA fragmentation [293]. It was reported that hybridomas expressing murine γδ TCR were found to produce cytokines in response to cardiolipin and structurally related anionic phospholipids. Cardiolipin can be presented by CD1d to subsets of γδ T cells in the spleen and liver of mice [294,295]. Chemotherapy induced stress could upregulate MHC class-I-related chain A/B (MICA/B) which has been proved as TCR ligand in human [296]. Tumors have been shown to overexpress HSP while chemotherapy could induce some HSP expression on the membrane of stressed tumor cells. Intracellular overexpression of HSP could inhibit apoptosis and exhibit cytoprotective activity while membrane expression of HSP could be potently immunostimulatory. Around 10-20% of normal splenic and lymph node γδ T cells have been reported responding to HSP60 [297]. γδ T cells could elicit cytotoxic activity toward macrophages and neutrophils that express Hsp60 and Hsp70 respectively [298]. It would be interesting to investigate the tumor derived molecules elicited by chemotherapy which could engage γδ TCR to facilitate γδ T17 activation.

Contribution of AhR, CCR6/CCL20, TGF-β and IL-6 pathway during chemotherapy

Aryl hydrocarbon receptor (AhR) is highly expressed on Th17 [299-301] and γδ T17 cells [251]. This transcriptional factor is closely related to the induction of IL-22. It remains

unclear how AhR contributes to Th17 differentiation, but Th17 cells are functionally underdeveloped in the absence of AhR as noted in the substantially decreased incidence of EAE pathology in AhR deficient mice [302]. AhR can cooperate with RORγt to induce maximal amounts of IL-17 and IL-22 production and to inhibit TGFβ-induced Foxp3 expression [303]. Based on the effects of TCDD, an AhR agonist, neutrophils, macrophages, NK cells and dendritic cells have been suggested to possess AhR [304]. Langerhans cells were shown to express high levels of AhR and absence of AhR resulted in impaired maturation and antigen presenting capacity [305]. AhR could show a negative regulatory role in inflammatory responses of macrophages induced by LPS through forming a complex with STAT1 and NF-κB [306]. In mice expressing LysMCre mediated AhR deletion in the myeloid lineage, macrophages exerted hyperreactive IL-1 responses to LPS challenge, resulting in increased susceptibility to septic shock [307].

AhR was significantly upregulated in the context of DX-induced tumor growth retardation (Figure a). By blocking AhR with its pure antagonist CH-223191, we showed that AhR activation was contributive to the success of anthracycline-based therapy. CH-223191 had no cell-autonomous effects on the tumor cells, alone or in combination with anthracyclines (Figure g). We noticed that AhR inhibition significantly reduced IL-17 production by γδ T cells stimulated by cytokines with or without CD3 crosslinking, which may partially account to the AhR-dependency of chemotherapeutic effect. In contrast, activation of γδ T cells was not interfered by AhR blockade as indicated by CD69 staining (Figure h). It remains possible that AhR may play a role in regulating the function of antigen presenting cells or other immune cells. Due to the widely expression of AhR, it could exert anti-tumor or pro-inflammotory roles in the same context or at different phases.

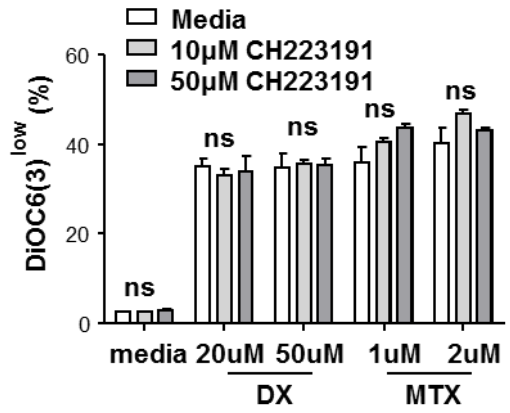

Figure g. ***The impact of the AhR-blocking agent, CH-223191, on the proapoptotic effect of DX or MTX against MCA205.*** A reduction in mitochondrial membrane potential indicated by decreased DiOC6(3) fluorescence was used to measure imminent apoptosis.

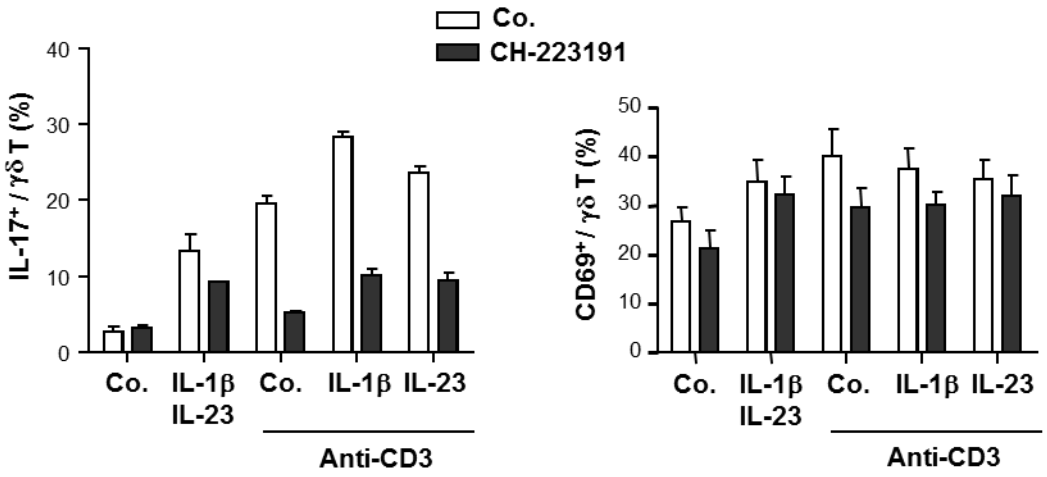

Figure h. ***The impact of CH-223191 on the activation and IL-17 production by γδ T cells.*** Single cells suspension from peripheral LNs of naive C57BL/6 mice were seeded in 96 well plates, stimulated with cytokines with or without anti-CD3 crosslinking in the presence of GolgiStop (BD Bioscience) for 6 hrs. IL-17 production and CD69 expression by γδ T cells with or without CH-223191 are depicted.

Expression of CCR6 is a phenotypic and functional hallmark of Th17 cells during some inflammatory processes. Since CCL20 was abundant in tumor tissues post-chemotherapy (data not shown), we therefore analyzed the role of CCR6 in the efficacy of chemotherapy and whether γδ T17 cells could be recruited in a CCL20/CCR6-dependent manner. The tumoricidal activity of doxorubicin against CT26 was not affected by repetitive systemic

injections of neutralizing anti-CCL20 mAb before and during anthracycline treatment (shown in published Figure S3B). Consistently, anthracycline treatment against established MCA205 sarcoma remained efficient in CCR6 loss-of-function mice. Moreover, CCR6 deficiency did not block tumor infiltration by γδ T17 (Figure i), suggesting CCR6/CCL20 are dispensable for the IL-17A/IFN-γ-dependent chemotherapy against tumor. IL-6 and TGF-β also failed to play a role in the immunogenicity or therapeutic effects of anthracyclines (shown in published S3C-D).

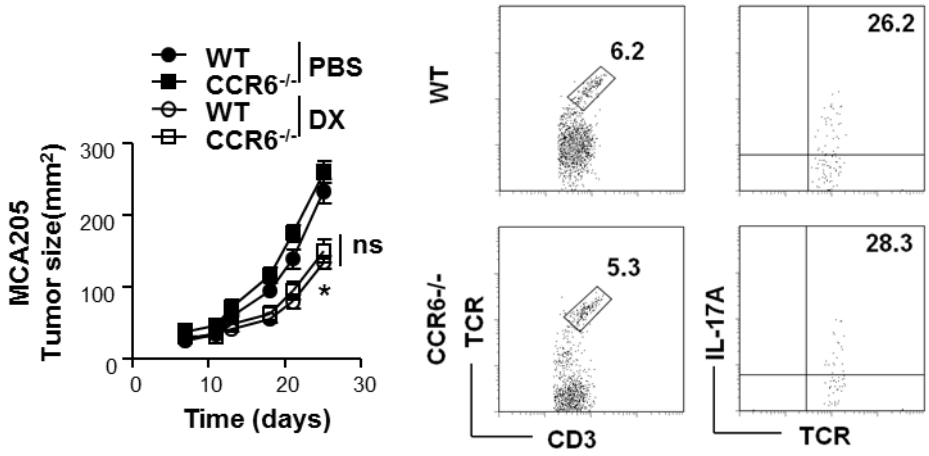

Figure i. ***CCR6 is dispensable for the efficacy of chemotherapy and the infiltration of γδ T17 in tumor bed.*** Tumor growth was monitored in WT and CCR6$^{-/-}$ mice after PBS or DX treatment (left). Flow cytometry analyses of the γδ T17 cells in the gate of live, CD45.2+, CD3+ T cells invading MCA205 tumors at day 8 post-DX in WT versus CCR6 loss of function mice (right).

Strategies to improve anti-cancer therapy with γδ T cells

Recent advances in the characterization of the functional capabilities of human resident Vδ1 and circulating Vδ2 T cells indicate that they are suitable candidates as anti-tumor effectors. MHC class I chain-related molecules A/B (MICA/MICB) and UL-16-binding proteins (ULBP) are constitutively expressed or induced to variable levels on many epithelial tumor cells. Vδ1 T cell could recognize these tumor-derived ligands through TCR or its activating natural killer receptors while Vδ2 T cells could also recognize phosphoantigens, ectopically expressed mitochondrial ATPase and isopentenyl

pyrophosphate (IPP) [248]. The application of synthetic phosphoantigens and aminobisphosphonates (N-BPs) is intended to enhance the expansion, cytotoxicity and IFN-γ production of mainly Vγ9Vδ2 cells, directly or indirectly through accumulating intermediate metabolites IPP which is assumed as tumor antigens in target cells. Recent clinical trials show that these drugs could enhance anti-tumor therapy, but with moderate success. Accumulating knowledge of γδ T cells, concerning their unique features of antigen specificities, requirements for antigen recognition, activation, polarization, migration and tissue distribution, should be integrated for their optimized therapeutic use. To translate our finding from bench to bedside, we propose these strategies to facilitate CTL priming with γδ T17 cells.

A. Adoptive cell transfer of autologous γδ T17 cells expanded in vitro.

Adoptive transfer strategy normally requires supraphysiological numbers of γδ T cells which restrain tumor outgrowth through their potent cytotoxicity and IFN-γ secretion. Importantly, in vitro expanded Vγ9Vδ2 T cells maintained their antitumor activity in vivo as shown in a xenografted human tumor model [308]. Autologous Vγ9Vδ2 cells could be reprogrammed to manifest Th17 polarization in vitro with phospholipids and cytokines. Such protocol has recently been developed by Caccamo and colleagues. Coculture naive Vγ9Vδ2 cells with phosphoantigens and a cocktail of cytokines (IL-1β, TGF-β, IL-6 and IL-23) leads to selective expression of the transcription factor RORγt and polarization towards IL-17 production. These cells shared similar phenotype with IL-17$^+$ Vγ9Vδ2 lymphocytes observed in the peripheral blood and at the site of disease of meningitis children [309]. Similar to our finding, these IL-17$^+$ cells retain their cytotoxicity, expressing Granzyme B, FasL and TRAIL and they vigorously kill tumor cells though TRAIL. To expand and polarize γδ T17 cells for adoptive transfer, tumor-infiltrating γδ T cells may be a better choice than circulating ones since they are enriched for tumor reactive clones and probably maintain their tumor-homing properties. Our preliminary experiments show peripheral LN derived γδ T cells (mostly Vγ4$^+$) expanded with TCR crosslinking (clone UC7-13D5, 5 μg/ml coated plate) and rIL-2 (100 U/ml) remained as potent IL-17 producers 8 days after in vitro culture. Using the same culture system, γδ T cells purified

from thymus (which poorly produced IL-17 originally) could significantly upregulate IL-17 secretion, suggesting a preferential expansion of IL-17 producers or activation/maturation of γδ T17 progenitors (not shown). Another challenging issue is how to guide infused γδ T17 towards tumor vicinity. IL-17$^+$ Vγ9Vδ2 T cells preferentially express CCR6 while IFN-γ$^+$ Vγ9Vδ2 T cells express CXCR3 and CCR5, indicating that they could be recruited to tumor bed through different chemokines [309]. Leukotriene B4 (LTB4), a potent chemokine for DC [310], has been recently reported also capable of attracting γδ T cells [311]. Further investigation on tumor-derived chemotactic factors triggered by chemotherapy will shed light on the optimal combination of chemotherapy with γδ T17 cells infusion.

B. Educating or activating γδ T17 in vivo.

Another alternative strategy is to create a favorable microenvironment in vivo for γδ T17 activation. Unacceptable toxicities and side effects can be encountered already at very low doses of cytokine delivery, preventing escalation to therapeutically active concentrations. Therefore, this treatment should be applied in a well-controlled manner (eg, timing, dosing and specificity for γδ T17).

γδ TCR-targeted delivery of pro-Th17 cytokines is a feasible choice. Antibody-based targeted delivery of cytokines (eg. IL-1β, IL-23), which has been termed as “immunocytokines” [312], could enhance the therapeutic index of recombinant cytokines. Cytokines-based fusion proteins-containing antibody targeting γδ TCR could be a promising option to activate/polarize γδ T17 cells in vivo. Nanoparticle-mediated delivery system may well suit this delicate demand. The polymer poly(lactide-co-glycolide) (PLG) is already approved by the FDA for drug delivery applications due to its safety, excellent biocompatibility, and “tunable” release rates. This technology is well-suited toward stimulation and manipulation of immune cell. Nanoparticles can be coated with antibody targeting γδ T cells surface molecules or phospholipids mimicking TCR ligand. The presentation of multiple targeting ligands per nanoparticle can ensure high valency and avidity of preferential contact with γδ T cells. And multiple pro-Th17 cytokine molecules can be encapsulated within the same nanoparticle to maximize their synergistic effect.

This biorecognition dependent delivery ensures relatively high concentration of cytokine precisely within the microenvironment of the targeted cell, avoiding systemic exposure to the therapeutic cytokine [313]. To specifically target TILs and release bioactive component locally, it is possible to take advantage of the properties of tumor microenvironment. These cytokines can be encapsulated in pH sensitive drug delivery vehicles, such as polyketal nanoparticles, which could targets to the acidic environments of tumors [314] and spare other tissues. Nanoparticles-based technology also facilitates sustained release at the desired period, perhaps shortly after chemotherapy.

C. γδ T17 activation through DC.

As we have shown, dying tumor cells could induce IL-1β production by DC, which is indispensible for γδ T17 and Tc1 activation. It would be interesting to test whether autologous DC loaded with polyclonal primary tumor cells pretreated with immunogenic chemo drugs could be used for adoptive transfer. It remains a fascinating possibility since these DC could induce and coordinate both innate γδ T17 and adaptive tumor specific Tc1 response. Several PRR, such as TLR1, TLR2 and dectin-1, are specifically expressed on $CCR6^+$ IL-17-producing γδ T cells [251]. They can be targeted directly to induce IL-17 production by γδ T cells, or indirectly to enhance this process through triggering IL-23 production from DC. Thus, TLR2 or dectin-1 ligands should be considered as candidate components of nanoparticles targeting γδ T cells or used during autologous DC generation for infusion.

D. Tumor-infiltrating or circulating γδ T17 as a potential beneficial prognostic factor.

We discovered that γδ T17 induction in tumor bed appears shortly after chemotherapy, sustained and correlated with the priming of Tc1 cells within tumor bed, both of which are critical for the efficacy of chemotherapy. It would be interesting to analyze whether tumor infiltrating or circulating γδ T17 could be an early prognostic factor after chemotherapy and whether correlated γδ T17 and Tc1 infiltration predict superior clinical outcome.

Part II. Optimized therapeutic application of TLR3 agonist by uncoupling tumor derived chemokines

This works has been published in Cancer Reasearch (2010 Jan 15; 70(2):490-500. PMID: 20068181) as attached in annex 3.

Tumors do not spontaneously release much danger signals. Therefore, they could not elicit strong immune reactions. Synthetic molecules have been developed which mimic pathogen invasion at the tumour site [56], among which TLR agonists are most widely used to boost immunity. Noticeably, neoplastic process may subvert antitumor TLR signaling to proinflammatory and/or immunosuppressive pathway and advance cancer progression. Thus, TLR agonists act like a double-edged sword during cancer treatment [315].

TLR3 ligands, such as polyinosinic:polycytidylic acids (Poly (I:C)) mimicing the viral double-stranded RNA, could boost innate immunity (eg. cytotoxicity of NK, antigen cross-presentation of DC) and augment adaptive immune responses (eg. antigen-specific $CD8^+$ T cells) and they are being evaluated for the treatment of cancer [316,317]. Combined CD40 and Poly (I:C) could decrease L-arginase activity of tumor infiltrating DC, enhance their production of type I IFN and IL-12, thus transform them from immunosuppressive to immunostimulatory cells [318]. Poly (I:C) could signal through TLR3 and cytosolic receptors retinoic acid-inducible gene-I (RIG-I) [319] and melanoma differentiation-associated Gene 5 (MDA5) [320], to trigger IL-12, type I IFN and IFN-γ production from immune cells [321-324].

Preclinical study and clinical application of Poly (I:C) have been limited by its high toxicity [317,325]. A non-toxic synthesized dsRNA Polyadenylic:polyuridylic acid (Poly (A:U)), which signals uniquely through TLR3, has also been utilized in preclinical and clinical studies. When combined with a candidate protein or viral antigen in mice, Poly(A:U) can promote antigen specific Th1-immune responses and boost antibody production [326,327]. When combined with radiotherapy, chemotherapy or used as alone, Poly(A:U)

showed moderate success for treating breast gastric cancers without toxic side effect [328-330].

Surprisingly, various functional TLRs are also expressed on a wild variety of tumor cells and their biological function remains to be fully understood [331]. Some of the TLR, especially TLR4 and TLR9, activation on tumor cells could promote tumor proliferation and resistance to apoptosis, induce inflammatory factors secretion by tumor cells, as well as enhance tumor invasion and metastasis [331]. TLR3 expression has been found on hepatocellular carcinoma [332], melanoma [333,334] and breast cancer [335]. TLR3 signaling could directly inhibit tumor proliferation, reduce metastasis [334,336] and induce apoptosis of tumor cells [335,337]. TLR3-mediated cell death involves the activation of caspases and engages both extrinsic and intrinsic apoptotic pathways [338]. This effect is mediated either dependent or independent of type I IFN [334,339]. Interestingly, Poly (I:C) induced TLR3 activation on prostate cancer cells could increase HIF-α expression and its nuclear accumulation, which renders tumor cells resistant to apoptosis and augments their VEGF production [340]. In Poly (A:U) based treatment of breast cancer patients, high TLR3 expression level on tumor cells has been suggested as the biomarker for decreased risk of metastatic relapse [341]. While in a cohort of recurrenced breast carcinoma, TLR3 was significantly upregulated on tumor cell and associated with higher probability of metastasis [342]. These conflicting data suggest that different stages of malignancy, hypoxic gene expression, the intrinsic property of triggered systematic inflammatory reaction could influence the beneficial responsiveness to TLR3 agonists. Therefore, it is of utmost importance to uncouple the pro-tumoral and anti-tumoral effect of TLR agonists to achieve their maximum beneficial therapeutic effect.

This study is intended to dissect the effect of Poly (A:U) on TLR3-expressing tumor cells and that on host immune system. We are focused in analyzing key factors which mediate the crosstalk between tumor and host immunity initiated by TLR3 agonist and their contribution to anti-tumor effect. We chose two TLR3-expressing murine tumor models, the highly aggressive metastatic B16OVA melanoma and highly tumorigenic GL26 glioma. Implanted B16OVA tumors did not respond to chemo drug oxaliplatine, tumor vaccine or poly (A:U) monotherapy. Neither could these tumors be controlled by the combination of

either two of these therapies. Interestingly, sequential administration of a tumor vaccine followed by oxaliplatin and poly (A:U), which we termed VCT, are necessary and sufficient to significantly retard tumor growth and prolong survival of tumor-bearing mice. This anti-tumor effect relies on intact T cells and TLR3-TRIF signal pathway of the host as its therapeutic effect was completely abolished in athymic *nu/nu* and $TRIF^{-/-}$ mice (shown in published Figure 1). Importantly, around 11% of all the wild type mice became tumor free after VCT treatment and 2/3 of them gained long time immunity against tumor rechallenge.

Our group previously showed that doxorubicin (DX) induced cell death was immunogenic in various tumor cell lines including B16OVA. DX treated B16OVA could elicit the recruitment of antigen specific IFN-γ-producing $CD8^{+}$T cells into the draining LNs when injected into footpad. B16F10A2/gp100 cells (melanoma cells present peptides from the human gp100 tumor antigen in the context of HLA class I A2) treated with DX could also significantly delay tumor growth when inoculated subcutaneously into “humanized” mice expressing an HLA.A2 transgene [116]. Therefore, we also compared the efficacy of dying tumor cell-based vaccines with that composed of adjuvant (CpG) admixed with model antigen ovalbumin (OVA) in our VCT therapy. DX could only induce around 20-30% cell death (8-20% apoptotic) as tested by Annexin/PI staining after 24hrs of treatment in B16OVA cell lines. To improve the lethality of DX, we combine it with type I IFN since this natural adjuvant could also induce cell cycle arrest, inhibit proliferation and trigger apoptotic cell death in various cell types including tumor [343-347]. Although we could not find any synergistic effect between type I IFN and DX to induce cell death, tumor cells under this treatment completely lose its tumorigenicity after inoculated into the mice. And these cells could vaccinate mice and prevent tumor outgrowth after rechallenged whileas tumor cells treated by repeated freeze and thawn were poorly immunogenic (shown in published Figure 2A, 2B). Type I IFN+DX based tumor cell vaccine was as potent as CpG admixed with OVA in terms of control tumor growth and prolong survival (shown in published Figure 2C, 2D). And type I IFN plus DX could better induce immunogenic cell death than DX alone as it afforded better efficacy in VCT therapy (not shown). Similarly, our tumor cell vaccine and VCT treatment could prevent outgrowth of GL26 in both the

prophylactic setting and the therapeutic setting. Importantly, the success of VCT therapy depended on intact TRIF signaling in the host as well as that in tumor cells. Knockdown TRIF expression using lentivirus-based shRNA abolished the efficacy of VCT (shown in published Figure 3A-C).

TRIF is so far the sole adaptor for TLR3 signaling pathway in response to double-stranded RNA [348,349] and Poly (A:U) signals uniquely through TLR3. Interestingly, we found that Poly (A:U) induced copious amount of CCL5 (RANTES) and CXCL10 (IP-10) from TLR3 expressing-tumor B16OVA in a dose dependent manner. Albeit type I IFN alone did not trigger CCL5 and CXCL10 production, pre-incubation with type I IFN could enhance this effect when combined with Poly (A:U). Type I IFN, particularly IFN-β could up-regulate TLR3 expression in both murine and human. IFN-β-induced TLR3 up-regulation on murine macrophages requires IFNAR1, STAT1, and in part IRF-1 [350]. GM-CSF, which can be spontaneously secreted by tumor cells [351-353], is able to upregulate TLR3 expression through activation of mitogen-activated protein kinase (MAPK) and phosphotidylinositol 3-kinase (PI3K)/Akt signaling pathways [354]. Indeed, we confirmed that type I IFN upregulated TLR3 expression on both B16F10 (parental cell line of B16OVA) and GL26 by immunochemistry staining (shown in published Figure S1).

Interestingly, B16OVA spontaneously produce huge amount of CXCL1 (KC) while it is completely abolished by Poly (A:U) with or without type I IFN pretreatment. Similar phenomenon has been found in GL26 glioma (shown in published Figure 4A, 4B). It has been previously reported that TLR3 agonist Poly (I:C) can induce CCL5, CXCL10 and TLR2 agonist Pam3Cys could induce CXCL1 production by bone marrow derived macrophage. Albeit CXCL10 and CCL5 production depend on TRIF instead of MyD88, CXCL1 production relies on MyD88 but not TRIF [355]. Macrophages from IRF3-deficient mice showed complete inhibition of CCL5 (RANTES) and CXCL10 (IP-10) production, but no effect on CXCL2 (MIP-2) or CXCL1 in response to *P. aeruginosa* stimulation [356]. Our results indicate that activation of TLR3-TRIF signaling pathway potentiate CCL5 and CXCL10 production and switch off or counteract with MyD88 mediated CXCL1 production by tumor cells. This change could modulate the tumor microenvironment and perhaps the

profile of immune cells infiltrating tumor.

To validate this finding in vivo, we performed VCT treatment in B16OVA tumor model and tested these cytokines within tumor lysate by ELISA before and after each procedure of VCT treatment. We noticed that CCL5 dramatically increased shortly after each Poly (A:U) injection. Production of CCL5 was a transient event as its level dropped quickly before the next Poly (A:U) injection was given 3-4 days later. CXCL10 in tumor bed gradually decrease as tumor progress in control (NaCl) group. Interestingly, after chemotherapy (oxaliplatine) was given, VCT treatment substantially increase CXCL10 production at all the time points observed. To dissect the relative contribution of host and that of tumor cells to chemokine production, we purified $CD45^+$ TILs and also $CD45^-$ cells from dissociated tumor and dosed CCL5 and CXCl10 in each fraction before and after each Poly (A:U) injection. In accordance with in vitro data, the increased tissular concentration of CXCL10 paralleled that of CCL5 in Poly (A:U)-based VCT treatment. We also confirmed that accumulating source of both chemokines resided in the tumor parenchyma (shown in published Figure 4C, 4D).

Certain chemokines are involved in forming immune contexture in situ within tumors. An improved prognosis is associated with the expression of specific chemoattractants and adhesion molecules. In a system biology study performed with a cohort of 108 CRC patients, the highest prediction score concerned the chemokine genes CX3CL1, CXCL9, and CXCL10. Indeed, when experimentally tested, high expression of these genes in the tumors correlated with high densities of Th1/cytotoxic memory T cells and with favorable prognosis [357]. To investigate the impact of CCR5/CCL5 on the synergistic effects of our immunochemotherapy, we performed the VCT treatment in wild type mice versus CCR5 loss of function mice. We noticed that tumor growth in CCR5 deficient mice was slightly slower (3-4 days delay, data not shown) than in wild type control. So we started the VCT treatment in both strain when B16OVA tumor reached the similar size. Interestingly, VCT worked more efficaciously when it was applied to $Ccr5^{-/-}$ mice rather than to WT mice. Consistently, when combined with VCT protocol, MetRantes (a pharmacologic inhibitor of CCL5) showed significant addictive effect on VCT therapy to retard tumor growth, even if we doubled the normal tumor cell inoculum. Importantly, when CCL5 expression in

B16OVA tumor was stably knockdown with shRNA by lentivirus transfection (shown in published Figure S6), VCT therapy provided superior tumor control and prolonged survival (shown in published Figure 5A-D). Altogether, these data suggest that tumor derived CCL5 and CCR5 expressing-host derived immune effector are deleterious for the outcome of VCT immunotherapy.

On the contrary, expression of the CXCL10 receptor CXCR3 in the tumor bearing mice is compulsory for the beneficial effect of VCT therapy. Footpad vaccination with CpG plus OVA could trigger antigen specific IFN-γ production, correlating with enriched IFN-γ-producing $CXCR3^+$ $CD8^+$ T cells in the draining LNs. But it did not induce T cell priming in the tumor draining LNs on the other side. We also observed increased $CXCR3^+$ $CD8^+$ cells infiltrating tumor bed after VCT (shown in published Figure 6A, 6B). These evidences imply the notion that tumor vaccine could prime antigen specific CXCR3-expressing $CD8^+$ T cells in the LNs. These cells can be recruited to tumor bed through tumor-derived CXCL10 in the presence of Poly (A:U) treatment and contribute to tumor control. In the absence of tumor vaccine or chemotherapy, the tumorigenicity of B16OVA tumor cells was greatly enhanced by 10 times if they are pretreated with type I IFN plus Poly (A:U) (shown in published Figure 6C). When the mice are immunized with CpG plus OVA, mice are protected from these type I IFN and Poly (A:U) pre-stimulated tumor cells. But this protection vanished if neutralizing antibody for CXCR3 was applied systematically.

Therefore, Poly (A:U) simultaneously enhances CCL5 and CXCL10 production by tumor cells which negatively and positively affect the anti-tumor immunity through host derived $CCR5^+$ and $CXCR3^+$ cells respectively (Figure j). This study emphasizes the contribution of tumor derived factors which initiate the crosstalk among tumor, tumor-associated stromal cells and tumor infiltrating TILs. A more intensive analysis of these factors and resulting modification of tumor microenironment is of great importance for better application of TLR3 agonist during anti-cancer therapies. Future studies should also explore the target cells responding to these tumor derived factors which beneficially or detrimentally influence tumor malignancy and anti-tumor immunity.

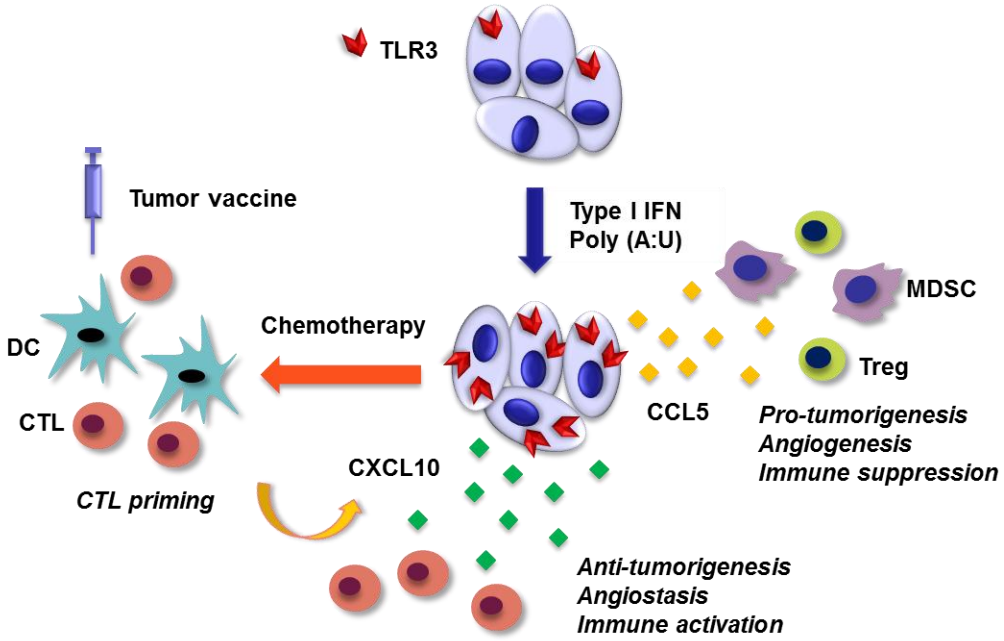

Figure j ***The protumoral and antitumoral effect of Poly (A:U) on TLR3 expressing cancer.*** TLR3 expression on tumor cells could be induced and/or upregulated by type I IFN. Activation of TLR3 on tumor cells leads to CCL5 secretion which activates tumor parenchyma and/or recruit CCR5 expressing tumor infiltrating cells (such as MDSC, Treg), facilitating tumor progression. Poly (A:U) treatment also induce CXCL10 secretion from tumor. When combined with vaccine and/or chemotherapy, both of which potentiate tumor antigen presentation and CTL priming, Poly (A:U) enables antigen specific CXCR3-expressing CTL recruitment into tumor bed to eradicate tumor.

Perspective

Every coin has two sides. Immune system could eliminate tumor cells but it also creates selection pressure for non-immunogenic tumor cells to survive and escape immunosurveillance. High doses broad inflammation could induce tumor cell eradication by specific immunity while low doses local inflammation facilitate tumor invasiveness, metastasis and immune suppression [358]. In the context of tumor, the defined stage of disease, local microenvironment and target cells/molecules/signal pathway should be taken into consideration to interpret the biological function of immune cells and effector molecules. Dying cells could stimulate a robust inflammatory response through releasing danger signals or. To boost the immunogenicity of tumor and reset the immune system against cancer with chemotherapy, radiotherapy and adjuvant, the following issues need to be explored:

1. What are the tumor cell derived danger signals (intracellular/hidden molecules) or proinflammatory molecules generated from extracellular components with the capacity to trigger inflammation? Are these molecules sufficient to trigger a robust anti-tumor response? How does chemotherapy induce the exposure of these molecules? Or how to compensate or maximize the effect of anti-cancer drugs in proper combination with other drugs?
2. How to modulate tumor metabolism and generate novel immunogenic epitope? For example, qualitative and quantitative changes in glycosylation are consistent features of malignancies. Tumor-specific somatic mutation in a chaperone gene Cosmc abolishes the activity of a glycosyltransferase and produces a tumor-specific antigen which induces the generation of a high-affinity antibody with antitumor activity [359].
3. The mechanisms of immune cells infiltrating tumor bed, sensing danger signals released by tumor cells and uptaking tumor associated antigen. How could the adaptive antitumor immunity be primed? What are the key effector molecules and immune cells during this process?
4. How to manipulate the stromal microenvironment of tumors to induce immune

recognition and destruction and to prevent recurrence?

5. How to improve coordinated immune response against cancer with immunoadjuvant and/or tumor vaccine? How to switch the balance between pro-tumoral and anti-tumoral inflammation? What are the molecules and pathways that can be targeted for therapeutic intervention?

Reference

1. Finn OJ: **Cancer immunology**. *N Engl J Med* 2008, **358**:2704-2715.

2. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K: **Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population**. *Lancet* 2000, **356**:1795-1799.

3. Birkeland SA, Storm HH, Lamm LU, Barlow L, Blohme I, Forsberg B, Eklund B, Fjeldborg O, Friedberg M, Frodin L, et al.: **Cancer risk after renal transplantation in the Nordic countries, 1964-1986**. *Int J Cancer* 1995, **60**:183-189.

4. Rama I, Grinyo JM: **Malignancy after renal transplantation: the role of immunosuppression**. *Nat Rev Nephrol* 2010, **6**:511-519.

5. Stewart T, Tsai SC, Grayson H, Henderson R, Opelz G: **Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation**. *Lancet* 1995, **346**:796-798.

6. Birkeland SA, Lokkegaard H, Storm HH: **Cancer risk in patients on dialysis and after renal transplantation**. *Lancet* 2000, **355**:1886-1887.

7. Ingvar A, Smedby KE, Lindelof B, Fernberg P, Bellocco R, Tufveson G, Hoglund P, Adami J: **Immunosuppressive treatment after solid organ transplantation and risk of post-transplant cutaneous squamous cell carcinoma**. *Nephrol Dial Transplant* 2010, **25**:2764-2771.

8. Dighe AS, Richards E, Old LJ, Schreiber RD: **Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors**. *Immunity* 1994, **1**:447-456.

9. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD: **Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice**. *Proc Natl Acad Sci U S A* 1998, **95**:7556-7561.

10. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD: **IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity**. *Nature* 2001, **410**:1107-1111.

11. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM, et al.: **A critical function for type I interferons in cancer immunoediting**. *Nat Immunol* 2005, **6**:722-729.

12. Swann JB, Hayakawa Y, Zerafa N, Sheehan KC, Scott B, Schreiber RD, Hertzog P, Smyth MJ: **Type I IFN contributes to NK cell homeostasis, activation, and antitumor function**. *J Immunol* 2007, **178**:7540-7549.

13. Engel AM, Svane IM, Mouritsen S, Rygaard J, Clausen J, Werdelin O: **Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression**. *APMIS* 1996, **104**:629-639.

14. Engel AM, Svane IM, Rygaard J, Werdelin O: **MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice**. *Scand J Immunol* 1997, **45**:463-470.

15. Smyth MJ, Crowe NY, Godfrey DI: **NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma**. *Int Immunol* 2001, **13**:459-463.

16. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA: **Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma**. *J Exp Med* 2000, **192**:755-760.

17. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI: **Differential tumor surveillance by natural killer (NK) and NKT cells**. *J Exp Med* 2000, **191**:661-668.

18. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC: **Regulation of cutaneous malignancy by gammadelta T cells**. *Science* 2001, **294**:605-609.

19. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, Craft J, Yin Z: **Gamma delta T cells provide an early source of interferon gamma in tumor immunity**. *J Exp Med* 2003, **198**:433-442.

20. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ: **Natural innate and adaptive immunity to cancer**. *Annu Rev Immunol* 2011, **29**:235-271.

21. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD: **Cancer immunoediting: from immunosurveillance to tumor escape**. *Nat Immunol* 2002, **3**:991-998.

22. Dunn GP, Old LJ, Schreiber RD: **The three Es of cancer immunoediting**. *Annu Rev Immunol* 2004, **22**:329-360.

23. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD: **Adaptive immunity maintains occult cancer in an equilibrium state**. *Nature* 2007, **450**:903-907.

24. Aguirre-Ghiso JA: **Models, mechanisms and clinical evidence for cancer dormancy**. *Nat Rev Cancer* 2007, **7**:834-846.

25. Schreiber RD, Old LJ, Smyth MJ: **Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion**. *Science* 2011, **331**:1565-1570.

26. Grivennikov SI, Greten FR, Karin M: **Immunity, inflammation, and cancer**. *Cell* 2010, **140**:883-899.

27. Aggarwal BB, Vijayalekshmi RV, Sung B: **Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe**. *Clin Cancer Res* 2009, **15**:425-430.

28. Medzhitov R: **Origin and physiological roles of inflammation**. *Nature* 2008, **454**:428-435.

29. Balkwill F, Mantovani A: **Inflammation and cancer: back to Virchow?** *Lancet* 2001, **357**:539-545.

30. Ostrand-Rosenberg S: **Immune surveillance: a balance between protumor and antitumor immunity**. *Curr Opin Genet Dev* 2008, **18**:11-18.

31. Grivennikov SI, Karin M: **Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage**. *Ann Rheum Dis* 2011, **70 Suppl 1**:i104-108.

32. Li N, Grivennikov SI, Karin M: **The Unholy Trinity: Inflammation, Cytokines, and STAT3 Shape The Cancer Microenvironment**. *Cancer Cell* 2011, **19**:429-431.

33. Zeisler H, Tempfer C, Joura EA, Sliutz G, Koelbl H, Wagner O, Kainz C: **Serum interleukin 1 in ovarian cancer patients**. *Eur J Cancer* 1998, **34**:931-933.

34. Mayerhofer K, Bodner K, Bodner-Adler B, Schindl M, Kaider A, Hefler L, Zeillinger R, Leodolter S, Joura EA, Kainz C: **Interleukin-8 serum level shift in patients with ovarian carcinoma undergoing paclitaxel-containing chemotherapy**. *Cancer* 2001, **91**:388-393.

35. Lokshin AE, Winans M, Landsittel D, Marrangoni AM, Velikokhatnaya L, Modugno F, Nolen BM, Gorelik E: **Circulating IL-8 and anti-IL-8 autoantibody in patients with ovarian cancer**. *Gynecol Oncol* 2006, **102**:244-251.

36. Deans DA, Wigmore SJ, Gilmour H, Paterson-Brown S, Ross JA, Fearon KC: **Elevated tumour interleukin-1beta is associated with systemic inflammation: A marker of reduced survival in gastro-oesophageal cancer**. *Br J Cancer* 2006, **95**:1568-1575.

37. Scambia G, Testa U, Benedetti Panici P, Foti E, Martucci R, Gadducci A, Perillo A, Facchini V, Peschle C, Mancuso S: **Prognostic significance of interleukin 6 serum levels in patients with ovarian cancer**. *Br J Cancer* 1995, **71**:354-356.

38. Tempfer C, Zeisler H, Sliutz G, Haeusler G, Hanzal E, Kainz C: **Serum evaluation of interleukin 6 in ovarian cancer patients**. *Gynecol Oncol* 1997, **66**:27-30.

39. Gastl G, Plante M: **Bioactive interleukin-6 levels in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer**. *Methods Mol Med* 2001, **39**:121-123.

40. Kryczek I, Grybos M, Karabon L, Klimczak A, Lange A: **IL-6 production in ovarian carcinoma is associated with histiotype and biological characteristics of the tumour and influences local immunity**. *Br J Cancer* 2000, **82**:621-628.

41. Crohns M, Saarelainen S, Laine S, Poussa T, Alho H, Kellokumpu-Lehtinen P: **Cytokines in bronchoalveolar lavage fluid and serum of lung cancer patients during radiotherapy - Association of interleukin-8 and VEGF with survival**. *Cytokine* 2010, **50**:30-36.

42. Apte RN, Dotan S, Elkabets M, White MR, Reich E, Carmi Y, Song X, Dvozkin T, Krelin Y, Voronov E: **The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions**. *Cancer Metastasis Rev* 2006, **25**:387-408.

43. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L: **IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis**. *FASEB J* 2003, **17**:2115-2117.

44. Vidal-Vanaclocha F, Amezaga C, Asumendi A, Kaplanski G, Dinarello CA: **Interleukin-1 receptor blockade reduces the number and size of murine B16 melanoma hepatic metastases**. *Cancer Res* 1994, **54**:2667-2672.

45. Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN: **IL-1 is required for tumor invasiveness and angiogenesis**. *Proc Natl Acad Sci U S A* 2003, **100**:2645-2650.

46. Nazarenko I, Marhaba R, Reich E, Voronov E, Vitacolonna M, Hildebrand D, Elter E, Rajasagi M, Apte RN, Zoller M: **Tumorigenicity of IL-1alpha- and IL-1beta-deficient fibrosarcoma cells**. *Neoplasia* 2008, **10**:549-562.

47. Balkwill F: **Tumour necrosis factor and cancer**. *Nat Rev Cancer* 2009, **9**:361-371.

48. Szlosarek P, Charles KA, Balkwill FR: **Tumour necrosis factor-alpha as a tumour promoter**. *Eur J*

Cancer 2006, **42**:745-750.

49. Yoshida N, Ikemoto S, Narita K, Sugimura K, Wada S, Yasumoto R, Kishimoto T, Nakatani T: **Interleukin-6, tumour necrosis factor alpha and interleukin-1beta in patients with renal cell carcinoma**. *Br J Cancer* 2002, **86**:1396-1400.

50. Ferrajoli A, Keating MJ, Manshouri T, Giles FJ, Dey A, Estrov Z, Koller CA, Kurzrock R, Thomas DA, Faderl S, et al.: **The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia**. *Blood* 2002, **100**:1215-1219.

51. Bozcuk H, Uslu G, Samur M, Yildiz M, Ozben T, Ozdogan M, Artac M, Altunbas H, Akan I, Savas B: **Tumour necrosis factor-alpha, interleukin-6, and fasting serum insulin correlate with clinical outcome in metastatic breast cancer patients treated with chemotherapy**. *Cytokine* 2004, **27**:58-65.

52. Yu H, Kortylewski M, Pardoll D: **Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment**. *Nat Rev Immunol* 2007, **7**:41-51.

53. Yu H, Pardoll D, Jove R: **STATs in cancer inflammation and immunity: a leading role for STAT3**. *Nat Rev Cancer* 2009, **9**:798-809.

54. Kortylewski M, Yu H: **Role of Stat3 in suppressing anti-tumor immunity**. *Curr Opin Immunol* 2008, **20**:228-233.

55. Sgambato A, Cittadini A: **Inflammation and cancer: a multifaceted link**. *Eur Rev Med Pharmacol Sci* 2010, **14**:263-268.

56. Mantovani A, Romero P, Palucka AK, Marincola FM: **Tumour immunity: effector response to tumour and role of the microenvironment**. *Lancet* 2008, **371**:771-783.

57. Egeblad M, Nakasone ES, Werb Z: **Tumors as organs: complex tissues that interface with the entire organism**. *Dev Cell* 2010, **18**:884-901.

58. Ladoire S, Mignot G, Dabakuyo S, Arnould L, Apetoh L, Rebe C, Coudert B, Martin F, Bizollon MH, Vanoli A, et al.: **In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival**. *J Pathol* 2011 Feb 3 [Epub ahead of print].

59. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, et al.: **Effector memory T cells, early metastasis, and survival in colorectal cancer**. *N Engl J Med* 2005, **353**:2654-2666.

60. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M,

Berger A, Wind P, et al.: **Type, density, and location of immune cells within human colorectal tumors predict clinical outcome**. *Science* 2006, **313**:1960-1964.

61. Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, Lagorce C, Wind P, Marliot F, Bruneval P, et al.: **In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer**. *J Clin Oncol* 2009, **27**:5944-5951.

62. Camus M, Tosolini M, Mlecnik B, Pages F, Kirilovsky A, Berger A, Costes A, Bindea G, Charoentong P, Bruneval P, et al.: **Coordination of intratumoral immune reaction and human colorectal cancer recurrence**. *Cancer Res* 2009, **69**:2685-2693.

63. Bindea G, Mlecnik B, Fridman WH, Pages F, Galon J: **Natural immunity to cancer in humans**. *Curr Opin Immunol* 2010, **22**:215-222.

64. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH: **Immune infiltration in human tumors: a prognostic factor that should not be ignored**. *Oncogene* 2010, **29**:1093-1102.

65. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, et al.: **Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer**. *J Clin Oncol* 2010, **28**:105-113.

66. Pittet MJ: **Behavior of immune players in the tumor microenvironment**. *Curr Opin Oncol* 2009, **21**:53-59.

67. Waldhauer I, Steinle A: **NK cells and cancer immunosurveillance**. *Oncogene* 2008, **27**:5932-5943.

68. Coronella JA, Spier C, Welch M, Trevor KT, Stopeck AT, Villar H, Hersh EM: **Antigen-driven oligoclonal expansion of tumor-infiltrating B cells in infiltrating ductal carcinoma of the breast**. *J Immunol* 2002, **169**:1829-1836.

69. Hansen MH, Nielsen H, Ditzel HJ: **The tumor-infiltrating B cell response in medullary breast cancer is oligoclonal and directed against the autoantigen actin exposed on the surface of apoptotic cancer cells**. *Proc Natl Acad Sci U S A* 2001, **98**:12659-12664.

70. Dong HP, Elstrand MB, Holth A, Silins I, Berner A, Trope CG, Davidson B, Risberg B: **NK- and B-cell infiltration correlates with worse outcome in metastatic ovarian carcinoma**. *Am J Clin Pathol* 2006, **125**:451-458.

71. de Visser KE, Korets LV, Coussens LM: **De novo carcinogenesis promoted by chronic**

inflammation is B lymphocyte dependent. *Cancer Cell* 2005, **7**:411-423.

72. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, Korets L, Lam J, Tawfik D, DeNardo DG, et al.: **FcRgamma activation regulates inflammation-associated squamous carcinogenesis**. *Cancer Cell* 2010, **17**:121-134.

73. Wong SC, Puaux AL, Chittezhath M, Shalova I, Kajiji TS, Wang X, Abastado JP, Lam KP, Biswas SK: **Macrophage polarization to a unique phenotype driven by B cells**. *Eur J Immunol* 2010, **40**:2296-2307.

74. Smyth MJ, Godfrey DI: **NKT cells and tumor immunity--a double-edged sword**. *Nat Immunol* 2000, **1**:459-460.

75. Smyth MJ, Crowe NY, Hayakawa Y, Takeda K, Yagita H, Godfrey DI: **NKT cells - conductors of tumor immunity?** *Curr Opin Immunol* 2002, **14**:165-171.

76. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, Hayakawa Y, Godfrey DI, Smyth MJ: **Differential antitumor immunity mediated by NKT cell subsets in vivo**. *J Exp Med* 2005, **202**:1279-1288.

77. Swann J, Crowe NY, Hayakawa Y, Godfrey DI, Smyth MJ: **Regulation of antitumour immunity by CD1d-restricted NKT cells**. *Immunol Cell Biol* 2004, **82**:323-331.

78. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, Godfrey DI, Ostrand-Rosenberg S, Smyth MJ, Berzofsky JA: **A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance**. *J Exp Med* 2005, **202**:1627-1633.

79. Zou W: **Regulatory T cells, tumour immunity and immunotherapy**. *Nat Rev Immunol* 2006, **6**:295-307.

80. Cao X: **Regulatory T cells and immune tolerance to tumors**. *Immunol Res* 2009, **46**:79-93.

81. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH: **Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer**. *Cancer Res* 2001, **61**:4766-4772.

82. Sugihara AQ, Rolle CE, Lesniak MS: **Regulatory T cells actively infiltrate metastatic brain tumors**. *Int J Oncol* 2009, **34**:1533-1540.

83. Mougiakakos D, Johansson CC, Trocme E, All-Ericsson C, Economou MA, Larsson O, Seregard S, Kiessling R: **Intratumoral forkhead box P3-positive regulatory T cells predict poor survival**

in cyclooxygenase-2-positive uveal melanoma. *Cancer* 2010, **116**:2224-2233.

84. Darrasse-Jeze G, Bergot AS, Durgeau A, Billiard F, Salomon BL, Cohen JL, Bellier B, Podsypanina K, Klatzmann D: **Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice**. *J Clin Invest* 2009, **119**:2648-2662.

85. Yaqub S, Henjum K, Mahic M, Jahnsen FL, Aandahl EM, Bjornbeth BA, Tasken K: **Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner**. *Cancer Immunol Immunother* 2008, **57**:813-821.

86. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, et al.: **Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma**. *J Immunol* 2002, **169**:2756-2761.

87. Menetrier-Caux C, Gobert M, Caux C: **Differences in tumor regulatory T-cell localization and activation status impact patient outcome**. *Cancer Res* 2009, **69**:7895-7898.

88. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, Smyth MJ, Hamann A, Huehn J, Sparwasser T: **Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma**. *Cancer Res* 2010, **70**:7788-7799.

89. Li X, Kostareli E, Suffner J, Garbi N, Hammerling GJ: **Efficient Treg depletion induces T-cell infiltration and rejection of large tumors**. *Eur J Immunol* 2010, **40**:3325-3335.

90. Ostrand-Rosenberg S, Sinha P: **Myeloid-derived suppressor cells: linking inflammation and cancer**. *J Immunol* 2009, **182**:4499-4506.

91. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V: **Myeloid-derived suppressor cell heterogeneity and subset definition**. *Curr Opin Immunol* 2010, **22**:238-244.

92. Nagaraj S, Gabrilovich DI: **Tumor escape mechanism governed by myeloid-derived suppressor cells**. *Cancer Res* 2008, **68**:2561-2563.

93. Gabrilovich DI, Nagaraj S: **Myeloid-derived suppressor cells as regulators of the immune system**. *Nat Rev Immunol* 2009, **9**:162-174.

94. Groh V, Wu J, Yee C, Spies T: **Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation**. *Nature* 2002, **419**:734-738.

95. Teng MW, Swann JB, von Scheidt B, Sharkey J, Zerafa N, McLaughlin N, Yamaguchi T, Sakaguchi S,

Darcy PK, Smyth MJ: **Multiple antitumor mechanisms downstream of prophylactic regulatory T-cell depletion**. *Cancer Res* 2010, **70**:2665-2674.

96. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, Corzo A, Cho HI, Celis E, Lennox B, et al.: **Lipid accumulation and dendritic cell dysfunction in cancer**. *Nat Med* 2010, **16**:880-886.

97. Pedroza-Gonzalez A, Xu K, Wu TC, Aspord C, Tindle S, Marches F, Gallegos M, Burton EC, Savino D, Hori T, et al.: **Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation**. *J Exp Med* 2011, **208**:479-490.

98. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale FV, van Bruggen N, et al.: **Bv8 regulates myeloid-cell-dependent tumour angiogenesis**. *Nature* 2007, **450**:825-831.

99. Cao W, Bover L, Cho M, Wen X, Hanabuchi S, Bao M, Rosen DB, Wang YH, Shaw JL, Du Q, et al.: **Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction**. *J Exp Med* 2009, **206**:1603-1614.

100. Villablanca EJ, Raccosta L, Zhou D, Fontana R, Maggioni D, Negro A, Sanvito F, Ponzoni M, Valentinis B, Bregni M, et al.: **Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses**. *Nat Med* 2010, **16**:98-105.

101. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, Murphy WJ, Azuma M, Anderson AC, Kuchroo VK, et al.: **Co-expression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia**. *Blood* 2011.

102. Marotta D, Karar J, Jenkins WT, Kumanova M, Jenkins KW, Tobias JW, Baldwin D, Hatzigeorgiou A, Alexiou P, Evans SM, et al.: **In vivo profiling of hypoxic gene expression in gliomas using the hypoxia marker EF5 and laser-capture microdissection**. *Cancer Res* 2011, **71**:779-789.

103. Semenza GL: **Targeting HIF-1 for cancer therapy**. *Nat Rev Cancer* 2003, **3**:721-732.

104. Hockel M, Vaupel P: **Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects**. *J Natl Cancer Inst* 2001, **93**:266-276.

105. Hockel M, Vaupel P: **Biological consequences of tumor hypoxia**. *Semin Oncol* 2001, **28**:36-41.

106. Vaupel P, Kelleher DK, Hockel M: **Oxygen status of malignant tumors: pathogenesis of hypoxia**

and significance for tumor therapy. *Semin Oncol* 2001, **28**:29-35.

107. Liu Y, Laszlo C, Liu W, Chen X, Evans SC, Wu S: **Regulation of G(1) arrest and apoptosis in hypoxia by PERK and GCN2-mediated eIF2alpha phosphorylation**. *Neoplasia* 2010, **12**:61-68.

108. De Milito A, Fais S: **Tumor acidity, chemoresistance and proton pump inhibitors**. *Future Oncol* 2005, **1**:779-786.

109. Martinez D, Vermeulen M, Trevani A, Ceballos A, Sabatte J, Gamberale R, Alvarez ME, Salamone G, Tanos T, Coso OA, et al.: **Extracellular acidosis induces neutrophil activation by a mechanism dependent on activation of phosphatidylinositol 3-kinase/Akt and ERK pathways**. *J Immunol* 2006, **176**:1163-1171.

110. Zhang Z, Liu Q, Che Y, Yuan X, Dai L, Zeng B, Jiao G, Zhang Y, Wu X, Yu Y, et al.: **Antigen presentation by dendritic cells in tumors is disrupted by altered metabolism that involves pyruvate kinase M2 and its interaction with SOCS3**. *Cancer Res* 2010, **70**:89-98.

111. Wei J, Wu A, Kong LY, Wang Y, Fuller G, Fokt I, Melillo G, Priebe W, Heimberger AB: **Hypoxia potentiates glioma-mediated immunosuppression**. *PLoS One* 2011, **6**:e16195.

112. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, et al.: **Inhibitory effect of tumor cell-derived lactic acid on human T cells**. *Blood* 2007, **109**:3812-3819.

113. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, Mordoh J, Fainboim L, Podhajcer OL, Rabinovich GA: **Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege**. *Cancer Cell* 2004, **5**:241-251.

114. Jinushi M, Hodi FS, Dranoff G: **Enhancing the clinical activity of granulocyte-macrophage colony-stimulating factor-secreting tumor cell vaccines**. *Immunol Rev* 2008, **222**:287-298.

115. Perez CA, Fu A, Onishko H, Hallahan DE, Geng L: **Radiation induces an antitumour immune response to mouse melanoma**. *Int J Radiat Biol* 2009, **85**:1126-1136.

116. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, Schmitt E, Hamai A, Hervas-Stubbs S, Obeid M, et al.: **Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death**. *J Exp Med* 2005, **202**:1691-1701.

117. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G,

Ullrich E, et al.: **Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors**. *Nat Med* 2009, **15**:1170-1178.

118. Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, Yu P, Fu YX, Weichselbaum RR, Rowley DA, et al.: **Induced sensitization of tumor stroma leads to eradication of established cancer by T cells**. *J Exp Med* 2007, **204**:49-55.

119. Ma Y, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C, Tesniere A, Martins I, Ly A, Haynes NM, et al.: **Chemotherapy and radiotherapy: cryptic anticancer vaccines**. *Semin Immunol* 2010, **22**:113-124.

120. Tanaka H, Matsushima H, Mizumoto N, Takashima A: **Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells**. *Cancer Res* 2009, **69**:6978-6986.

121. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, et al.: **Calreticulin exposure dictates the immunogenicity of cancer cell death**. *Nat Med* 2007, **13**:54-61.

122. Ma Y, Conforti R, Aymeric L, Locher C, Kepp O, Kroemer G, Zitvogel L: **How to improve the immunogenicity of chemotherapy and radiotherapy**. *Cancer Metastasis Rev* 2011, **30**:71-82.

123. Kepp O, Galluzzi L, Lipinski M, Yuan J, Kroemer G: **Cell death assays for drug discovery**. *Nat Rev Drug Discov* 2011, **10**:221-237.

124. Green DR, Ferguson T, Zitvogel L, Kroemer G: **Immunogenic and tolerogenic cell death**. *Nat Rev Immunol* 2009, **9**:353-363.

125. Zitvogel L, Kepp O, Kroemer G: **Decoding cell death signals in inflammation and immunity**. *Cell* 2010, **140**:798-804.

126. Obeid M, Panaretakis T, Joza N, Tufi R, Tesniere A, van Endert P, Zitvogel L, Kroemer G: **Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis**. *Cell Death Differ* 2007, **14**:1848-1850.

127. Panaretakis T, Joza N, Modjtahedi N, Tesniere A, Vitale I, Durchschlag M, Fimia GM, Kepp O, Piacentini M, Froehlich KU, et al.: **The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death**. *Cell Death Differ* 2008, **15**:1499-1509.

128. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N,

Weissman IL: **CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis**. *Cell* 2009, **138**:271-285.

129. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ: **High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin**. *J Leukoc Biol* 2007, **81**:59-66.

130. Orlova VV, Choi EY, Xie C, Chavakis E, Bierhaus A, Ihanus E, Ballantyne CM, Gahmberg CG, Bianchi ME, Nawroth PP, et al.: **A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin**. *EMBO J* 2007, **26**:1129-1139.

131. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, et al.: **Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy**. *Nat Med* 2007, **13**:1050-1059.

132. Zhu XM, Yao YM, Liang HP, Xu CT, Dong N, Yu Y, Sheng ZY: **High mobility group box-1 protein regulate immunosuppression of regulatory T cells through toll-like receptor 4**. *Cytokine* 2011.

133. 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.

134. Tang D, Loze MT, Zeh HJ, Kang R: **The redox protein HMGB1 regulates cell death and survival in cancer treatment**. *Autophagy* 2010, **6**:1181-1183.

135. Gupta S, Deepti A, Deegan S, Lisbona F, Hetz C, Samali A: **HSP72 protects cells from ER stress-induced apoptosis via enhancement of IRE1alpha-XBP1 signaling through a physical interaction**. *PLoS Biol* 2010, **8**:e1000410.

136. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, Boireau W, Rouleau A, Simon B, Lanneau D, et al.: **Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells**. *J Clin Invest* 2010, **120**:457-471.

137. Spisek R, Dhodapkar MV: **Towards a better way to die with chemotherapy: role of heat shock protein exposure on dying tumor cells**. *Cell Cycle* 2007, **6**:1962-1965.

138. Murshid A, Gong J, Calderwood SK: **Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I**. *J Immunol*

2010, **185**:2903-2917.

139. Chan T, Chen Z, Hao S, Xu S, Yuan J, Saxena A, Qureshi M, Zheng C, Xiang J: **Enhanced T-cell immunity induced by dendritic cells with phagocytosis of heat shock protein 70 gene-transfected tumor cells in early phase of apoptosis**. *Cancer Gene Ther* 2007, **14**:409-420.

140. Basu S, Binder RJ, Ramalingam T, Srivastava PK: **CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin**. *Immunity* 2001, **14**:303-313.

141. Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, et al.: **Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation**. *Immunity* 2002, **17**:353-362.

142. Elsner L, Flugge PF, Lozano J, Muppala V, Eiz-Vesper B, Demiroglu SY, Malzahn D, Herrmann T, Brunner E, Bickeboller H, et al.: **The endogenous danger signals HSP70 and MICA cooperate in the activation of cytotoxic effector functions of NK cells**. *J Cell Mol Med* 2010, **14**:992-1002.

143. Junger WG: **Immune cell regulation by autocrine purinergic signalling**. *Nat Rev Immunol* 2011, **11**:201-212.

144. Trautmann A: **Extracellular ATP in the immune system: more than just a "danger signal"**. *Sci Signal* 2009, **2**:pe6.

145. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG: **ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors**. *Science* 2006, **314**:1792-1795.

146. Schenk U, Westendorf AM, Radaelli E, Casati A, Ferro M, Fumagalli M, Verderio C, Buer J, Scanziani E, Grassi F: **Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels**. *Sci Signal* 2008, **1**:ra6.

147. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K, et al.: **ATP drives lamina propria T(H)17 cell differentiation**. *Nature* 2008, **455**:808-812.

148. Dzhandzhugazyan KN, Kirkin AF, thor Straten P, Zeuthen J: **Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas**. *FEBS Lett* 1998, **430**:227-230.

149. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjyoji K, Linden J, Oukka

M, et al.: **Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression**. *J Exp Med* 2007, **204**:1257-1265.

150. Stagg J, Divisekera U, Duret H, Sparwasser T, Teng MW, Darcy PK, Smyth MJ: **CD73-Deficient Mice Have Increased Antitumor Immunity and Are Resistant to Experimental Metastasis**. *Cancer Res* 2011, **71**:2892-2900.

151. Scherf DB, Dally H, Muller P, Werle-Schneider G, Jager B, Edler L, Tuengerthal S, Fischer JR, Drings P, Bartsch H, et al.: **Single nucleotide polymorphisms in matrix metalloproteinase genes and lung cancer chemotherapy response and prognosis**. *Eur Respir J* 2010, **35**:381-390.

152. Ma Y, Aymeric L, Locher C, Kroemer G, Zitvogel L: **The dendritic cell-tumor cross-talk in cancer**. *Curr Opin Immunol* 2010, **23**:146-152.

153. Villadangos JA, Schnorrer P: **Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo**. *Nat Rev Immunol* 2007, **7**:543-555.

154. Ali OA, Emerich D, Dranoff G, Mooney DJ: **In situ regulation of DC subsets and T cells mediates tumor regression in mice**. *Sci Transl Med* 2009, **1**:8ra19.

155. Zhang B, Zhang Y, Bowerman NA, Schietinger A, Fu YX, Kranz DM, Rowley DA, Schreiber H: **Equilibrium between host and cancer caused by effector T cells killing tumor stroma**. *Cancer Res* 2008, **68**:1563-1571.

156. Spiotto MT, Rowley DA, Schreiber H: **Bystander elimination of antigen loss variants in established tumors**. *Nat Med* 2004, **10**:294-298.

157. Zhang B, Karrison T, Rowley DA, Schreiber H: **IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers**. *J Clin Invest* 2008, **118**:1398-1404.

158. Schietinger A, Philip M, Liu RB, Schreiber K, Schreiber H: **Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase**. *J Exp Med* 2010, **207**:2469-2477.

159. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L, et al.: **Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures**. *J Clin Oncol* 2008, **26**:4410-4417.

160. Wolf MJ, Seleznik GM, Zeller N, Heikenwalder M: **The unexpected role of lymphotoxin beta**

receptor signaling in carcinogenesis: from lymphoid tissue formation to liver and prostate cancer development. *Oncogene* 2010, **29**:5006-5018.

161. Lee Y, Chin RK, Christiansen P, Sun Y, Tumanov AV, Wang J, Chervonsky AV, Fu YX: **Recruitment and activation of naive T cells in the islets by lymphotoxin beta receptor-dependent tertiary lymphoid structure**. *Immunity* 2006, **25**:499-509.

162. Nelson BH: **CD20+ B cells: the other tumor-infiltrating lymphocytes**. *J Immunol* 2010, **185**:4977-4982.

163. Grabner R, Lotzer K, Dopping S, Hildner M, Radke D, Beer M, Spanbroek R, Lippert B, Reardon CA, Getz GS, et al.: **Lymphotoxin beta receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE-/- mice**. *J Exp Med* 2009, **206**:233-248.

164. Kim S, Han S, Kim MY: **Heterogeneity of IL-22-producing Lymphoid Tissue Inducer-like Cells in Human and Mouse**. *Immune Netw* 2010, **10**:115-119.

165. Gil-Guerrero L, Dotor J, Huibregtse IL, Casares N, Lopez-Vazquez AB, Rudilla F, Riezu-Boj JI, Lopez-Sagaseta J, Hermida J, Van Deventer S, et al.: **In vitro and in vivo down-regulation of regulatory T cell activity with a peptide inhibitor of TGF-beta1**. *J Immunol* 2008, **181**:126-135.

166. Gaffen SL: **An overview of IL-17 function and signaling**. *Cytokine* 2008, **43**:402-407.

167. Korn T, Bettelli E, Oukka M, Kuchroo VK: **IL-17 and Th17 Cells**. *Annu Rev Immunol* 2009, **27**:485-517.

168. Onishi RM, Gaffen SL: **Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease**. *Immunology* 2010, **129**:311-321.

169. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y: **IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis**. *J Immunol* 2006, **177**:566-573.

170. van den Berg WB, Miossec P: **IL-17 as a future therapeutic target for rheumatoid arthritis**. *Nat Rev Rheumatol* 2009, **5**:549-553.

171. Garrett-Sinha LA, John S, Gaffen SL: **IL-17 and the Th17 lineage in systemic lupus erythematosus**. *Curr Opin Rheumatol* 2008, **20**:519-525.

172. Tokura Y, Mori T, Hino R: **Psoriasis and other Th17-mediated skin diseases**. *J UOEH* 2011, **32**:317-328.

173. Ahern PP, Izcue A, Maloy KJ, Powrie F: **The interleukin-23 axis in intestinal inflammation**. *Immunol Rev* 2008, **226**:147-159.

174. Chen L, Ahmed E, Wang T, Wang Y, Ochando J, Chong AS, Alegre ML: **TLR signals promote IL-6/IL-17-dependent transplant rejection**. *J Immunol* 2009, **182**:6217-6225.

175. Shilling RA, Wilkes DS: **Role of Th17 cells and IL-17 in lung transplant rejection**. *Semin Immunopathol* 2011.

176. Kappel LW, Goldberg GL, King CG, Suh DY, Smith OM, Ligh C, Holland AM, Grubin J, Mark NM, Liu C, et al.: **IL-17 contributes to CD4-mediated graft-versus-host disease**. *Blood* 2009, **113**:945-952.

177. van de Veerdonk FL, Gresnigt MS, Kullberg BJ, van der Meer JW, Joosten LA, Netea MG: **Th17 responses and host defense against microorganisms: an overview**. *BMB Rep* 2009, **42**:776-787.

178. Kanwar B, Favre D, McCune JM: **Th17 and regulatory T cells: implications for AIDS pathogenesis**. *Curr Opin HIV AIDS* 2011, **5**:151-157.

179. Wilke CM, Kryczek I, Wei S, Zhao E, Wu K, Wang G, Zou W: **Th17 Cells in Cancer: Help or Hindrance?** *Carcinogenesis* 2011.

180. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I: **Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells**. *Blood* 2008, **112**:2340-2352.

181. Kryczek I, Wu K, Zhao E, Wei S, Vatan L, Szeliga W, Huang E, Greenson J, Chang A, Rolinski J, et al.: **IL-17+ Regulatory T Cells in the Microenvironments of Chronic Inflammation and Cancer**. *J Immunol* 2011, **186**:4388-4395.

182. Res PC, Piskin G, de Boer OJ, van der Loos CM, Teeling P, Bos JD, Teunissen MB: **Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis**. *PLoS One* 2010, **5**:e14108.

183. He D, Wu L, Kim HK, Li H, Elmets CA, Xu H: **CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses**. *J Immunol* 2006, **177**:6852-6858.

184. Kish DD, Li X, Fairchild RL: **CD8 T cells producing IL-17 and IFN-gamma initiate the innate immune response required for responses to antigen skin challenge**. *J Immunol* 2009,

182:5949-5959.

185. Rachitskaya AV, Hansen AM, Horai R, Li Z, Villasmil R, Luger D, Nussenblatt RB, Caspi RR: **Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion**. *J Immunol* 2008, **180**:5167-5171.

186. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, Berzins SP, Smyth MJ, Godfrey DI: **Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population**. *Proc Natl Acad Sci U S A* 2008, **105**:11287-11292.

187. Roark CL, Simonian PL, Fontenot AP, Born WK, O'Brien RL: **gammadelta T cells: an important source of IL-17**. *Curr Opin Immunol* 2008, **20**:353-357.

188. Meyer C, Zeng X, Chien YH: **Ligand recognition during thymic development and gammadelta T cell function specification**. *Semin Immunol* 2010, **22**:207-213.

189. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, Milder M, Le Bourhis L, Soudais C, Treiner E, et al.: **Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells**. *Blood* 2010, **117**:1250-1259.

190. Passos ST, Silver JS, O'Hara AC, Sehy D, Stumhofer JS, Hunter CA: **IL-6 promotes NK cell production of IL-17 during toxoplasmosis**. *J Immunol* 2010, **184**:1776-1783.

191. Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, Strieter RM, Rosin DL, Okusa MD: **IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury**. *J Clin Invest* 2009, **120**:331-342.

192. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, Olivenstein R, Elias J, Chakir J: **IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines**. *J Allergy Clin Immunol* 2001, **108**:430-438.

193. Sawa S, Cherrier M, Lochner M, Satoh-Takayama N, Fehling HJ, Langa F, Di Santo JP, Eberl G: **Lineage relationship analysis of RORgammat+ innate lymphoid cells**. *Science* 2010, **330**:665-669.

194. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H: **Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2**. *Immunity* 2010, **33**:752-764.

195. Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, Fibbe WE, Cornelissen JJ, Spits H: **Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells**. *Nat Immunol* 2009, **10**:66-74.

196. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov, II, Littman DR, O'Shea JJ: **Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22**. *J Exp Med* 2009, **206**:35-41.

197. Crispin JC, Tsokos GC: **Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype**. *J Immunol* 2009, **183**:4675-4681.

198. Cowley SC, Meierovics AI, Frelinger JA, Iwakura Y, Elkins KL: **Lung CD4-CD8- double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with Francisella tularensis live vaccine strain**. *J Immunol* 2010, **184**:5791-5801.

199. Lambert C, Genin C: **CD3 bright lymphocyte population reveal gammadelta T cells**. *Cytometry B Clin Cytom* 2004, **61**:45-53.

200. Beatty GL, Paterson Y: **Regulation of tumor growth by IFN-gamma in cancer immunotherapy**. *Immunol Res* 2001, **24**:201-210.

201. Ikeda H, Old LJ, Schreiber RD: **The roles of IFN gamma in protection against tumor development and cancer immunoediting**. *Cytokine Growth Factor Rev* 2002, **13**:95-109.

202. Blankenstein T, Qin Z: **The role of IFN-gamma in tumor transplantation immunity and inhibition of chemical carcinogenesis**. *Curr Opin Immunol* 2003, **15**:148-154.

203. Malmberg KJ, Levitsky V, Norell H, de Matos CT, Carlsten M, Schedvins K, Rabbani H, Moretta A, Soderstrom K, Levitskaya J, et al.: **IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism**. *J Clin Invest* 2002, **110**:1515-1523.

204. Muranski P, Restifo NP: **Does IL-17 promote tumor growth?** *Blood* 2009, **114**:231-232.

205. Ngiow SF, Smyth MJ, Teng MW: **Does IL-17 suppress tumor growth?** *Blood* 2010, **115**:2554-2555; author reply 2556-2557.

206. Murugaiyan G, Saha B: **Protumor vs antitumor functions of IL-17**. *J Immunol* 2009, **183**:4169-4175.

207. Tartour E, Fossiez F, Joyeux I, Galinha A, Gey A, Claret E, Sastre-Garau X, Couturier J, Mosseri V,

Vives V, et al.: **Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice**. *Cancer Res* 1999, **59**:3698-3704.

208. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK, et al.: **IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis**. *J Immunol* 2005, **175**:6177-6189.

209. Numasaki M, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, Robbins PD, Tahara H, Lotze MT: **Interleukin-17 promotes angiogenesis and tumor growth**. *Blood* 2003, **101**:2620-2627.

210. Hirahara N, Nio Y, Sasaki S, Minari Y, Takamura M, Iguchi C, Dong M, Yamasawa K, Tamura K: **Inoculation of human interleukin-17 gene-transfected Meth-A fibrosarcoma cells induces T cell-dependent tumor-specific immunity in mice**. *Oncology* 2001, **61**:79-89.

211. Yang Z, Zhang B, Li D, Lv M, Huang C, Shen GX, Huang B: **Mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment via IL-17 pathway in murine hepatocarcinoma model**. *PLoS One* 2010, **5**:e8922.

212. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H: **IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway**. *J Exp Med* 2009, **206**:1457-1464.

213. Kortylewski M, Xin H, Kujawski M, Lee H, Liu Y, Harris T, Drake C, Pardoll D, Yu H: **Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment**. *Cancer Cell* 2009, **15**:114-123.

214. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, et al.: **Tumor-specific Th17-polarized cells eradicate large established melanoma**. *Blood* 2008, **112**:362-373.

215. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C: **T helper 17 cells promote cytotoxic T cell activation in tumor immunity**. *Immunity* 2009, **31**:787-798.

216. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, et al.: **Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments**. *Blood* 2009, **114**:1141-1149.

217. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y, Munn DH: **Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in**

tumor-draining lymph nodes. *Blood* 2009, **113**:6102-6111.

218. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerk B, Wrzesinski C, Borman ZA, Muranski P, Restifo NP: **Type 17 CD8+ T cells display enhanced antitumor immunity**. *Blood* 2009, **114**:596-599.

219. Kwong BY, Roberts SJ, Silberzahn T, Filler RB, Neustadter JH, Galan A, Reddy S, Lin WM, Ellis PD, Langford CF, et al.: **Molecular analysis of tumor-promoting CD8+ T cells in two-stage cutaneous chemical carcinogenesis**. *J Invest Dermatol* 2009, **130**:1726-1736.

220. Maniati E, Soper R, Hagemann T: **Up for Mischief? IL-17/Th17 in the tumour microenvironment**. *Oncogene* 2010, **29**:5653-5662.

221. Girardi M: **Immunosurveillance and immunoregulation by gammadelta T cells**. *J Invest Dermatol* 2006, **126**:25-31.

222. Gomes AQ, Martins DS, Silva-Santos B: **Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application**. *Cancer Res* 2010, **70**:10024-10027.

223. Braakman E, van de Winkel JG, van Krimpen BA, Jansze M, Bolhuis RL: **CD16 on human gamma delta T lymphocytes: expression, function, and specificity for mouse IgG isotypes**. *Cell Immunol* 1992, **143**:97-107.

224. Kuziel WA, Lewis J, Nixon-Fulton J, Tigelaar RE, Tucker PW: **Murine epidermal gamma/delta T cells express Fc gamma receptor II encoded by the Fc gamma R alpha gene**. *Eur J Immunol* 1991, **21**:1563-1566.

225. Chen Z, Freedman MS: **CD16+ gammadelta T cells mediate antibody dependent cellular cytotoxicity: potential mechanism in the pathogenesis of multiple sclerosis**. *Clin Immunol* 2008, **128**:219-227.

226. Hao J, Wu X, Xia S, Li Z, Wen T, Zhao N, Wu Z, Wang P, Zhao L, Yin Z: **Current progress in gammadelta T-cell biology**. *Cell Mol Immunol* 2010, **7**:409-413.

227. Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, Bukowski JF: **MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function**. *Immunity* 2001, **15**:83-93.

228. Brandes M, Willimann K, Bioley G, Levy N, Eberl M, Luo M, Tampe R, Levy F, Romero P, Moser B: **Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell**

responses. *Proc Natl Acad Sci U S A* 2009, **106**:2307-2312.

229. Meuter S, Eberl M, Moser B: **Prolonged antigen survival and cytosolic export in cross-presenting human gammadelta T cells**. *Proc Natl Acad Sci U S A* 2010, **107**:8730-8735.

230. Brandes M, Willimann K, Moser B: **Professional antigen-presentation function by human gammadelta T Cells**. *Science* 2005, **309**:264-268.

231. Havran WL, Chien YH, Allison JP: **Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors**. *Science* 1991, **252**:1430-1432.

232. Huber H, Descossy P, van Brandwijk R, Knop J: **Activation of murine epidermal TCR-gamma delta+ T cells by keratinocytes treated with contact sensitizers**. *J Immunol* 1995, **155**:2888-2894.

233. Carding SR, Egan PJ: **The importance of gamma delta T cells in the resolution of pathogen-induced inflammatory immune responses**. *Immunol Rev* 2000, **173**:98-108.

234. Kirby AC, Newton DJ, Carding SR, Kaye PM: **Pulmonary dendritic cells and alveolar macrophages are regulated by gammadelta T cells during the resolution of S. pneumoniae-induced inflammation**. *J Pathol* 2007, **212**:29-37.

235. Braun RK, Ferrick C, Neubauer P, Sjoding M, Sterner-Kock A, Kock M, Putney L, Ferrick DA, Hyde DM, Love RB: **IL-17 producing gammadelta T cells are required for a controlled inflammatory response after bleomycin-induced lung injury**. *Inflammation* 2008, **31**:167-179.

236. Huang Y, Jin N, Roark CL, Aydintug MK, Wands JM, Huang H, O'Brien RL, Born WK: **The influence of IgE-enhancing and IgE-suppressive gammadelta T cells changes with exposure to inhaled ovalbumin**. *J Immunol* 2009, **183**:849-855.

237. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, et al.: **Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury**. *Nat Med* 2009, **15**:946-950.

238. Hayday AC: **Gammadelta T cells and the lymphoid stress-surveillance response**. *Immunity* 2009, **31**:184-196.

239. Girardi M, Glusac E, Filler RB, Roberts SJ, Propperova I, Lewis J, Tigelaar RE, Hayday AC: **The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T**

cells to different stages of chemically induced skin cancer. *J Exp Med* 2003, **198**:747-755.

240. Takeuchi A, Dejima T, Yamada H, Shibata K, Nakamura R, Eto M, Nakatani T, Naito S, Yoshikai Y: **IL-17 production by gammadelta T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guerin treatment against bladder cancer**. *Eur J Immunol* **41**:246-251.

241. Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, Kitamura H, Nishimura T: **Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis**. *Eur J Immunol* 2010, **40**:1927-1937.

242. Ke Y, Kapp LM, Kapp JA: **Inhibition of tumor rejection by gammadelta T cells and IL-10**. *Cell Immunol* 2003, **221**:107-114.

243. Seo N, Tokura Y, Takigawa M, Egawa K: **Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells**. *J Immunol* 1999, **163**:242-249.

244. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF: **Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway**. *Immunity* 2007, **27**:334-348.

245. Couzi L, Levaillant Y, Jamai A, Pitard V, Lassalle R, Martin K, Garrigue I, Hawchar O, Siberchicot F, Moore N, et al.: **Cytomegalovirus-induced gammadelta T cells associate with reduced cancer risk after kidney transplantation**. *J Am Soc Nephrol* 2009, **21**:181-188.

246. Dechanet J, Merville P, Lim A, Retiere C, Pitard V, Lafarge X, Michelson S, Meric C, Hallet MM, Kourilsky P, et al.: **Implication of gammadelta T cells in the human immune response to cytomegalovirus**. *J Clin Invest* 1999, **103**:1437-1449.

247. van Burik JA, Carter SL, Freifeld AG, High KP, Godder KT, Papanicolaou GA, Mendizabal AM, Wagner JE, Yanovich S, Kernan NA: **Higher risk of cytomegalovirus and aspergillus infections in recipients of T cell-depleted unrelated bone marrow: analysis of infectious complications in patients treated with T cell depletion versus immunosuppressive therapy to prevent graft-versus-host disease**. *Biol Blood Marrow Transplant* 2007, **13**:1487-1498.

248. Kabelitz D, Wesch D, He W: **Perspectives of gammadelta T cells in tumor immunology**. *Cancer*

Res 2007, **67**:5-8.

249. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, et al.: **Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer**. *Cancer Res* 2007, **67**:7450-7457.

250. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP: **Gammadelta T cells for immune therapy of patients with lymphoid malignancies**. *Blood* 2003, **102**:200-206.

251. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M: **Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals**. *Immunity* 2009, **31**:321-330.

252. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH: **Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity**. *Immunity* 2009, **31**:331-341.

253. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, Steinman L, Saito T, Locksley RM, Davis MM, et al.: **Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma**. *Immunity* 2008, **29**:90-100.

254. Jensen KD, Chien YH: **Thymic maturation determines gammadelta T cell function, but not their antigen specificities**. *Curr Opin Immunol* 2009, **21**:140-145.

255. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, Girardi M, Borst J, Hayday AC, Pennington DJ, et al.: **CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets**. *Nat Immunol* 2009, **10**:427-436.

256. Shibata K, Yamada H, Nakamura R, Sun X, Itsumi M, Yoshikai Y: **Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers**. *J Immunol* 2008, **181**:5940-5947.

257. Haas JD, Gonzalez FH, Schmitz S, Chennupati V, Fohse L, Kremmer E, Forster R, Prinz I: **CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells**. *Eur J Immunol* 2009, **39**:3488-3497.

258. Kisielow J, Kopf M, Karjalainen K: **SCART scavenger receptors identify a novel subset of adult gammadelta T cells**. *J Immunol* 2008, **181**:1710-1716.

259. Stagg J, Smyth MJ: **Extracellular adenosine triphosphate and adenosine in cancer**. *Oncogene* 2010, **29**:5346-5358.

260. Heilig JS, Tonegawa S: **Diversity of murine gamma genes and expression in fetal and adult T lymphocytes**. *Nature* 1986, **322**:836-840.

261. Pereira P, Boucontet L: **Rates of recombination and chain pair biases greatly influence the primary gammadelta TCR repertoire in the thymus of adult mice**. *J Immunol* 2004, **173**:3261-3270.

262. O'Brien RL, Born WK: **gammadelta T cell subsets: a link between TCR and function?** *Semin Immunol* 2010, **22**:193-198.

263. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL: **Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells**. *J Immunol* 2007, **179**:5576-5583.

264. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y: **Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production**. *J Immunol* 2007, **178**:4466-4472.

265. Hamada S, Umemura M, Shiono T, Tanaka K, Yahagi A, Begum MD, Oshiro K, Okamoto Y, Watanabe H, Kawakami K, et al.: **IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver**. *J Immunol* 2008, **181**:3456-3463.

266. Liu Z, Eltoum IE, Guo B, Beck BH, Cloud GA, Lopez RD: **Protective immunosurveillance and therapeutic antitumor activity of gammadelta T cells demonstrated in a mouse model of prostate cancer**. *J Immunol* 2008, **180**:6044-6053.

267. Do JS, Fink PJ, Li L, Spolski R, Robinson J, Leonard WJ, Letterio JJ, Min B: **Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism**. *J Immunol* 2010, **184**:1675-1679.

268. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A, Waisman A, Kuchroo VK, Glimcher LH, Oukka M: **IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections**. *J Immunol* 2010, **184**:1710-1720.

269. Awasthi A, Riol-Blanco L, Jager A, Korn T, Pot C, Galileos G, Bettelli E, Kuchroo VK, Oukka M: **Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells**. *J Immunol* 2009, **182**:5904-5908.

270. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y, et al.: **IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection**. *J Immunol* 2007, **178**:3786-3796.

271. Lin Y, Ritchea S, Logar A, Slight S, Messmer M, Rangel-Moreno J, Guglani L, Alcorn JF, Strawbridge H, Park SM, et al.: **Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis**. *Immunity* 2009, **31**:799-810.

272. Xu S, Han Y, Xu X, Bao Y, Zhang M, Cao X: **IL-17A-producing gammadeltaT cells promote CTL responses against Listeria monocytogenes infection by enhancing dendritic cell cross-presentation**. *J Immunol* 2010, **185**:5879-5887.

273. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, Prinz I, Hemmer B, Kuchroo VK, Oukka M, et al.: **gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism**. *Immunity* 2010, **33**:351-363.

274. Carmi Y, Rinott G, Dotan S, Elkabets M, Rider P, Voronov E, Apte RN: **Microenvironment-derived IL-1 and IL-17 interact in the control of lung metastasis**. *J Immunol* 2011, **186**:3462-3471.

275. Cua DJ, Tato CM: **Innate IL-17-producing cells: the sentinels of the immune system**. *Nat Rev Immunol* 2010, **10**:479-489.

276. Andrews DM, Chow MT, Ma Y, Cotterell CL, Watt SV, Anthony DA, Akira S, Iwakura Y, Trapani JA, Zitvogel L, et al.: **Homeostatic defects in interleukin 18-deficient mice contribute to protection against the lethal effects of endotoxin**. *Immunol Cell Biol* 2011.

277. Lalor SJ, Dungan LS, Sutton CE, Basdeo SA, Fletcher JM, Mills KH: **Caspase-1-Processed Cytokines IL-1{beta} and IL-18 Promote IL-17 Production by {gamma}{delta} and CD4 T Cells That Mediate Autoimmunity**. *J Immunol* 2011.

278. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A: **Control of epithelial cell function by interleukin-22-producing RORgammat+ innate lymphoid cells**. *Immunology* 2011,

132:453-465.

279. Ma HL, Liang S, Li J, Napierata L, Brown T, Benoit S, Senices M, Gill D, Dunussi-Joannopoulos K, Collins M, et al.: **IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation**. *J Clin Invest* 2008, **118**:597-607.

280. Tang Q, Ahn YO, Southern P, Blazar BR, Miller JS, Verneris MR: **Development of IL-22-producing NK lineage cells from umbilical cord blood hematopoietic stem cells in the absence of secondary lymphoid tissue**. *Blood* 2011, **117**:4052-4055.

281. Hughes T, Becknell B, Freud AG, McClory S, Briercheck E, Yu J, Mao C, Giovenzana C, Nuovo G, Wei L, et al.: **Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue**. *Immunity* 2010, **32**:803-814.

282. Colonna M: **Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity**. *Immunity* 2009, **31**:15-23.

283. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kiss EA, Hoyler T, Flach M, Bengsch B, Thimme R, Holscher C, et al.: **Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes**. *Immunity* 2010, **33**:736-751.

284. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R: **IL-22 increases the innate immunity of tissues**. *Immunity* 2004, **21**:241-254.

285. Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW, Wynn TA: **Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent**. *J Exp Med* 2010, **207**:535-552.

286. Simonian PL, Wehrmann F, Roark CL, Born WK, O'Brien RL, Fontenot AP: **gammadelta T cells protect against lung fibrosis via IL-22**. *J Exp Med* 2010, **207**:2239-2253.

287. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C: **IL-17 and IL-22: siblings, not twins**. *Trends Immunol* 2010, **31**:354-361.

288. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D: **Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A**. *J Exp Med* 2010, **207**:1293-1305.

289. Champagne E: **gammadelta T cell receptor ligands and modes of antigen recognition**. *Arch Immunol Ther Exp (Warsz)* 2011, **59**:117-137.

290. Kreslavsky T, von Boehmer H: **gammadeltaTCR ligands and lineage commitment**. *Semin Immunol* 2010, **22**:214-221.

291. Conklin KA: **Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness**. *Integr Cancer Ther* 2004, **3**:294-300.

292. Schlattner U, Tokarska-Schlattner M, Ramirez S, Bruckner A, Kay L, Polge C, Epand RF, Lee RM, Lacombe ML, Epand RM: **Mitochondrial kinases and their molecular interaction with cardiolipin**. *Biochim Biophys Acta* 2009, **1788**:2032-2047.

293. Sorice M, Circella A, Misasi R, Pittoni V, Garofalo T, Cirelli A, Pavan A, Pontieri GM, Valesini G: **Cardiolipin on the surface of apoptotic cells as a possible trigger for antiphospholipids antibodies**. *Clin Exp Immunol* 2000, **122**:277-284.

294. Born WK, Vollmer M, Reardon C, Matsuura E, Voelker DR, Giclas PC, O'Brien RL: **Hybridomas expressing gammadelta T-cell receptors respond to cardiolipin and beta2-glycoprotein 1 (apolipoprotein H)**. *Scand J Immunol* 2003, **58**:374-381.

295. Dieude M, Striegl H, Tyznik AJ, Wang J, Behar SM, Piccirillo CA, Levine JS, Zajonc DM, Rauch J: **Cardiolipin Binds to CD1d and Stimulates CD1d-Restricted {gamma}{delta} T Cells in the Normal Murine Repertoire**. *J Immunol* 2011, **186**:4771-4781.

296. Wu J, Groh V, Spies T: **T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells**. *J Immunol* 2002, **169**:1236-1240.

297. O'Brien RL, Fu YX, Cranfill R, Dallas A, Ellis C, Reardon C, Lang J, Carding SR, Kubo R, Born W: **Heat shock protein Hsp60-reactive gamma delta cells: a large, diversified T-lymphocyte subset with highly focused specificity**. *Proc Natl Acad Sci U S A* 1992, **89**:4348-4352.

298. Hirsh MI, Junger WG: **Roles of heat shock proteins and gamma delta T cells in inflammation**. *Am J Respir Cell Mol Biol* 2008, **39**:509-513.

299. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B: **The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins**. *Nature* 2008, **453**:106-109.

300. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, Caccamo M, Oukka M, Weiner HL: **Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor**. *Nature* 2008, **453**:65-71.

301. Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T: **Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells**. *Proc Natl Acad Sci U S A* 2008, **105**:9721-9726.

302. Stockinger B, Hirota K, Duarte J, Veldhoen M: **External influences on the immune system via activation of the aryl hydrocarbon receptor**. *Semin Immunol* 2011, **23**:99-105.

303. Zhou L, Littman DR: **Transcriptional regulatory networks in Th17 cell differentiation**. *Curr Opin Immunol* 2009, **21**:146-152.

304. Kerkvliet NI: **AHR-mediated immunomodulation: the role of altered gene transcription**. *Biochem Pharmacol* 2009, **77**:746-760.

305. Jux B, Kadow S, Esser C: **Langerhans cell maturation and contact hypersensitivity are impaired in aryl hydrocarbon receptor-null mice**. *J Immunol* 2009, **182**:6709-6717.

306. Kimura A, Naka T, Nakahama T, Chinen I, Masuda K, Nohara K, Fujii-Kuriyama Y, Kishimoto T: **Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses**. *J Exp Med* 2009, **206**:2027-2035.

307. Sekine H, Mimura J, Oshima M, Okawa H, Kanno J, Igarashi K, Gonzalez FJ, Ikuta T, Kawajiri K, Fujii-Kuriyama Y: **Hypersensitivity of aryl hydrocarbon receptor-deficient mice to lipopolysaccharide-induced septic shock**. *Mol Cell Biol* 2009, **29**:6391-6400.

308. Kabelitz D, Wesch D, Pitters E, Zoller M: **Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo**. *J Immunol* 2004, **173**:6767-6776.

309. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, Sireci G, Fournie JJ, Dieli F: **Differentiation, phenotype and function of interleukin-17-producing human V{gamma}9V{delta}2 T cells**. *Blood* 2011.

310. Del Prete A, Shao WH, Mitola S, Santoro G, Sozzani S, Haribabu B: **Regulation of dendritic cell migration and adaptive immune response by leukotriene B4 receptors: a role for LTB4 in up-regulation of CCR7 expression and function**. *Blood* 2007, **109**:626-631.

311. Costa MF, de Souza-Martins R, de Souza MC, Benjamim CF, Piva B, Diaz BL, Peters-Golden M, Henriques MG, Canetti C, Penido C: **Leukotriene B4 mediates gammadelta T lymphocyte migration in response to diverse stimuli**. *J Leukoc Biol* 2010, **87**:323-332.

312. Kaspar M, Trachsel E, Neri D: **The antibody-mediated targeted delivery of interleukin-15 and**

GM-CSF to the tumor neovasculature inhibits tumor growth and metastasis. *Cancer Res* 2007, **67**:4940-4948.

313. Park J, Gao W, Whiston R, Strom TB, Metcalfe S, Fahmy TM: **Modulation of CD4+ T lymphocyte lineage outcomes with targeted, nanoparticle-mediated cytokine delivery**. *Mol Pharm* 2010, **8**:143-152.

314. Heffernan MJ, Murthy N: **Polyketal nanoparticles: a new pH-sensitive biodegradable drug delivery vehicle**. *Bioconjug Chem* 2005, **16**:1340-1342.

315. Killeen SD, Wang JH, Andrews EJ, Redmond HP: **Exploitation of the Toll-like receptor system in cancer: a doubled-edged sword?** *Br J Cancer* 2006, **95**:247-252.

316. Seya T, Matsumoto M: **The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer**. *Cancer Immunol Immunother* 2009, **58**:1175-1184.

317. Cheng YS, Xu F: **Anticancer function of polyinosinic-polycytidylic acid**. *Cancer Biol Ther* 2011, **10**:1219-1223.

318. Scarlett UK, Cubillos-Ruiz JR, Nesbeth YC, Martinez DG, Engle X, Gewirtz AT, Ahonen CL, Conejo-Garcia JR: **In situ stimulation of CD40 and Toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells**. *Cancer Res* 2009, **69**:7329-7337.

319. Hausmann S, Marq JB, Tapparel C, Kolakofsky D, Garcin D: **RIG-I and dsRNA-induced IFNbeta activation**. *PLoS One* 2008, **3**:e3965.

320. De Miranda J, Yaddanapudi K, Hornig M, Lipkin WI: **Astrocytes recognize intracellular polyinosinic-polycytidylic acid via MDA-5**. *FASEB J* 2009, **23**:1064-1071.

321. Sugiyama T, Hoshino K, Saito M, Yano T, Sasaki I, Yamazaki C, Akira S, Kaisho T: **Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7**. *Int Immunol* 2008, **20**:1-9.

322. Perrot I, Deauvieau F, Massacrier C, Hughes N, Garrone P, Durand I, Demaria O, Viaud N, Gauthier L, Blery M, et al.: **TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA**. *J Immunol* 2010, **185**:2080-2088.

323. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, et al.: **Differential roles of MDA5 and RIG-I helicases in the recognition of RNA**

viruses. *Nature* 2006, **441**:101-105.

324. McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, Murphy KM, Colonna M: **Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells**. *J Exp Med* 2009, **206**:2967-2976.

325. Stevenson HC, Abrams PG, Schoenberger CS, Smalley RB, Herberman RB, Foon KA: **A phase I evaluation of poly(I,C)-LC in cancer patients**. *J Biol Response Mod* 1985, **4**:650-655.

326. Wang L, Smith D, Bot S, Dellamary L, Bloom A, Bot A: **Noncoding RNA danger motifs bridge innate and adaptive immunity and are potent adjuvants for vaccination**. *J Clin Invest* 2002, **110**:1175-1184.

327. Bot A, Smith D, Phillips B, Bot S, Bona C, Zaghouani H: **Immunologic control of tumors by in vivo Fc gamma receptor-targeted antigen loading in conjunction with double-stranded RNA-mediated immune modulation**. *J Immunol* 2006, **176**:1363-1374.

328. Lacour J, Lacour F, Spira A, Michelson M, Petit JY, Delage G, Sarrazin D, Contesso G, Viguier J: **Adjuvant treatment with polyadenylic-polyuridylic acid (Polya.Polyu) in operable breast cancer**. *Lancet* 1980, **2**:161-164.

329. Laplanche A, Alzieu L, Delozier T, Berlie J, Veyret C, Fargeot P, Luboinski M, Lacour J: **Polyadenylic-polyuridylic acid plus locoregional radiotherapy versus chemotherapy with CMF in operable breast cancer: a 14 year follow-up analysis of a randomized trial of the Federation Nationale des Centres de Lutte contre le Cancer (FNCLCC)**. *Breast Cancer Res Treat* 2000, **64**:189-191.

330. Jeung HC, Moon YW, Rha SY, Yoo NC, Roh JK, Noh SH, Min JS, Kim BS, Chung HC: **Phase III trial of adjuvant 5-fluorouracil and adriamycin versus 5-fluorouracil, adriamycin, and polyadenylic-polyuridylic acid (poly A:U) for locally advanced gastric cancer after curative surgery: final results of 15-year follow-up**. *Ann Oncol* 2008, **19**:520-526.

331. Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H: **TLR signaling by tumor and immune cells: a double-edged sword**. *Oncogene* 2008, **27**:218-224.

332. Yoneda K, Sugimoto K, Shiraki K, Tanaka J, Beppu T, Fuke H, Yamamoto N, Masuya M, Horie R, Uchida K, et al.: **Dual topology of functional Toll-like receptor 3 expression in human hepatocellular carcinoma: differential signaling mechanisms of TLR3-induced NF-kappaB activation and apoptosis**. *Int J Oncol* 2008, **33**:929-936.

333. Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, Ryffel B, Ferrantini M, Uppaluri R, Schreiber R, et al.: **Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands**. *Cancer Res* 2010, **70**:490-500.

334. Besch R, Poeck H, Hohenauer T, Senft D, Hacker G, Berking C, Hornung V, Endres S, Ruzicka T, Rothenfusser S, et al.: **Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells**. *J Clin Invest* 2009, **119**:2399-2411.

335. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T: **TLR3 can directly trigger apoptosis in human cancer cells**. *J Immunol* 2006, **176**:4894-4901.

336. Zhang Y, Sun R, Liu B, Deng M, Zhang W, Li Y, Zhou G, Xie P, Li G, Hu J: **TLR3 activation inhibits nasopharyngeal carcinoma metastasis via downregulation of chemokine receptor CXCR4**. *Cancer Biol Ther* 2009, **8**:1826-1830.

337. Weber A, Kirejczyk Z, Besch R, Potthoff S, Leverkus M, Hacker G: **Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells**. *Cell Death Differ* 2010, **17**:942-951.

338. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P: **Toll-like receptor 3 expressed by melanoma cells as a target for therapy?** *Clin Cancer Res* 2007, **13**:4565-4574.

339. Chiron D, Pellat-Deceunynck C, Amiot M, Bataille R, Jego G: **TLR3 ligand induces NF-{kappa}B activation and various fates of multiple myeloma cells depending on IFN-{alpha} production**. *J Immunol* 2009, **182**:4471-4478.

340. Paone A, Galli R, Gabellini C, Lukashev D, Starace D, Gorlach A, De Cesaris P, Ziparo E, Del Bufalo D, Sitkovsky MV, et al.: **Toll-like receptor 3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor 1 alpha**. *Neoplasia* 2010, **12**:539-549.

341. Salaun B, Zitvogel L, Asselin-Paturel C, Morel Y, Chemin K, Dubois C, Massacrier C, Conforti R, Chenard MP, Sabourin JC, et al.: **TLR3 as a biomarker for the therapeutic efficacy of double-stranded RNA in breast cancer**. *Cancer Res* 2011, **71**:1607-1614.

342. Gonzalez-Reyes S, Marin L, Gonzalez L, Gonzalez LO, del Casar JM, Lamelas ML,

Gonzalez-Quintana JM, Vizoso FJ: **Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis**. *BMC Cancer* 2011, **10**:665.

343. Lechner J, Malloth N, Seppi T, Beer B, Jennings P, Pfaller W: **IFN-alpha induces barrier destabilization and apoptosis in renal proximal tubular epithelium**. *Am J Physiol Cell Physiol* 2008, **294**:C153-160.

344. Bekisz J, Baron S, Balinsky C, Morrow A, Zoon KC: **Antiproliferative Properties of Type I and Type II Interferon**. *Pharmaceuticals (Basel)* 2011, **3**:994-1015.

345. Pokrovskaja K, Panaretakis T, Grander D: **Alternative signaling pathways regulating type I interferon-induced apoptosis**. *J Interferon Cytokine Res* 2005, **25**:799-810.

346. Chen Q, Gong B, Mahmoud-Ahmed AS, Zhou A, Hsi ED, Hussein M, Almasan A: **Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma**. *Blood* 2001, **98**:2183-2192.

347. Henry T, Brotcke A, Weiss DS, Thompson LJ, Monack DM: **Type I interferon signaling is required for activation of the inflammasome during Francisella infection**. *J Exp Med* 2007, **204**:987-994.

348. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, et al.: **Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway**. *Science* 2003, **301**:640-643.

349. Takeda K, Akira S: **TLR signaling pathways**. *Semin Immunol* 2004, **16**:3-9.

350. Heinz S, Haehnel V, Karaghiosoff M, Schwarzfischer L, Muller M, Krause SW, Rehli M: **Species-specific regulation of Toll-like receptor 3 genes in men and mice**. *J Biol Chem* 2003, **278**:21502-21509.

351. Takeda K, Hatakeyama K, Tsuchiya Y, Rikiishi H, Kumagai K: **A correlation between GM-CSF gene expression and metastases in murine tumors**. *Int J Cancer* 1991, **47**:413-420.

352. Garrity T, Pandit R, Wright MA, Benefield J, Keni S, Young MR: **Increased presence of CD34+ cells in the peripheral blood of head and neck cancer patients and their differentiation into dendritic cells**. *Int J Cancer* 1997, **73**:663-669.

353. Morales JK, Kmieciak M, Knutson KL, Bear HD, Manjili MH: **GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells**. *Breast Cancer Res Treat* 2010,

123:39-49.

354. Yang H, Wei J, Zhang H, Lin L, Zhang W, He S: **Upregulation of Toll-like receptor (TLR) expression and release of cytokines from P815 mast cells by GM-CSF**. *BMC Cell Biol* 2009, **10**:37.

355. Johnson AC, Li X, Pearlman E: **MyD88 functions as a negative regulator of TLR3/TRIF-induced corneal inflammation by inhibiting activation of c-Jun N-terminal kinase**. *J Biol Chem* 2008, **283**:3988-3996.

356. Carrigan SO, Junkins R, Yang YJ, Macneil A, Richardson C, Johnston B, Lin TJ: **IFN regulatory factor 3 contributes to the host response during Pseudomonas aeruginosa lung infection in mice**. *J Immunol* 2010, **185**:3602-3609.

357. Mlecnik B, Tosolini M, Charoentong P, Kirilovsky A, Bindea G, Berger A, Camus M, Gillard M, Bruneval P, Fridman WH, et al.: **Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer**. *Gastroenterology* 2010, **138**:1429-1440.

358. Apte RN, Voronov E: **Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy?** *Immunol Rev* 2008, **222**:222-241.

359. Schietinger A, Philip M, Yoshida BA, Azadi P, Liu H, Meredith SC, Schreiber H: **A mutant chaperone converts a wild-type protein into a tumor-specific antigen**. *Science* 2006, **314**:304-308.

Annex 1: Protocols

Protocol 1: RNA extraction, reverse transcription into cDNA and quantitative RT-PCR analysis of gene expression.

1. Tumor samples are freshly collected, washed briefly by RPMI1640 media (GIBCO).
2. Cut tissue samples to a maximum thickness in any one dimension of 0.5 cm and place the fresh tissue in 5 volumes of RNAlater® (Sigma Aldrich). Samples can be stored at room temperature if processed for RNA extraction on the same day or stored at 4°C for less than 1 month.
3. RNA extraction from each tumor (up to 30 mg/sample) is performed using RNeasy Mini Kit (QIAGEN) following the instructions.
4. RNA concentration and purity is tested by NanoDrop Spectrophotometers (Thermo Scientific).
5. Reverse transcription:
   1) mRNA (containing 2.5-5 µg RNA in 30 µl RNase and DNase free water) is incubated at 65°C 10 min, then placed on ice for 2 min. Maintain the mRNA on ice until use.
   2) Prepare master mix (from Promega or Invitrogen)

Reagent	Stock	working	Qte (µl)
dN6	50 ng/µl	3 ng/ul	3
dNTP	10 mM	1 mM	5
Buffer	5X	1X	10
RNAsin	40 U/µl	40U	1
Superscript® III RT	200 U/µl	200U	1
Total			20

   3) Add 20 µl of the master mix into the processed mRNA sample. Incubate at 50°C for 1h and then inactivate the reaction at 75°C for 15 min.

6. qRT-PCR:

   1) Dilute the cDNA of each sample at 1:3 with DNase free water. And then 5ul of diluted cDNA is used for qRT-PCR.

   2) For each sample, add

Reagent	Qte (µl)
H_2O	4
TaqMan® Assay Mix (20X)	1
TaqMan® Gene Expression Master Mix (2X)	10
Diluted cDNA	5
Total	20

   3) Perform 45 cycles with standard PCR program using StepOnePlus™ system (Applied Biosystems).

Protocol 2: Generating BMDC of C57bl/6 origin with J558 supernatant, GM-CSF+IL-4 or Flt3 ligand

1. Prepare the red cell lysate buffer (ACK solution: 8.29g NH_4Cl, 0.037g EDTA, 1g $KHCO_3$ in 1L ddH_2O) and IMDM culture media (10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 5×10^{-5} M β-mercaptoethanol complemented with 3% of J558 supernatant (equal to 40ng/ml GM-CSF final), 10 ng/ml rmGM-SCF and 10 ng/ml rmIL-4 (R&D system), or 100ng/ml rFlt3L (PeproTech)).
2. Femurs and Tibia are removed from the hind legs of the C57bl/6 mice. The skin is peeled off and the muscle tissue is removed with tweezers and disposable scalpels. The bones are decontaminated with 70% alcohol briefly and maintained in RPMI1640 on ice before use.
3. The ends of the bones are cut to expose the bone marrow (BM) and BM cells are flushed thoroughly with syringes loaded with RPMI1640 using 21-22.5 gauge needles. The BM is pipetted gently and filtered through 100 μM cell strainer to create single cell suspensions.
4. The BM cells suspension is mixed with equal volume of ACK buffer, kept at room temperature for 3 min before centrifugation at 200×g, 10 min to lyse red blood cells. The cell pellet is washed with IMDM culture media once to remove remaining ACK solution.
5. Femurs and tibias from 8 week old mice should yield 10-15 million BM cells per bone. The BM cells are counted and seeded at 10 million cells in 20 ml media per 150 mm^2 petri-dish for J558 BMDC on day 0, or 2 million cells in 4 ml media for GM-CSF/IL-4 and Flt3L DC.
6. On day 3, 7 and 10, cell culture are gently resuspended and collected. The cells remaining on original culture dish are briefly digested with cold 0.5% typsin and combined with the suspension cells. Cells are counted and seeded in fresh complete media into new dishes at the original density for J558 DC and GM-CSF/IL-4 DC. For Flt3L DC, all cells are added back into the original plate.

7. BMDC can be used since day 7 to day 12. The purity of culture should be at least above 70% as tested by FACS with CD11c and MHC II staining. And less than 30% of the DC should be MHC II^{high}.

Annex 2：Publication and Patent Application

1. **Ma Y**, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, Boucontet L, Apetoh L, Ghiringhelli F, Casares N, et al.: **Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy**. *J Exp Med* 2011, **208**:491-503.
2. **Ma Y**, Conforti R, Aymeric L, Locher C, Kepp O, Kroemer G, Zitvogel L: **How to improve the immunogenicity of chemotherapy and radiotherapy**. *Cancer Metastasis Rev* 2011, **30**:71-82.
3. Andrews DM, Chow MT, **Ma Y**, Cotterell CL, Watt SV, Anthony DA, Akira S, Iwakura Y, Trapani JA, Zitvogel L, et al.: **Homeostatic defects in interleukin 18-deficient mice contribute to protection against the lethal effects of endotoxin**. *Immunol Cell Biol* 2011.
4. Zitvogel L, Kepp O, Aymeric L, **Ma Y**, Locher C, Delahaye NF, Andre F, Kroemer G: **Integration of host-related signatures with cancer cell-derived predictors for the optimal management of anticancer chemotherapy**. *Cancer Res* 2010, **70**:9538-9543.
5. **Ma Y**, Aymeric L, Locher C, Kroemer G, Zitvogel L: **The dendritic cell-tumor cross-talk in cancer**. *Curr Opin Immunol* 2011, **23**:146-152.
6. Locher C, Conforti R, Aymeric L, **Ma Y**, Yamazaki T, Rusakiewicz S, Tesniere A, Ghiringhelli F, Apetoh L, Morel Y, et al.: **Desirable cell death during anticancer chemotherapy**. *Ann N Y Acad Sci* 2010, **1209**:99-108.
7. **Ma Y**, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C, Tesniere A, Martins I, Ly A, Haynes NM, et al.: **Chemotherapy and radiotherapy: cryptic anticancer vaccines**. *Semin Immunol* 2010, **22**:113-124.
8. Conforti R, **Ma Y (co-first author)**, Morel Y, Paturel C, Terme M, Viaud S, Ryffel B, Ferrantini M, Uppaluri R, Schreiber R, et al.: **Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands**. *Cancer Res* 2010, **70**:490-500.
9. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, **Ma Y**, Ortiz C, Vermaelen K, Panaretakis T,

Mignot G, Ullrich E, et al.: **Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors**. *Nat Med* 2009, **15**:1170-1178.

Patent Application:

Title: Compounds and uses thereof to induce an immunogenic cancer cell death in a subject

Inventors: Zitvogel Laurence, Kroemer Guido, Delahaye Nicolas, **Ma Yuting**, Kepp Oliver.

Assignee: IGR

Priority patent application n° PCT/EP2010/055404 filed on April 24, 2010 and PCT/FR2010/051470 filed on July 13, 2010

International patent application n° PCT/EP2011/055134filed on April 1, 2011

Abstract: The present invention relates to the fields of genetics, immunology and medicine. The present invention more specifically relates to in vitro or ex vivo methods for determining the susceptibility to a cancer treatment of a subject having a tumour. These methods comprise a step of determining the ability of the treatment, of the subject and/or of the tumour to induce an anticancer immune response, the inability of at least one of the treatment, the subject and the tumor to induce an anticancer immune response being indicative of a resistance of the subject to the therapeutic treatment of cancer.

Inventors in particular identify genes specific of a human subject or of cancerous cells which can be used to predict or assess the sensitivity of a subject to a treatment of cancer.

The invention also relates to particular compounds capable of activating or enhancing the immune system of a particular subject, when the subject is exposed to a therapeutic treatment of cancer or before such an exposition. It further relates to uses of such compounds, in particular to prepare a pharmaceutical composition to allow or improve the efficiency of a therapy of cancer in a subject in need thereof.

The present invention in addition provides kits, methods for selecting a compound of interest, as well as pharmaceutical compositions and uses thereof.

Annex 3：First-authored Papers

Published March 7, 2011

JEM

Article

Contribution of IL-17–producing γδ T cells to the efficacy of anticancer chemotherapy

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By triggering immunogenic cell death, some anticancer compounds, including anthracyclines and oxaliplatin, elicit tumor-specific, interferon-γ–producing CD8$^+$ αβ T lymphocytes (Tc1 CTLs) that are pivotal for an optimal therapeutic outcome. Here, we demonstrate that chemotherapy induces a rapid and prominent invasion of interleukin (IL)-17–producing γδ (Vγ4$^+$ and Vγ6$^+$) T lymphocytes (γδ T17 cells) that precedes the accumulation of Tc1 CTLs within the tumor bed. In T cell receptor δ$^{-/-}$ or Vγ4/6$^{-/-}$ mice, the therapeutic efficacy of chemotherapy was compromised, no IL-17 was produced by tumor-infiltrating T cells, and Tc1 CTLs failed to invade the tumor after treatment. Although γδ T17 cells could produce both IL-17A and IL-22, the absence of a functional IL-17A–IL-17R pathway significantly reduced tumor-specific T cell responses elicited by tumor cell death, and the efficacy of chemotherapy in four independent transplantable tumor models. Adoptive transfer of γδ T cells restored the efficacy of chemotherapy in IL-17A$^{-/-}$ hosts. The anticancer effect of infused γδ T cells was lost when they lacked either IL-1R1 or IL-17A. Conventional helper CD4$^+$ αβ T cells failed to produce IL-17 after chemotherapy. We conclude that γδ T17 cells play a decisive role in chemotherapy-induced anticancer immune responses.

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Abbreviations used: AhR, aryl hydrocarbon receptor; BMDC, BM-derived DC; CIg, isotype control Ig; DX, doxorubicin; γδ T17, IL-17A–producing γδ T cell; MTX, mitoxantrone; OX, oxaliplatin; Tc1, IFN-γ–producing CD8$^+$ T cell; TIL, tumor-infiltrating lymphocyte; TR, tumor regression.

The current management of cancer patients relies upon the therapeutic use of cytotoxic agents that are supposed to directly destroy cancer cells through a diverse array of cell death pathways.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 3 491-503
www.jem.org/cgi/doi/10.1084/jem.20100269

Supplemental Material can be found at:
http://jem.rupress.org/content/suppl/2011/03/04/jem.20100269.DC1.html

Published March 7, 2011

Nonetheless, several lines of evidence point to a critical contribution of the host immune system to the therapeutic activity mediated by tumoricidal agents (Nowak et al., 2002, 2003). Indeed, in some instances, the cell death triggered by chemotherapy or radiotherapy allows recognition of dying (anthracycline-treated or irradiated) tumor cells by antigen-presenting cells, thus eliciting a tumor-specific cognate immune response for tumor resolution. Whether cell death is immunogenic or not depends on the presence of tumor-specific antigens, as well as on the lethal hit. Thus, oxaliplatin (OX) and anthracyclines induce immunogenic cell death, whereas other chemotherapeutic agents such as cisplatin and alkylating agents tend to induce nonimmunogenic cell death (Casares et al., 2005; Obeid et al., 2007). Stressed and dying tumor cells may emit a particular pattern of "danger signals," and these cell death–associated molecules are either exposed on the surface of dying cells or secreted into the microenvironment. The combined action of "find-me" and "eat-me" signals, together with the release of hidden molecules that are usually secluded within live cells may influence the switch between silent corpse removal and inflammatory reactions that stimulate the cellular immune response (Zitvogel et al., 2010). We initially described the crucial importance of an eat-me signal represented by the early translocation of the endoplasmic reticulum resident calreticulin–ERp57 complex to the plasma membrane for the immunogenicity of tumor cell death (Obeid et al., 2007; Panaretakis et al., 2008, 2009). Next, we showed that the nuclear alarmin HMGB1 must be released into the tumor microenvironment to engage TLR4 on host DCs to facilitate antigen processing and presentation (Apetoh et al., 2007). We also reported that ATP released from dying tumor cells could trigger the purinergic P2RX7 receptor on host DC, stimulating the release of IL-1β, which in turn facilitates the priming of $CD8^+$ tumor-specific T cells for IFN-γ production that is indispensable for the success of chemotherapy (Ghiringhelli et al., 2009).

Although the contribution of IFN-γ to tumor surveillance and anticancer immune responses is clearly established, that of the IL-17A–IL-17R pathway remains controversial (Martin-Orozco and Dong, 2009; Muranski and Restifo, 2009; Ngiow et al., 2010). In tumor models where $CD4^+$ T cells are the source of IL-17, this cytokine could induce Th1-type chemokines, recruiting effector cells to the tumor microenvironment (Kryczek et al., 2009) or promote IL-6–mediated Stat3 activation, acting as a protumorigenic trigger (Kortylewski et al., 2009; Wang et al., 2009). Tumor-specific Th17 exhibited stronger therapeutic efficacy than Th1 cells upon adoptive transfer, and converted into effective IFN-γ producers (Muranski et al., 2008) and/or triggered the expansion, differentiation, and tumor homing of tumor-specific $CD8^+$ T cells (Martin-Orozco et al., 2009). IL-17–producing $CD8^+$ T cells also reduced the volume of large established tumors and could differentiate into long-lasting IFN-γ producers (Hinrichs et al., 2009). In contrast, Kwong et al. (2010) described a tumor-promoting, IL-17–producing TCR $\alpha\beta^+CD8^+$ cell subset. Therefore, the heterogeneous source (and perhaps the targets) of IL-17 in the tumor microenvironment may determine whether this cytokine negatively or positively affects tumor growth. Whether conventional anticancer therapies such as chemotherapy and radiotherapy modulate IL-17 secretion and/or Th17 polarization remains to be explored (Maniati et al., 2010).

Similarly, the contribution of γδ T cells in tumor immunosurveillance is still elusive (Hayday, 2009). In humans, $V\delta1^+$ γδ T cells have been shown to mediate immunosuppressive activities (Peng et al., 2007) or, on the contrary, to be associated with a reduced occurrence of cancers in transplanted patients bearing a CMV infection (Déchanet et al., 1999; Couzi et al., 2010) and with long-term relapse-free survival after BM transplantation (Godder et al., 2007). $V\delta2^+$ γδ T cells can be activated by various synthetic ligands to produce Th1-like cytokines, exhibit cytotoxic functions against tumors (Kabelitz et al., 2007), and mediate antitumor effects in patients (Wilhelm et al., 2003; Dieli et al., 2007). Although various γδ T cell subsets are capable of producing IL-17 during microbial infection or autoimmune disorders of mice (Shibata et al., 2007; O'Brien et al., 2009), very little is known about the incidence and functional relevance of IL-17–producing γδ T cells (that we termed γδ T17) in cancer (Gonçalves-Sousa et al., 2010). γδ T17 cells have been reported to share most phenotypic markers with Th17 cells (expressing CCR6, RORγt, aryl hydrocarbon receptor [AhR], IL-23R, IL-17A, and IL-22; Martin et al., 2009). γδ T17 cells depend upon TGF-β but not IL-23 or IL-6 for their development and maintenance (Do et al., 2010) and can be activated by IL-1β plus IL-23 (Sutton et al., 2009). They are unrestricted by Vγ usage (although they are mostly Vγ4 in the context of mycobacteria [Martin et al., 2009] and experimental autoimmune encephalitis [Sutton et al., 2009]). Recent work suggests that thymic selection does little to constrain γδ T cell antigen specificities, but instead determines their effector fate. When triggered through the TCR, ligand-experienced cells secrete IFN-γ, whereas ligand-naive γδ T cells produce IL-17 (Jensen et al., 2008). $CD27^+$ γδ thymocytes expressed LTβR and genes associated with a Th1 phenotype, in contrast to $CD27^-$ γδ thymocytes which give rise to IL-17–producing γδ cells (Ribot et al., 2009).

Therapy-induced immunogenic tumor cell death that stimulates a therapeutic anticancer immune response can be expected to influence the composition and/or the architecture of tumor immune infiltrates, which in turn contribute to the control of residual tumor cells. Here, we demonstrate that both IL-17A/IL-17RA signaling and γδ T cells are required for optimal anticancer responses and that the source of IL-17A is the γδ T population during immunogenic chemotherapy and radiotherapy. We show that an early tumor infiltration by γδ T17 cells is a prerequisite for optimal tumor colonization of IFN-γ–producing $CD8^+$ T cells. γδ T cell activation depends on IL-1R1 and IL-1β (but not IL-23) produced by DCs in response to immunogenic dying tumor cells. Finally, the adoptive transfer of WT γδ T17 cells can restore the therapeutic efficacy of anticancer chemotherapy that is compromised in IL-17A$^{-/-}$ hosts.

Published March 7, 2011

RESULTS

A marked Th1 pattern 8 d after chemotherapy

Anthracyclines induce immune responses that culminate in CD8$^+$ T cell– and IFN-γ/IFN-γR–dependent antitumor effects (Ghiringhelli et al., 2009). To further study chemotherapy-induced immune effectors at the site of tumor retardation, we performed quantitative RT-PCR to compare the transcription profile of 40 immune gene products expressed in MCA205 tumors, which were controlled by the anthracycline doxorubicin (DX) 8 d after treatment (Fig. 1 A, top), with that of progressing, sham-treated (PBS) tumors (Fig. 1 A, bottom). Several Th1-related gene products were specifically induced in regressing tumors (Fig. 1 B). In particular, the Th1 transcription factors Eomes and Tbx21 (also called T-bet) and their target, IFN-γ, were increased by 4–5 fold in DX versus PBS-treated tumors (Fig. 1 C, left). Unsupervised hierarchical clustering indicated that IFN-γ production correlated with that of the quintessential Th1 transcription factor, Tbx21. By day 8, the protein levels of IFN-γ also increased in DX-treated MCA205 sarcomas (Fig. 1 D, left). Other surrogate markers of Th1 responses (lymphotoxin-β, Ccl5, Cxcl10, Cxcl9, and TNF) were also significantly induced at the mRNA level after DX treatment (Fig. 1, B and C, left). Another set of gene products was also overexpressed in the context of DX-induced tumor regression. These genes encoded IL-7R, IL-21, AhR, Cxcl2, and Foxp3, suggesting that inflammation and/or tissue repair occurred in the tumor bed (Fig. 1, B and C, right). Indeed, by day 3 after chemotherapy, the protein levels of the inflammatory cytokine IL-17 were significantly increased within tumor homogenates (Fig. 1 D, right).

Reinforcing this finding, we found that AhR, a sensor of small chemical compounds, is involved in the success of anthracycline-based therapy in this model. AhR is recognized as a transcriptional regulator for the optimal IL-17–associated immune response, promoting the differentiation and/or maintenance of IL-17–producing cells (Esser et al., 2009). CH-223191 is a pure antagonist of AhR because it does not have any agonist actions up to 100 μM (Kim et al., 2006). Blocking AhR with CH-223191 markedly reduced the efficacy of DX on established cancers in vivo (Fig. S1 A). This contrasts with the observation that CH-223191 had no cell autonomous effects on the tumor cells, alone or in combination with anthracyclines (Fig. S1 B).

DX (compared with PBS) induced a threefold increase in the proportions of both IFN-γ– and IL-17–producing tumor-infiltrating lymphocytes (TILs) as tested by flow cytometry (FACS; Fig. 1 E). To identify the cellular source of IFN-γ and IL-17, TILs were immunophenotyped by cell surface staining and intracellular detection of the cytokines with FACS. Careful analyses revealed that the major source of IFN-γ was CD8$^+$ T cells, whereas that of IL-17 was mostly TCR δ$^+$ T cells rather than CD4$^+$ Th17 cells 8 d after chemotherapy in MCA205 sarcomas (Fig. 1 F). We further analyzed the IFN-γ and IL-17 production by each subset of TILs. CD4$^+$ T cells could secrete IFN-γ, but rarely IL-17. CD8$^+$ T and γδ T cells were polarized to become potent producers of IFN-γ and IL-17, respectively. DX-based chemotherapy substantially enhanced IFN-γ production by CD8$^+$ and CD4$^+$ TILs, as well as IL-17 production by γδ TILs (Fig. 1 G).

γδ T17 cells preceded and predicted the accumulation of Tc1 CTLs in tumor beds after chemotherapy

Kinetic experiments revealed that γδ TILs invaded MCA205 tumor beds and produced IL-17 shortly after chemotherapy, with significant increases (~9-fold) over the background 4 d after DX injection (Fig. 2 A, left). γδ TILs still rapidly divided (as indicated by the expression of Ki67) 8 d after DX treatment (Fig. 2 B). This early induction of IL-17–producing γδ T cells (Fig. 2 C, left) contrasted with the comparatively late induction of IFN-γ–producing CD8$^+$ T cells, which emerged sharply 8 d after chemotherapy (Fig. 2 C, right) and rapidly proliferated (Fig. 2 B). Altogether, anthracyclines induced an early Th17-biased inflammation together with a marked Th1 polarization in MCA205 tumor beds, associated with a brisk infiltration of γδ T17 cells followed by Tc1 effectors.

To generalize these findings, we systematically immunophenotyped TILs in CT26 colon cancer treated by a single intratumoral injection of DX, which significantly retarded tumor growth (Fig. 3 A). Indeed, the majority of IL-17A$^+$ TILs were CD45$^+$CD3^{bright}. They failed to express CD4, but were positively stained with anti-TCR δ–specific antibodies (Fig. S2 A). Consistently, chemotherapy dramatically increased the frequency of IFN-γ–producing CD8$^+$ T lymphocytes (Tc1; Fig. 3 B) and IL-17A–producing γδ T cells (γδ T17; Fig. 3 C) in the tumor microenvironment. Next, we monitored transplantable TS/A mammary carcinomas treated with local radiotherapy, which operates in a T cell–dependent manner (Apetoh et al., 2007). Irradiation of TS/A tumors led either to tumor regression or to no response, and hence tumor progression (Fig. 3 D). An accumulation of both Tc1 (Fig. 3 E) and γδ T17 (Fig. 3 F) lymphocytes was found in those tumors that responded to radiotherapy, but not in those that continued to progress or in untreated controls. Importantly, in each of the three tumor models that we tested, a clear correlation was observed between tumor invading γδ T17 and Tc1 cells (Fig. 3 G).

γδ T17 TILs were preponderantly CD44$^+$ CD62L$^-$ CD69$^+$ and Granzyme B$^+$. They did not express CD24, c-kit, NKG2D, CD27 (a thymic determinant for IFN-γ–producing γδ T cells; Ribot et al., 2009), SCART2 (a specific marker for peripheral IL-17–producing cells which can be down-regulated upon activation; Kisielow et al., 2008), or CD122 (a marker for self antigen-experienced γδ T cells with potential to produce IFN-γ (Jensen et al., 2008; unpublished data). FACS indicated that ~60% of γδ T17 used Vγ4 chain (nomenclature of Vγ genes according to Heilig and Tonegawa [1986]), but expression of Vγ1 and Vγ7 chain was rarely found (Fig. S2 B). We then sorted Vγ1$^-$Vγ4$^-$Vγ7$^-$ γδ T17 TILs (Fig. S2 C) and performed single-cell PCRs and sequencing (Pereira and Boucontet, 2004) to examine their Vγ chain usage. The majority of these cells (21 of 23 clones) contained functional

Published March 7, 2011

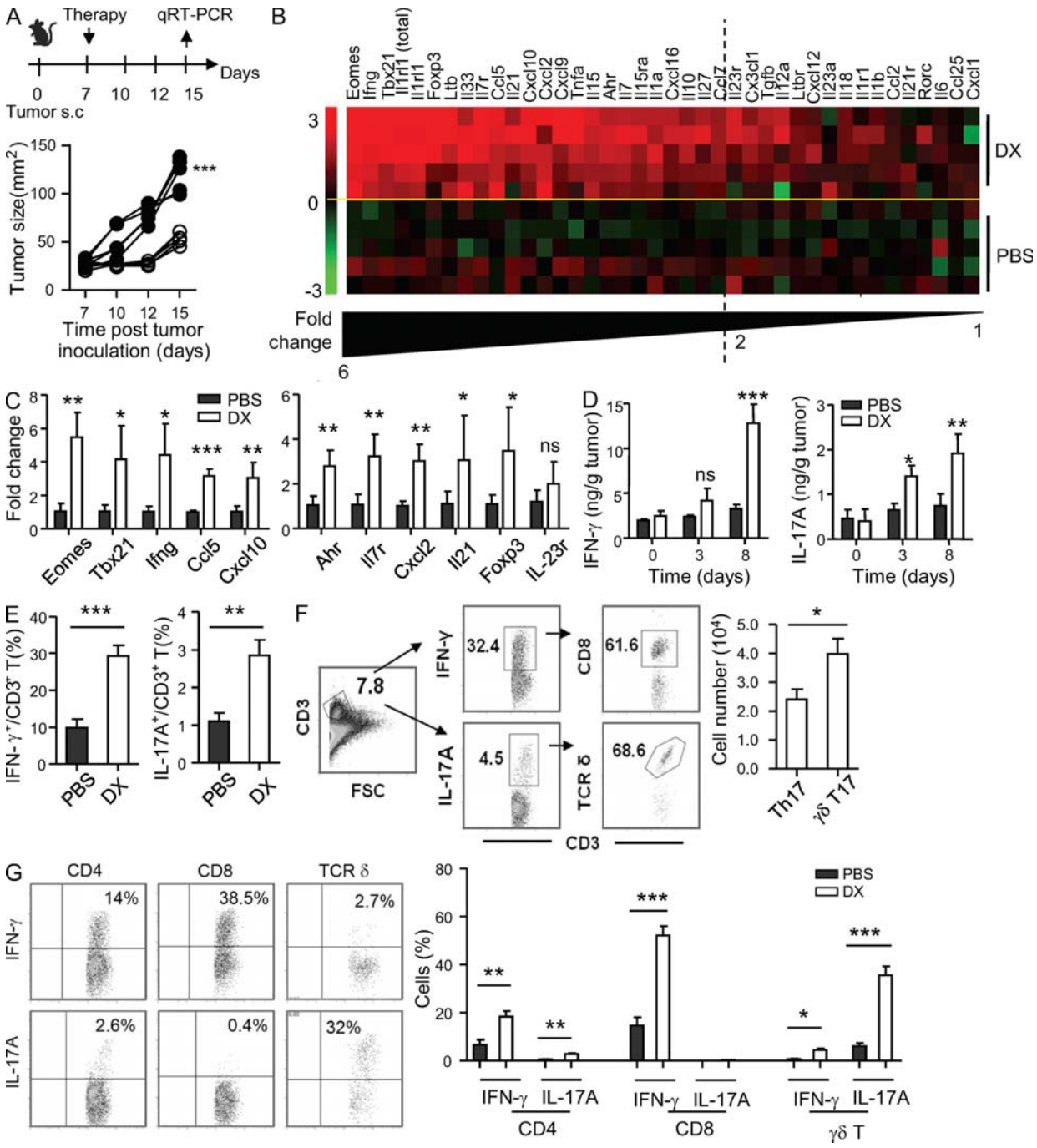

Figure 1. Th1 and Th17 immune response in tumors after chemotherapy. (A) Mice bearing MCA205 tumors were treated with PBS (solid symbols) or DX (open symbols) intratumorally at day 7 after tumor inoculation. Tumor growth was monitored at the indicated time points. (B and C) 8 d after chemotherapy (day 15 after tumor inoculation), tumor homogenates in PBS and DX groups were tested by quantitative RT-PCR (qRT-PCR). (B) Fold changes of gene expression are shown as a heat map. (C) Th1- and Th17-related gene expression in DX versus PBS groups (with a fold change >2) are listed. (D) Measurements of IFN-γ and IL-17A protein in tumor homogenates by ELISA at the indicated time points. (E and F) Single-cell suspension of MCA205 tumors (day 8 after DX) were analyzed by FACS. (E) Expression of IFN-γ and IL-17A in TILs was tested by intracellular staining gated on live, CD45⁺ and CD3⁺ cells. (F) IFN-γ⁺ and IL-17A⁺ cells were gated, and the proportions of CD3⁺ CD8⁺ cells and CD3⁺ TCR δ⁺ cells were examined in DX-treated tumors. A typical dot plot analysis (left) and the absolute numbers of Th17 and γδ T17 cells in the whole tumors (right) are shown. (G) IFN-γ and IL-17A production by total CD4⁺, CD8⁺, and TCR δ⁺ TILs. Representative FACS plots in DX-treated tumors (left) and the percentages in PBS- or DX-treated tumors (right) are shown. Each group contained at least five mice, and each experiment was performed at least twice, yielding similar results. Graphs depict mean ± SEM of fold change of gene expression (C), protein content (D), percentages, or absolute numbers of positive cells (E and G). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Published March 7, 2011

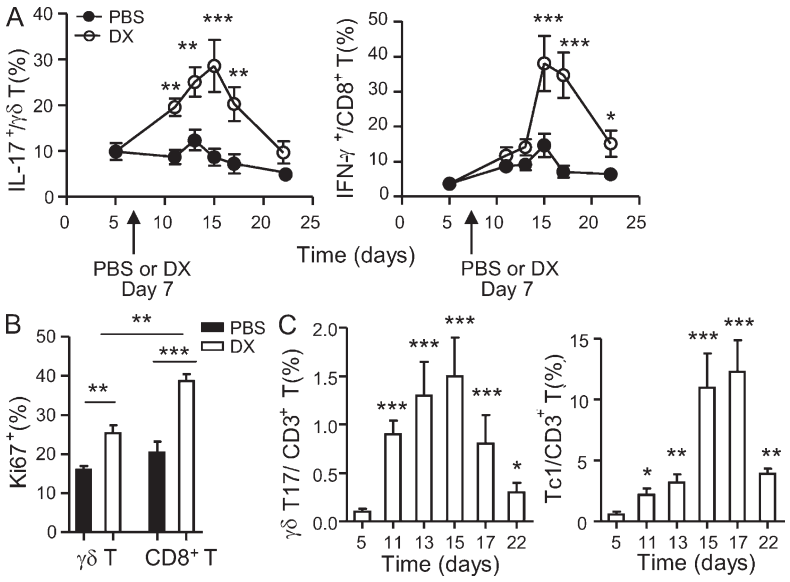

Figure 2. γδ T17 cells preceded Tc1 CTL into tumors after chemotherapy. (A) The percentages of IL-17– and IFN-γ–producing cells among all tumor infiltrating γδ T cells and $CD8^+$ T cells, respectively, are plotted before and at the indicated time points after tumor inoculation. Mice were treated with PBS (filled symbols) or DX (open symbols) at day 7. (B) Ki67 expression on γδ T and $CD8^+$ TILs 8 d after treatment. (C) The percentages of γδ T17 and Tc1 among all $CD3^+$ TILs at the indicated time points after tumor inoculation. DX was given at day 7. These experiments were performed twice on 5–10 tumors at each time point. *, $P < 0.05$; **, $P < 0.01$.

Vγ6 rearrangements identical to those found in fetal γδ T cells (Lafaille et al., 1989). These experiments show that most γδ T17 TILs express Vγ4 or Vγ6 chains (Fig. S2, D and E).

Thus, chemotherapy and radiotherapy could trigger the accumulation of cytokine producing TILs in the tumor bed. This applies to distinct subsets of γδ T cells that rapidly invaded tumor and become IL-17 producers, correlating with the accumulation of Tc1 cells, which contribute to the chemotherapy-induced anticancer immune response.

The IL-17A–IL-17R pathway is involved in the immunogenicity of cell death

Because both Tc1 and γδ T17 cells accumulated within tumors after chemotherapy or radiotherapy in a coordinated fashion, we determined whether neutralizing their signature cytokines IFN-γ and IL-17A could mitigate the efficacy of anticancer therapies. Antibody-mediated neutralization of either IFN-γ or IL-17A negatively affected the growth-retarding effect of DX against MCA205 tumors (Fig. 4 A). The mandatory role of the IL-17A–IL-17RA pathway was confirmed using neutralizing anti–IL-17RA antibodies and IL-$17A^{-/-}$ mice in the same tumor model (Fig. 4 B), in DX-treated MCA2 sarcomas (Fig. 4 C), as well as in OX-treated, OVA-expressing EG7 thymomas or CT26 colon cancers (Fig. 4, D and E).

To rationalize the sequential recruitment of γδ T17 and Tc1 cells into the tumor bed after chemotherapy, we hypothesized that γδ T17 might act as helper cells for Tc1 priming. We previously reported that specific antitumor immune responses rely on Tc1 cells primed by tumor cells undergoing immunogenic cell death by using a system in which IFN-γ production by OVA-specific T cells could be triggered by OX-treated EG7 cells (Ghiringhelli et al., 2009). We used this system to check whether IL-17 is involved in initiating the specific antitumor response, comparing normal WT with IL-$17RA^{-/-}$ mice. In this assay, the absence of IL-17RA fully abolished antigen-specific T cell priming in response to dying cells, yet had no negative effect on T cell priming by OVA holoprotein admixed with CpG oligodeoxynucleotides (Fig. 5 A, left). Consistently, a neutralizing anti–IL-17A antibody, but not the isotype control Ig (CIg), markedly impaired the OVA-specific T cell induced by OX-treated EG7 (Fig. 5 A, right). Because Th1/Tc1 immune responses against dying tumor cells mediate a prophylactic protection against rechallenge with live tumor cells (Apetoh et al., 2007; Ghiringhelli et al., 2009), we addressed the functional relevance of the IL-17A–IL-17RA pathway in this setting. Subcutaneous injection of mitoxantrone (MTX)-treated MCA205 sarcoma cells could protect WT mice, but not athymic nude mice, against rechallenge with live MCA205 tumor cells (Fig. 5 B). The efficacy of this vaccination was attenuated in IL-$17RA^{-/-}$ mice. Because IL-17 was not significantly produced by $CD4^+$ or $CD8^+$ T cells, neither in tumor beds during chemotherapy (Fig. 1 G) nor in the tumor draining LNs (unpublished data), we refrained from investigating Th17 cells and rather focused on γδ T and NKT cells as potential IL-17 producers (Mills, 2008; Pichavant et al., 2008) that might contribute to the anticancer vaccination by dying tumor cells. Although $CD1d^{-/-}$ mice, which lack all NKT population (Godfrey et al., 2010), were undistinguishable from WT controls in their ability to resist live tumor cells rechallenge after a dying tumor cell vaccine, Vγ4/6$^{-/-}$ mice (Sunaga et al., 1997) exhibited a reduced capacity to mount this anticancer immune response (Fig. 5 B). These results suggest that IL-17A, IL-17R, and γδ T17 cells all play a partial role in the afferent phase of the immune response against dying tumor cells, which includes T cell priming for IFN-γ production.

IL-1β–dependent, but not IL-23–dependent, activation of γδ T lymphocytes

The IL-1β–IL-1R1 pathway is mandatory for eliciting Tc1 immune responses and for the efficacy of chemotherapy (Ghiringhelli et al., 2009). Moreover, we found an IL-1–related gene expression signature after chemotherapy in tumor beds (Fig. 1 B), prompting us to address its role in the activation of γδ T17 cells.

To explore the molecular requirements for γδ T17 activation in situ, we sorted γδ T cells from the skin-draining LNs

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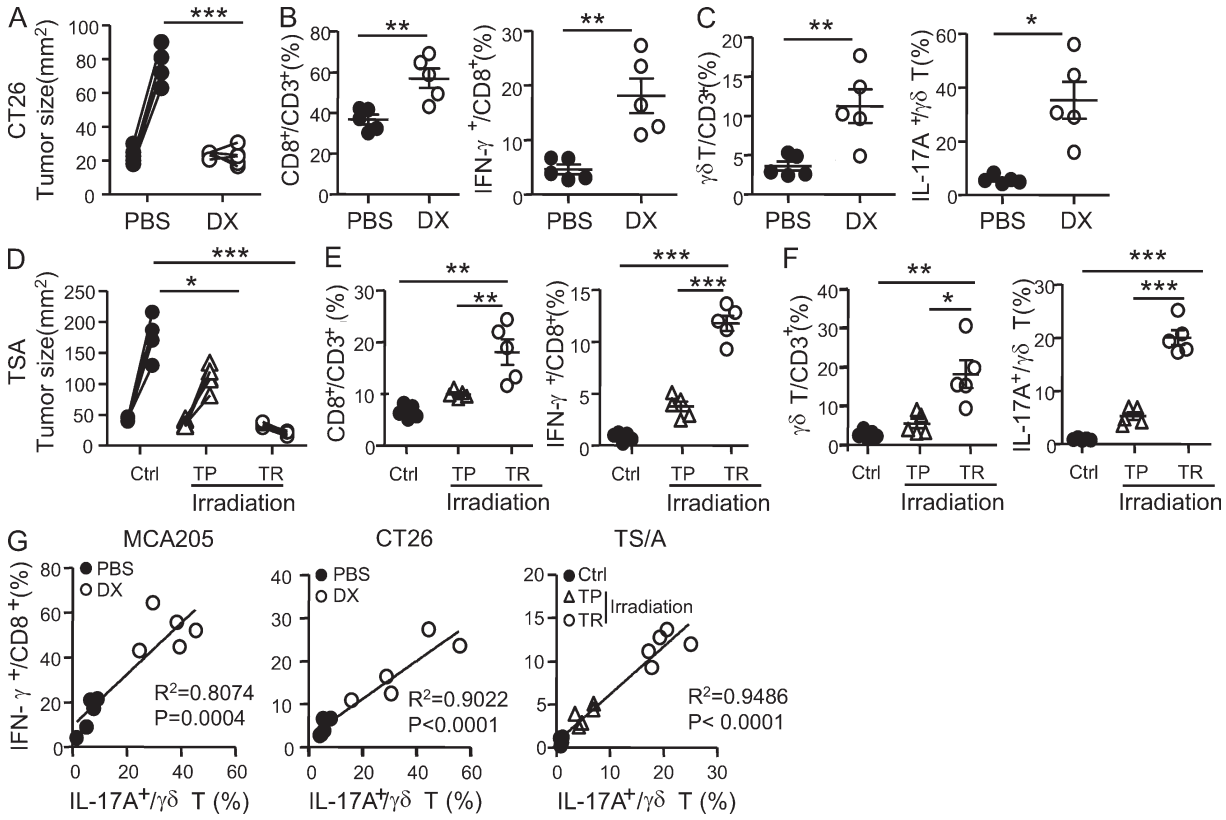

Figure 3. Recruitment of both Tc1 and γδ T17 cells in CT26 and TS/A tumors that correlate with better tumor control. (A–C) CT26 colon cancer treated with anthracyclines. (A) Tumor size before and 8 d after treatment with PBS (filled symbols) or DX (open symbols). (B) The percentage of $CD8^+$ T cells among $CD3^+$ cells and of IFN-γ–producing cells among $CD8^+$ T cells. (C) The percentage of γδ T cells among $CD3^+$ cells and of IL-17A–producing cells among $CD3^+$ γδ T cells. Data are presented as mean ± SEM with five tumors/group. (D–F) TS/A mammary cancer treated with x rays. (D) Established TS/A tumors were treated with local irradiation (open symbols) on day 10. Mice were segregated into nonresponders (tumor progression [TP], triangles) and responders (tumor regression [TR], circles) 22 d after radiotherapy (n = 5). (E) The percentage of $CD8^+$ T cells among $CD3^+$ cells and of IFN-γ-producing cells among $CD8^+$ T cells; (F) The percentage of γδ T cells among $CD3^+$ cells and of IL-17A–producing cells among $CD3^+$ γδ T cells are indicated as mean ± SEM. (G) The correlation between the percentages of γδ T17 and Tc1 TILs in all tumors (treated or not) was plotted for MCA205, CT26, and TS/A tumors (each dot representing one mouse). Data are representative of two to three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of naive mice (around 1–2% of the LN T cell pool). Among these γδ T cells, ∼70% harbored the Vγ4 TCR. Moreover, these cells vigorously produced IL-17A (but not IFN-γ) upon stimulation with PMA/ionomycin (Fig. S2 F; Do et al., 2010). In contrast to Th17 cells (Ivanov et al., 2006), LN-resident γδ T cells failed to produce IL-17 in response to TGF-β or IL-6 alone, or in combination with IL-1β. However, they potently secreted IL-17 and IL-22 in response to the combined stimulation of IL-1β plus IL-23 (unpublished data; Sutton et al., 2009). TCR engagement also synergized with IL-1β (and to a lesser extent with IL-23) to trigger IL-17 and IL-22 secretion by LN-resident γδ T cells (unpublished data). It is noteworthy that these stimuli specifically activated IL-17A, but not IFN-γ production by γδ T cells. Because γδ T17 cells were activated (as indicated by their $Ki67^+$, GzB^+, $CD69^+$, and $IL\text{-}17^+$ phenotype) after chemotherapy, we addressed whether dying tumor cells could directly promote the activation of γδ T17. Although DX-treated MCA205 cells failed to directly induce IL-17 secretion by γδ T cells, they did so indirectly. Thus, BM-derived DCs (BMDCs) that had been loaded with DX-treated MCA205 (Fig. 5 C; or CT26, not depicted), but not with live tumor cells, produced IL-1β and markedly stimulated the release of IL-17 and IL-22 by γδ T cells (Fig. 5 C). As a quality control for in vitro–generated DCs, the expression of CD11c, MHC class II, CD11b, and F4/80 was assessed. Only qualified DC preparations that contain functional DCs (>80% $CD11c^+MHCII^+$) rather than macrophages (>70% $CD11b^+F4/80^+CD11c^-$) could activate γδ T cells for IL-17A production when they encountered DX-treated tumor cells. $CD11b^+Gr1^+$ neutrophils reportedly produce IL-17 and promote downstream IL-12/IFN-γ contributing to reperfusion injury (Li et al., 2010). Interestingly, $CD11b^+Gr1^+$ cells sorted from DX-treated tumor beds bearing the IL-1β messenger RNA failed to secrete IL-1β or IL-17A protein and failed to activate γδ T cells for IL-17A production in vitro (unpublished data). IL-17 production by γδ T cells was dependent on IL-1β because the IL-1R1 antagonist IL-1RA entirely abrogated the DC/γδ T cell cross talk in the presence

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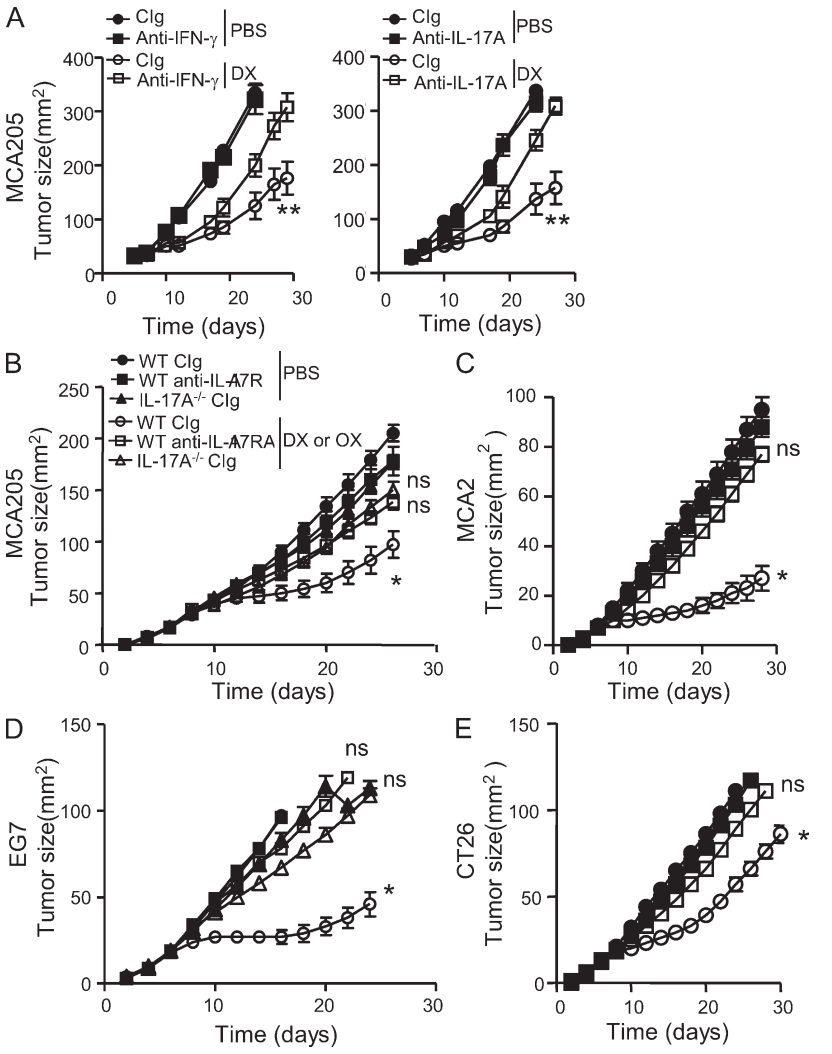

Figure 4. A mandatory role for the IL-17A–IL-17RA pathway in the efficacy of chemotherapy. (A) Mice bearing established MCA205 sarcomas were treated with local PBS (filled symbols) or DX (open symbols) 7 d after tumor inoculation and with systemic neutralizing antibodies against mouse IFN-γ (left), IL-17A (right), or control Ig (Clg) i.p. every 2 d (3 injections, 200 µg/mouse) starting on the day of DX. (B–E) WT (circles or squares) or IL-17A$^{-/-}$ (triangles) mice bearing established MCA205 sarcomas (B), MCA2 (C), EG7 (D), or CT26 (E) tumors were treated with PBS (B-E, solid symbols), DX (B and C, open symbols), or OX (D and E, open symbols) together with systemic administration of neutralizing antibodies against IL-17RA (squares) or Clg. Tumor sizes are plotted as mean ± SEM for 5–15 mice/group, and each experiment was repeated at least 2 times, yielding similar results. *, $P < 0.05$; **, $P < 0.01$.

of dying cells. The neutralization of IL-18R, IL-23, or IL-23R failed to abolish IL-17 production by γδ T cells co-cultured with DCs (Fig. 5 D). IL-22 production was completely abolished by blocking the IL-1β–IL-1R or IL-23–IL-23R pathways but not affected by IL-18R blockade. Interestingly, chemotherapy lost part of its anticancer activity in IL-1R1–deficient mice, yet maintained its efficacy in mice treated with IL-23p19–neutralizing antibodies or in IL-23p19$^{-/-}$ mice (Fig. 5, E–G). IL-1β–activated γδ T cells produced IL-17 and IL-22 (Fig. 5, C and D). However, IL-22 did not play an essential role in the antitumor effects promoted by chemotherapy (Fig. S3 A). It is of note that the antibody we used in this experiment could block the bioactivity of IL-22 in a lung bacterial infection model (Aujla et al., 2008), and IL-22 mRNA in the bulk TILs was below the detection limit of quantitative RT-PCR. Collectively, these results underscore the importance of IL-1β and IL-17 for the immune-dependent anticancer effects of chemotherapy, yet suggest that both IL-23 and IL-22 are dispensable for such effects.

γδ T lymphocytes are indispensable for the immune-dependent effects of chemotherapy

To further evaluate the contribution of γδ T cells to the therapeutic action of DX on established MCA205 sarcomas, such tumors were implanted into age- and sex-matched WT, TCR δ$^{-/-}$, Vγ4/6$^{-/-}$ mice, and then subjected to chemotherapy. As compared with WT controls, the absence of the TCR δ chain, as well as that of Vγ4 and Vγ6 γδ T cells, greatly reduced the efficacy of chemotherapy (Fig. 6 A). At day 8 after chemotherapy, when γδ T17 and Tc1 massively infiltrated tumor beds in WT mice, these cytokine-producing TILs were either absent or greatly reduced in Vγ4/6$^{-/-}$ mice (Fig. 6 B), suggesting that the presence of Vγ4 and Vγ6 γδ T cells are critical for the optimal Tc1 response in tumor beds.

Expression of CCR6 is a phenotypic and functional hallmark of Th17 cells (Reboldi et al., 2009) during some inflammatory processes. We therefore analyzed the role of CCR6 in the efficacy of chemotherapy. Because CCL20 was detectable in tumor tissues before and after chemotherapy (unpublished data), we assessed whether γδ T17 cells could be recruited in a CCL20/CCR6-dependent manner. The tumoricidal activity of DX against CT26 was not affected by repetitive systemic injections of neutralizing anti-CCL20 antibody before and during anthracycline treatment (Fig. S3 B). Consistently, anthracycline treatment against established MCA205 sarcoma remained efficient in CCR6 loss-of-function mice. Moreover, CCR6 deficiency did not influence tumor infiltration by γδ T17 (unpublished data). Therefore, Vγ4 and Vγ6 γδ T cells contribute to the immune-mediated action of anticancer agents in a CCR6-independent fashion.

Next, we determined the contribution of adoptively transferred γδ T cells to the efficacy of chemotherapy. The infusion of γδ T cells derived from skin-draining LNs (from naive

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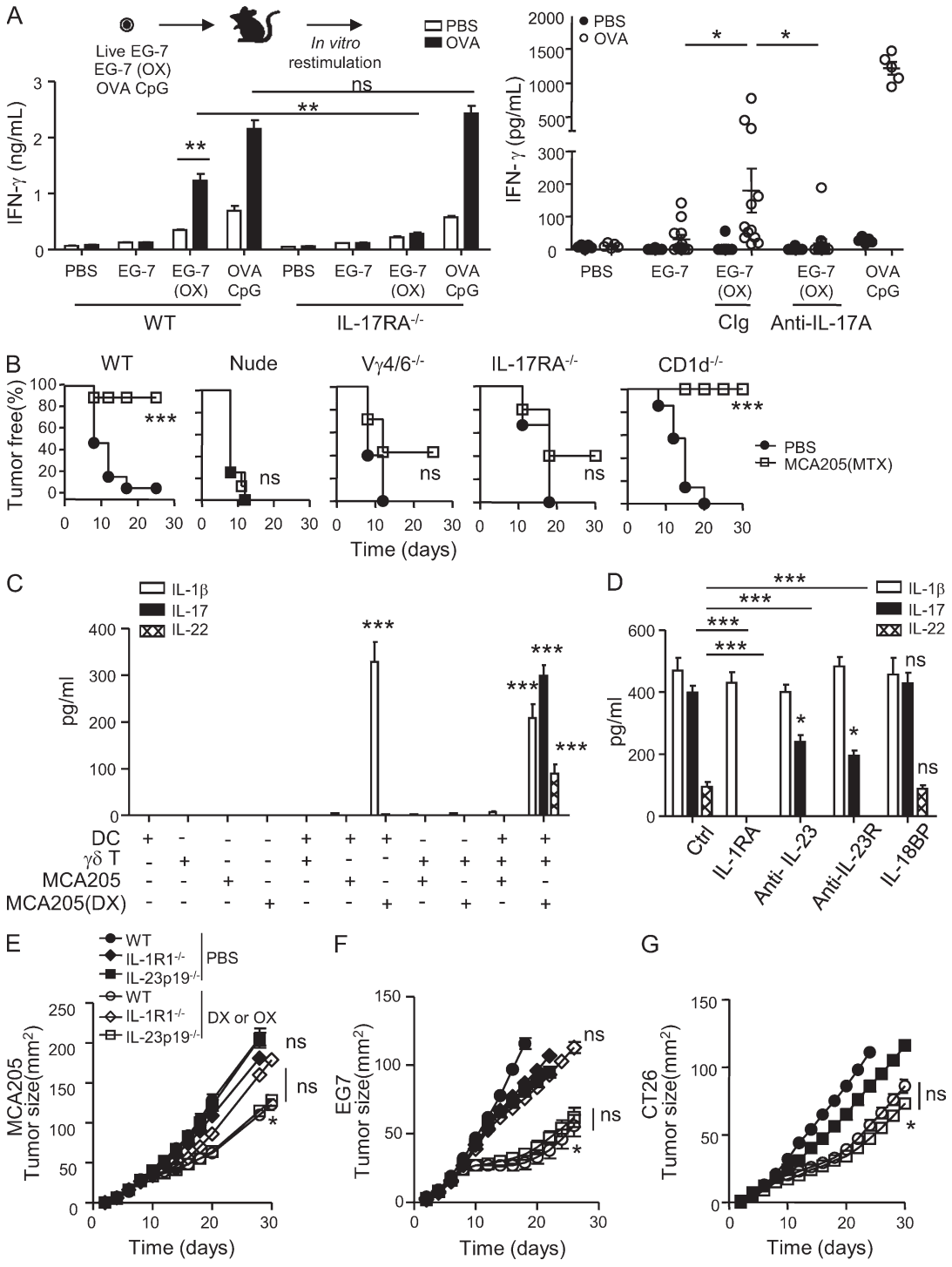

Figure 5. Role of γδ T17 in the priming of T cell responses during an immunogenic cell death and regulation by IL-1β. (A) OX-treated EG-7 cells were inoculated in the footpad of WT versus IL-17RA$^{-/-}$ mice (n = 5; left) along with anti–IL-17A neutralizing antibody (or Clg; right panel). OVA-specific IFN-γ secretion by draining LN cells was measured in vitro by ELISA after stimulation with OVA protein (1 mg/ml). OVA/CpG immunization was used as positive control. (B) Immunization with MTX-treated MCA205 and rechallenge with a tumorigenic dose of live MCA205 were performed at day 0 and day 7, respectively in WT C57Bl6 (n = 10), nude (n = 10), Vγ4/6$^{-/-}$ (n = 15), IL-17RA$^{-/-}$ (n = 8), and CD1d$^{-/-}$ (n = 6) mice. The percentages of tumor-free mice were scored at the indicated time points. Experiments in A and B were performed twice with similar results. (C) Production of IL-1β, IL-17A, and IL-22 from mixed co-cultures of LN-derived γδ T cells and/or BMDCs loaded or not loaded with live or DX-treated MCA205 was monitored by ELISA. Data are shown as mean ± SEM (D) Co-cultures of DX-treated MCA205/BMDC/γδ T were performed in the presence of 20 μg/ml IL-1RA (Amgen), anti–IL-23, or

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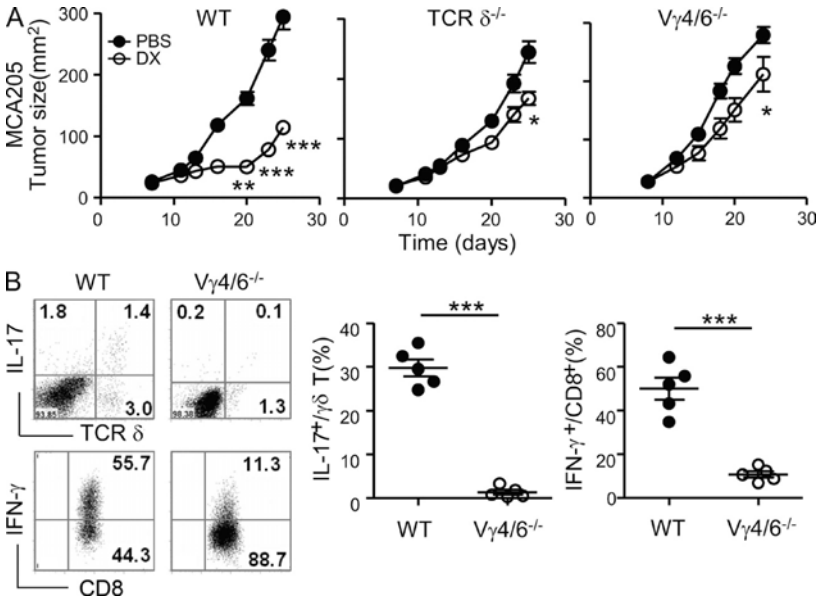

Figure 6. The therapeutic activity of anthracyclines and tumor colonization of Tc1 depend upon Vγ4 Vγ6 γδ T cells. (A) WT, TCR $\delta^{-/-}$, or V$\gamma4/6^{-/-}$ mice with established MCA205 tumors were injected intratumorally with PBS or DX. Tumor size was measured at the indicated time and plotted as mean ± SEM (n = 8/group). (B) Percentage of IL-17A– or IFN-γ–expressing cells within $CD3^+$ $TCR\delta^+$ and $CD3^+$ $CD8^+$ TILs, respectively, in WT or V$\gamma4/6^{-/-}$ mice. A typical dot plot is shown (left) and statistical analysis was performed with combined data from two independent experiments (right). *, P < 0.05; ***, P < 0.001.

WT mice) into tumor beds 2 d after DX potentiated the growth-retarding effect of chemotherapy, yet had no effect on PBS-treated tumors (Fig. 7 A). Importantly, synergistic antitumor effects of DX and adoptively transferred γδ T cells were lost when the γδ T cells were obtained from IL-17A$^{-/-}$ or IL-1R1$^{-/-}$ donors (Fig. 7, B and C), emphasizing the role of IL-1β responses and IL-17 production in the function of γδ T cells. Moreover, the adoptive transfer of WT γδ T cells could restore the antitumor efficacy of chemotherapy in IL-17A–deficient mice (Fig. 7 D). Collectively, these results emphasize the important contribution of γδ T17 cells to the immune-dependent effects of anticancer chemotherapy.

DISCUSSION

Our results highlight a role of γδ T cells, particularly the Vγ4- and Vγ6-expressing subsets that produce the effector cytokine IL-17A, in the anticancer immune response induced by cytotoxic chemotherapeutics. We demonstrated that the IL-17A–IL-17RA signaling pathway is required for the priming of IFN-γ–secreting, antigen-specific T cells by tumor cells exposed to chemotherapy. This tumor-specific, Tc1-mediated immune response is essential for anticancer immunity because the protective effect of dying tumor cell vaccination is lost in athymic nude mice or when $CD8^+$ T cells are depleted (Casares et al., 2005), and chemotherapy fails to work when the IFN-γ–IFN-γR system is blocked (Ghiringhelli et al., 2009). Accordingly, we found that the absence of the IL-17A–IL-17RA pathway reduced the capacity of mice to mount a protective antitumor response.

When exploring the source of IL-17A elicited by dying tumor cells, we found that γδ T cells were the quantitatively and functionally most important IL-17A producers, based on several observations. First, in the context of chemotherapy, IL-17–producing cells accumulated in tumors, and most of them were positive for γδ T markers. Second, antigen-specific $CD4^+$ T cells in LNs draining the dying tumor cells showed a Th1 (IL-2 and IFN-γ) instead of a Th17 cytokine pattern (Ghiringhelli et al., 2009). $CD4^+$ and $CD8^+$ TILs were polarized to produce IFN-γ instead of IL-17. Also, IL-6 and TGF-β, two key regulatory cytokines essential for the differentiation of Th17 cells (Ivanov et al., 2006; Veldhoen et al., 2006), were dispensable for the efficacy of chemotherapy or vaccination with dying tumor cells (Fig. S3, C and D), suggesting that Th17 cells may not be required for the anticancer immune response after chemotherapy. Third, when popliteal LNs were recovered from mice that had been injected with dying (but not live) tumor cells through footpad, the restimulation of LN-resident cells using anti-CD3ε plus IL-23 readily enhanced IL-17 production (unpublished data), a feature common to memory T cells (van Beelen et al., 2007), innate NKT (Rachitskaya et al., 2008), and γδ T cells (Sutton et al., 2009). Fourthly, the subset of NKT cells capable of producing IL-17 in LNs ($CD103^+CD4^-NK1.1^-CCR6^+CD1d$ tetramer$^+$; Doisne et al., 2009) did not appear to be specifically triggered by dying cells in vivo (unpublished data). Moreover, $CD1d^{-/-}$ mice, which lack NKT cells, were indistinguishable from WT mice when the efficacy of chemotherapy was assessed in prophylactic vaccination settings. Fifthly, knockout of Vγ4/6 or TCR δ attenuated the protective antitumor vaccination with dying tumor cells and reduced the efficacy of the anthracycline-based chemotherapy on established tumors. Finally, the adoptive transfer of WT γδ T cells into IL-17A$^{-/-}$ hosts could restore the clinical response to chemotherapy and improve

IL-23R neutralizing antibodies, or 10 µg/ml IL-18BP. Experiments in C and D were repeated three to six times. (E and G) Tumor size was monitored in WT (circles), IL-1R1$^{-/-}$ (diamonds), and IL-23p19$^{-/-}$ (squares) mice treated with PBS (filled symbols) or DX (open symbols; E and F), or in WT mice treated with systemic anti–IL-23 neutralizing antibodies (squares) or Clg (circles; G). Data are representative of 2 experiments with 6–10 mice/group. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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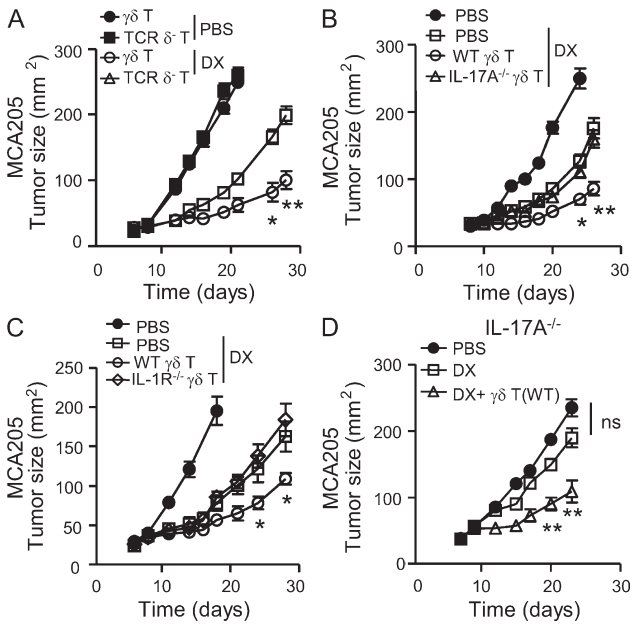

Figure 7. Role of γδ T cell–derived IL-17A during chemotherapy. $CD3^+$ TCR δ^+ or $CD3^+$ TCR δ^- T cells from WT mice (A), $CD3^+$ TCR δ^+ T cells from IL-$17A^{-/-}$ (B), or IL-$1R1^{-/-}$ (C) mice were injected intratumorally into MCA205-bearing WT mice (A–C) or IL-$17A^{-/-}$ mice (D) 2 d after PBS or DX treatment. Tumor sizes are plotted as mean ± SEM for five mice/group. Experiments were repeated two to three times with similar results. *, $P < 0.05$; **, $P < 0.01$.

the response in WT hosts, and this latter effect was lost when γδ T cells from IL-$17A^{-/-}$ (rather than WT) donors were used.

In the context of immunogenic chemotherapy, it appears clear that IL-1β plays a major role in stimulating IL-17 production and the anticancer function of γδ T cells. The key role of IL-1β in regulating γδ T cells function was shown by using IL-1RA in co-cultures of DCs/γδ T cells in the presence of dying tumor cells. Also, γδ T cells that lack IL-1R1 lose the capacity to amplify the tumoricidal action of anthracyclines. Interestingly, inflammasome-dependent IL-1β secretion from DCs was also found to be mandatory for the polarization of $CD8^+$ T cells toward a Tc1 pattern (Ghiringhelli et al., 2009), suggesting that a connection between DCs, γδ T17 cells, and Tc1 cells might be important for optimal anticancer immune responses. We noticed a strong correlation between γδ T17 and Tc1 cells after chemotherapy in three different tumor models. We also noticed that the production of IL-17 production preceded that of IFN-γ by TILs. It is well possible that besides helping the development of Tc1 response, γδ T17 cells might enhance the chemoattraction of effector Tc1 into the tumor beds. These results are compatible with observations obtained in a cancer-unrelated context, microbial infection, in which γδ T17 associated with Th1 responses exert protective immune response (Umemura et al., 2007). As IL-17 could not directly induce IFN-γ production or enhance proliferation of $CD8^+$ T cells (unpublished data), our results imply a causal relationship between the presence of γδ T17 cells and the recruitment of antitumor effector Tc1 cells into tumor beds.

γδ T cells represent a major source of IL-17 during lung infection by *Mycobacterium tuberculosis* (Lockhart et al., 2006; Umemura et al., 2007) and liver infection by *Lysteria* (Hamada et al., 2008). γδ T cell-derived IL-17 is critical for the recruitment of neutrophil recruitment into the peritoneal cavity after *Escherichia coli* inoculation (Shibata et al., 2007). γδ T cells can be directly stimulated through TLR2, TLR1, and/or dectin-1 in response to *Mycobacterium tuberculosis* and *Candida albicans* to produce IL-17 in synergy with IL-23 (Martin et al., 2009). As to the mechanisms that link chemotherapy-elicited tumor cell death to the accumulation of γδ T17 cells, our data suggest that IL-1β acts as a major trigger. One previous report demonstrated the pivotal function of IL-1β in regulating γδ T17 cells in experimental autoimmune encephalomyelitis (EAE; Sutton et al., 2009). In that model, IL-1β synergized with IL-23 to promote IL-17 production by γδ T, which in turn, stimulated the differentiation of pathogenic Th17 cells.

Our data can be interpreted to support the contention that the context and immune orchestration at the site of cell death may be critical for an optimal contribution of the immune system to the efficacy of anticancer therapies. The present data introduces the idea that γδ T17 cells are part of the innate immune response that facilitates the subsequent cognate anticancer T cell responses. It remains a formidable challenge for investigating further how the innate and cognate immune effectors develop a dialog within the three-dimensional architecture of the tumor composed of dying and live tumor cells, as well as multiple stromal elements. Should γδ T17 cells also be recruited into human tumor beds after chemotherapy, it would be of the utmost importance to determine their TCR Vδ usage to propose combination therapy of phosphoantigens (for $V\delta2^+$) or other ligands or innate cytokines (for $V\delta2^-$) and anthracyclines to increase therapeutic benefit in neoadjuvant settings or prevent metastases.

MATERIALS AND METHODS

Mice. WT C57BL1/6 (H-2^b) and BALB/c (H-2^d) mice aged between 7 and 12 wk were purchased from Harlan. Nude mice were bred in the animal facility of Institut Gustave Roussy. TCR $\delta^{-/-}$, IL-$1R1^{-/-}$, and IL-$17RA^{-/-}$ (H-2^b) mice were bred at Cryopréservation, Distribution, Typage, et Archivage Animal (Orléans, France) by B. Ryffel (CNRS, Orleans, France) and P. Pereira (Institut Pasteur, Paris, France; TCR $\delta^{-/-}$ was bred in the same manner). IL-$23p19^{-/-}$ and IL-$17A^{-/-}$ (H-2^b) were provided by M.J. Smyth (Peter MacCallum Cancer Centre, Victoria, Australia). $V\gamma4\gamma6^{-/-}$ mice (H-2^b) were provided by G. Matsuzaki (University of the Ryukyus, Okinawa, Japan) and K. Ikuta (Kyoto University, Kyoto, Japan). CD$1d^{-/-}$ and $CCR6^{-/-}$ (H-2^b) mice were bred at St. Vincent de Paul Hospital AP-HP (Paris, France) and provided by K. Benlagha. The experimental protocols were approved by the Animal Care and Use Committee in the animal facility of Institut Gustave Roussy.

Cell lines and reagents. CT26 (H-2^d) colon cancer, MCA205 (H-2^b) and MCA2 (H-2^d) sarcoma, TS/A mammalian cancer (H-2^d), and EG7 thymoma (H-2^b) were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 10 mM

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Hepes at 37°C, 5% CO_2. All media were purchased from Invitrogen. Recombinant mouse IL-1β, IL-23, IL-6, TGF-β, and IL-18 BPd/Fc were purchased from R&D Systems. AhR antagonist CH223191 was obtained from EMD. DX hydrochloride (D1515), MTX dihydrochloride (M6545), and $DiOC_6(3)$ were obtained from Sigma-Aldrich. Mouse IL-17A, IL-1β, and IL-23p19 ELISA kits were purchased from eBioscience. Mouse ELISA kits and neutralizing antibody for IL-22 (AF582; AB108C as isotype control) were purchased from R&D system. Antibodies for CD45.2 (104), CD3ε (145-2C11), CD4 (GK1.5), CD8α (53–6.7), TCR δ (GL-3), CD69 (H1.2F3), IL-17A (TC11-18H10), or IFN-γ (XMG1.2) were purchased from BD or eBioscience. Anti-SCART2 polyclonal serum was provided by J. Kisielow (Swiss Federal Institute of Technology, Zurich, Switzerland). Neutralizing antibodies for IL-17A (MAB421), IFN-γ (XMG1.2), CCL20 (MAB760), IL-23 (AF1619), IL-23R (MAB1686), IL-6 (MAB406), and IL-22 (AF582) were purchased from R&D Systems. CpG oligodeoxynucleotide 1668 was obtained from MWG Biotech AG. Anti–TGF-β peptide P17 and control peptide were obtained from J.J. Lasarte (University of Navarra, Pamplona, Spain; Dotor et al., 2007),

Tumor models and chemo/radiotherapy. 8×10^5 MCA205, EG7, CT26, TS/A, or MCA2 tumor cells were inoculated s.c. near the thigh into syngeneic mice. Chemotherapy was performed in MCA205 and CT26 models by intratumoral injection of DX (2 mM, 50 µl) or OX (5 mg/kg body weight, i.p) when tumors reached 25-45 mm^2. Radiotherapy was performed by local x-ray irradiation (10 Gy; RT250; Phillips) at the unshielded tumor area when TS/A tumor reached 40–60 mm^2.

Gene expression assays. Whole RNA was extracted using RNEasy Mini kit (QIAGEN) from tumor homogenates. 5 µg of RNA from each sample were reverse-transcribed using QuantiTect Reverse Transcription kit (QIAGEN). Gene expression assays were performed with custom TaqMan Low Density Arrays using StepOnePlus Real-Time PCR System. PPIA was chosen as the endogenous control to perform normalization between different samples.

Tumor dissection and FACS analysis. Tumor burdens were carefully removed, cut into small pieces, and digested in 400 U/ml Collagenase IV and 150 U/ml DNase I for 30 min at 37°C. Single-cell suspension was obtained by grinding the digested tissue and filtering through a 70-µM cell strainer. Cells were blocked with 10 µg/ml anti-CD16/CD32 (eBioscience) before surface staining (2.5 µg/ml of each antibody). LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen) was used to distinguish live and dead cells. For intracellular staining, freshly isolated cells were treated with 50 ng/ml PMA, 1 µg/ml ionomycin, and GolgiStop (BD) for 4 h at 37°C in RPMI containing 2% mouse serum (Janvier). Cells were then stained with anti–IFN-γ and anti–IL-17 using a Cytofix/Cytoperm kit (BD).

Protein extraction. Tumors were mechanically dissociated with lysis buffer (T-PER Tissue Protein Extraction Reagent; Thermo Fisher Scientific) containing protease inhibitor (complete Mini EDTA-free; Roche). Tumor lysate was then centrifuged at 10000 *g* for 5 min at 4°C to obtain supernatant.

Purification and adoptive transfer of γδ T cells. The skin-draining LNs (inguinal, popliteal, superficial cervical, axillary, and brachial LNs) were harvested from naive mice (8–12 wk). Dead cells were removed from single-cell suspension (Dead Cell Removal kit) before γδ T cell purification (TCRγ/δ^+ T Cell Isolation kit) using AutoMACS Separator (Miltenyi Biotec) with recommended programs. Purity of this isolation normally reached >95%. The TCR δ^- $CD3^+$ cell fraction was also collected and used as control for some experiments. Day 2 after chemotherapy, 2.5×10^5 cells were injected directly into the tumor with insulin syringes for the adoptive transfer setting.

T cell priming and tumor vaccination. EG7 cells pretreated with 5 µg/ml OX overnight or left untreated were washed thoroughly and injected at 1 million/50 µl into the foodpad of naive syngeneic mice. CpG/OVA (5 µg CpG+1 mg OVA/mouse) and PBS injection were used as positive and negative controls. In some setting, neutralizing antibody (200 µg/mouse) for IL-17A or CIg was injected i.p. 5 d later, the popliteal LN cells were harvested, seeded in a 96-well plate at 3×10^5/well and restimulated with 1 mg/ml OVA protein. IFN-γ secretion was measured by OptEIA Mouse IFN-γ ELISA kit (BD). MCA205 cells were treated with 2 µM MTX overnight, washed thoroughly, and injected into left flank s.c. at 3×10^5/mouse. PBS was used as control. Mice were rechallenged with 5×10^4 live MCA205 cells in the right flank 7 d later. Tumor growth was monitored every 2–3 d.

DC-tumor mixed lymphocyte cultures. DCs were propagated in Iscoves's medium (Sigma-Aldrich) with J558 supernatant (40 ng/ml GM-CSF), 10% FCS, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol (Sigma-Aldrich) and used between day 8 and 12 when the proportion of $CD11c/MHC\ class\ II^+$ cells was >80%. In mixed co-cultures, DCs were seeded at 10^5/100 µl/well in U-bottom 96-well plates. Tumor cells were treated overnight with 25 µM DX or left untreated, washed, and used at 7.5×10^4/100 µl/well. 2×10^4/50 µl γδ T cells were added 12 h later. Supernatant was collected 36 h later.

Statistical analyses of experimental data. All results are expressed as mean ± SEM, or as ranges when appropriate. For two groups, normal distributions were compared by unpaired Student's *t* test. Non-normal samplings were compared using the Mann-Whitney test or Wilcoxon matched paired test when appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curve. Statistical analyses were performed using Prism 5 software (GraphPad). P values of <0.05 were considered significant.

Online supplemental material. Fig. S1 shows the effect of AhR antagonist on the efficacy of chemotherapy (DX). Fig. S2 depicts the Vγ chain usage of tumor-infiltrating γδ T17 and γδ T cells in the LNs of naive mice. Fig. S3 shows the effect of neutralizing IL-22, CCL20, IL-6, or blocking TGF-β on the efficacy of chemotherapy or vaccine. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100269/DC1.

This work was supported by LIGUE labellisée (L. Zitvogel and G. Kroemer), INFLACARE FP7 EU grant, INCa, Fondation pour la Recherche Médicale ,and Fondation de France. Y. Ma was supported by the China Scholarship Council. N.F. Delahaye was supported by INCa and INFLACARE FP7. L. Apetoh was supported by the Agence Nationale pour la Recherche [ANR-10-PDOC-014-01].

The authors have no conflicting financial interests.

Submitted: 8 February 2010
Accepted: 4 February 2011

REFERENCES

Apetoh, L., F. Ghiringhelli, A. Tesniere, M. Obeid, C. Ortiz, A. Criollo, G. Mignot, M.C. Maiuri, E. Ullrich, P. Saulnier, et al. 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med.* 13:1050–1059. doi:10.1038/nm1622

Aujla, S.J., Y.R. Chan, M. Zheng, M. Fei, D.J. Askew, D.A. Pociask, T.A. Reinhart, F. McAllister, J. Edeal, K. Gaus, et al. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* 14:275–281. doi:10.1038/nm1710

Casares, N., M.O. Pequignot, A. Tesniere, F. Ghiringhelli, S. Roux, N. Chaput, E. Schmitt, A. Hamai, S. Hervas-Stubbs, M. Obeid, et al. 2005. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J. Exp. Med.* 202:1691–1701. doi:10.1084/jem.20050915

Couzi, L., Y. Levaillant, A. Jamai, V. Pitard, R. Lassalle, K. Martin, I. Garrigue, O. Hawchar, F. Siberchicot, N. Moore, et al. 2010. Cytomegalovirus-induced gammadelta T cells associate with reduced cancer risk after kidney transplantation. *J. Am. Soc. Nephrol.* 21:181–188. doi:10.1681/ASN.2008101072

Déchanet, J., P. Merville, A. Lim, C. Retière, V. Pitard, X. Lafarge, S. Michelson, C. Méric, M.M. Hallet, P. Kourilsky, et al. 1999. Implication of gammadelta T cells in the human immune response to cytomegalovirus. *J. Clin. Invest.* 103:1437–1449. doi:10.1172/JCI5409

Dieli, F., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, G. Cicero, A. Roberts, S. Buccheri, M. D'Asaro, N. Gebbia, et al. 2007. Targeting

Published March 7, 2011

JEM

human gammadelta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 67:7450–7457. doi:10.1158/0008-5472.CAN-07-0199

Do, J.S., P.J. Fink, L. Li, R. Spolski, J. Robinson, W.J. Leonard, J.J. Letterio, and B. Min. 2010. Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism. *J. Immunol.* 184:1675–1679. doi:10.4049/jimmunol.0903539

Doisne, J.M., C. Becourt, L. Amniai, N. Duarte, J.B. Le Luduec, G. Eberl, and K. Benlagha. 2009. Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J. Immunol.* 183:2142–2149. doi:10.4049/jimmunol.0901059

Dotor, J., A.B. López-Vázquez, J.J. Lasarte, P. Sarobe, M. García-Granero, J.I. Riezu-Boj, A. Martínez, E. Feijoó, J. López-Sagaseta, J. Hermida, et al. 2007. Identification of peptide inhibitors of transforming growth factor beta 1 using a phage-displayed peptide library. *Cytokine.* 39:106–115. doi:10.1016/j.cyto.2007.06.004

Esser, C., A. Rannug, and B. Stockinger. 2009. The aryl hydrocarbon receptor in immunity. *Trends Immunol.* 30:447–454. doi:10.1016/j.it.2009.06.005

Ghiringhelli, F., L. Apetoh, A. Tesniere, L. Aymeric, Y. Ma, C. Ortiz, K. Vermaelen, T. Panaretakis, G. Mignot, E. Ullrich, et al. 2009. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat. Med.* 15:1170–1178. doi:10.1038/nm.2028

Godder, K., P.J. Henslee-Downey, J. Mehta, B.S. Park, K.Y. Chiang, S. Abhyankar, and L.S. Lamb. 2007. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant.* 39:751–757.

Godfrey, D.I., D.G. Pellicci, O. Patel, L. Kjer-Nielsen, J. McCluskey, and J. Rossjohn. 2010. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin. Immunol.* 22:61–67.

Gonçalves-Sousa, N., J.C. Ribot, A. deBarros, D.V. Correia, I. Caramalho, and B. Silva-Santos. 2010. Inhibition of murine gammadelta lymphocyte expansion and effector function by regulatory alphabeta T cells is cell-contact-dependent and sensitive to GITR modulation. *Eur. J. Immunol.* 40:61–70.

Hamada, S., M. Umemura, T. Shiono, K. Tanaka, A. Yahagi, M.D. Begum, K. Oshiro, Y. Okamoto, H. Watanabe, K. Kawakami, et al. 2008. IL-17A produced by gammadelta T cells plays a critical role in innate immunity against *Listeria monocytogenes* infection in the liver. *J. Immunol.* 181:3456–3463.

Hayday, A.C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity.* 31:184–196.doi:10.1016/j.immuni.2009.08.006

Heilig, J.S., and S. Tonegawa. 1986. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature.* 322:836–840. doi:10.1038/322836a0

Hinrichs, C.S., A. Kaiser, C.M. Paulos, L. Cassard, L. Sanchez-Perez, B. Heemskerk, C. Wrzesinski, Z.A. Borman, P. Muranski, and N.P. Restifo. 2009. Type 17 CD8+ T cells display enhanced antitumor immunity. *Blood.* 114:596–599. doi:10.1182/blood-2009-02-203935

Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 126:1121–1133. doi:10.1016/j.cell.2006.07.035

Jensen, K.D., X. Su, S. Shin, L. Li, S. Youssef, S. Yamasaki, L. Steinman, T. Saito, R.M. Locksley, M.M. Davis, et al. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity.* 29:90–100. doi:10.1016/j.immuni.2008.04.022

Kabelitz, D., D. Wesch, and W. He. 2007. Perspectives of gammadelta T cells in tumor immunology. *Cancer Res.* 67:5–8. doi:10.1158/0008-5472.CAN-06-3069

Kim, S.H., E.C. Henry, D.K. Kim, Y.H. Kim, K.J. Shin, M.S. Han, T.G. Lee, J.K. Kang, T.A. Gasiewicz, S.H. Ryu, and P.G. Suh. 2006. Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazophenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor. *Mol. Pharmacol.* 69:1871–1878. doi:10.1124/mol.105.021832

Kisielow, J., M. Kopf, and K. Karjalainen. 2008. SCART scavenger receptors identify a novel subset of adult gammadelta T cells. *J. Immunol.* 181:1710–1716.

Kortylewski, M., H. Xin, M. Kujawski, H. Lee, Y. Liu, T. Harris, C. Drake, D. Pardoll, and H. Yu. 2009. Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment. *Cancer Cell.* 15:114–123. doi:10.1016/j.ccr.2008.12.018

Kryczek, I., M. Banerjee, P. Cheng, L. Vatan, W. Szeliga, S. Wei, E. Huang, E. Finlayson, D. Simeone, T.H. Welling, et al. 2009. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood.* 114:1141–1149. doi:10.1182/blood-2009-03-208249

Kwong, B.Y., S.J. Roberts, T. Silberzahn, R.B. Filler, J.H. Neustadter, A. Galan, S. Reddy, W.M. Lin, P.D. Ellis, C.F. Langford, et al. 2010. Molecular analysis of tumor-promoting CD8+ T cells in two-stage cutaneous chemical carcinogenesis. *J. Invest. Dermatol.* 130:1726–1736. doi:10.1038/jid.2009.362

Lafaille, J.J., A. DeCloux, M. Bonneville, Y. Takagaki, and S. Tonegawa. 1989. Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell.* 59:859–870. doi:10.1016/0092-8674(89)90609-0

Li, L., L. Huang, A.L. Vergis, H. Ye, A. Bajwa, V. Narayan, R.M. Strieter, D.L. Rosin, and M.D. Okusa. 2010. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J. Clin. Invest.* 120:331–342. doi:10.1172/JCI38702

Lockhart, E., A.M. Green, and J.L. Flynn. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J. Immunol.* 177:4662–4669.

Maniati, E., R. Soper, and T. Hagemann. 2010. Up for Mischief? IL-17/Th17 in the tumour microenvironment. *Oncogene.* 29:5653–5662. doi:10.1038/onc.2010.367

Martin, B., K. Hirota, D.J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity.* 31:321–330. doi:10.1016/j.immuni.2009.06.020

Martin-Orozco, N., and C. Dong. 2009. The IL-17/IL-23 axis of inflammation in cancer: friend or foe? *Curr. Opin. Investig. Drugs.* 10:543–549.

Martin-Orozco, N., P. Muranski, Y. Chung, X.O. Yang, T. Yamazaki, S. Lu, P. Hwu, N.P. Restifo, W.W. Overwijk, and C. Dong. 2009. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity.* 31:787–798. doi:10.1016/j.immuni.2009.09.014

Mills, K.H. 2008. Induction, function and regulation of IL-17-producing T cells. *Eur. J. Immunol.* 38:2636–2649. doi:10.1002/eji.200838535

Muranski, P., and N.P. Restifo. 2009. Does IL-17 promote tumor growth? *Blood.* 114:231–232. doi:10.1182/blood-2009-04-215541

Muranski, P., A. Boni, P.A. Antony, L. Cassard, K.R. Irvine, A. Kaiser, C.M. Paulos, D.C. Palmer, C.E. Touloukian, K. Ptak, et al. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood.* 112:362–373. doi:10.1182/blood-2007-11-120998

Ngiow, S.F., M.J. Smyth, and M.W. Teng. 2010. Does IL-17 suppress tumor growth? *Blood.* 115:2554–2555, author reply :2556–2557. doi:10.1182/blood-2009-11-254607

Nowak, A.K., B.W. Robinson, and R.A. Lake. 2002. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res.* 62:2353–2358.

Nowak, A.K., R.A. Lake, A.L. Marzo, B. Scott, W.R. Heath, E.J. Collins, J.A. Frelinger, and B.W. Robinson. 2003. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J. Immunol.* 170:4905–4913.

O'Brien, R.L., C.L. Roark, and W.K. Born. 2009. IL-17-producing gammadelta T cells. *Eur. J. Immunol.* 39:662–666. doi:10.1002/eji.200839120

Obeid, M., A. Tesniere, F. Ghiringhelli, G.M. Fimia, L. Apetoh, J.L. Perfettini, M. Castedo, G. Mignot, T. Panaretakis, N. Casares, et al. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* 13:54–61. doi:10.1038/nm1523

Published March 7, 2011

Panaretakis, T., N. Joza, N. Modjtahedi, A. Tesniere, I. Vitale, M. Durchschlag, G.M. Fimia, O. Kepp, M. Piacentini, K.U. Froehlich, et al. 2008. The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death Differ.* 15:1499–1509. doi:10.1038/cdd.2008.67

Panaretakis, T., O. Kepp, U. Brockmeier, A. Tesniere, A.C. Bjorklund, D.C. Chapman, M. Durchschlag, N. Joza, G. Pierron, P. van Endert, et al. 2009. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J.* 28:578–590. doi:10.1038/emboj.2009.1

Peng, G., H.Y. Wang, W. Peng, Y. Kiniwa, K.H. Seo, and R.F. Wang. 2007. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity*. 27:334–348. doi:10.1016/j.immuni.2007.05.020

Pereira, P., and L. Boucontet. 2004. Rates of recombination and chain pair biases greatly influence the primary gammadelta TCR repertoire in the thymus of adult mice. *J. Immunol*. 173:3261–3270.

Pichavant, M., S. Goya, E.H. Meyer, R.A. Johnston, H.Y. Kim, P. Matangkasombut, M. Zhu, Y. Iwakura, P.B. Savage, R.H. DeKruyff, et al. 2008. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J. Exp. Med*. 205:385–393. doi:10.1084/jem.20071507

Rachitskaya, A.V., A.M. Hansen, R. Horai, Z. Li, R. Villasmil, D. Luger, R.B. Nussenblatt, and R.R. Caspi. 2008. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J. Immunol*. 180:5167–5171.

Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat. Immunol*. 10:514–523. doi:10.1038/ni.1716

Ribot, J.C., A. deBarros, D.J. Pang, J.F. Neves, V. Peperzak, S.J. Roberts, M. Girardi, J. Borst, A.C. Hayday, D.J. Pennington, and B. Silva-Santos. 2009. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat. Immunol*. 10:427–436. doi:10.1038/ni.1717

Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J. Immunol*. 178:4466–4472.

Sunaga, S., K. Maki, Y. Komagata, J. Miyazaki, and K. Ikuta. 1997. Developmentally ordered V-J recombination in mouse T cell receptor gamma locus is not perturbed by targeted deletion of the Vgamma4 gene. *J. Immunol*. 158:4223–4228.

Sutton, C.E., S.J. Lalor, C.M. Sweeney, C.F. Brereton, E.C. Lavelle, and K.H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. 31:331–341. doi:10.1016/j.immuni.2009.08.001

Umemura, M., A. Yahagi, S. Hamada, M.D. Begum, H. Watanabe, K. Kawakami, T. Suda, K. Sudo, S. Nakae, Y. Iwakura, and G. Matsuzaki. 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *J. Immunol*. 178:3786–3796.

van Beelen, A.J., Z. Zelinkova, E.W. Taanman-Kueter, F.J. Muller, D.W. Hommes, S.A. Zaat, M.L. Kapsenberg, and E.C. de Jong. 2007. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity*. 27:660–669. doi:10.1016/j.immuni.2007.08.013

Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 24:179–189. doi:10.1016/j.immuni.2006.01.001

Wang, L., T. Yi, M. Kortylewski, D.M. Pardoll, D. Zeng, and H. Yu. 2009. IL-17 can promote tumor growth through an IL-6–Stat3 signaling pathway. *J. Exp. Med*. 206:1457–1464. doi:10.1084/jem.20090207

Wilhelm, M., V. Kunzmann, S. Eckstein, P. Reimer, F. Weissinger, T. Ruediger, and H.P. Tony. 2003. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. *Blood*. 102:200–206. doi:10.1182/blood-2002-12-3665

Zitvogel, L., O. Kepp, and G. Kroemer. 2010. Decoding cell death signals in inflammation and immunity. *Cell*. 140:798–804. doi:10.1016/j.cell.2010.02.015

Published March 7, 2011

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SUPPLEMENTAL MATERIAL

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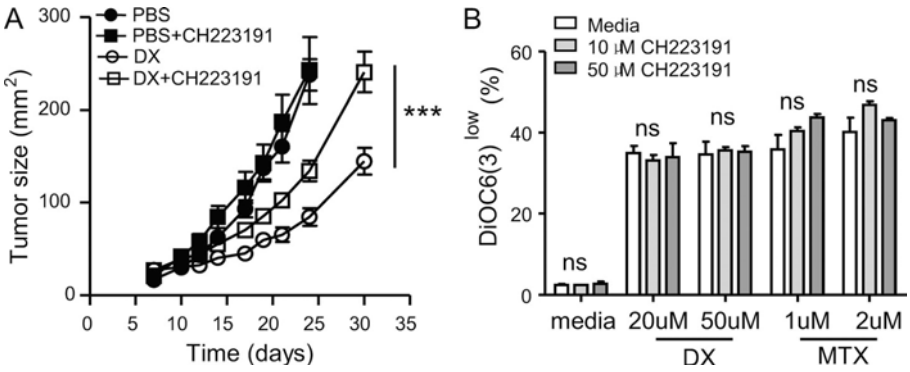

Figure S1. AhR antagonist partially impaired the efficacy of anthracyclines. (A) AhR antagonist CH223191 was dissolved with DMSO and diluted in olive oil. Mice treated with either PBS or DX received a daily systemic inoculation (i.p.) of CH223191 (2 mM, 100 µl) for 4 d from the day of PBS or DX treatment. Tumor size was measured at the indicated time points. One representative experiment out of three is shown. (B) Apoptosis of MCA205 cells treated with media, DX, or MTX with or without the indicated concentration of AhR inhibitor CH-223191. Apoptosis is indicated by a reduction in mitochondrial membrane potential detected by decreased DiOC6(3) fluorescence. This experiment was performed twice with similar results. ***, $P < 0.001$. ns, not significant.

Published March 7, 2011

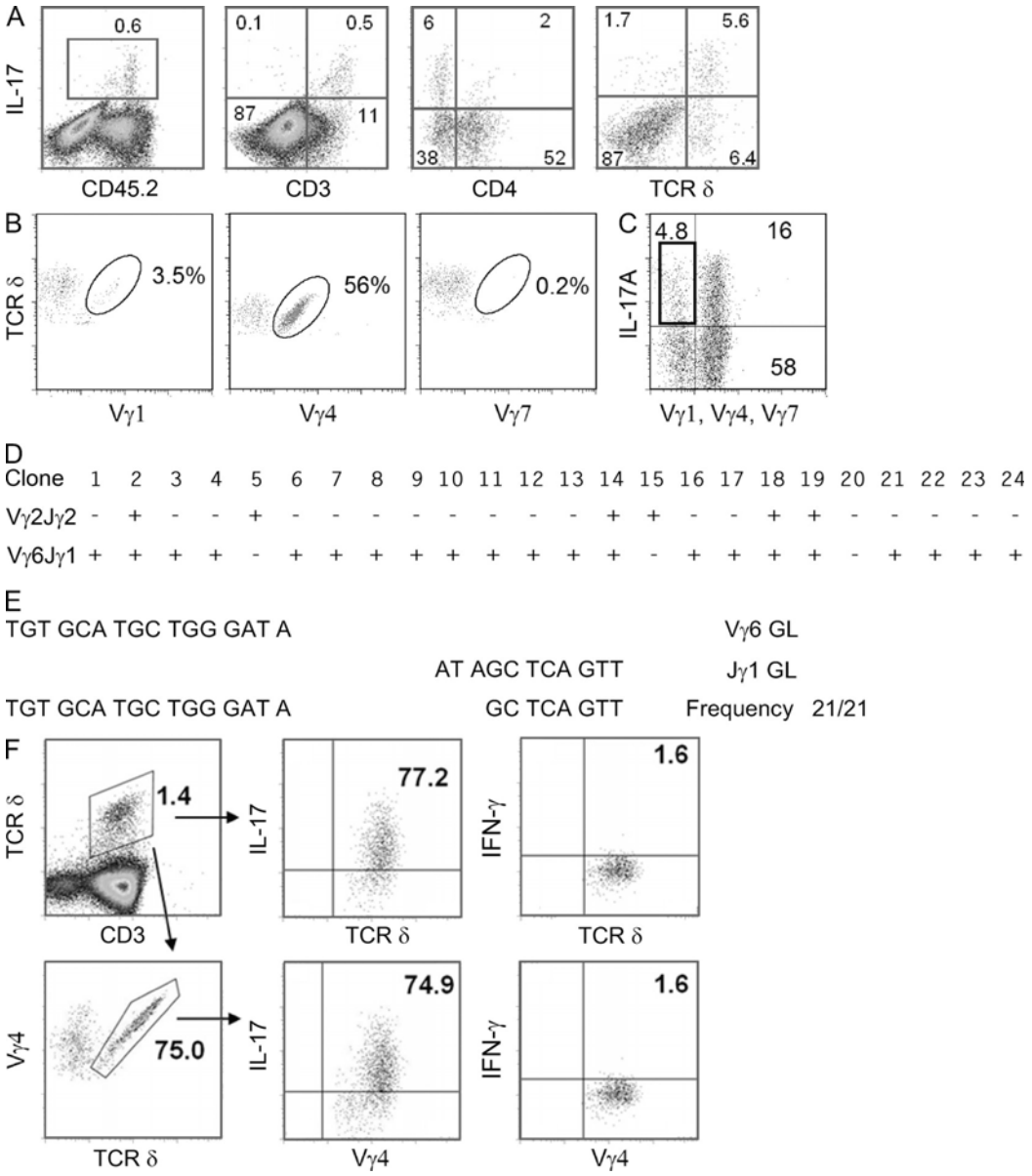

Figure S2. Vγ chain usage by γδ T17 cells in tumor bed and skin draining LNs of naive mice. (A) CD45, CD3, CD4, and TCR δ expression by IL-17A–producing cells from tumor beds of mice 8 d after chemotherapy. (B) Vγ usage in live CD45$^+$ CD3$^+$ TCR δ$^+$ IL-17$^+$ cells in tumor beds after DX. Numbers represent the percentage of Vγ1$^+$, Vγ4$^+$, or Vγ7$^+$ cells among TCR δ$^+$ IL-17$^+$ cells as indicated. One experiment representative of three is shown. (C–E) Individual Vγ1$^-$Vγ4$^-$Vγ7$^-$ γδ T17 TILs as gated in (C) were sorted in PCR plates, and DNA was amplified with primers specific for Vγ2-Jγ2 or Vγ6-Jγ1 rearrangements. (D) Presence (+) or absence (−) of specific amplification bands in 24 clones analyzed. (E) Junctional sequences of the Vγ6-Jγ1 amplifications present in 21 clones in D. GL denotes the germline sequences of the Vγ6 and Jγ1 ends as indicated. (F) LN cells from naive mice were stimulated with PMA/ionomycin, and total γδ T cells (top) or Vγ4$^+$ cells (bottom) were gated and analyzed for intracellular IL-17 (middle) or IFN-γ (right) by FACS. Numbers indicate the percentage of cells inside the gates. One experiment representative of four is shown.

Published March 7, 2011

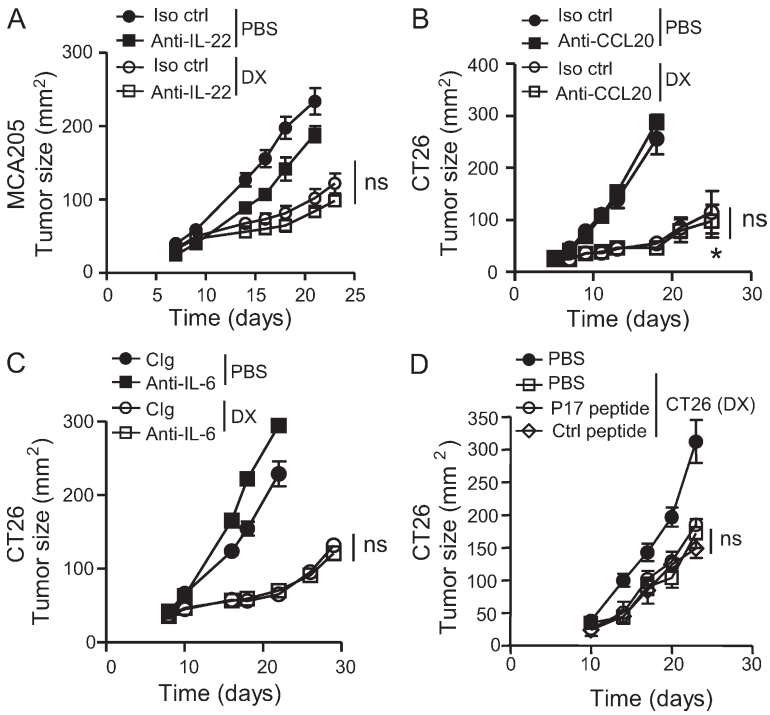

Figure S3. Dispensable roles of IL-22, CCL20, IL-6, and TGF-β for the efficacy of chemotherapy. (A and B) Neutralizing antibodies against IL-22 (50 µg/mouse; A), CCL20 (200 µg/mouse; B), or Clg were administered i.p. every other day for 1 wk starting at the day of chemotherapy in MCA205- or CT26-bearing WT mice. Tumor growth was measured at the indicated time points. One representative experiment out of two is shown. (C) Subcutaneous CT26 colon cancers were treated with DX in the presence of systemic administration of neutralizing antibody against IL-6 (300 µg/mouse) or Clg. (D) Mice were immunized with DX-treated CT26 (injected s.c. into the right flank) and concomitantly challenged with live CT26 tumor cells (injected into the opposite flank at day 0). In parallel, anti–TGF-β or a control peptide (100 µg/mouse) was administered locally (on the site of the vaccination) daily from day 0 to 10. Tumor size was measured at the indicated time points (n = 5 mice/group). The experiment was performed twice with similar results. ns, not significant.

Cancer Research

Opposing Effects of Toll-like Receptor (TLR3) Signaling in Tumors Can Be Therapeutically Uncoupled to Optimize the Anticancer Efficacy of TLR3 Ligands

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Abstract

Many cancer cells express Toll-like receptors (TLR) that offer possible therapeutic targets. Polyadenylic-polyuridylic acid [poly(A:U)] is an agonist of the Toll-like receptor TLR3 that displays anticancer properties. In this study, we illustrate how the immunostimulatory and immunosuppressive effects of this agent can be uncoupled to therapeutic advantage. We took advantage of two TLR3-expressing tumor models that produced large amounts of CCL5 (a CCR5 ligand) and CXCL10 (a CXCR3 ligand) in response to type I IFN and poly(A:U), both *in vitro* and *in vivo*. Conventional chemotherapy or *in vivo* injection of poly(A:U), alone or in combination, failed to reduce tumor growth unless an immunochemotherapeutic regimen of vaccination against tumor antigens was included. CCL5 blockade improved the efficacy of immunochemotherapy, whereas CXCR3 blockade abolished its beneficial effects. These findings show how poly(A:U) can elicit production of a range of chemokines by tumor cells that reinforce immunostimulatory or immunosuppressive effects. Optimizing the anticancer effects of TLR3 agonists may require manipulating these chemokines or their receptors. *Cancer Res; 70(2); 490–500. ©2010 AACR.*

Introduction

Agonists of Toll-like receptors (TLR) are being evaluated for the treatment of cancer (1, 2). Preclinical studies revealed that systemic administration of TLR agonists can boost innate immunity, augment antibody-dependent effector functions, and enhance adaptive immune responses (1–3). TLR3 is the critical sensor of viral double-stranded RNA (4). The synthetic polyinosinic:polycytidylic acid [poly(I:C)] is a TLR3 ligand (TLR3L) that mediates potent adjuvant effects in thus far that it strongly enhances antigen-specific $CD8^+$ T-cell responses (5, 6), promotes antigen cross-presentation by dendritic cells (7), and directly acts on effector $CD8^+$ T and natural killer (NK) cells to augment IFN-γ release (8). Poly(I:C) is recognized by both the endosomal receptor TLR3 and cytosolic receptors, including RNA helicases such as RIG-I and the *melanoma differentiation–associated gene 5* (*MDA5*). In the poly(I:C)-induced immune responses *in vivo, MDA5* is critical for IFN-γ induction, whereas TLR3 is mandatory for IL-12p40 release (9).

Another synthetic double-stranded RNA, polyadenylic:polyuridylic acid [poly(A:U)], which only signals through TLR3, has also been widely used in preclinical and clinical studies. When combined with a candidate protein or viral antigen in mice, poly(A:U) can promote antigen-specific Th1-immune responses and boost antibody production (10, 11). Poly(A:U) has been safely used with moderate success for treating breast or gastric cancers as a monotherapy (12–14). Retrospective analyses highlighted that TLR3-expressing breast cancers may be selectively sensitive to the antitumor effects of poly(A:U). Indeed, TLR3 is not only expressed by immune cells but also by some epithelial (15) or endothelial cells (16). Intracellular staining for TLR3 was reported for human breast cancers (17) and melanoma (18) and its expression can be induced by type I IFNs. TLR3 signaling can directly inhibit the proliferation of carcinoma cells (19) or can induce apoptosis when combined with protein synthesis inhibitors or type I IFN (17, 18). Besides these beneficial effects on established cancers, TLR3 signaling may also

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-1890

participate in proinflammatory reactions contributing to tumorigenesis, suggesting that exploiting the TLR system in cancer might be a doubled-edged sword (20–22). Consequently, there is a need for a fine dissection of the direct (on tumor cells) versus the indirect (on immune cells) effects of TLR agonists as their potential anticancer effects are being evaluated.

Taking advantage of two murine tumor models expressing TLR3, we show that poly(A:U) acts not only in host cells but also in the tumor parenchyma to generate the opposite action of two chemokines, CXCL10 and CCL5, which are favorable and deleterious for the clinical outcome, respectively. These findings support the idea that manipulating TLR3 signaling for cancer therapy will benefit from uncoupling chemokine receptor signaling at the tumor/host interface.

Materials and Methods

Reagents. Poly(A:U) was from Innate Pharma. The murine type I IFN was produced by M. Ferrantini (Istituto Superiore di Sanità). Human IFN α2b and ELISA kits for CCL5 and CXCL10 were from R&D Systems. Ovalbumin was from Calbiochem. CpG oligodeoxynucleotide (ODN) 1668 was from MWG Biotech AG. Methionylated RANTES (MetRantes) was provided by Amanda Proudfoot (Merck Serono Geneva Research Center, Geneva, Switzerland).

Mice and cell lines. B16-OVA murine melanoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 IU/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acids, and 10 mmol/L HEPES. Murine GL26 glioma cells (H-2b) were maintained in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin/streptomycin, 10 mmol/L HEPES, and 50 μmol/L β-mercaptoethanol. Human breast cancer primary cultures were established at Institut Gustave Roussy from metastatic patients suffering from ascitis; patients provided informed consent. Cells were used after three passages of propagation in AIM-V culture medium.

C57BL/6 mice were purchased from Charles River. C57BL/6 nude mice were obtained from animal facility of Institut Gustave Roussy. *Trif*$^{-/-}$, *Cxcr3*$^{-/-}$, and *Ccr5*$^{-/-}$ green fluorescent protein (GFP) mice were bred at Centre National de la Recherche Scientifique IEM 2815, Orléans, France, and Institut National de la Santé et de la Recherche Médicale, U543, Paris, France. The experimental protocols were approved by the Animal Care and Use Committee of Institut Gustave Roussy.

In vitro *tumor stimulation assays.* B16-OVA (or GL26; 5×10^4) or primary human breast cancer cells (2×10^5) were seeded in 24-well plates, treated with 1,000 IU/mL of type I IFN for 18 h, and then treated with poly(A:U) for 48 h. Supernatants were collected to dose chemokine production.

Tumor models and immunotherapy. B16-OVA (3×10^5 or 6×10^5) and GL26 (6×10^5) cells were inoculated s.c. into the left flank of mice. Vaccines were injected into the right footpad [for CpG+OVA: CpG ODN 1668 (5 μg/mouse) plus ovalbumin (1 mg/mouse)] or right flank [or cell vaccines: 10^6 B16-OVA or GL26 pretreated with type I IFN (1,000 IU/mL) for 18 h and then doxorubicin (20 μmol/L) for 24 h for each mouse]. Chemotherapy (oxaliplatin) was applied i.p. at 5 mg/kg. Poly(A:U) was injected i.p. at 100 μg per mouse in B16-OVA model and at 500 μg per mouse in the GL26 model. MetRantes (10 μg/mouse) was injected i.p. daily for 3 wk to block CCL5. Necrotic cells (F/T) were obtained following two consecutive cycles of freezing (liquid nitrogen) and thawing (37°C). For preimmunization, OVA-CpG vaccine was injected into the right footpad 7 d before inoculation of tumor cells. To block CXCR3, anti–CXCR3-173 neutralizing monoclonal antibody (mAb) or the control mAb (PIP) were injected i.p. at 200 μg per mouse every other day for 12 d since 5 d before tumor cell inoculation.

Lentivirus-based short hairpin RNA construction. The lentivirus construction and viral particles were designed and produced by Vectalys SA. As for the lentivirus carrying the short hairpin RNA (shRNA) knocking down CCL5, the forward primer 5′-CGCGACGTCAAGGAGTATTTCTATTCAAGAGATAGAAATACTCCTTGACGTTTTTTTGCA-3′ and the reverse primer 3′-TGCAGTTCCTCATAAAGATAAGTTCTCTATCTTTATGAGGAACTGCAAAAAA-5′ were annealed and ligated into vector [pLV-H1-EF1-PURO-IRES-GFP (pV2.3.127)] by cohesive *Mlu*I/*Nsi*I ligation. A similar approach was used to knockdown Lamin A/C and TRIF expression targeting sequences 5′-GAAGGAGGGTGACCTGATA-3′ and 5′-GGAAAGCAGTGGCCTATTA-3′, respectively.

Flow cytometry. Cells from tumor, tumor draining lymph node (DLN), or vaccine DLN were isolated by mechanical dissociation and filtered through a 70-μm cell strainer. CD3ε-PerCP, CD8-FITC (BD Pharmingen), CXCR3-PE (R&D System), NK1.1-Pacific Blue (eBioscience), and isotype control antibodies (2.5 μg/mL) were used for the surface staining at 4°C for 30 min. Hydroxystilbamidine (Molecular Probes, Invitrogen) was used to exclude dead cells. For intracellular staining, freshly isolated cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate, 1 μg/mL ionomycin, and Golgi-stop (BD Pharmingen) for 4 h at 37°C in RPMI containing 2% mouse serum (Janvier). Cells were then fixed, permeabilized, and stained with IFN-γ–allophycocyanin (BD Pharmingen) with fixation/permeabilization kits (BD Bioscience).

Protein extraction. Tumors were mechanically dissociated with lysis buffer (T-PER Tissue Protein Extraction Reagent, Pierce) containing a protease inhibitor (complete Mini EDTA-free, Roche). Tumor lysate was then centrifuged at 10,000 × *g* for 5 min at 4°C to obtain supernatant. Alternatively, tumors were digested with 400 U/mL Collagenase IV and 150 U/mL DNase I for 30 min. Single-cell suspension was sorted using AutoMACS (Miltenyi Biotec) to obtain $CD45^+$ and $CD45^-$ fractions, and whole-cell protein was extracted using lysis buffer (1×10^6 cells/100 μL buffer).

Statistical analyses. Comparison of continuous data and categorical data were achieved by the Mann-Whitney *U* test and by χ^2 as appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curves. Statistical analyses were performed using GraphPad Prism 5.0. All *P* values are two-tailed. All *P* values <0.05 were considered statistically significant for all experiments. *, **, and *** indicated *P* values of <0.05, <0.01, and <0.001, respectively.

Results

Synergistic effects between vaccines, chemotherapy, and poly(A:U). To characterize the relative importance of direct effects of poly(A:U) on tumor parenchyma versus indirect, immune-mediated effects, we took advantage of the B16-OVA, which expresses TLR3 (data not shown), such as the parental cell line B16F10 (Supplementary Fig. S1) as well as the model antigen ovalbumin (OVA). Albeit mediating significant cytostatic effects on B16-OVA tumor cells *in vitro* (Supplementary Fig. S2), oxaliplatin-based chemotherapy failed to hamper tumor progression *in vivo* when it was administered alone or combined with the poly(A:U) (Fig. 1*B*), following the protocol detailed in Fig. 1*A*. However, the administration of a vaccine composed of OVA plus the adjuvant CpG before the combination of oxaliplatin and poly(A:U) significantly retarded tumor growth (Fig. 1*B*) and prolonged the survival of tumor-bearing C57BL/6 mice (Fig. 1*C*). This vaccine, when applied in the

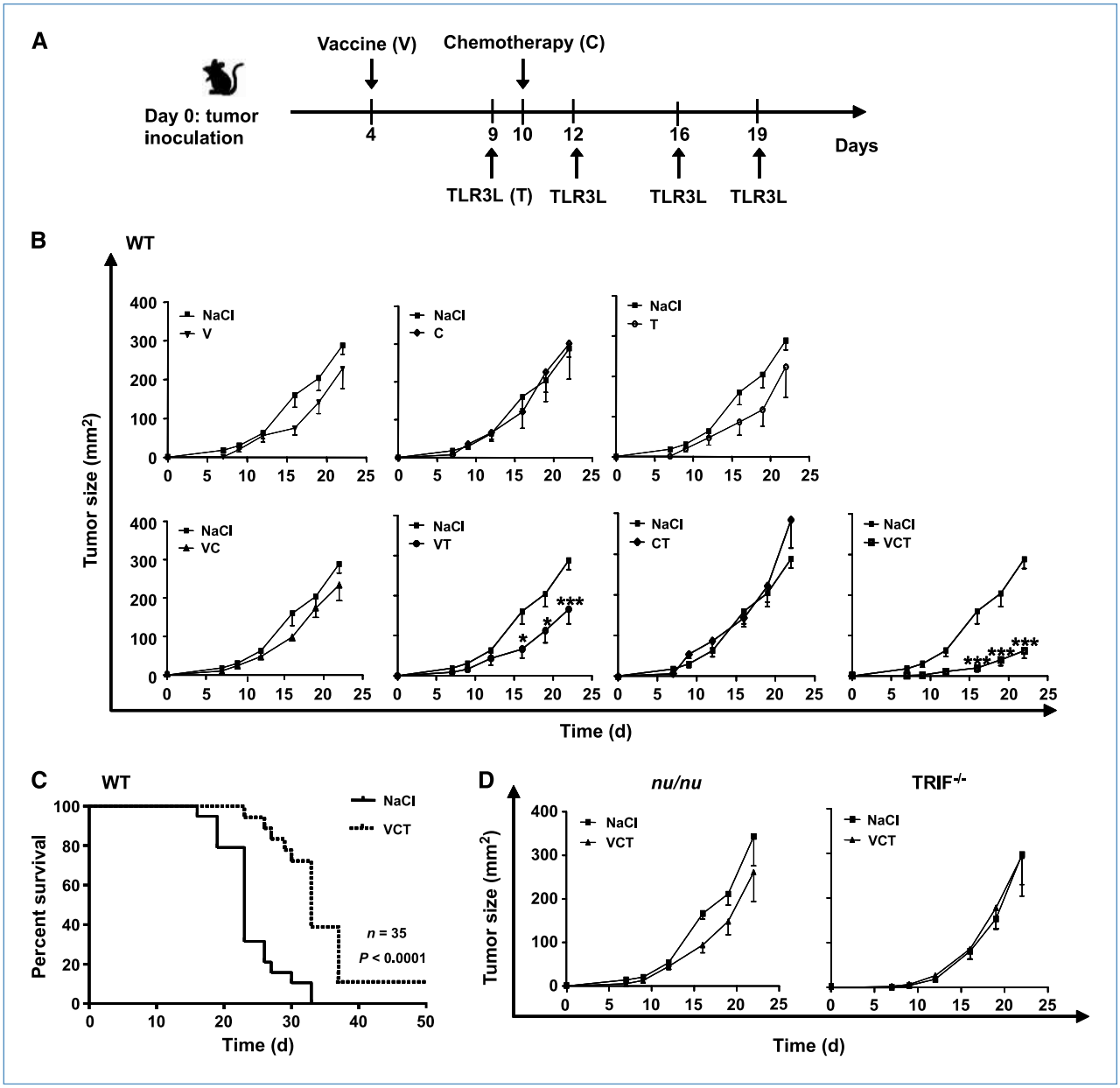

Figure 1. Sequential immunochemotherapy is efficient against established melanoma. *A,* therapeutic setting of VCT treatment is shown as a scheme. *B* and *C,* B16OVA tumor growth was monitored in WT mice receiving single-agent therapy (*V, C*, or *T*), two agent–based therapy (*VC, VT*, or *CT*), or sequential tritherapy (*VCT*). *Points,* mean of tumor size from one representative experiment out of five (n = 5 mice per group); *bars,* SEM (*B*). The survival curve shows 35 mice in each group (*C*). *D,* tumor growth curve in *nu/nu* (*left*) or TRIF$^{-/-}$ (*right*) C57Bl/6 mice treated with or without VCT.

footpad opposite to the flank where the tumor was growing, stimulated an OVA-specific Th1 immune response in the DLN (Supplementary Fig. S3). It is noteworthy that B16-OVA did not express TLR9 and did not respond to CpG ODN *in vitro* (data not shown). The antitumor effects of the sequential administration of a vaccine followed by oxaliplatin and TLR3L was well reproducible in immunocompetent wild-type (WT) C57BL/6 mice, yet failed to be observed in *nu/nu* and *Trif*$^{-/-}$ mice (Fig. 1*D*), indicating the obligate contribution of T cells and TRIF-dependent signals to the therapeutic effect. Altogether, 11% of WT mice were completely protected from melanoma by the sequential therapeutic regimen (Fig. 1*C*), and 67% among the tumor-free mice developed long-term protective immunity and hence became resistant to a later challenge with live tumor cells (data not shown).

We observed a similar antitumor effect when chemotherapy and poly(A:U) injections were combined with a cell-based anticancer vaccine. The freeze-thawing technique aimed at mediating the nonimmunogenic cell death (necrosis) in contrast to anthracycline-induced tumor cell death that generates an endoplasmic reticulum stress response (23). In accordance with our previous reports, type I IFN and doxorubicin induced immunogenic cell death of B16-OVA cells and injection of dying cells induced a protective immunity against later rechallenge with live B16-OVA cells (Fig. 2*A* and *B*). This cell-based vaccine boosted the antitumor activity of the combination of oxaliplatin plus poly(A:U) (Fig. 2*C*) and enhanced survival (Fig. 2*D*) when used in a therapeutic setting after the implantation of tumors. Very similar results were obtained when B16-OVA melanoma cells were replaced by another TLR3-expressing cell line, GL26 glioblastoma (Supplementary Fig. S1), which only bears natural tumor antigens. Vaccination of immunocompetent mice with GL26 cells that were dying in response to type I IFN and doxorubicin was efficient in preventing tumor outgrowth in the prophylactic setting (Fig. 3*A*) and also in the therapeutic setting only if the vaccination was combined with oxaliplatin and TLR3L following a regimen identical to that presented in Fig. 1*A* (Fig. 3*B*). To further show the importance of the TLR3 agonist on tumor parenchyma during vaccine+chemotherapy +TLR3L (VCT) therapy, we selectively knocked down the TRIF adaptor molecule in GL26 glioblastoma (Lamin as a negative control). Interestingly, VCT therapy failed to control the tumor outgrowth of TRIF knockdown GL26 *in vivo* (Fig. 3*C*).

Altogether, it seems that poly(A:U) could mediate synergistic antitumor effects with chemotherapy against established TLR3-expressing tumors, provided that this combined therapy was preceded by anticancer vaccination. For the sake of brevity, we will refer to this therapeutic schedule as "immunochemotherapy."

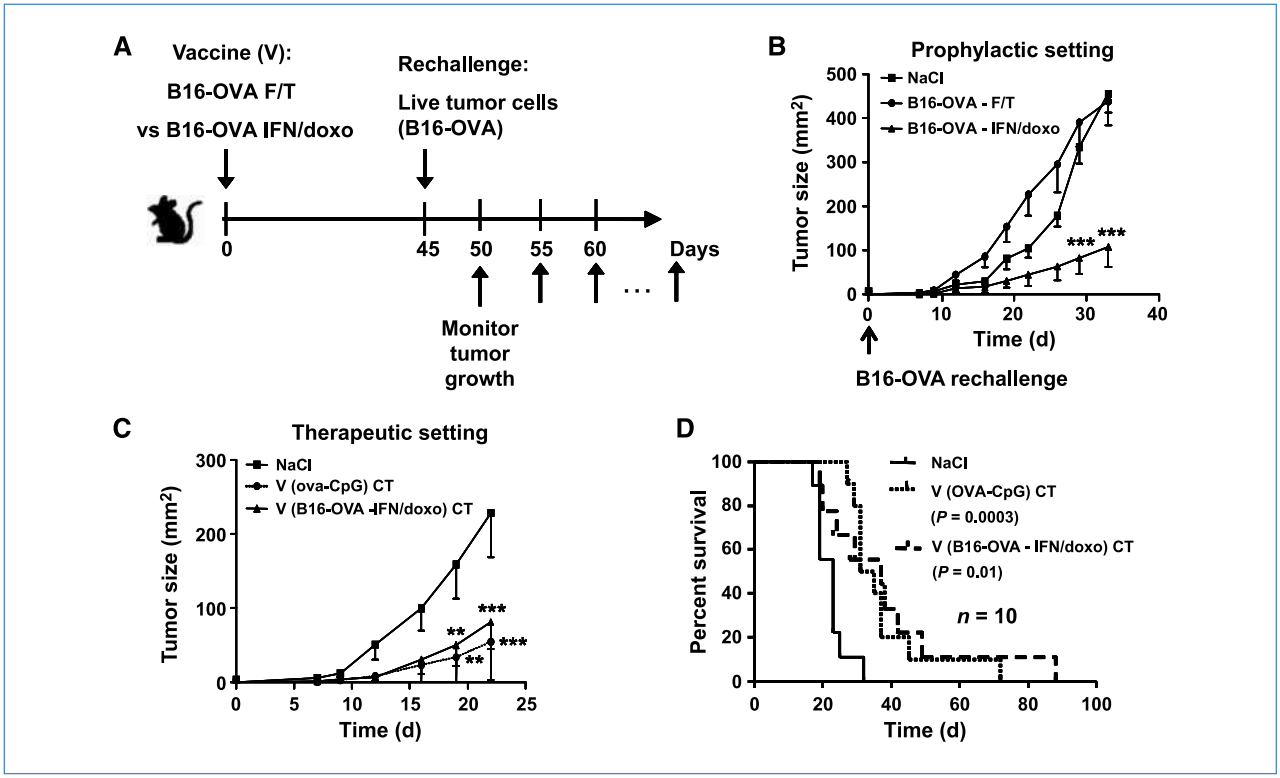

Figure 2. Immunochemotherapy of melanoma with cell-based vaccines inhibits tumor outgrowth. *A,* prophylactic setting in a schematic view. Naïve C57b/6 mice were vaccinated with B16-OVA pretreated with type I IFN plus doxorubicin (*doxo*) or freeze-thawed (*F/T*). Forty-five days later, mice were rechallenged with live syngeneic tumor cells. *B,* tumor growth is depicted with five mice per group following prophylactic setting. *C* and *D,* the therapeutic regimen depicted in Fig. 1*A* was performed with two different vaccines, OVA-CpG or the cell-based vaccine (same as in *A*), and tumor growth was monitored (*C*). Survival curve with 10 mice per group; the *P* value indicates the comparison between each treated and control group (*D*).

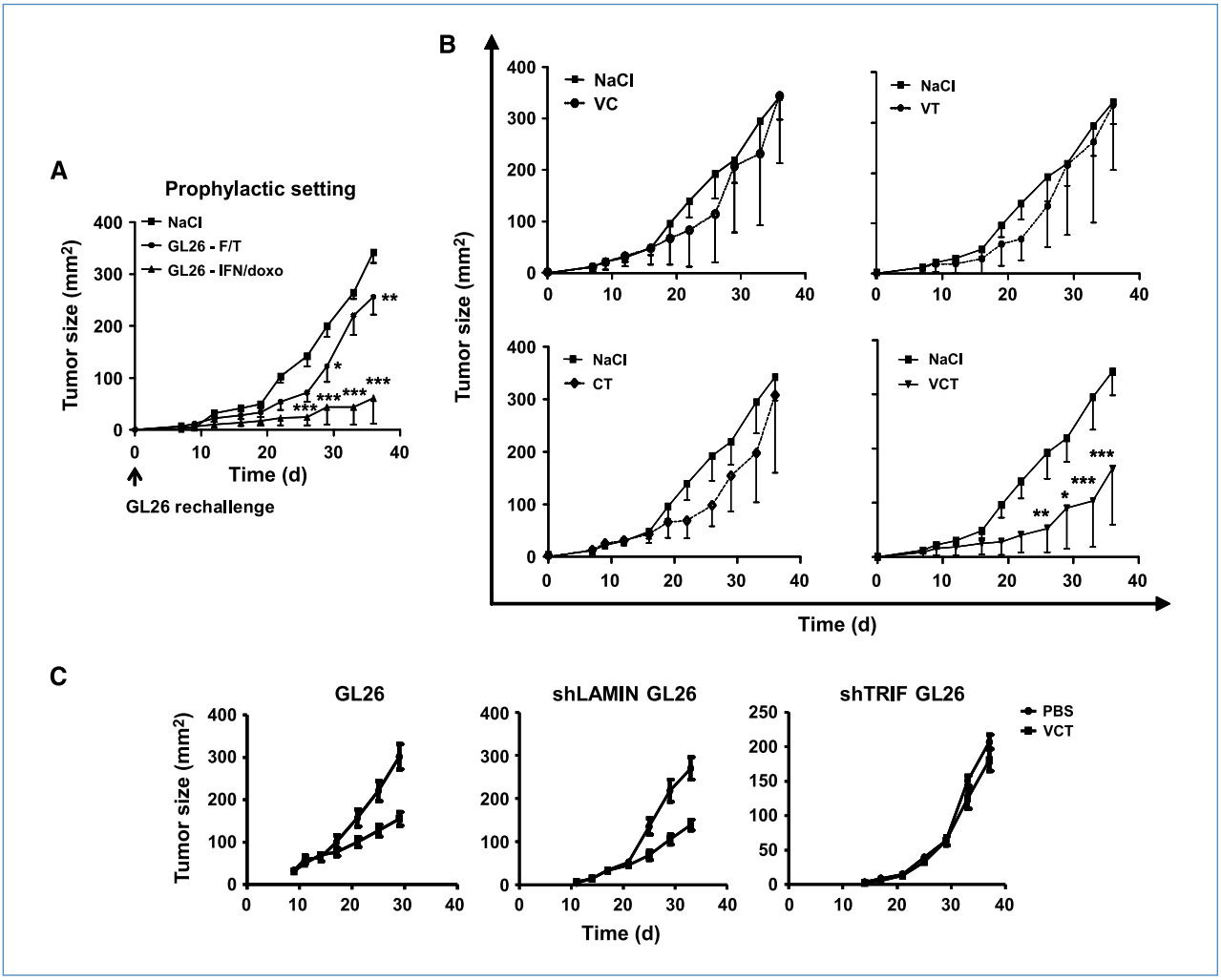

Figure 3. Immunochemotherapy is efficient against established glioblastoma. *A,* naïve C57bl/6 mice were vaccinated with GL26 tumor cells pretreated with type I IFN plus doxorubicin and rechallenged with live syngeneic tumor cells 7 d later. The kinetics of tumor outgrowth are monitored. *B,* the cell-based vaccine was then assessed for its therapeutic efficacy in the VCT setting outlined in Fig. 1*A*. *C,* after knockdown TRIF expression in GL26 (Lamin as a control), established tumors were treated with the VCT protocol starting from day 15. Tumor growth kinetics are shown from one representative experiment with five mice per group.

TLR3-expressing tumors directly responded to poly(A:U). The finding that TRIF must be intact both in the host's immune system and the tumor parenchyma for full antitumor effects (Figs. 1*D* and 3*C*) suggested that poly(A:U) might exert direct effects on the tumor parenchyma. When added to B16-OVA cells *in vitro*, poly(A:U) induced the secretion of copious amounts of both CCL5/RANTES and CXCL10/IP-10. This effect could be further enhanced by preincubation with type I IFN (Fig. 4*A*). Type I IFN plus poly(A:U) showed an additive effect on CCL5 secretion by both GL26 (Fig. 4*B*) and human breast cancer cells (in three of four primary cultures; Supplementary Fig. S4). GL26 cells also secreted more CXCL10 when treated with type I IFN plus poly(A:U) compared with either treatment alone (Fig. 4*B*). TRIF knockdown GL26 cells lost their response to poly(A:U) stimulation, whereas Lamin knockdown GL26 behaved like parental cells (Supplementary Fig. S5). Interestingly, the secretion of CXCL1 by B16-OVA was abolished by poly(A:U) (Fig. 4*A*).

To validate these findings *in vivo*, we studied the concentration of CCL3/MIP-1α, CCL5, and CXCL10 within tumor beds at each single step of the tritherapy in B16-OVA model. We observed a significant production of CCL5 at baseline before chemotherapy. This CCL5 production dropped after the first TLR3L injection but increased again after the third injection of poly(A:U) (Fig. 4*C, top left*), whereas no CCL3 was produced (data not shown). In accordance with *in vitro* data, the tissular concentration of CXCL10 paralleled that of CCL5 *in vivo* after oxaliplatin injection and the third injection of poly(A:U) (Fig. 4*C, bottom right*). To further dissect whether chemokine production originated from leukocytes or tumor cells, we sorted $CD45^+$ versus $CD45^-$ cells from dissociated tumor beds after each poly(A:U) injection and observed that

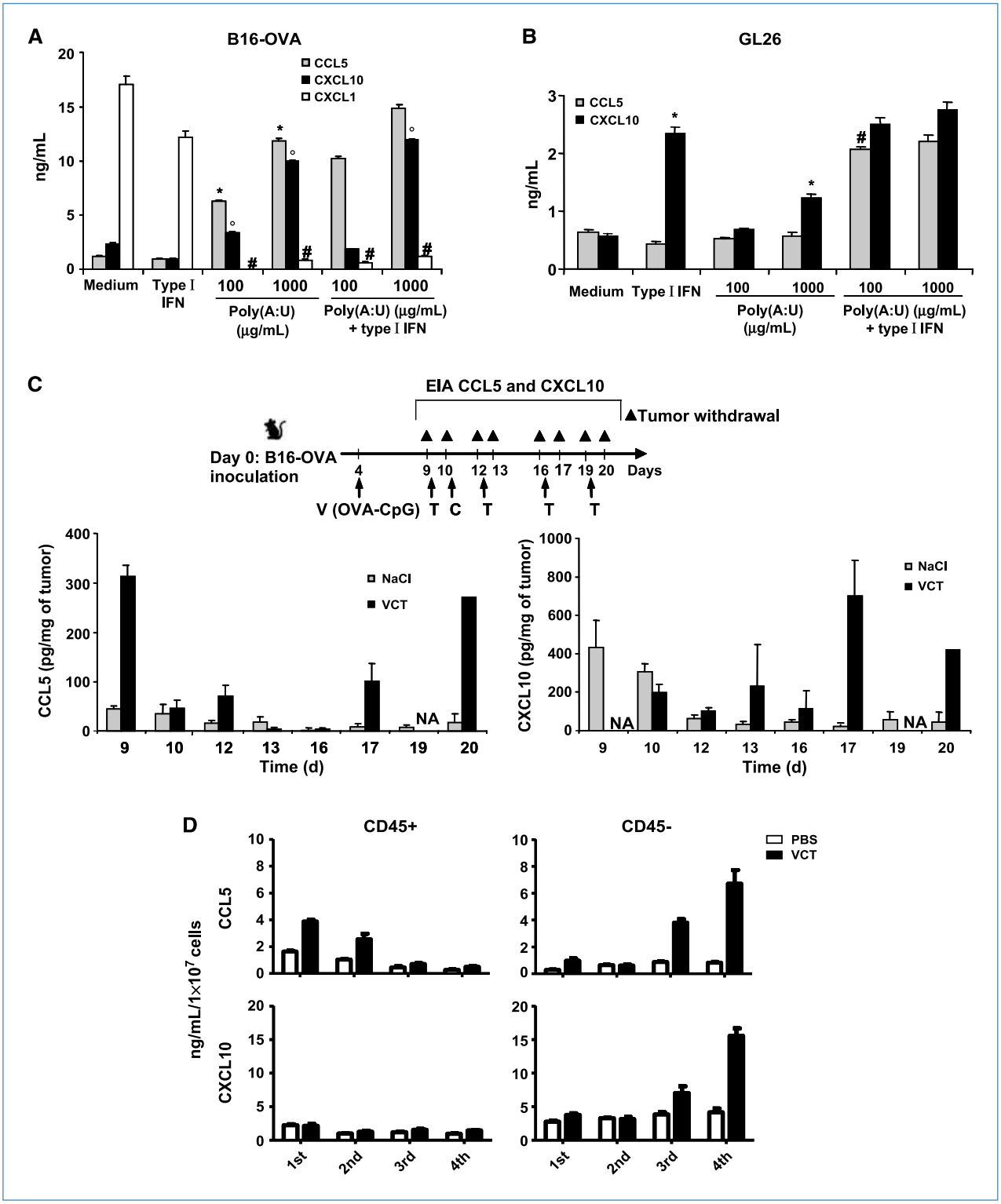

Figure 4. CXCL10 and CCL5 release upon stimulation with poly(A:U). B16-OVA (*A*) and GL26 (*B*) were treated with type I IFN and poly(A:U) and the supernatants were harvested to dose the chemokine secretion. *Columns,* mean of two triplicated experiments (#, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$); *bars,* SEM. Established B16-OVA tumors from the NaCl and VCT groups were harvested at various time points and either were dissociated to measure their contents of CCL5 and CXCL10 (*NA,* not available due to limited tumor size; *C*) or cell sorted after tumor dissociation on the basis of CD45 staining to monitor their chemokine content 36 h after each poly(A:U) injection (*D*). *Columns,* means of chemokine per milligram of tumor (*C*) or per milliliter per 1×10^7 cells (*D*); *bars,* SEM.

the accumulating source of chemokines resided in the tumor parenchyma (Fig. 4*D*).

Altogether, these results indicate that poly(A:U) can directly act on tumor cells to stimulate the production of chemokines, both *in vitro* and *in vivo*.

Deleterious role of CCL5 and CCR5. TLR3 stimulation can trigger the release of a variety of chemokines, including CCL5 (24, 25), as confirmed for the tumors studied in this article, whereas the role of CCR5 (CCL5 receptor) in cancer remains controversial. CCR5 expression in tumor epithelia has been associated with tumorigenesis (26) although some cancer immunotherapies require a functional CCR5 pathway (5, 27, 28). Therefore, we investigated the impact of CCR5 on the synergistic effects of our immunochemotherapy. Surprisingly, the tritherapy was more efficacious when it was applied to *Ccr5*$^{-/-}$ mice rather than to WT mice (Fig. 5*A*

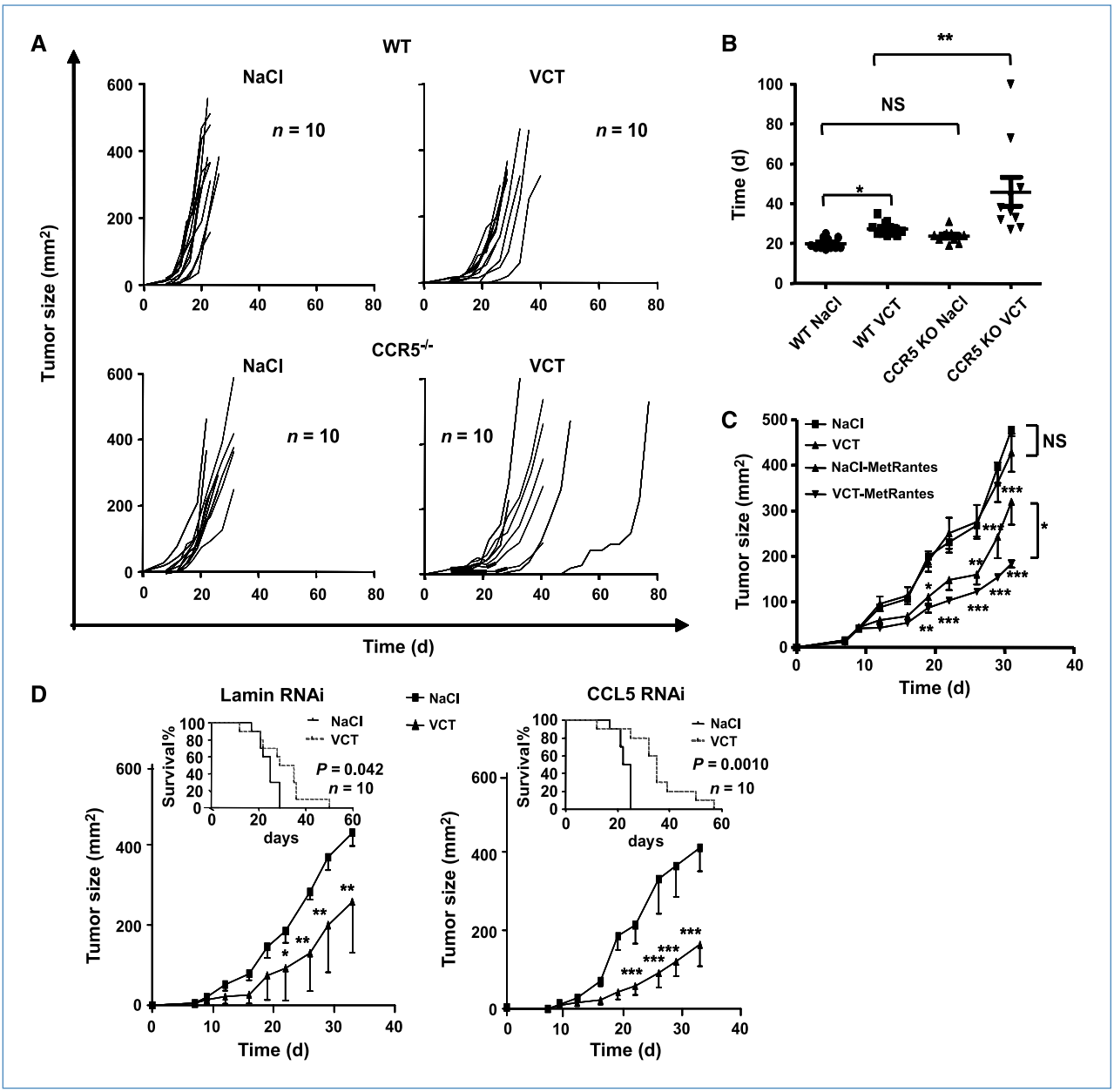

Figure 5. CCR5 signaling antagonized the efficacy of immunochemotherapy. B16-OVA tumor growth was compared in WT versus *Ccr5*$^{-/-}$ mice with or without VCT treatment. Each curve features one single animal (*A*); *NS,* not significant. The time needed for tumors to reach the size of 200 mm^2 was shown for each group (*B*). *C,* 0.6 × 10^6 B16-OVA were inoculated and VCT was performed along with daily administration of MetRantes for 3 wk. *D,* the efficacy of VCT was compared between CCL5 and Lamin knockdown B16-OVA. All experiments were conducted with five mice per group at least twice, yielding identical results.

and *B*). We corroborated these data using a pharmacologic inhibitor recombinant MetRantes that could inhibit agonist-induced activities (29). MetRantes significantly improved tumor growth retardation caused by the immunochemotherapy in the B16-OVA model (Fig. 5*C*). This result was also confirmed in the GL26 glioblastoma (data not shown).

To further show that the source of the deleterious CCL5 was indeed the tumor cells stimulated by poly(A:U) during our sequential therapy, we carried out CCL5 knockdown in B16-OVA by lentiviruses carrying a shRNA-targeting CCL5 (Lamin as a control). This infection induced a significant suppression of the poly(A:U)-induced CCL5 production *in vitro* (Supplementary Fig. S6). The tritherapy mediated enhanced antitumor activity and long-term survival against established B16-OVA–shRNA CCL5 compared with established B16-OVA–shRNA Lamin control (Fig. 5*D*), whereas the spontaneous growth of each transfectant was comparable *in vitro* (data not shown).

Altogether, these results support the idea that the interaction between CCL5 that originated from tumors and CCR5 that was expressed in the host-derived immune effector has a negative impact on the outcome of immunochemotherapy.

CXCR3 as a positive mediator of immunochemotherapy. The OVA-CpG vaccine, which elicited potent IFN-γ–polarized T-cell responses in WT mice (Supplementary Fig. S3), failed to promote the tumoricidal activity when combined with chemotherapy and TLR3L in *nu/nu* C57BL/6 mice (Fig. 1*D*), suggesting that IFN-γ–producing T lymphocytes are required for the antitumor effects. Knowing that IFN-γ–polarized T cells express CXCR3 (30) and TLR3L promotes CXCL10 secretion (a CXCR3 ligand) by tumor cells (31), we compared the efficacy of the immunochemotherapy in WT versus *Cxcr3*$^{-/-}$ mice carrying B16-OVA tumors. In contrast to WT littermate controls, in which immunochemotherapy yielded a significant delay in tumor growth, no beneficial effect was observed for the control of tumors growing in *Cxcr3*$^{-/-}$ mice (Fig. 6*A*). Therefore, the chemokine receptor CXCR3, which is widely expressed in NK cells and activated Th1 and CTLs, is mandatory for the therapeutic success of the combined therapy. Accordingly, functional immunophenotyping revealed that immunochemotherapy induced augmented recruitment of CD8$^+$ CXCR3$^+$ T lymphocytes in the vaccine DLN but not in the tumor DLN (data not shown). These lymphocytes were able to produce IFN-γ upon restimulation with OVA (Supplementary Fig. S3; Fig. 6*B*). NK cells did not express CXCR3 in these settings (data not shown). Importantly, the percentage of CD8$^+$ CXCR3$^+$ T cells increased among tumor-infiltrating lymphocytes (TIL) after immunochemotherapy (Fig. 6*B*), supporting the notion that this T-cell subset contributes to the anticancer efficacy of immunochemotherapy.

Next, we incubated B16-OVA with type I IFN and poly(A:U) (which both mediated cytostatic effects on B16-OVA *in vitro* as shown in Supplementary Fig. S2) and inoculated these tumor cells into WT animals. This pretreatment reduced the minimal tumorigenic dose (the number of cells that had to be inoculated to generate a tumor; Fig. 6*C*). This gain of tumorigenicity was lost when the animals were immunized with the OVA-CpG vaccine (Fig. 6*D*), indicating that the direct effect of poly(A:U) stimulation of the tumor cells is beneficial only when the host has been immunized (when specific CTL against tumoral antigen are present within the host). The beneficial effect of prophylactic immunization with OVA-CpG was abrogated if the tumor cells were injected together with an anti-CXCR3 neutralizing antibody (Fig. 6*D*). Altogether, these results underscore the importance of the chemokine receptor CXCR3 for allowing immune effectors to control tumor growth *in vivo*.

Discussion

Although TLR agonists may contribute to the activation of anticancer responses, they may also directly increase the tumorigenic potential of TLR-expressing tumor cells (3, 15). The aim of this study was to weigh the relative impact of individual components of the chemokine cascade resulting from chronic stimulation of the tumor epithelium with the TLR3L *in vivo*. Our findings revealed that poly(A:U) triggers the concomitant secretion of both CCL5 and CXCL10 from TLR3-expressing tumor *in vitro* and *in vivo* (Fig. 4), and interfering with CCR5 engagement on host hematopoietic cells enhanced the efficacy of an immunogenic treatment that stimulated a T-cell– and CXCR3-dependent anticancer immune response (Figs. 1*D* and 6*A* and *D*). These results suggest that the optimization of anticancer therapies relying on TLR adjuvants may require uncoupling of the chemokine cascade.

It is known that systemic administration of poly(A:U) can exert immunoadjuvant effects through TLR3 and TLR7 (32). Although both TLR3 and TLR7 were required for the clonal expansion of antigen-specific CD8$^+$ T cells, only TLR3 was mandatory to generate IFN-γ–producing CD8$^+$ T cells (32). Our biweekly administration of poly(A:U) was not able to trigger potent immunoadjuvant effects when poly(A:U) was given alone. However, combined with vaccines and chemotherapy, poly(A:U) triggers an efficient T-cell–dependent and TRIF-dependent antitumor response. TRIF signaling leads to type I IFN production by host allophycocyanin, which might directly act on tumor cells to upregulate TLR3 expression (33, 34) and/or synergize with TLR3 to stimulate the release of chemokines (Fig. 4). Of note, we could measure increased levels of CXCL10 and CCL5 in tumor beds only after three systemic administrations of poly(A:U), supporting that host factors (such as type I IFN) may cooperate with poly(A:U) to stimulate the induction of chemokines by tumor cells.

As shown by other groups (35), combinations of specific tumor vaccines with chemotherapy may significantly ameliorate progression-free survival. Surprisingly, although two different vaccines could elicit prophylactic antitumor effects (Figs. 2*B* and 3*A*) and IFN-γ–producing T cells on their own (Supplementary Fig. S3 or data not shown), we could not achieve significant synergistic effects by associating such vaccines with taxanes or oxaliplatin for the treatment of melanoma (Fig. 1 and data not shown). One possible explanation for this absence of synergy might be the failure of tumor beds to produce chemokines that attract polarized effector CD8$^+$

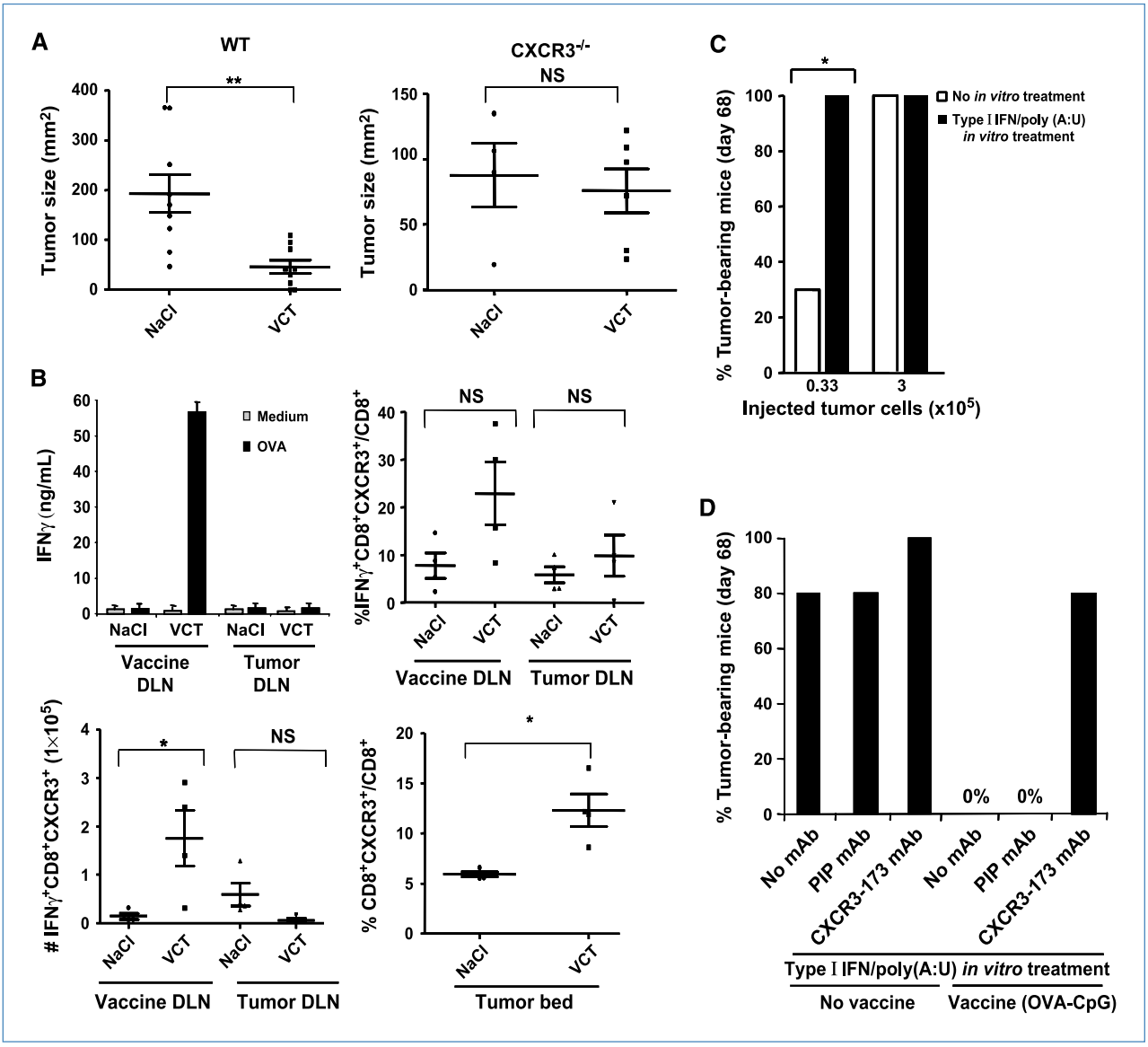

Figure 6. CXCR3-dependent antitumor effects mediated by VCT therapy associated with CXCR3$^+$ TILs. *A,* the mean tumor size at day 19 in VCT or control group is compared between *Cxcr3*$^{-/-}$ versus WT mice. *B,* DLNs from the vaccine site or the contralateral site were collected at day 13 from VCT or control group. Cells were restimulated with OVA protein (or PBS) either for 48 h to monitor the OVA-specific IFN-γ production in the supernatants by ELISA (*top left*) or for 12 h before intracellular stainings showing IFN-γ production by CD8$^+$CXCR3$^+$ T cells (*percentages* and *absolute numbers*). Tumors from NaCl versus VCT-treated mice were dissociated at day 16 and analyzed for the percentage of CXCR3$^+$ cells among CD8$^+$ cells (*bottom right*). *C,* B16-OVA cells were pretreated with type I IFN followed by poly(A:U) and the minimal tumorigenic dose of B16-OVA cells was determined. The percentages of tumor-bearing mice at day 68 are depicted. *D,* mice were preimmunized with OVA-CpG and challenged 7 d later with the minimal tumorigenic dose of B16-OVA tumor cells (0.33 × 10^5) presensitized with poly(A:U) and type I IFN. CXCR3-173 neutralizing mAb or control PIP mAb were applied. The graph depicts the percentages of tumor-bearing mice at day 68 in one of two experiments.

T cells (Fig. 4*C*). Indeed, some reports (36, 37) supported the notion that intratumoral chemokines (such as lymphotactin/XCL1 or CXCL10) could enhance the trafficking of effector T cell to tumors and ameliorate the anticancer efficacy of adoptively transferred T lymphocytes.

Although highly activated CD8$^+$ T cells can coexist with autoantigen-expressing hepatocytes without causing overt tissue damage (38), engagement of TLR3 could break this immunoprivileged state by triggering IFN-γ and tumor necrosis factor-α–dependent CXCL9 expression in the liver and by recruiting CXCR3$^+$ autoreactive CTLs (38). Indeed, a TLR3 agonist could induce the VLA-4–dependent homing of specific CTL into central nervous system tumors (39). Accordingly, several reports described that TLR3 signaling in astrocytes or glioma induced multiple proinflammatory cytokines and chemokines, including IP-10, IL-8, or GROα (39, 40). However, the theory that TLR3 agonists augment trafficking of CTL into tumor beds has been challenged by a recent report

showing that injections of double-stranded RNA [poly(I:C)] into mesotheliomas did not stimulate the recruitment of newly primed antitumor T cells and rather reactivated local $CD8^+$ T cells in a type I IFN–dependent manner (41). However, it has not been clarified whether mesothelioma cells express TLR3 and it remains formally possible that poly(I:C) may activate TLR3-independent signaling pathways that improve clinical outcome by alternative mechanisms of action.

Secretion of CC chemokines is a major determinant for chemoattraction of macrophages, neutrophils, and lymphocytes into tumor beds in human carcinogenesis (42). In breast cancer for instance, mesenchymal stem cells produce CCL5, which enhances the metastatic potential of tumors and correlates with disease progression (43, 44). Moreover, tumor-infiltrating leukocytes may express high levels of the CCL5 receptors CCR1 and CCR5 (45). Injection of a CCL5 antagonist can reduce the migration of macrophages to tumor beds and facilitate tumor regression (45). In WT animals, CXCR3 expression in tumor-specific IFN-γ–producing T cells was enhanced, which facilitates their trafficking to the tumor beds (Fig. 6*B*), whereas in *Ccr5*$^{-/-}$ mice, we failed to observe an exaggerated accumulation of Tc1 cells (data not shown). Although concanavalin A–treated *Ccr5*$^{-/-}$ mice suffered from severe hepatitis related to pronounced recruitment and activation of IFN-γ–producing NK cells into the liver (46), we failed to monitor an enhanced proportion of $CXCR3^+$ NK cells in the tumor or DLNs (data not shown). It remains conceivable that CXCR3 can be downregulated in NK cells upon engagement with local chemokines. Therefore, the beneficial effect of CCR5 inhibition may be most likely related to the disappearance of subsets of immunosuppressive cells rather than to the recruitment or activation of effector IFN-γ–producing $CD8^+$ T cells.

Within the hematopoietic system, CCR5 is expressed in regulatory T cells (47) and myeloid-derived suppressor cells (MDSC; ref. 5), making them potential candidates for immune suppressors. However, we failed to improve the efficacy of the immunochemotherapy either by using metronomic dosages of cyclophosphamide that reduce functionally active regulatory T cells (48), or by administering sildenafil, a phosphodiesterase-5 inhibitor known to downregulate the principal immunosuppressive effectors (arginase-1 and NOS-2) of MDSC (data not shown; ref. 49). These results suggested that Treg and MDSCs may not be the $CCR5^+$-immunosuppressive subsets to be identified.

Our results support two important conclusions. First, TLR3 agonists can promote $TLR3^+$ tumor cells to produce chemokines that accumulate locally to physiologically relevant concentrations. Second, these intratumoral chemokines likewise are not neutral in their clinical significance and need to be uncoupled to boost the efficacy of immunochemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Amanda Proudfoot for kindly providing us with MetRantes.

Grant Support

Innate Pharma, INCa, and Ligue Nationale contre le Cancer (équipes labelisées by L. Zitvogel); Innate Pharma (R. Conforti); and China Scholarship Council (Y. Ma).

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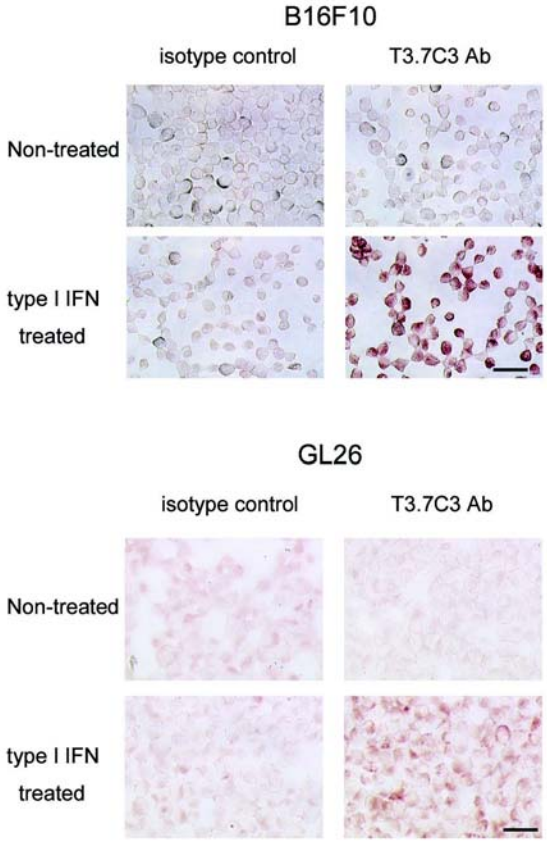
Received 5/24/09; revised 11/15/09; accepted 11/17/09; published OnlineFirst 1/12/10.

References

1. Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. Oncogene 2008;27:161–7.
2. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat Med 2007;13:552–9.
3. Killeen SD, Wang JH, Andrews EJ, Redmond HP. Exploitation of the Toll-like receptor system in cancer: a doubled-edged sword? Br J Cancer 2006;95:247–52.
4. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
5. Umemura N, Saio M, Suwa T, et al. Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol 2008;83:1136–44.
6. Cui Z, Qiu F. Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. Cancer Immunol Immunother 2006;55:1267–79.
7. Schulz O, Diebold SS, Chen M, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;433:887–92.
8. Tabiasco J, Devevre E, Rufer N, et al. Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor. J Immunol 2006;177:8708–13.
9. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–5.
10. Wang L, Smith D, Bot S, Dellamary L, Bloom A, Bot A. Noncoding RNA danger motifs bridge innate and adaptive immunity and are potent adjuvants for vaccination. J Clin Invest 2002;110:1175–84.
11. Bot A, Smith D, Phillips B, Bot S, Bona C, Zaghouani H. Immunologic control of tumors by *in vivo* Fc γ receptor-targeted antigen loading in conjunction with double-stranded RNA-mediated immune modulation. J Immunol 2006;176:1363–74.
12. Lacour J, Lacour F, Spira A, et al. Adjuvant treatment with polyadenylic-polyuridylic acid (Polya.Polyu) in operable breast cancer. Lancet 1980;2:161–4.
13. Laplanche A, Alzieu L, Delozier T, et al. Polyadenylic-polyuridylic acid plus locoregional radiotherapy versus chemotherapy with CMF in operable breast cancer: a 14 year follow-up analysis of a randomized trial of the Federation Nationale des Centres de Lutte contre le Cancer (FNCLCC). Breast Cancer Res Treat 2000;64:189–91.
14. Jeung HC, Moon YW, Rha SY, et al. Phase III trial of adjuvant 5-fluorouracil and Adriamycin versus 5-fluorouracil, Adriamycin, and

polyadenylic-polyuridylic acid (poly A:U) for locally advanced gastric cancer after curative surgery: final results of 15-year follow-up. Ann Oncol 2008;19:520–6.
15. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. Cancer Immunol Immunother 2008;57:1271–8.
16. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452:591–7.
17. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006; 176:4894–901.
18. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin Cancer Res 2007;13:4565–74.
19. Stewart WE II, De Clercq E, Billiau A, Desmyter J, De Somer P. Increased susceptibility of cells treated with interferon to the toxicity of polyriboinosinic-polyribocytidylic acid. Proc Natl Acad Sci U S A 1972;69:1851–4.
20. Huang B, Zhao J, Li H, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer Res 2005;65:5009–14.
21. Huang B, Zhao J, Shen S, et al. *Listeria monocytogenes* promotes tumor growth via tumor cell toll-like receptor 2 signaling. Cancer Res 2007;67:4346–52.
22. Kelly MG, Alvero AB, Chen R, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 2006;66:3859–68.
23. Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007;13: 54–61.
24. Orinska Z, Bulanova E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. Blood 2005;106:978–87.
25. Takahashi N, Yamada T, Narita N, Fujieda S. Double-stranded RNA induces production of RANTES and IL-8 by human nasal fibroblasts. Clin Immunol 2006;118:51–8.
26. Azenshtein E, Luboshits G, Shina S, et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res 2002;62:1093–102.
27. Lavergne E, Combadiere C, Iga M, et al. Intratumoral CC chemokine ligand 5 overexpression delays tumor growth and increases tumor cell infiltration. J Immunol 2004;173:3755–62.
28. Nakazaki Y, Hase H, Inoue H, et al. Serial analysis of gene expression in progressing and regressing mouse tumors implicates the involvement of RANTES and TARC in antitumor immune responses. Mol Ther 2006;14:599–606.
29. Proudfoot AE, Power CA, Hoogewerf AJ, et al. Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. J Biol Chem 1996;271:2599–603.
30. Chen J, Vistica BP, Takase H, et al. A unique pattern of up- and down-regulation of chemokine receptor CXCR3 on inflammation-inducing Th1 cells. Eur J Immunol 2004;34:2885–94.
31. Loos T, Dekeyzer L, Struyf S, et al. TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in autoimmune arthritis. Lab Invest 2006;86:902–16.
32. Sugiyama T, Hoshino K, Saito M, et al. Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. Int Immunol 2008;20:1–9.
33. Hoebe K, Janssen EM, Kim SO, et al. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat Immunol 2003;4:1223–9.
34. Siren J, Pirhonen J, Julkunen I, Matikainen S. IFN-α regulates TLR-dependent gene expression of IFN-α, IFN-β, IL-28, and IL-29. J Immunol 2005;174:1932–7.
35. Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? Cancer Res 2004;64:6827–30.
36. Huang H, Li F, Gordon JR, Xiang J. Synergistic enhancement of antitumor immunity with adoptively transferred tumor-specific CD4+ and CD8+ T cells and intratumoral lymphotactin transgene expression. Cancer Res 2002;62:2043–51.
37. Huang H, Liu Y, Xiang J. Synergistic effect of adoptive T-cell therapy and intratumoral interferon γ-inducible protein-10 transgene expression in treatment of established tumors. Cell Immunol 2002; 217:12–22.
38. Lang KS, Georgiev P, Recher M, et al. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. J Clin Invest 2006;116:2456–63.
39. Zhu X, Nishimura F, Sasaki K, et al. Toll like receptor-3 ligand poly-ICLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. J Transl Med 2007;5:10.
40. Park C, Lee S, Cho IH, et al. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. Glia 2006;53:248–56.
41. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BW. Targeting the effector site with IFN-αβ-inducing TLR ligands reactivates tumor-resident CD8 T cell responses to eradicate established solid tumors. J Immunol 2008;180:1535–44.
42. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.
43. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449: 557–63.
44. Luboshits G, Shina S, Kaplan O, et al. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res 1999; 59:4681–7.
45. Robinson SC, Scott KA, Wilson JL, Thompson RG, Proudfoot AE, Balkwill FR. A chemokine receptor antagonist inhibits experimental breast tumor growth. Cancer Res 2003;63:8360–5.
46. Ajuebor MN, Wondimu Z, Hogaboam CM, Le T, Proudfoot AE, Swain MG. CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. Am J Pathol 2007;170:1975–88.
47. Moreira AP, Cavassani KA, Massafera Tristao FS, et al. CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression. J Immunol 2008;180:3049–56.
48. Taieb J, Chaput N, Schartz N, et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 2006;176:2722–9.
49. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 2006;203:2691–702.


B16F10
isotype control
T3.7C3 Ab
Non-treated
type I IFN
treated
GL26
isotype control
T3.7C3 Ab
Non-treated
type I IFN
treated

upplemental Figure 2

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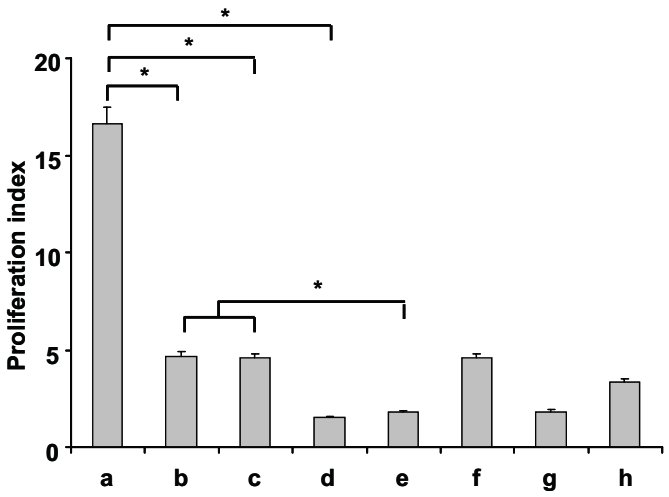
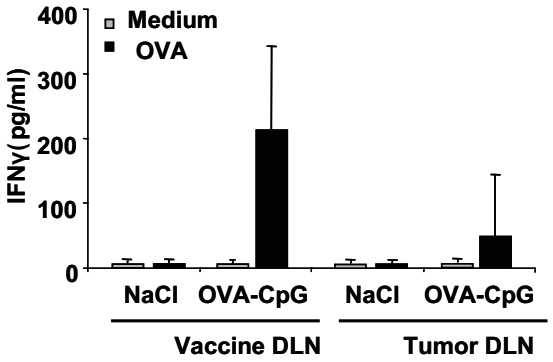
SEQUENTIAL TREATMENT	1st incubation (18hrs)	2nd incubation (48hrs)	
	medium	medium	a
	type I IFN	medium	b
	medium	poly(A:U)	c
	medium	oxaliplatin for the last 24hrs	d
	type I IFN	poly(A:U)	e
	type I IFN	oxaliplatin for the last 24hrs	f
	medium	poly(A:U) + oxaliplatin for the last 24hrs	g
	type I IFN	poly(A:U) + oxaliplatin for the last 24hrs	h



Supplemental Figure 3



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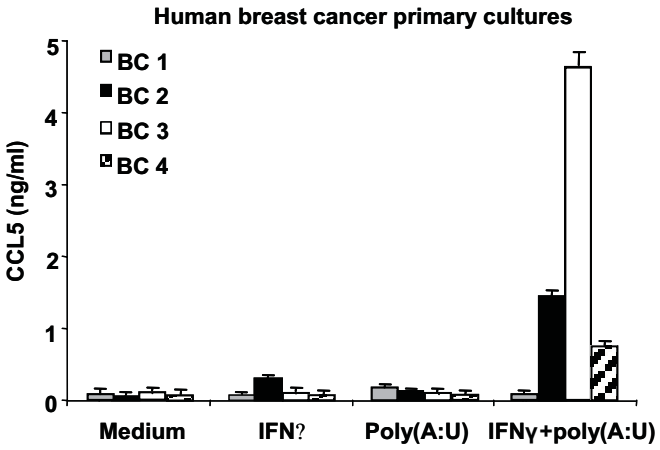
Supplemental Figure 4



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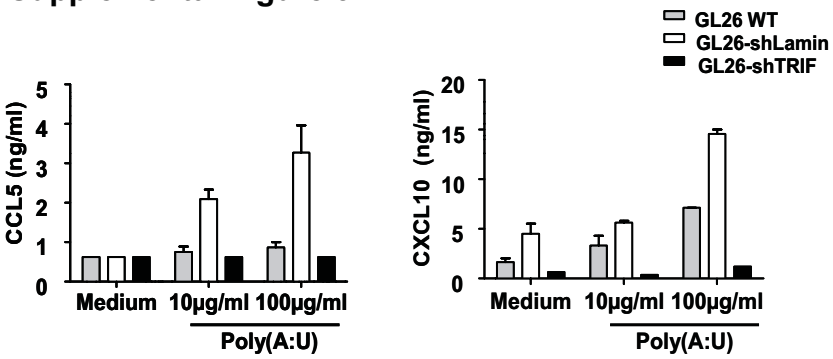
Supplemental Figure 5



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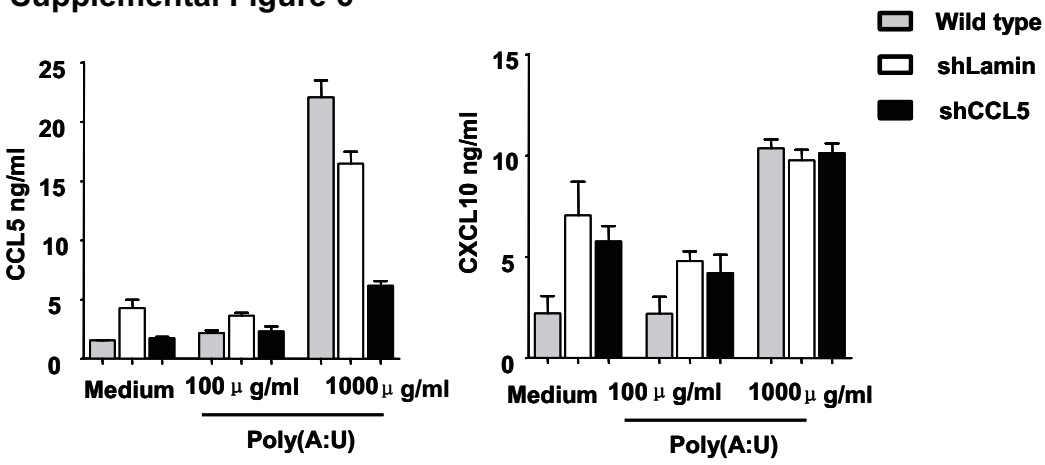
Supplemental Figure 6



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Seminars in Immunology 22 (2010) 113–124

Contents lists available at ScienceDirect

Seminars in Immunology

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Review

Chemotherapy and radiotherapy: Cryptic anticancer vaccines

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ARTICLE INFO

Keywords:
Chemotherapy
Cancer
Inflammasome
Interleukin-1β
Interferon-γ

ABSTRACT

An attractive, yet hitherto unproven concept predicts that the promotion of tumor regression should elicit the host's immune response against residual tumor cells to achieve an optimal therapeutic effect. In a way, chemo- or radiotherapy must trigger "danger signals" emitted from immunogenic cell death and hence elicit "danger associated molecular patterns" to stimulate powerful anticancer immune responses. Here, based on the recent experimental and clinical evidence, we will discuss the molecular identity of the multiple checkpoints that dictate the success of "immunogenic chemotherapy" at the levels of the drug, of the tumor cell and of the host immune system.

1. Introduction

In the 1990s, the genetic engineering of recombinant viral vectors facilitated the emergence of a novel concept of anticancer vaccines that prevailed until recently. Indeed, irradiated genetically modified autologous or allogeneic tumor cells were broadly utilized in preclinical studies and clinical trials to elicit tumor-specific humoral and cellular immune responses that were occasionally associated with tumor regression [1]. To enhance the potency of antitumor immunity, several groups devised strategies to augment the uptake and cross-presentation of dying tumor cells by dendritic cells (DCs). Among these, one of the most successful was that developed by Dranoff and colleagues, who vaccinated with irradiated tumor cells (cell lines or autologous dissociated tumor pieces) that were engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) by means of recombinant retroviruses or adenoviruses. Such dying cells were able to mobilize DC, plasma cells, invariant NKT cells and tumor reactive $CD4^+$ and $CD8^+$ T cells, both in mice and cancer patients, alone or in conjunction with anti-CTLA4 Ab [2]. Importantly, GM-CSF expressing dying tumor cells could promote tumor destruction, necrosis and fibrosis correlating with humoral immune responses and favorable clinical outcome [3,4]. The comprehensive analyses of the specificities recognized by the post-vaccine IgG antibodies revealed key autoantigens involved in cell cycle regulation, cellular stress and oncogenesis [5–9].

Unfortunately, the state-of-the-art GMP conditions required to freeze–thaw irradiated tumor cells before injection into patients in Phase III trials, jeopardizing the immunogenic potential of the vaccine while suggesting that the specific characteristics of dying cells dictate the clinical outcome (Pardoll and Dranoff, personal communications). Indeed, Albert et al. reported that apoptotic cell death could be immunogenic by facilitating antigen cross-presentation by DC, a phenomenon that could be relevant to the pathogenesis of autoimmune paraneoplastic syndromes [10,11]. These findings inaugurated the debate on how cellular death, whether necrotic, apoptotic, autophagic, senescent or associated with mitotic castastrophe, may generate tolerance, ignorance or immunity [12,13] and how this knowledge might be exploited to generate optimal cancer vaccines.

2. Cell death inducers do not always mediate immunosuppression and can synergize with immunomodulators

The effects of anticancer drugs on the immune system have been detailed in previous reviews [14,15]. As a reminder, radia-

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doi:10.1016/j.smim.2010.03.001

tion therapy (either as a single or fractionated dose) can induce tumor-specific Th1 and Tc1 cells in the draining lymph nodes of the irradiated tumor and even favors the trafficking of effector cells into tumors in an IFN-γ-dependent manner [16,17]. Moreover, potent synergistic effects against established tumors between passively transferred CTLs [18], intratumoral DC [19] or TLR9 ligands [20] and ionizing radiation have been reported. A recent translational study performed on colon and prostate cancer patients undergoing radiation therapy (plus or minus chemotherapy) revealed that survivin-specific $CD8^+$ T cell responses, which were already detectable in 50% cases prior to therapy, increased significantly post-therapy in the vast majority of patients. Moreover, in those responding to the therapeutic regimen, higher levels of nuclear (rather than cytoplasmic) survivin were detected in tumor beds [21].

Interestingly, not only cancer cells but also stromal cells can be directly targeted by chemotherapy-induced CTLs [22]. Hans Schreiber and coworkers elegantly showed that in cases of low antigen expression by tumor cells, antigen transfer to stromal cells is mandatory for complete cure. Treating advanced tumors with local chemotherapy or radiotherapy caused the transfer of tumor-specific MHC/peptide complexes to stromal cells, allowing adoptively transferred CTLs to efficiently attack the tumor [22].

The host immune system also contributes to the effects of so-called "targeted" therapies that have been recently added to the oncological armamentarium. In combination with intratumor inoculation of adenoviruses engineered to express IL-12 and 4-1BB, daily administration of the tyrosine kinase inhibitor sunitinib significantly improved long-term survival in mice bearing large tumor burdens while each therapeutic approach alone failed to mediate antitumor efficacy [23].

Finally, classic immunization and chemotherapeutical strategies can synergize. Using DNA-based-vaccination targeting an oncogenic protein involved in tumor maintenance, Chiarle et al. demonstrated that plasmids encoding the cytoplasmic domain of anaplastic lymphoma kinase (ALK) can immunize mice against anaplastic large cell lymphoma (ALCL) in a $CD8^+$ and IFN-γ-dependent manner and cure animals bearing advanced ALCL when combined with doxorubicin [24].

Correale et al. pioneered the field of chemoimmunotherapy in Phase II trials launched in metastatic colon cancer by combining immunogenic chemotherapy (gemcitabine + oxaliplatine) with GM-CSF and IL-2 [25,26] and showed that tumor antigen-specific immune responses and autoimmune side effects can accompany encouraging clinical outcome [27].

These examples illustrate that radiotherapy or chemotherapy can elicit anticancer immune responses or cooperate with tumor vaccines, in line with the notion that conventional anti-neoplastic therapies may be compatible with therapeutically relevant antitumor immune responses.

3. Checkpoints for tumor immunogenicity at the drug level

Some apoptosis-inducing agents or cytotoxic anticancer drugs may directly or indirectly boost the immune system, in three different ways [14,15]. Firstly, some therapeutic regimen can elicit specific cellular responses that render tumor cell death immunogenic. Secondly, some drugs may have off-target effects that stimulate the immune system, for instance by transient lymphodepletion, by the subversion of immunosuppressive mechanisms, or by direct or indirect stimulatory effects on immune effectors. Thirdly, some drugs can sensitize tumor cells to lysis by CTL or NK cells. Here, we will focus our discussion on drugs that elicit T cell responses against tumor cells.

3.1. Systematic screenings

Tanaka et al. examined the biological effects of 54 chemotherapeutic agents on DC functions (maturation and APC function, survival and growth) using a DC biosensor system (DC line XS106 expressing the yellow fluorescent protein under the control of the IL-1β promoter) [28]. This unbiased functional screen unveiled a striking diversity among anticancer drugs. Most topoisomerase inhibitors and antimicrotubule agents promoted DC maturation. In contrast, alkylating agents, antimetabolites, platinum-based compounds and hormonal agents failed to do so. The *Vinca* alkaloid vinblastine was the most efficacious in inducing CD40, CD80, CD86 and MHC class II expression on mouse and human DC and in stimulating the secretion of IL-1β, IL-6 and IL-12p40. At low dosages (0.1–1 μM), vinblastine markedly improved the uptake of FITC-dextran, antigen cross-presentation and allogeneic or tumor antigen-specific T cell responses *in vivo*, specifically in tumor bearing hosts [28,29]. Vinblastine mediated more pronounced antitumor effects against B16 melanoma in immunocompetent mice than in immunocompromised littermates, while the antitumor effects of cisplatin were indistinguishable in both groups [29]. These results suggest that partial or temporal disruption of the intracellular microtubule network may be sensed by DC as an immunostimulatory signal.

Our groups also performed a systematic screening of anticancer compounds for their ability to induce immunogenic cancer cell death. For this study, CT26 colon cancer cells were treated with a panel of chemotherapeutic agents that all induced 70 ± 10% of apoptosis (assessed by staining with and Annexin V). Then, the dying or dead cells were inoculated subcutaneously, in the absence of any adjuvant, into one flank of immunocompetent syngeneic BALB/c mice, which were rechallenged one week later with injection of live CT26 cells in the opposite flank. The absence of tumor growth was then scored as an indication of a productive anticancer immune response. Some 20 different apoptosis-inducing agents that operate through distinct modes of action failed to induce immunogenic cancer cell death. This applied to drugs that kill cancer cells through mitochondria, lysosomal stress, as well as tyrosine kinase inhibitors, proteasome inhibitors or DNA-damaging agents (alkylating agents or topoisomerase inhibition). In sharp contrast, anthracyclines (daunorubicin, idarubicin, mitoxanthrone) were the most potent inducers of immunogenic cell death, not only in CT26 tumors, but also in EL4 thymomas and MCA205 sarcomas [30,31]. Anthracyclines, whose chemical structure is based on samine and tetra-hydro-naphthacene-dione, inhibit DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication of rapidly growing cancer cells. They also create iron-mediated free oxygen radicals that damage the DNA and cell membranes. We found that doxorubicin could elicit immunogenic signals on enucleated tumor cells [31], suggesting that the immunologically relevant changes induced by anthracyclines are cytoplasmic. Indeed, we found that anthracyclines can elicit the rapid (within hours) phosphorylation of the eukaryotic (translation) initiation factor 2α (eIF2α), through the activation of the eIF2α kinase PERK [32] and the dissociation of the eIF2α phosphatase complex composed by PP1 and GADD34 [33]. In general, it appears that chemotherapeutic agents that stimulate eIF2α phosphorylation, which is a sign of endoplasmic reticulum (ER) stress, are particularly efficient in eliciting immunogenic cell death. Based on these considerations, as well as on the molecular identification of immunogenic signals emanating from dying cells, we are currently devising high-throughput screens for the identification of drugs that can induce immunogenic (as opposed to non-immunogenic) cell death (Figs. 1 and 2).

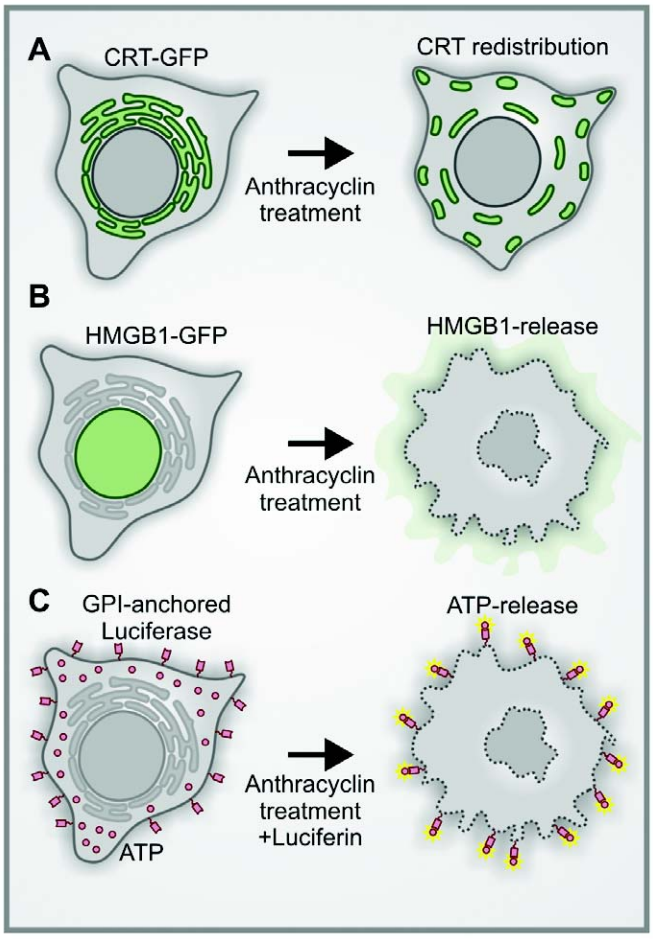

Fig. 1. Biosensor cell lines for the measurement of immunogenic signals. (A) Schematic representation of the redistribution of GFP-CRT fusion protein from a diffuse perinuclear localizaton within the endoplasmic reticulum to a peripheral cytoplasmic puncta. Note that this redistribution of GFP-CRT can occur before cells manifest morphological signs of apoptosis, in which case it predicts immunogenic cell death. (B) Redistribution of GFP-HMGB1 from the nucleus to the cytoplasm and to the extracellular milieu. The release of GFP-HMGB1 occurs when secondary necrosis (necrosis after apoptosis) becomes manifest and the plasma membrane is permeabilized. (C) Release of ATP allowing a luciferase enzyme tethered via a GPI anchor to the plasma membrane to emit photons upon hydrolysis of luciferin.

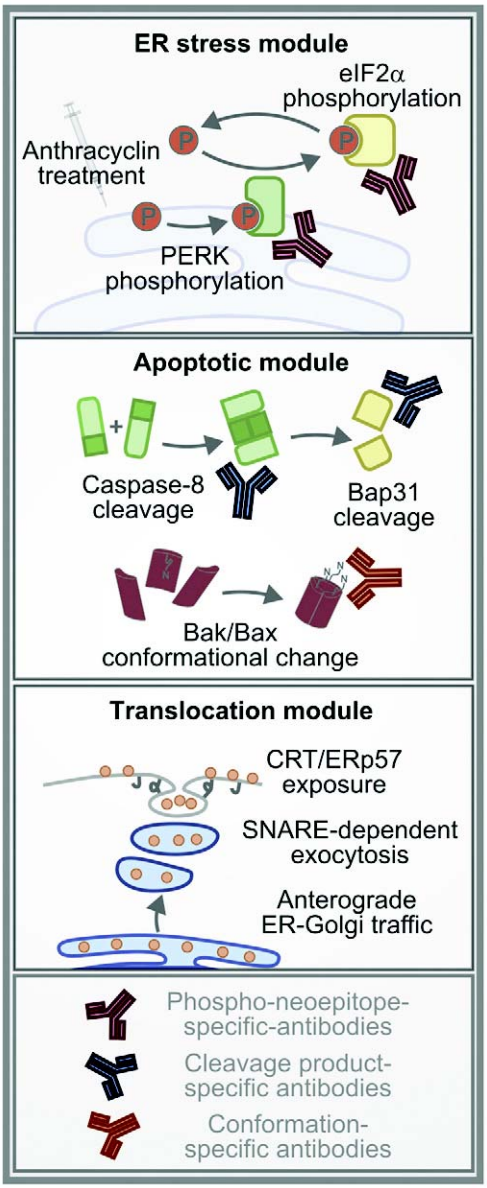

Fig. 2. Biochemical pathways leading to the translocation of calreticulin (CRT) and ERp57 to the plasma membrane. Depicted are key steps of the process that may be detected in chemotherapy-exposed tumor cells. Thus, antibodies that recognize phospho-neoepitopes present on phosphorylated PERK and serine 57-phosphorlated eIF2α, as well antibodies that recognize cleaved caspase-8 and cleaved BAP31 might be used for the immuno(histo)chemical detection of molecular events that predict CRT/ERp57 exposure. Moreover, antibodies specific for the activated conformation of Bax and Bak may be useful for this purpose.

3.2. Metronomic cyclophosphamide

In mice, low (so-called "metronomic") doses of the alkylating agent cyclophosphamide potentiate delayed-type hypersensitivity (DTH) responses by acting on a cyclophosphamide-sensitive suppressor T cell subset [34]. Metronomic cyclophosphamide decreases the number and inhibitory function of $CD4^+CD25^+$ regulatory T (T_{reg}) cells [35,36]. The cyclophosphamide-stimulated IFNα production might account for the augmented antibody responses and the persistence of memory T cells [37,38]. All these effects may contribute to the eradication of immunogenic tumors in synergy with specific immunotherapies [39–41]. Early clinical trials performed on a limited number of patients indicate that the combination of metronomic dosing of cyclophosphamide with vaccines do augment DTH responses [42], decrease the frequency of circulating $CD4^+2H4^+$ (CD45) suppressor T cells [43] and prolong the survival of metastatic cancer patients [44]. This contrasts with recent trials using intravenous 300 mg/m^2/day of cyclophosphamide that failed to reduce the number or functional activity of tumor-induced regulatory T cells [45]. Nonetheless, one daily administration of oral cyclophosphamide for one month to end-stage cancer patients significantly reduced peripheral T_{reg} numbers and inhibited the suppressive action of T_{reg} cells on both T and NK cells [46]. Interestingly, another alkylating agent, dacarbazine, was shown to enhance memory $CD8^+$ T cell responses to peptide vaccines in melanoma patients, suggesting that a diverse array of alkylating agents may mediate immunostimulatory functions [47].

3.3. Gemcitabine

Gemcitabine, a synthetic pyrimidine nucleoside analogue, induced sizeable T cell responses against established hemagglutinin (HA)-transfected AB1 tumors in BALB/c mice [48] and mediates synergistic antitumor effects with a CD40 ligand [49]. Likewise, gemcitabine acts at two levels to mediate tumor immuno-

genicity. Gemcitabine facilitates antigen cross-presentation by dendritic cells and decreases the expansion of tumor-induced myeloid derived suppressor cells in the spleen, leading to the induction of antitumor immunity not only in mice [50], but also in humans [51].

3.4. Ionizing irradiation

Local irradiation of a single tumor site can induce the reduction of non-irradiated metastases located at a distant site, a phenomenon known as "the abscopal effect", which is mediated by the immune system. Low doses of ionizing irradiation modulate the repertoire of tumor-derived peptides [18] upregulate the expression of MHC class I molecules, tumor-associated antigens [52] and CD95/Fas on tumor cells, thereby boosting CTL activity [53], and finally promote T cell trafficking towards irradiated tumor sites [16]. At present, it is not known which among these multiple effects of ionizing irradiation accounts for the abscopal effect.

3.5. Tyrosine kinase inhibitors

Our own studies indicate that the paradigmatic c-kit tyrosine kinase inhibitor imatinib mesylate (IM) boosts IFN-γ secretion by NK cells, both in mice and in GIST patients [54,55]. The effects of IM on cognate immune responses have been reviewed elsewhere [56] stressing that IM does not prevent and even boosts peptide specific vaccines in chronic myeloid leukemia. Other groups investigated the immunological effects of the two novel multi-kinase inhibitors, sorafenib and sunitinib, which both have been successfully introduced in the treatment of patients with renal cell carcinoma (RCC). While sorafenib inhibited the function of DC and markedly reduced antigen-specific T cell responses *in vivo* [57], sunitinib reduced the numbers of circulating T_{reg} [57,58] and myeloid derived suppressor cells, restored the production of IFN-γ by T cells, and downregulated the suppressive microenvironment of tumor beds [23,59]. However, the clinical responses of RCC patients to sunitinib did not correlate with any of the immunological parameters investigated thus far [58]. Therefore, it remains to be determined whether sunitinib might exert part of its therapeutic effect via the immune system.

4. Checkpoints for the immunogenicity of cell death at the tumor level

Chemotherapy might fail because tumor cells do not die in response to the therapeutic insult or because cell death occurs in a non-immunogenic manner, meaning that the immune system is not mobilized by the tumor cell distress. The molecular dialogue between cellular damage and innate effectors, which culminates in tumor antigen-specific cognate T cell responses, is being progressively unraveled.

4.1. Eat-me signal: calreticulin

Obeid et al. demonstrated that anthracyclines, oxaliplatin and ionizing irradiation have the potential to trigger immunogenic cell death by regulating the translocation of an ER resident protein complex (composed of calreticulin (CRT) and the disulfide isomerase ERp57) to the plasma membrane of tumor cells [31,59]. CRT/ERp57 is considered as an eat-me signal that is required for DC to engulf dying tumor cells, thereby eventually inducing T cell-dependent chemotherapeutic effects against tumors [31,60,61]. CRT exposure occurs well before the cells exhibit phosphatidylserine residues, and is abolished by blockade of ER calcium efflux [62] or caspase inhibition [31]. CRT exposure results from an ER stress response that results in the phosphorylation of eIF2α (see above). Downstream of the ER stress response, a subapoptotic event causes partial caspase-8 activation, Bap31 cleavage and conformational changes in Bax and Bak that are usually associated with apoptosis. Next, CRT/ERp57 complexes appear at the cell surface as a result of their SNARE-dependent exocytosis following an anterograde ER-Golgi trafficking of CRT/ERp57-containing vesicles [32]. Therefore, an entirely new class of proteins that have no significant impact on cell death, yet determine whether immunogenic CRT exposure occurs, could influence the clinical outcome of chemotherapy. When ERp57-deficient tumors (which cannot expose CRT at the cell surface) are implanted in mice, they are resistant against anthracycline-based chemotherapy unless exogenous CRT is injected [59]. This contrasts with the cell-autonomous response of ERp57-deficient cancer cells, which respond normally to anthracylines *in vitro*. Hence, the failure to emit immunogenic signals can result in ineffictive chemotherapy responses. We suspect that the expression levels and phosphorylation status of key players of the CRT exposure pathway (such as PERK, eIF2alpa, caspase-8, Bap31 and others) and apoptotic regulation (such as Bcl-2 family proteins and IAP proteins) might ultimately lead to an algorithm that predicts anticancer immune responses elicited by chemotherapy or radiotherapy.

4.2. Don't eat me signal: CD47

CD47 is an Ig-like protein known to functionally interact with integrins and thrombospondin-1. It is also interacting with its receptor SIRP-α on macrophages to negatively regulate phagocytosis [63]. CD47 is constitutively upregulated on mouse and human myeloid leukemias, and overexpression of CD47 favors disease dissemination by evading macrophage-mediated phagocytosis [64]. It has been shown that the pro-phagocytic effects of plasma membrane-exposed CRT are counteracted by the expression of CD47 on the same cell that exposes CRT [65]. Interestingly, cross-linking of CD47 on a chronic lymphocytic leukemia could induce caspase-independent cell death [65,66]. Moreover, an antibody that blocks anti-CD47 was able to elicit macrophage-mediated phagocytosis of non-apoptotic $CD47^{hi}$ human acute lymphoblastic leukemia cells [67]. On theoretical grounds, preventing the interaction between CD47 and SIRP-α, could enforce the phagocytosis of tumor cells by professional APC such as DC. Whether this is the case requires urgent confirmation. Moreover, it remains an open conundrum whether inhibition of CD47 can stimulate an anticancer immune response.

4.3. Anti-inflammatory factor: milk fat globule epidermal growth factor VIII

Through studies performed in GM-CSF-deficient mice, Dranoff and Tahara identified milk fat globule epidermal growth factor VIII (MFG-E8, also called lactadherin) as a critical determinant of the pro- versus anti-inflammatory properties of GM-CSF [68]. GM-CSF induces the secretion of MFG-E8 from resting (that is non TLR-induced) phagocytes. MFG-E8 binds to phosphatidylserine-expressing dying cells, and signals through αvβ3 and αvβ5 integrins to promote the uptake of apoptotic cells and the secretion of TGF-β and CCL22 by myeloid cells, all contributing to the maintenance of $Foxp3^+$ T_{reg}. In addition, MFG-E8 expression and secretion is induced in tumor cells exposed to cytotoxic compounds and represents a potent anti-apoptotic event. Blocking antibodies to MFG-E8 could subserve four independent functions that might explain their marked synergistic anticancer effects when combined with chemotherapy, radiotherapy and molecular targeted compounds [69]. First, anti-MFG-E8 antibodies increase the susceptibility of tumor cells to drug-induced apoptosis [69]. Second, they facilitate the FcγR-mediated uptake of dying cells by DC, thereby

promoting cross-presentation of tumor antigens to $CD4^+$ and $CD8^+$ T cells [69]. Third, they may evoke Th_1 reactivities and enhance tumor infiltration by CTL by virtue of their capacity to suppress the accumulation of $Foxp3^+$ T_{reg} cells [69]. Fourth, they may interfere with the intrinsic aggressiveness of a variety of tumor cells [70]. As a result, it may be important to monitor the MFG-E8 release from tumor cells before and after anticancer therapy to appreciate the functional outcome of the drug/tumor/host interaction.

4.4. DAMP: HMGB1

High mobility group box 1 protein (HMGB1) is an abundant nuclear protein that is tightly associated with chromatin and acts as a transcription factor, when present in the nucleus, as well as a pro-inflammatory cytokine, when it is released from cells [71]. Damaged and necrotic cells were primarily shown to release HMGB1 into the extracellular milieu, where it triggers an inflammatory response to necrosis. Necrotic fibroblasts derived from *hmgb1*$^{-/-}$ mice failed to induce the maturation of DC, in conditions in which necrotic cells from wild type mice were able to trigger DC maturation. Inhibitors of HMGB1 (such as neutralizing Ab or the HMGB1 inhibitory fragment box A) hampered the reactivity of APC to necrotic cells. Moreover, apoptotic lymphoma cells were poorly immunogenic unless they were combined with supernatants from necrotic fibroblasts. The "adjuvanticity" of the necrotic cell-derived supernatants was partially ablated by HMGB1 blockade [72]. RAGE was reported to be the receptor for HMGB1 in these experiments. Recently, several groups reported that apoptotic cells also extrude HMGB1 into the extracellular milieu [73–75], thereby mediating an immunogenic cell death pathway. Indeed, binding of HMGB1 to TLR4 on DC facilitates the processing and presentation of tumor antigens by DC-derived MHC class I molecules. This effect could be attributed to the TLR4-dependent inhibition of the lysosome-dependent degradation of the phagocytic cargo, resulting in improved tumor antigen cross-presentation [75].

In the context of impaired phagocytosis of apoptotic cells (clearance deficiencies), secondary necrosis can occur and HMGB1-nucleosome complexes are released from dead cells. Such HMGB1-nucleosome complexes cause the maturation of macrophages and DC (secretion of IL-1β, IL-6, TNFa, IL-10), thus breaking tolerance to dsDNA in a TLR2-dependent manner [76]. Adding some more complexity, Kazama et al. succeeded in switching tolerogenic into immunogenic cell death by inducing post-transcriptional modifications in HMGB1 using a ROS scavenger. Indeed, in splenocytes undergoing apoptosis, activated caspase-3 and caspase-7 cleave the p75 kDa subunit of the respiratory complex leading to production of ROS which oxidize Cys106 in HMGB1, disabling its potential to activate DC [76]. Hence, the avoidance of HMGB1 oxidation may have immunostimulatory effects. The clinical relevance of HMGB1 and its post-transcriptional changes remain to be established in patients undergoing anticancer chemotherapy.

4.5. DAMP: uric acid

In certain conditions of cell stress, an endogenous adjuvant activity is delivered to the environment of the damaged tissues, influencing the inflammatory and immune outcome. Shi et al. pioneered the field demonstrating that monosodium urate crystals are danger signals that are released by dying mammalian cells and then stimulate DC and promote antigen-specific $CD8^+$ T cell responses [77]. During chemotherapy by bleomycine, a selective inhibitor of DNA synthesis used to treat a variety of human malignancies, oxidative damage and cell death of alveolar macrophages and epithelial cells create acute lung injury culminating in interstitial pulmonary fibrosis. Gasse et al. showed that uric acid is the danger signal activating the Nlrp3 inflammasome leading to IL-1β release and IL-1R1/Myd88-dependent lung fibrosis [78,79]. In spite of these insights, it remains elusive whether uric acid has a positive or negative effect on chemotherapy-induced anticancer immune responses.

4.6. DAMP: HSP70–HSP90

A common adaptive response to cell stress, including that induced by chemotherapy, is the transcriptional activation of a series of molecular chaperones that belong to the class of inducible heat-shock proteins (HSPs). Such HSPs protect against cell death by refolding damaged proteins, by directing damaged proteins to proteasome-mediated degradation and finally by inhibiting apoptosis [80]. HSPs can also stimulate the immune system by acting on the scavenger receptor CD91 on the surface of DCs, thereby transmitting a maturation signal [81] or by chaperoning tumor-specific antigens to MHC class I and II pathways for efficient T cell activation as detailed elsewhere [15,82]. In human myeloma cells treated with the proteasome inhibitor bortezomib [83], HSP90 appears on the surface of tumor cells and serves as a contact-dependent activation signal for autologous DCs.

4.7. Pro-inflammatory and find-me signal: ATP

The systematic screening of various anticancer drugs inducing cell death with distinct mechanisms on a variety of cancer cell lines revealed that cell death is accompanied by a reduction of intracellular ATP concentrations and an accumulation of extracellular ATP [84]. Chemotherapy affects ATP levels at the pre-apoptotic level, before and during the entry of cells into the step-wise process leading to apoptosis and secondary necrosis [85]. It remains to be determined whether ATP is passively or actively exocytosed (via vesicular trafficking) from cancer cells undergoing the chemotherapeutic hit and as such, ATP release may indeed represent a checkpoint to the immunogenicity of chemotherapy. Irrespective of these incognita, it appears clear that depletion of ATP from dying cells by inhibition of ATP synthesis or by addition of the ATP-degrading enzyme apyrase abolishes the immunogenicity of cancer cell death [84]. Indeed, ATP released from tumor cells acts on the purinergic P2RX7 receptor present on DC to facilitate anticancer immune responses (see below).

ATP and UTP can also play the role of non-redundant find-me signals that are released by apoptotic cells for their efficient clearance by monocytes/macrophages that express the purinergic receptor P2RY2 [86]. Forced expression of CD39 (NTPDase-1), an ecto-apyrase responsible for the degradation of NTP by immune cells *in vivo* [87] abrogated the chemoattractant activity of apoptotic cells [86], suggesting that tumor cells could control their clearance via the expression of CD39 or other enzymes that degrade ATP [88]. CD39 is overexpressed on some cancer cell types such as melanomas [89], and it will be interesting to correlated the expression of CD39 (and other ATP-degrading enzymes) with anticancer immune responses elicited by chemotherapy or radiotherapy.

4.8. Pro-tolerogenic factors: Gas6

The receptor tyrosine kinase Mer is involved in the phagocytosis of apoptotic cells by certain macrophage subpopulations [90]. The role of Mer in the immunoregulation of TLR signaling [91] and in the apoptosis-induced inactivation of $CD11c^+CD8\alpha^+$ dendritic cells has been established [92]. Mutant mice lacking the three receptor tyrosine kinases TAM (Tyro3, Axl, Mer) show defective clearance of apoptotic bodies and develop severe lymphoproliferative disorders accompanied by broad spectrum autoimmunity [93]. The growth arrest specific gene 6 (Gas6) detectable on the surface of dying cells is a phosphatidylserine opsonin and a ligand for Mer (and Axl).

Blocking Gas6 prevents the inhibitory effects of apoptotic cells on $CD11c^+CD8\alpha^+$ DC, restoring their activation and T cell stimulatory activity [92]. Axl/Gas6 signaling has been shown to regulate survival, proliferation and migration of a variety of tumor cell lines of epithelial, mensenchymal and hematopoietic origin, to be inducible by chemotherapy and to confer drug resistance [94,95]. Overexpression of Axl/Gas6 in renal cell carcinoma or glioblastoma or leukemia correlated with poor prognosis [96–98]. It remains to be established whether the TAM/Gas6 interaction affects clinical outcome through a tumor cell-autonomous pathway or rather through an effect on the dialogue between tumor cells and phagocytes.

5. Checkpoints for the immunogenicity of cell death at the host level

As outlined above, chemotherapy may fail because it is intrinsically unable to stimulate immunogenic cell death or because cells fail to emit the appropriate set of immunogenic signals as they die. In addition, chemotherapy may fail because the immune system is unable to perceive immunogenic signals or because it has been subjected to local or systemic immunosuppression.

5.1. The role of DC and T cells

If the host immune system plays a role in the antitumor effects mediated by cytotoxic agents, then, certain defects in genes encoding immune functions should subvert the clinical efficacy of these anticancer compounds. The first *in vivo* studies performed in 1973, comparing immunocompetent versus compromised mice, indicated that part of the antitumor activity of anthracyclines could be attributed to the host's immune system [99]. These findings were corroborated in various experimental models, and anthracyclines were shown to enhance innate and cognate immune functions *in vivo* [100,101]. Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells [30,102]. However, for historical reasons, drug discovery programs for cancer therapy have overlooked the possibility that immune reactions might contribute to the success of treatment. Indeed, in 1976, the National Cancer Institute (NCI) edited guidelines for drug screening, prompting investigators to validate their strategy using human tumor cells xenotransplanted into immunodeficient mice [103].

Recently, we discovered that the oxaliplatin-mediated tumoricidal activity against EL-4 was completely abolished in mice deficient for the recombination activating protein 2 (Rag2, which lack both B and T cells), in athymic *nu*/*nu* mice (which lack T cells), and in wild type mice depleted from $CD8^+$ lymphocytes [84]. Similarly, the antitumor efficacy of 10 Gy-irradiation against the breast cancer TS/A was severely compromised in *nu*/*nu* mice [75]. Neutralizing antibodies directed against anti-$CD4^+$ and $CD8^+$ lymphocytes also abrogated the immune response against dying tumor cells [30]. Using CD11c-DTR transgenic mice in which diptheria toxin depletes conventional DC, it was found that dendritic cells mobilized by doxorubicin-treated tumor cells are indispensable to elicit CTL responses that protect mice against rechallenge with live tumor cells [30]. Accordingly, tumor antigens derived from doxorubicin or oxaliplatin-treated cells can be cross-presented by host DC to MHC class I-restricted Tc1 lymphocytes [31,84]. Thus, cross-presentation of tumor antigens by DC may be decisive for dying cancer cells to elicit specific immune responses.

Since the mouse $CD8\alpha^+$ DC excels at cross-presenting antigens [104]. Sancho et al. went on studying myeloid C type lectins uniquely expressed on this subset and their role in the immunogenicity of cell death. They showed that mouse $CD8\alpha^+$ DC take advantage of one of their surface molecules, DC/NK lectin group receptor-1 (DNGR-1, also called CLEC9A), to regulate cross-presentation of necrotic cells by signaling via SYK kinase [105]. The CLEC9A receptor handles dying cells resulting from secondary necrosis promoted through UV light, anthracyclines, freeze-thawing, or serum deprivation, but does not function in the phagocytosis of latex particles. Rather, the CLEC9A/SYK pathway may activate DC in response to dead cells, presumably in coordination with other danger receptors because targeting this DC receptor with antigen epitopes covalently coupled to a specific antibody requires adjuvant to elicit T cell priming [104]. Moreover, it is currently unknown whether CLEC9A contributes to anticancer immune responses.

5.2. The coordinated action of TLR4 and P2RX7

The systematic screening of the danger receptors such as Toll-like (TLR) and Nod-like receptors (NLR) revealed a major role for TLR4 and NLRP3 in the immunogenicity and efficacy of chemotherapy or radiotherapy in mice. Mutations in TLR4 that affect receptor signaling markedly decreased the efficacy of conventional anticancer therapies applied to a series of tumors growing on syngenic mice. This applies to X-rays used for the cure of established TS/A mammary cancers, oxaliplatin employed against EL-4 thymoma and GOS osteosarcoma, as well as doxorubicin administered against CT26 colon cancers [75]. Accordingly, dying tumor cells failed to elicit antigen-specific Tc1 immune responses in $TLR4^{-/-}$ mice unless they were loaded onto bone marrow-derived DC bearing a TLR4 WT genotype. These results suggested that TLR4 must function within host DC for optimal efficacy of chemotherapy [75]. TLR4 signaling in DC involved Myd88 (but not TRIF) adaptor molecules and appeared to be critical for the dynamic of the endocytic compartments, the processing of the phagocytic cargo and the presentation of antigens by MHC class I molecules. TLR4 engagement by HMGB1 acted in coordination with the Nlrp3 inflammasome complex to induce the processing and maturation of IL-1β in DC [84]. Indeed, DC loaded with oxaliplatin-treated tumor cells secreted IL-1β in an Nlrp3-, ASC- and caspase-1-dependent fashion, and IL-1β secretion was blocked by neutralizing HMGB1 [84]. Therefore, the efficacy of doxorubicin or oxaliplatin against established tumors (whether transplantable or methycholanthrene-induced) was markedly impaired in animals bearing genetic defects in the Nlrp3 → ASC → Casp-1 → IL-1β → IL-1R1 axis and mice treated with neutralizing anti-IL-1β antibodies (Fig. 3). In contrast, IL-1α and IL-18 did not contribute to the antitumor effects of these therapies [84].

Purinergic receptors P2RX7 expressed on DC were found to be strictly required for the activation of the Nlrp3 inflammasome and for the immune response against dying tumor cells [84]. P2RX7 receptors sense extracellular ATP. Removal of ATP from dying cells, scavenging of ATP by the ATP-degrading enzyme apyrase, or excess amounts of P2X receptor antagonists prevented the priming of tumor antigen-specific T cells by dying tumor cells and the prophylactic effects of vaccines composed of mitoxanthrone or doxorucin-treated CT26 against rechallenge with live cells [84].

Why is IL-1β produced by DC encountering dying tumor cells so critical for the efficacy of chemotherapy? The immune response ensuing in the draining lymph nodes of a tumor, 5–7 days post-exposure to X-rays or local doxorubicin or systemic oxaliplatin, requires tumor antigen-specific $CD8^+$ T cells that produce IFN-γ in an TLR4-, caspase-1- and IL-1R1-dependent manner [84]. However, these IFN-γ producing T cells did not exhibit potent cytolytic activities against tumor cells. Accordingly, we found that signaling through IFN-γR and IFN-γ was mandatory for the efficacy of chemotherapy against a variety of different tumors, while IL-12Rβ2, perforin or TRAIL were dispensable [84]. Finally, IL-1β was shown to be a key cytokine gearing the polarization of TCR-triggered $CD8^+$ (but not $CD4^+$) T lymphocytes *in vitro*, yet lack-

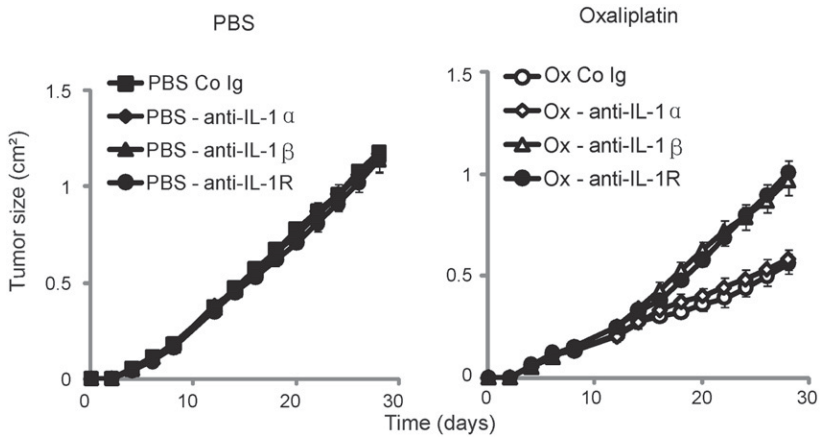

Fig. 3. IL-1β-dependent antitumor effects of chemotherapy. CT26 colon adenocarcinomas were established in BALB/c mice and treated on days 7 and 14 with PBS or oxaliplatin (5 mg/kg i.p.) in the presence of control immunoglobulin (Co Ig), neutralizing anti-IL-1α, anti-IL-1β, or anti-IL-1R antibodies (250 μg i.p./mouse, twice weekly from days 4 to 21). The growth kinetics was monitored twice a week. A representative experiment of 5 mice/group repeated twice with identical results is depicted as means ± SEM of tumor sizes over time. $^{*}p < 0.05$.

ing the potential to modulate antigen processing or activation in DC.

Altogether, these results support the contention that two DAMP receptors, TLR4 (which senses HMGB1) and P2RX7 (which senses ATP), have to be activated in a concerted fashion to allow for anticancer immune responses to be efficient. Thus, a defined spatiotemporary pattern of cell death-associated DAMPs (CRT exposure, HMGB1 release, ATP release) functions like a "key" to open the "lock" that usually precludes an immune response, through the action of defined receptor present on the surface of DC.

6. Clinical data supporting the key/lock paradigm

A polymorphism in human TLR4 (rs4986790) resulting in a single-nucleotide exchange (896A/G) in the *tlr4* gene and in an amino acid substitution (Asp299Gly) in the extracellular domain of TLR4 has been associated with decreased responses to inhaled lipopolysaccharide [106]. This substitution not only decreased the binding of HMGB1 to TLR4 but also resulted in a weaker activation of the transcription factor NF-κB ([107] and unpublished data) as well as in a profound alteration of the capacity of monocyte derived-human DC to cross-present melanoma tumor antigens from dying melanoma cell lines [75]. In a retrospective study, we analyzed the time to metastatic progression in a cohort of 280 patients that had been treated for non-metastatic breast cancer with local lymph node invasion, following a standard protocol of local surgery, local radiotherapy and systemic anthracycline injections (FEC protocol). Patients carrying TLR4 Asp299Gly allele (about 17%) did not differ from patients displaying the normal TLR4 allele for all classical prognostic factors. However, patients bearing the TLR4 Asp299Gly allele developed metastasis more rapidly than patients bearing the normal TLR4 allele, establishing TLR4 Asp299Gly as an independent predictive factor of early disease progression [75].

Next, we investigated whether the same loss-of-function allele of *tlr4* could affect the progression-free survival (PFS) of metastatic colorectal cancer (CRC) patients ($n = 338$) undergoing an oxaliplatin-based regimen. Patients that were heterozygous or homozygous for the *tlr4* Asp299Gly/Thr399Ile allele ($n = 48$) did not differ from patients bearing the normal TLR4 allele ($n = 290$) with respect to prognostic parameters relevant in CRC. Once again, patients bearing the normal TLR4 allele manifested an increased PFS (Hazard ratio 0.73, CI [0.53; 1.00], $p \leq 0.05$) and overall survival (OS) (Hazard ratio 0.72, CI [0.52; 1.01], $p = 0.05$), as compared to patients bearing the loss-of-function allele of TLR4. In contrast, in a cohort of stage II CRC patients ($n = 258$) who were treated with surgical removal of the primary tumor in a curative intent, without any adjuvant chemotherapy, no statistical differences in the terms of disease-free survival among patients bearing the normal or variant allele of *tlr4*. This result suggests that *tlr4*Asp299Gly is not a prognostic factor but rather a predictive factor of the response to oxaliplatin [108].

More recently, we investigated the prognostic value of a single-nucleotide polymorphism in the ligand-gated cation channel P2RX7 at nucleotide position 1513 (1513A > C) changing a glutamic acid to alanine at aa 496 (Glu496Ala) which abrogates the ATP-induced Ca^{2+} and ethidium influx (and the K^{+} efflux) and severely retards the ATP-dependent IL-1β release from monocytes [109]. We analyzed a cohort of 225 sporadic breast cancer patients that were stratified according to the *P2RX7* genotype (normal (64%) versus variant (36%) *P2RX7*). While there was no significant differences in classical prognostic factors between the normal and variant groups of patients, the *P2RX7* loss-of-function allele had a significant negative prognostic impact on metastatic disease-free survival (Log rank test; $p = 0.02$). A multivariate Cox regression model revealed a significant effect, both for the tumor grade and for the *P2RX7* genotype.

Altogether, these data suggest that selective immune defects (in the DC-mediated presentation of antigen from dying cells or in IL-1β release) can compromise the response to anticancer radiotherapy and chemotherapy, at least in node positive (N+) breast cancers treated with adjuvant anthracyclines. However, it is noteworthy that *in vitro* studies indicated that homozygous loss-of-function of P2RX7 are accompanied with a marked defect in IL-1β release and that P2RX7 also initiates downstream events such as the stimulation of a metalloproteinase causing the shedding of L-selectin from monocytes and lymphocytes [109]. Therefore, prospective long-term studies correlating immune functions with loss-of-function SNPs and time to progression are needed.

7. Immunological prospects for personalized chemotherapy

7.1. Promoting the immune response following tumor cell death

Several strategies have been attempted in preclinical studies that have been reviewed elsewhere [110,111]. Some recent findings will be reported below.

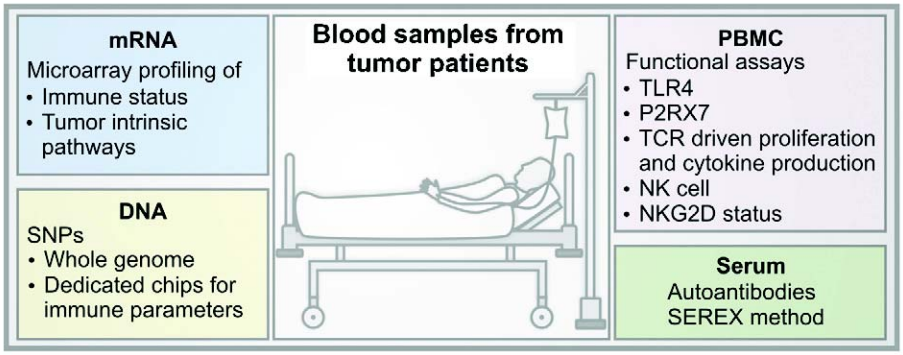

Fig. 4. Checkpoints of immunogenic cell death at the level of the host. Determining whether genetic defects in the relevant immune genes (SNPs search using whole genome CHIPs or dedicated immune CHIPs), and innate or acquired transcriptional and functional defects residing at various levels (B, T, NK, DC, TLR4, P2RX7, etc.) will require systematic prospective and translational studies on a large series of patients; correlations between these parameters and the clinical outcome will shed some light into the relevant immunological pathways during given therapies.

Inducing cell death by targeting TRAIL receptors may be a reasonable strategy not only to bypass tumor resistance to mitochondrial membrane permeabilizing agents, but also to generate an immunogenic cell death pathway [112]. When comparing the immunogenicity of B16F10 killed by a specific TRAIL-expressing DC cell subpopulation versus perforin/Granzyme B expressing NK cells, we found that tumor cells are particularly effective in eliciting prophylactic antitumor activity [113]. Accordingly, TRAIL can stimulate CRT exposure on tumor cells [32]. Low dose cyclophosphamide may induce TRAIL expression on T and/or NK effectors and promote the eradication of TRAIL-sensitive tumors [114]. When combined with TLR2/4 agonists, cyclophosphamide induced TRAIL expression on DC and DC became tumor killers leading to antigen cross-presentation and T cell and TRAIL-dependent antitumor effects [115]. Finally, antibodies targeting not only DR5 but also costimulatory molecules expressed by DC (such as anti-CD40 or anti-CD1d Ab) and T cells (such as anti-CD137 mAb) mediated potent synergistic antitumor effects against TRAIL-sensitive tumors [116]. In TRAIL-resistant tumors, the combination of doxorubicin or gemcitabine with anti-CD1d and anti-CD137 agonistic Ab improved antitumor activity [117]. Finally, it is feasible to improve targeted therapies of ErbB-2/HER2$^+$ breast cancer by using a combination of anti-DR5 and anti-ErbB-2 antibodies, which both significantly suppressed the growth of advanced spontaneous tumors arising in ErbB-2/neuT transgenic mice, in a CD11b$^+$ and CD8$^+$ T cell-dependent manner [118].

In genetically engineered Hgf-Cdk4^{R24C} mice where sporadic melanomas develop, complete cure of primary and metastatic disease could only be achieved by a combination of four strategies, i.e. (i) chemotherapy (alkylating agents), (ii) the adoptive transfer of p-mel specific CD8$^+$ T cells, (iii) adenoviral vectors engineered to express the gp100 antigen and (iv) immunostimulatory nucleic acids in the tumor microenvironment, all culminating in expansion, differentiation and survival of IFN-γ producing CTLs, sparing healthy tissues [119].

Neutralizing immunosuppressive pathways together with chemotherapy has also been successful. Combining anti-CTLA4 with a conditioning regimen for allogeneic hematopoietic stem cell transplantation proved safe and efficient for 3/29 patients in relapse, without causing overt graft-versus-host disease [120]. Combining anti-PD-1 Ab with gemcitabine was synergistic in a mouse model of pancreatic cancer [121]. Chemoimmunotherapy associating chemotherapy (paclitaxel, gemcitabine or cyclophosphamide) with the D stereoisomer of 1-methyl-tryptophan (inhibitors of indoleamine 2,3-dioxygenase) was more efficacious than each agent alone [122]. Promoting the exhaustion and apoptosis of intratumoral T_{reg} using a combination of cyclophosphamide and agonistic anti-OX40 Ab may result in potent synergistic antitumor effects against B16F10 melanoma [123].

These examples illustrate the possibility to combine therapies that induce immunogenic cell death with immunostimulatory regimes to mediate an "immunochemotherapeutic" synergy.

7.2. Compensating defects at the level of the tumor and of the host

Pinpointing the molecular defects at the level of the tumor might result in a specific therapeutic intervention. Thus, restoring the capacity of a tumor to expose CRT can be achieved by manipulating the PERK → eIF2a axis and the PP1-GADD34 complex using specific ER stress response modifiers or specific inhibitory compounds, respectively [32,33]. Local injection of recombinant CRT into tumors that lack essential compounds of the CRT translocation machinery (such as Erp57) can also re-establish the sensitivity to chemotherapy [59].

Identifying the immunological defects at the level of the host may also facilitate a targeted compensation (Fig. 4). This has been achieved by using chloroquine in mice deficient for TLR4 [75] or by coadministering TLR3 or TLR9 agonists [107]. In the former case, the lysosomotropic chloroquine given at the time of cell death may restore the dynamics of the endocytic compartments in DC, thereby favoring antigen cross-presentation and T cell priming [75]. In the latter case, TLR3 or TLR9 likewise stimulate the Myd88 pathway that fails to be activated by deficient TLR4. In mice that lacked P2RX7, elements of the Nlrp3 inflammasome (Nlrp3, caspase-1) or IL-1β, we could show that exogenous recombinant IL-1β or rIL-12 restored the Tc1 immune response triggered by cell death inducers [84]. However, exogenous IL-1β was unable to restore the failing anticancer immune response in mice lacking TLR4. These results underscore the importance of diagnosing immune defects in order to proceed to a case-specific therapeutic restoration of the immune response.

8. Concluding remarks

An ideal vaccination strategy against tumors should rely on specific antigens required for tumor maintenance, such as those involved in the oncogenic process or the autoantigens dictating cell cycle regulation or cell stress. Strong preclinical and clinical evidence supports that X ray-induced death of tumor cells genetically modified to express GM-CSF do mount humoral and cellular immune responses while in parallel, the contribution of the host

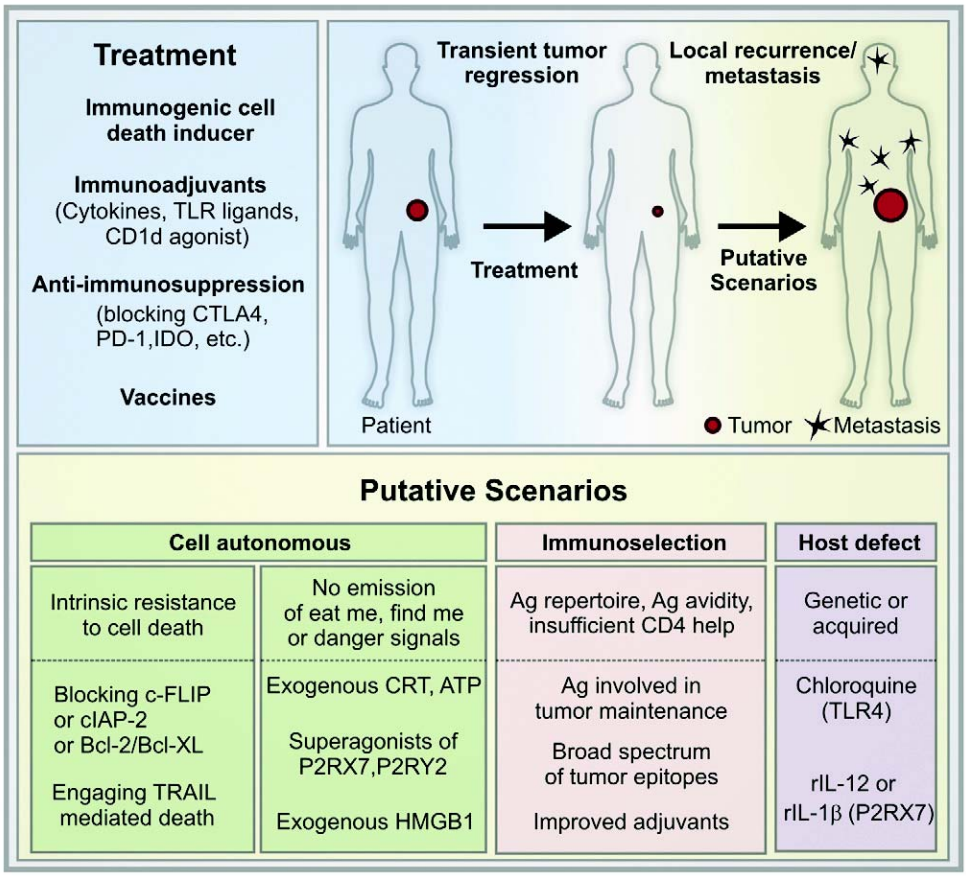

Fig. 5. Accounting for treatment failure. Revisiting the reasons. On the basis of about 10% responders for all types of vaccines and 30–40% of long-term survivors for all chemotherapies, one might consider that treatment is not efficient. However, predicting upfront the subset of complete responders would allow a 100% success. By selecting only patients not harbouring a mutation in P2RX7 nor TLR4 and a therapeutic regimen mediating "an immunogenic ER stress response and cell death", and a tumor inducible for cell stress, one should expect that a full antigen spreading will allow an efficient and broad repertoire of T cell responses which will synergize with the cell death inducer. We depict the scenarios whereby one of these checkpoints might jeopardize the success of therapy and suggest potential compensatory strategies.

immune system in the efficacy of some chemotherapies is being demonstrated. In all cases, the clinical success remains limited or the "immunochemotherapy" approach is adequate or suitable for a limited subset of tumors and/patients that remains to be identified based on the molecular dialogue between dying tumor cells and immune effectors.

Prospective translational studies are required to elucidate which among the theoretical checkpoints dictating the immunogenic cell death and residing at the level of the tumor, of the drug and of the host will prevail in controlling humoral and cellular immune responses, as well as the clinical outcome. We anticipate that the validation of at least some of these checkpoints will allow (i) to device algorithms predictive of clinical responses, (ii) to personalize therapy with cell death inducers, and (iii) to re-orient immunization strategies according the pre-existing immune status of the patients (Fig. 5).

Acknowledgements

The authors are supported by grants from the Ligue Nationale contre le Cancer (G.K. and L.Z.), China Scholarship Council (Y.M.), the Fondation pour la Recherche Médicale (to L. Apetoh., G.K., and L.Z.), the European Union (INFLACARE grant), the Association For International Cancer Research (to G.K.), Cancéropôle Ile-de-France, Institut National du Cancer (L.Z. and G.K.), Agence Nationale pour la Recherche (to G.K.), the European Molecular Biology Organization (L. Apetoh.), INSERM (A.T.), and the National Health, National Health and Medical Research Council of Australia and The Victorian Cancer Agency (M.J.S. and N.M.H.).

References

[1] Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004;4:11–22.
[2] Hodi FS, Dranoff G. Combinatorial cancer immunotherapy. Adv Immunol 2006;90:341–68.
[3] Soiffer R, Hodi FS, Haluska F, Jung K, Gillessen S, Singer S, et al. Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. J Clin Oncol 2003;21:3343–50.
[4] Salgia R, Lynch T, Skarin A, Lucca J, Lynch C, Jung K, et al. Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. J Clin Oncol 2003;21:624–30.
[5] Fonseca C, Soiffer R, Ho V, Vanneman M, Jinushi M, Ritz J, et al. Protein disulfide isomerases are antibody targets during immune-mediated tumor destruction. Blood 2009;113:1681–8.
[6] Sittler T, Zhou J, Park J, Yuen NK, Sarantopoulos S, Mollick J, et al. Concerted potent humoral immune responses to autoantigens are associated with tumor destruction and favorable clinical outcomes without autoimmunity. Clin Cancer Res 2008;14:3896–905.
[7] Schmollinger JC, Vonderheide RH, Hoar KM, Maecker B, Schultze JL, Hodi FS, et al. Melanoma inhibitor of apoptosis protein (ML-IAP) is a target for immune-mediated tumor destruction. Proc Natl Acad Sci USA 2003;100:3398–403.
[8] Hodi FS, Schmollinger JC, Soiffer RJ, Salgia R, Lynch T, Ritz J, et al. ATP6S1 elicits potent humoral responses associated with immune-mediated tumor destruction. Proc Natl Acad Sci USA 2002;99:6919–24.
[9] Jinushi M, Hodi FS, Dranoff G. Therapy-induced antibodies to MHC class I chain-related protein A antagonize immune suppression and stimulate anti-tumor cytotoxicity. Proc Natl Acad Sci USA 2006;103:9190–5.

[10] Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998;392:86–9.
[11] Albert ML, Darnell JC, Bender A, Francisco LM, Bhardwaj N, Darnell RB. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. Nat Med 1998;4:1321–4.
[12] Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191:423–34.
[13] Zitvogel L, Casares N, Pequignot MO, Chaput N, Albert ML, Kroemer G. Immune response against dying tumor cells. Adv Immunol 2004;84:131–79.
[14] Lake RA, Robinson BW. Immunotherapy and chemotherapy—a practical partnership. Nat Rev Cancer 2005;5:397–405.
[15] Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008;8:59–73.
[16] Lugade AA, Moran JP, Gerber SA, Rose RC, Frelinger JG, Lord EM. Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. J Immunol 2005;174:7516–23.
[17] Lugade AA, Sorensen EW, Gerber SA, Moran JP, Frelinger JG, Lord EM. Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. J Immunol 2008;180:3132–9.
[18] Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. J Exp Med 2006;203:1259–71.
[19] Teitz-Tennenbaum S, Li Q, Rynkiewicz S, Ito F, Davis MA, McGinn CJ, et al. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. Cancer Res 2003;63:8466–75.
[20] Chamoto K, Takeshima T, Wakita D, Ohkuri T, Ashino S, Omatsu T, et al. Combination immunotherapy with radiation and CpG-based tumor vaccination for the eradication of radio- and immuno-resistant lung carcinoma cells. Cancer Sci 2009;100:934–9.
[21] Schaue D, Comin-Anduix B, Ribas A, Zhang L, Goodglick L, Sayre JW, et al. T-cell responses to survivin in cancer patients undergoing radiation therapy. Clin Cancer Res 2008;14:4883–90.
[22] Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, et al. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. J Exp Med 2007;204:49–55.
[23] Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, et al. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. Cancer Res 2009;69:2514–22.
[24] Chiarle R, Martinengo C, Mastini C, Ambrogio C, D'Escamard V, Forni G, et al. The anaplastic lymphoma kinase is an effective oncoantigen for lymphoma vaccination. Nat Med 2008;14:676–80.
[25] Correale P, Cusi MG, Tsang KY, Del Vecchio MT, Marsili S, Placa ML, et al. Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. J Clin Oncol 2005;23:8950–8.
[26] Correale P, Tagliaferri P, Fioravanti A, Del Vecchio MT, Remondo C, Montagnani F, et al. Immunity feedback and clinical outcome in colon cancer patients undergoing chemoimmunotherapy with gemcitabine + FOLFOX followed by subcutaneous granulocyte macrophage colony-stimulating factor and aldesleukin (GOLFIG-1 Trial). Clin Cancer Res 2008;14:4192–9.
[27] Correale P, Del Vecchio MT, La Placa M, Montagnani F, Di Genova G, Savellini GG, et al. Chemotherapeutic drugs may be used to enhance the killing efficacy of human tumor antigen peptide-specific CTLs. J Immunother 2008;31:132–47.
[28] Tanaka H, Matsushima H, Mizumoto N, Takashima A. Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. Cancer Res 2009;69:6978–86.
[29] Tanaka H, Matsushima H, Nishibu A, Clausen BE, Takashima A. Dual therapeutic efficacy of vinblastine as a unique chemotherapeutic agent capable of inducing dendritic cell maturation. Cancer Res 2009;69:6987–94.
[30] 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;202:1691–701.
[31] 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.
[32] 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;28:578–90.
[33] Kepp O, et al. Cell Cycle 2009;8(23):3971–7. Epub 2009 Dec 28.
[34] Berche PA, North RJ. Non-H-2 restriction of expression of passively transferred delayed sensitivity. J Exp Med 1982;155:1334–43.
[35] Taieb J, Chaput N, Schartz N, Roux S, Novault S, Menard C, et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 2006;176:2722–9.
[36] Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;105:2862–8.
[37] Schiavoni G, Mattei F, Di Pucchio T, Santini SM, Bracci L, Belardelli F, et al. Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. Blood 2000;95:2024–30.
[38] Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels JP, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. J Exp Med 2005;201:1591–602.
[39] Glaser M. Regulation of specific cell-mediated cytotoxic response against SV40-induced tumor associated antigens by depletion of suppressor T cells with cyclophosphamide in mice. J Exp Med 1979;149:774–9.
[40] Castano AP, Mroz P, Wu MX, Hamblin MR. Photodynamic therapy plus low-dose cyclophosphamide generates antitumor immunity in a mouse model. Proc Natl Acad Sci USA 2008;105:5495–500.
[41] Ghiringhelli F, Manckoundia P, Pfitzenmeyer P. Acute tubulointerstitial nephritis due to *Salmonella typhimurium* infection. Eur J Intern Med 2004;15:401.
[42] Berd D, Maguire Jr HC, Mastrangelo MJ. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res 1986;46:2572–7.
[43] Berd D, Mastrangelo MJ. Effect of low dose cyclophosphamide on the immune system of cancer patients: depletion of $CD4^+$, $2H4^+$ suppressor-inducer T-cells. Cancer Res 1988;48:1671–5.
[44] MacLean GD, Miles DW, Rubens RD, Reddish MA, Longenecker BM. Enhancing the effect of THERATOPE STn-KLH cancer vaccine in patients with metastatic breast cancer by pretreatment with low-dose intravenous cyclophosphamide. J Immunother Emphasis Tumor Immunol 1996;19:309–16.
[45] Audia S, Nicolas A, Cathelin D, Larmonier N, Ferrand C, Foucher P, et al. Increase of CD4+ CD25+ regulatory T cells in the peripheral blood of patients with metastatic carcinoma: a Phase I clinical trial using cyclophosphamide and immunotherapy to eliminate CD4+ CD25+ T lymphocytes. Clin Exp Immunol 2007;150:523–30.
[46] Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+ CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56:641–8.
[47] Nistico P, Capone I, Palermo B, Del Bello D, Ferraresi V, Moschella F, et al. Chemotherapy enhances vaccine-induced antitumor immunity in melanoma patients. Int J Cancer 2009;124:130–9.
[48] Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, Collins EJ, et al. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J Immunol 2003;170:4905–13.
[49] Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. Cancer Res 2003;63:4490–6.
[50] Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 2005;11:6713–21.
[51] Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med 2005;201:1257–68.
[52] Hareyama M, Imai K, Ban T, Koshiba H, Kubo K, Shidou M, et al. Effect of radiation on the expression of carcinoembryonic antigen on the membranes of human gastric adenocarcinoma cells—immunological study using monoclonal antibodies. Nippon Igaku Hoshasen Gakkai Zasshi 1988;48:1572–4.
[53] Garnett CT, Palena C, Chakraborty M, Tsang KY, Schlom J, Hodge JW. Sublethal irradiation of human tumor cells modulates phenotype resulting in enhanced killing by cytotoxic T lymphocytes. Cancer Res 2004;64:7985–94.
[54] Borg C, Terme M, Taieb J, Menard C, Flament C, Robert C, et al. Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects. J Clin Invest 2004;114:379–88.
[55] Menard C, Blay JY, Borg C, Michiels S, Ghiringhelli F, Robert C, et al. Natural killer cell IFN-gamma levels predict long-term survival with imatinib mesylate therapy in gastrointestinal stromal tumor-bearing patients. Cancer Res 2009;69:3563–9.
[56] Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulos EB, et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 2004;103:1037–42.
[57] Hipp MM, Hilf N, Walter S, Werth D, Brauer KM, Radsak MP, et al. Sorafenib, but not sunitinib, affects function of dendritic cells and induction of primary immune responses. Blood 2008;111:5610–20.
[58] Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res 2009;15:2148–57.
[59] 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;15:1499–509.
[60] Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, et al. Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from "silent" to immunogenic. Cancer Res 2007;67:7941–4.
[61] 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;14:1848–50.

[62] Tufi R, Panaretakis T, Bianchi K, Criollo A, Fazi B, Di Sano F, et al. Reduction of endoplasmic reticulum Ca2+ levels favors plasma membrane surface exposure of calreticulin. Cell Death Differ 2008;15:274–82.
[63] Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol 2001;11:130–5.
[64] Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009;138:271–85.
[65] Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell 2005;123:321–34.
[66] Uno S, Kinoshita Y, Azuma Y, Tsunenari T, Yoshimura Y, Iida S, et al. Antitumor activity of a monoclonal antibody against CD47 in xenograft models of human leukemia. Oncol Rep 2007;17:1189–94.
[67] Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs Jr KD, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 2009;138:286–99.
[68] Jinushi M, Nakazaki Y, Dougan M, Carrasco DR, Mihm M, Dranoff G. MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and anti-inflammatory activities of GM-CSF. J Clin Invest 2007;117:1902–13.
[69] Jinushi M, Sato M, Kanamoto A, Itoh A, Nagai S, Koyasu S, et al. Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms. J Exp Med 2009;206:1317–26.
[70] Jinushi M, Nakazaki Y, Carrasco DR, Draganov D, Souders N, Johnson M, et al. Milk fat globule EGF-8 promotes melanoma progression through coordinated Akt and twist signaling in the tumor microenvironment. Cancer Res 2008;68:8889–98.
[71] Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007;220:35–46.
[72] Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazzon M, et al. HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep 2004;5:825–30.
[73] Bell CW, Jiang W, Reich CF, 3rd, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol 2006;291:C1318–25.
[74] Tesniere A, Panaretakis T, Kepp O, Apetoh L, Ghiringhelli F, Zitvogel L, et al. Molecular characteristics of immunogenic cancer cell death. Cell Death Differ 2008;15:3–12.
[75] 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;13:1050–9.
[76] Urbonaviciute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, et al. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. J Exp Med 2008;205:3007–18.
[77] Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 2003;425:516–21.
[78] Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, et al. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med 2009;179:903–13.
[79] Gasse P, Mary C, Guenon I, Noulin N, Charron S, Schnyder-Candrian S, et al. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. J Clin Invest 2007;117:3786–99.
[80] Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle 2006;5:2592–601.
[81] Somersan S, Larsson M, Fonteneau JF, Basu S, Srivastava P, Bhardwaj N. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. J Immunol 2001;167:4844–52.
[82] Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. Nat Immunol 2005;6:593–9.
[83] 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;109:4839–45.
[84] 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;15:1170–8.
[85] Martins I. Cell Cycle 2009;8(22):3723–8. Epub 2009 Nov 8.
[86] 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;461:282–6.
[87] Mizumoto N, Kumamoto T, Robson SC, Sevigny J, Matsue H, Enjyoji K, et al. CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. Nat Med 2002;8:358–65.
[88] Kunzli BM, Berberat PO, Giese T, Csizmadia E, Kaczmarek E, Baker C, et al. Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. Am J Physiol Gastrointest Liver Physiol 2007;292:G223–30.
[89] Dzhandzhugazyan KN, Kirkin AF, thor Straten P, Zeuthen J. Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas. FEBS Lett 1998;430:227–30.
[90] Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature 2001;411:207–11.
[91] Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell 2007;131:1124–36.
[92] Wallet MA, Sen P, Flores RR, Wang Y, Yi Z, Huang Y, et al. MerTK is required for apoptotic cell-induced T cell tolerance. J Exp Med 2008;205:219–32.
[93] Lu Q, Lemke G. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. Science 2001;293:306–11.
[94] Hong CC, Lay JD, Huang JS, Cheng AL, Tang JL, Lin MT, et al. Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. Cancer Lett 2008;268:314–24.
[95] Vajkoczy P, Knyazev P, Kunkel A, Capelle HH, Behrndt S, von Tengg-Kobligk H, et al. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. Proc Natl Acad Sci USA 2006;103:5799–804.
[96] Gustafsson A, Martuszewska D, Johansson M, Ekman C, Hafizi S, Ljungberg B, et al. Differential expression of Axl and Gas6 in renal cell carcinoma reflecting tumor advancement and survival. Clin Cancer Res 2009;15:4742–9.
[97] Hutterer M, Knyazev P, Abate A, Reschke M, Maier H, Stefanova N, et al. Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiform. Clin Cancer Res 2008;14:130–8.
[98] Rochlitz C, Lohri A, Bacchi M, Schmidt M, Nagel S, Fopp M, et al. Axl expression is associated with adverse prognosis and with expression of Bcl-2 and CD34 in de novo acute myeloid leukemia (AML): results from a multicenter trial of the Swiss Group for Clinical Cancer Research (SAKK). Leukemia 1999;13:1352–8.
[99] Schwartz HS, Grindey GB. Adriamycin and daunorubicin: a comparison of antitumor activities and tissue uptake in mice following immunosuppression. Cancer Res 1973;33:1837–44.
[100] Ho RL, Maccubbin D, Zaleskis G, Krawczyk C, Wing K, Mihich E, et al. Development of a safe and effective adriamycin plus interleukin 2 therapy against both adriamycin-sensitive and -resistant lymphomas. Oncol Res 1993;5:373–81.
[101] Fisk B, Ioannides CG. Increased sensitivity of adriamycin-selected tumor lines to CTL-mediated lysis results in enhanced drug sensitivity. Cancer Res 1998;58:4790–3.
[102] Salup RR, Back TC, Wiltrout RH. Successful treatment of advanced murine renal cell cancer by bicompartmental adoptive chemoimmunotherapy. J Immunol 1987;138:641–7.
[103] Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. Eur J Cancer 2004;40:827–36.
[104] Sancho D, Mourao-Sa D, Joffre OP, Schulz O, Rogers NC, Pennington DJ, et al. Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. J Clin Invest 2008;118:2098–110.
[105] Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, et al. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature 2009;458:899–903.
[106] Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187–91.
[107] 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.
[108] Tesniere A. Oncogene 2010;29(4):482–91. Epub 2009 Nov 2.
[109] Sluyter R, Shemon AN, Wiley JS. Glu496 to Ala polymorphism in the P2X7 receptor impairs ATP-induced IL-1 beta release from human monocytes. J Immunol 2004;172:3399–405.
[110] Haynes NM, van der Most RG, Lake RA, Smyth MJ. Immunogenic anti-cancer chemotherapy as an emerging concept. Curr Opin Immunol 2008;20:545–57.
[111] Demaria S, Formenti SC. Sensors of ionizing radiation effects on the immunological microenvironment of cancer. Int J Radiat Biol 2007;83:819–25.
[112] Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 2008;8:782–98.
[113] Ullrich E, Bonmort M, Mignot G, Jacobs B, Bosisio D, Sozzani S, et al. Trans-presentation of IL-15 dictates IFN-producing killer dendritic cells effector functions. J Immunol 2008;180:7887–97.
[114] van der Most RG, Currie AJ, Cleaver AL, Salmons J, Nowak AK, Mahendran S, et al. Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. PLoS One 2009;4:e6982.
[115] Roux S, Apetoh L, Chalmin F, Ladoire S, Mignot G, Puig PE, et al. CD4+ CD25+ Tregs control the TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in rodent models of colon cancer. J Clin Invest 2008;118:3751–61.
[116] Uno T, Takeda K, Kojima Y, Yoshizawa H, Akiba H, Mittler RS, et al. Eradication of established tumors in mice by a combination antibody-based therapy. Nat Med 2006;12:693–8.
[117] Teng MW, Sharkey J, McLaughlin NM, Exley MA, Smyth MJ. CD1d-based combination therapy eradicates established tumors in mice. J Immunol 2009;183:1911–20.
[118] Stagg J, Sharkey J, Pommey S, Young R, Takeda K, Yagita H, et al. Antibodies targeted to TRAIL receptor-2 and ErbB-2 synergize in vivo and induce an antitumor immune response. Proc Natl Acad Sci USA 2008;105:16254–9.

[119] Kohlmeyer J, Cron M, Landsberg J, Bald T, Renn M, Mikus S, et al. Complete regression of advanced primary and metastatic mouse melanomas following combination chemoimmunotherapy. Cancer Res 2009;69:6265–74.
[120] Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L, et al. CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. Blood 2009;113:1581–8.
[121] Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 lig-and/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res 2007;13:2151–7.
[122] Hou DY, Muller AJ, Sharma MD, DuHadaway J, Banerjee T, Johnson M, et al. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res 2007;67:792–801.
[123] Hirschhorn-Cymerman D, Rizzuto GA, Merghoub T, Cohen AD, Avogadri F, Lesokhin AM, et al. OX40 engagement and chemotherapy combination provides potent antitumor immunity with concomitant regulatory T cell apoptosis. J Exp Med 2009;206:1103–16.

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Current Opinion in Immunology

The dendritic cell–tumor cross-talk in cancer

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The question as to whether the tumor grows because of or despite the host immune system is being progressively addressed with refined technology, gene targeting in mice and human translational research. The productive interplay between major actors of the antitumor immunity is actively compromised by the tumor microenvironment subverting the links between innate and cognate immunity and/or generating devastating new players. The complexity of the host–tumor equilibrium could be dissected at the reduced level of the dialogue between professional antigen presenting cells (APC), more precisely dendritic cells, and tumor cells that may profoundly dictate the outcome of the neoplasma. This review will summarize the novel mechanisms by which tumor cells regulate DC recruitment, differentiation, activation and cross-presenting functions in tumor beds and how innate players might counterbalance these interactions. Finally, we will highlight interesting strategies that harness the DC potential to fight against cancer.

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Current Opinion in Immunology 2010, **23**:1–7

This review comes from a themed issue on
Antigen processing
Edited by Peter van den Elsen and James McCluskey

0952-7915/$ – see front matter
Published by Elsevier Ltd.

DOI 10.1016/j.coi.2010.09.008

Tumor microenvironment and deficient antigen presenting functions

Cancer-induced tolerance relies on the unresponsiveness of the host immune system to professional antigen presenting cells (APC) invading tumor beds or residing in the vicinity (tumor-draining lymph nodes) of developing tumors. Tumor progression induces defects in DC recruitment, differentiation, maturation and survival (Figure 1). The mechanisms of inadequate DC functions causing and/or resulting from tumor escape have been reviewed elsewhere [1,2]. It is noteworthy that the regulatory capacity of tumor infiltrating DC is not related to a defined DC subset but rather results from the influence of tumor microenvironment. Over the past decade, a series of immunosuppressive factors (such as GM-CSF and S100A9 associated with myeloid derived suppressor cells (MDSC), M-CSF, IL-6, VEGF, TGF-β, CXCL8, IL-10, gangliosides, altered glycosylation of tumor associated antigens, reactive oxygen species, indoleamine 2,3-deoxygenase (IDO) and extracellular adenosine) have been described to block DC recruitment and/or functions, mainly through activation of signal transduction and activator of transcription STAT3 [3].

Immunohistochemical analyses of breast cancer tissues revealed that plasmacytoid DC (pDC) invade 13% of primary tumors and predict short progression free survival [4]. In the same malignancy, Gobert *et al.* demonstrated a strong association between the DC-LAMP$^+$ DC and Tregs in a CCR4/CCL22 chemokine milieu also associated with poor prognosis if located in the periphery of tumors [5]. Interestingly, BST2 released from tumor cells can subvert pDC through ILT7 signaling and make pDC fail to respond to danger signals for type 1 IFN production. Pretreatment with IFN-α and TNF-α significantly increased BST2 secretion, suggesting that the inflammatory status of tumor microenvironment support this immunoregulatory pathway [6••].

Moreover, prostaglandin E_2 (PGE_2), the major cyclooxygenase 2 metabolite released by tumor cells, can interrupt the tumor > DC > T cell cascade by inducing an IL-10-dependent reduction of DC infiltration in tumors and of DC maturation, and PGE2 compromises CCR7-dependent DC migration in LN promoting abortive CD8$^+$ T cell responses [7]. These data extended the pioneering finding that tumor growing in PGE_2 receptor deficient hosts exhibited markedly enhanced DC differentiation and antitumor CTL responses compared with WT littermates [8]. In humans, maturation of DC in the presence of PGE_2 resulted in upregulation of CD25 and IDO culminating in DC-mediated T cell inhibition, a scenario compatible with the DC phenotype found in human tumors [9]. More recently, novel mechanisms of alterations of DC cross-presenting functions were unraveled. Herber *et al.* reported accumulation of triacylglycerol through exacerbated macrophage scavenger receptor 1 (MSR1)-mediated uptake in DC from tumor bearers. Lipid-laden DC were severely altered in their ability

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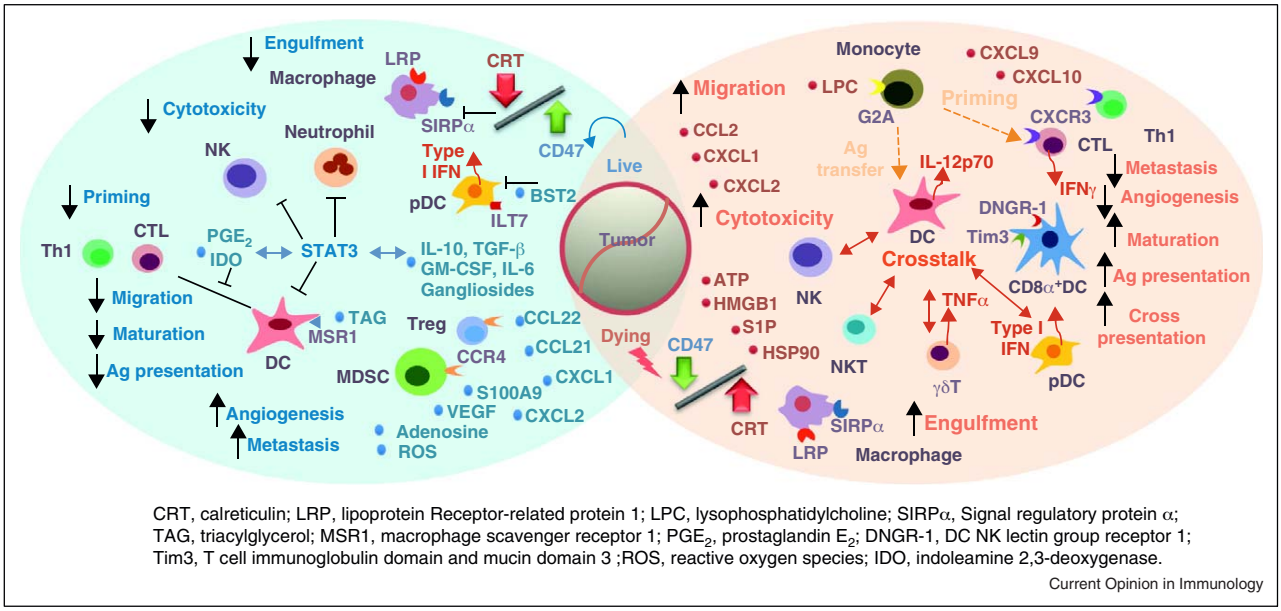
Figure 1



CRT, calreticulin; LRP, lipoprotein Receptor-related protein 1; LPC, lysophosphatidylcholine; SIRPα, Signal regulatory protein α; TAG, triacylglycerol; MSR1, macrophage scavenger receptor 1; PGE_2, prostaglandin E_2; DNGR-1, DC NK lectin group receptor 1; Tim3, T cell immunoglobulin domain and mucin domain 3 ;ROS, reactive oxygen species; IDO, indoleamine 2,3-deoxygenase.

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The yin/yang dialogue between tumor cells and immune players in cancer. Live tumor cells produce or express a variety of metabolites or proteins that subvert the capacity of bona fide antigen presenting cells to initiate tumor-specific T cell responses (at all levels: engulfment, recruitment, differentiation, migration, activation, cross-presentation) and/or contribute to activate MDSC or Treg competing against effector T cells and/or directly promoting angiogenesis or metastases (blue circle). Tumor cell death (intrinsically or extrinsically regulated) might either reenforce tumor-induced tolerance (via engulfment by inflammatory phagocytes and/or tolerogenic molecular pathways) or instead, reset immune responses by exposing appropriate 'cell death-associated molecular patterns' (rebooting APC functions and T cell polarization) and/or recruiting key innate effectors (red circle).

to cross-present soluble antigen or tumor associated antigens unless an inhibitor of acetyl-CoA carboxylase was added to DC *in vitro* and *in vivo* [10••]. Patients bearing head and neck tumors also presented with lipid accumulation in their circulating, tumoral and lymph node $Lin^{-}CD4^{+}$ DC [10••]. Human and mouse tumors releasing cholesterol metabolites could dampen the expression of CCR7 on maturing DCs by triggering liver X receptor (LXR), thereby impairing DC migration and allowing tumor escape [11••]. Interestingly, cancer stem cells may not differ from parental tumor cells in their spectrum of antigen expression [12] and the lack of immune tolerance to pluripotency antigen (such as the transcription factor octamer-binding protein 4 (OCT4)) in cancer patients suggests that tumor outgrowth may not result from a primary defect of DC recognition of tumor stem cells [13].

Come-and-get-me signals emanating from tumor cells

During tumor progression, there is constant and in some cases prominent apoptosis (such as Burkitt lymphoma) that is part of a vicious circle. Indeed, apoptotic cells express phosphatidylserine (PS) on their surface at late stages of apoptosis, and several PS receptors or adaptors have been described [such as T cell immunoglobulin and mucin domain-containing protein 4 (Tim4), Mer tyrosine kinase (MerTK), milk fat globule-EGF factor 8 protein (MFG-E8), brain-specific angiogenesis inhibitor 1 (BAI1)] on macrophages to ensure prompt clearance of apoptotic cells and elicitation of tolerance before full blown inflammatory necrosis [14–16,17••]. During the pre-apoptosis phase, cells upregulate sphingosine kinase 1 expression, allowing the release of sphingosine-1-phosphate (S1P) that causes cytoskeletal rearrangements and chemoattraction of macrophages, even at low nanomolar ranges [18]. However, *in vivo* studies showed that tumors expanding in $S1P_2^{-/-}$ (the G protein-coupled receptor for S1P) animals exhibited a higher infiltration with $CD11b^{+}Gr1^{-}CD34^{-}$ bone marrow cells including $F4/80^{+}$ macrophages in a high VEGF, TGF-β1, basic FGF and IL-1β stromal environment with augmented tumor angiogenesis and growth [19]. S1P may even synergize with other chemokine-like factors such as lysophosphatidylcholine (LPC) [20], IL-8 or CCL2 that can be released by dying cells [18]. The G protein-coupled receptors G2A, unlike its relative GPR4, is involved in the chemotaxis of monocytic cells and could be a phagocyte receptor for find me signals such as LPC secreted by dying cells [21].

However, contrasting with the theory whereby inflammatory phagocytes mediate a status of clearance and tolerance of tumor cell debris, a different view positioning

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macrophages as scavengers preventing dissemination of circulating and live tumor cells has recently emerged. Indeed, the pentaspanin integrin associated protein CD47 was found to be overexpressed on myeloid leukemia and migrating hematopoietic progenitors (and also solid tumors), resulting in reduced uptake by signal-regulatory protein alpha (SIRPα) expressing macrophages. The level of CD47 expression correlated with the ability to evade phagocytosis and macrophage-dependent immunosurveillance against developing leukemia [22•,23]. It is likely that an 'eat me' signal, like calreticulin, might be expressed in cis on leukemic cells to engage a counterreceptor mediating efficient uptake (via lipoprotein receptor-related protein 1 (LRP) [24]) by the phagocyte. However, peripheral tolerance is governed by efficient phagocytosis of apoptotic cells and cross-presentation of self-antigens by CD8α$^+$ DC [25]. This subset may not use CD36, DEC205, αvβ3/αvβ5 integrins for the clearance of dying self. Using UV-irradiated thymocytes, Nakayama *et al.* demonstrated that Tim3 expressed on CD8α$^+$ DC is crucial for the uptake of dying cells and prevention of autoimmunity [26]. Using Fas-expressing tumor cells, Qiu *et al.* discovered that blood borne cell associated antigens are cross-presented by marginal zone splenic CD8α$^+$CD103$^+$DEC207$^+$ DC to provide peripheral tolerance [27].

Tertiary lymphoid organogenesis (TLO): friends or foes?

The tumor microenvironment may contain lymphoid-like structures that could regulate local adaptive immune responses. Tumor infiltrating-bronchus associated lymphoid tissues (Ti-BALTs) have been recently described in non-small cell lung cancers endowed with very favorable clinical outcome [28]. Ti-BALTs appear to be orchestrated around DC-Lamp$^+$ DC that interact with CD4$^+$ T cells of a memory phenotype. The DC/CD4$^+$ T cell clusters were associated with CD8$^+$ T cells of a Th1 pattern (T-bet$^+$) residing in tumor nests and surrounded by B cell follicles containing germinal center DC. While concordant with previous reports showing the association between TLO and autoimmune flare up or atherogenesis, this clinical observation is contrasting with a mouse study unveiling the protumorigenic behaviour of LN paracortex stroma-like areas containing lymphoid tissue-inducer cells and high endothelial venules [29•]. Such structures were induced by tumor cells overexpressing CCL21, a CCR7 ligand promoting recruitment of Treg and MDSC. By contrast, CCL21-deficient tumors induced antitumor immunity. The tolerogenic switch triggered by the CCL21-driven mimicry of the LN stroma was associated with a recruitment of naïve T cells to peripheral sites (as opposed to memory T cells), CCL21 promoting their differentiation into Treg and inducing effector T cell senescence [30]. Moreover, the mouse study failed to mention B cell follicles (in contrast to the former setting) that could elicit a humoral anticancer immune response synergizing with T cell reactivities. Future studies will pinpoint the role of RORγt, LTβ, IL-7/IL-7R and NK22 cells, features associated with lymphoid tissue-inducer cells, in the induction or maintenance of long term protective antitumor immune responses.

Sensing of tumors by CD8α$^+$DC: tolerance or immunity?

CD8α$^+$ DC have been shown to be a masterpiece of peripheral tolerance to cell associated self/tumor antigens [25]. However, the same DC subset is pivotal for antitumor immunosurveillance against MCA induced sarcoma, which depended upon IFN type I and type II receptors and lymphocytes [31]. Deletion of the transcriptional factor Batf3 ablated development of CD8α$^+$ DC that provoked tumor outgrowth of very immunogenic tumor variants [32•]. Importantly, Reis e Sousa and coworkers identified DC NK lectin group receptor 1 (DNGR-1), a SYK-coupled C type lectin receptor, selectively expressed by CD8α$^+$ DC and involved in sensing and cross-presenting antigens from necrotic cells [33]. Despite the fact that DNGR-1 is an endocytic receptor, its main function may not be phagocytosis but rather to maintain the phagocytic cargo away from lysosomal compartments to allow retrieval of antigens for efficient cross-presentation. The DNGR-1-SYK pathway does not activate DC in response to dead cells and targeting of DNGR-1 with a specific antibody coupled to a tumor antigen requires TLR3 adjuvants to promote antitumor immunity [34••]. The putative human equivalents have been recently reported by several teams, showing that BDCA3^{high}DNGR-1$^+$ Lin$^-$DR$^+$ DC, constituting 0.1% of human spleen cellularity, express most of the mouse CD8α$^+$ DC markers (Necl2, CD205, CD207, BATF3, TLR3, IRF8), internalize dead cells and respond to TLR3 and TLR8 agonists to cross-present long peptides [35••]. Whether this novel human DC subset could be exploited for immunological interventions against cancer remains an open conundrum.

Alternatively, bona fide CD8α$^+$ DC residing in the LN can acquire MHC I/peptide complexes generated by cross-presentation from inflammatory monocyte-derived DC [36•]. Such cross-dressing can prime naïve CD4$^+$ and CD8$^+$ T cells against tumor antigens from a necrotic tumor (but not live tumor) that have been uptaken by monocyte-derived DC [37,38].

There are anatomical determinations as to whether CD8α$^+$ versus CD8α$^-$ DC might be more eligible for efficient cross-presentation. It remains unclear how the tumor microenvironment might dictate such events and how cell death (either spontaneous or therapy-induced) might edit the biological features of cross-presentation.

In as much as immunogenic tumors might be frequently invaded by effector memory TILs [39] and since DC also

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play a major role in reactivating memory $CD8^+$ T cell responses [40], one wonder whether tumor infiltrating DC (as opposed to LN-residing DC) contribute to the pool of tumor-reactive T cells. Arguing against that hypothesis, memory T cells appear to better respond to LN resident $CD8\alpha^+$ DC than migratory $CD8\alpha^-$ DC located in inflamed peripheral tissues during influenza virus infection (such as virally infected-skin or lung) [41].

It is noteworthy that other inflammatory phagocytes such as neutrophils, diverging ontogenically from DC, may be able to transport tumor antigens from peripheral tissues to lymphoid organs [42], and to cross-present tumor antigens to naïve T cells [43•]. However, neutrophils may contribute to tumor-induced tolerance as suggested by clinical reports associating the presence of intratumoral neutrophils with short recurrence-free survival in localized renal cell carcinoma [44].

Innate cells providing help for antitumor T cell immune responses

NK cell-killing of target cells is far more efficient at eliciting humoral and cellular immunity than the UV or gamma irradiation-driven cell death modality. The immunogenicity of the NK cell-killing pathway involves TRIF/Myd88 signaling and both type I and II IFNR [45]. Recognition of tumor cells by NK cells can occur via downregulation of MHC class I molecules as well as expression of stress associated ligands or costimulatory molecules (such as CD70 and CD80), all engaging NK cell signaling. The coinciding inflammatory signals with cytotoxicity appear to optimally gear the ensuing adaptive immune response. Indeed, the NK-DC cross-talk plays a dominant role in T cell priming, either by boosting DC maturation (and production of IL-12 and type 1 IFN [46,47]), or by eliminating DC to limit adaptive immunity (as shown in viral infections, [48]). Other innate subsets could do both, kill tumor cells and promote DC maturation. CD1d-restricted NKT cells can induce IL-12p70 production by DC in a CD40-dependent fashion [49] and were shown to promote help for DC cross-presentation to $CD8^+$ T cells in a CCL17/CCR4 dependent manner [50]. Vδ1 γδT cells can trigger DC maturation in a TNFα and CD1c-dependent manner, and synergize with LPS for the induction of naïve $CD4^+$ T cell responses [51].

Concluding remarks and novel prospects

Immunotherapeutic interventions may have not been very successful so far because of their inability to counteract tumor-induced immunosuppressive pathways and/or of their low capacity to elicit potent and coordinated interactions in the immune network. Resetting the DC/tumor dialogue may be approached in many advantageous ways [52]. A few novel strategies of active or passive immunization will be developed in this conclusion.

Can we ameliorate the coordination of the multiple DC subtypes?

The cooperative functional pathways existing between various DC subsets have been extensively reviewed [25]. Consequently, a spatiotemporal delivery by poly (lactic-co-glycolic acid) matrices of high levels of GM-CSF containing tumor lysates as well as polyethylenimine-condensed CpG ODN could provide a secondary immunostimulatory site of tumor antigen presentation eliciting efficient $CD8^+$ T cell responses and tumor eradication [53••]. Such matrices could recruit up to 1.2×10^6 pDC, 6×10^5 $CD8\alpha^+$ DC and 3×10^6 $CD11b^+$DC correlating with the local expansion of antitumor CTLs (and the proportional decrease of Tregs) and their recirculation to spleens. Importantly, this local orchestration of a DC network was concomitant with the accumulation of type 1 IFN and IL-12 (and the reduction of TGF-β and IL-10). The prophylactic antitumor efficacy of this vaccine was proportional to the presence of pDC, $CD8\alpha^+$ DC and IL-12.

How could we best handle $CD4^+$ T cell help?

Naïve $CD4^+$ T cells could become cytotoxic and highly contribute to tumor rejection. Adoptively transferred naïve $CD4^+$ T cell (ACT) specific for self/tumor antigens can differentiate into Th1 and LAMP1/GrzB/Pfr positive cells capable of eradicating large melanoma and inducing autoimmunity. Therapy was independent of prior vaccination, exogenous cytokine support, B or $CD8^+$T or NK or NKT lymphocytes but strongly relied upon common IL-2R γ chain [54]. It appeared that improper DC activation (i.e. low MHC class II and CD86 expression and low sensitivity of DC to respond to and to produce IL-12 and IL-15) will dramatically affect Th1 priming of the $CD4^+$ T cell based-ACT. Low IFN-γ may also contribute to reduced CXCL9 production by the tumor milieu causing a poor recruitment of CXCR3 expressing Th1 $CD4^+$ T cells [55]. Cytokine/antibody immune complexes to IL-15 or IL-2 or IL-7 may mimic the effects of lymphopenia required for such an efficient ACT of naïve $CD4^+$ T cells. These findings highlight the potential of harnessing host DC or *ex vivo* derived DC to better control ACT in the future.

Can chemotherapy reset tumor immunogenicity?

Certain tumor cell death modalities differentially triggered by routinely administered chemotherapies can be immunogenic, and act as cryptic vaccines [56]. We indeed characterized the molecular pathways associated with an immunogenic cell death during chemotherapy or radiotherapy of cancer. Anthracyclines, oxaliplatin and X Rays, by promoting an ER stress response before apoptosis, induce the exposure of calreticulin (CRT) [57] and the release of high-mobility group box 1 (HMGB1) from dying tumor cells [58]. CRT and HMGB1 play the role of an 'eat me' and a 'danger' signal, respectively, thereby facilitating engulfment and processing of apoptotic

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bodies by DC. In addition, dying tumor cells must release ATP to engage P2RX7 on host DC, triggering the activation of the inflammasome plateform Nlrp3, culminating in the release of IL-1β, which in turn elicits tumor-specific IFN-γ producing $CD8^+$ T cells that are indispensable for the success of chemotherapy [59].

TRAIL (and Fas)-mediated tumor cell death is accompanied by CRT exposure on dying tumor cells [60]. Interestingly, anti-DR5 antibody targeting mouse TRAIL promoted tumor clearance through a mechanism involving $CD11c^+$DC [61] suggesting a role for DC in cross-presentation of anti-TRAIL Ab-directed dying tumor cells.

Genetically modified tumor vaccines hold promise in breaking immune tolerance to the tumor by various interesting mechanisms [62] but their GMP manufacturing should take into account the 'cell death-associated molecular patterns' that appear crucial for an appropriate orchestration of immune effectors.

Acknowledgements

This work was supported by INFLA-CARE FP7 EU grant, INCa, Fondation pourl a Recherche Médicale, Fondation de France. YM was supported by China Scholarship Council.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Fricke I, Gabrilovich DI: **Dendritic cells and tumor microenvironment: a dangerous liaison**. *Immunol Invest* 2006, **35**:459-483.

2. Chaput N, Conforti R, Viaud S, Spatz A, Zitvogel L: **The Janus face of dendritic cells in cancer**. *Oncogene* 2008, **27**:5920-5931.

3. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mule J, Kerr WG *et al.*: **Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity**. *Nat Med* 2005, **11**:1314-1321.

4. Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP, Bremond A, Goddard S, Pin JJ, Barthelemy-Dubois C *et al.*: **Dendritic cell infiltration and prognosis of early stage breast cancer**. *Clin Cancer Res* 2004, **10**:7466-7474.

5. Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, Biota C, Doffin AC, Durand I, Olive D *et al.*: **Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome**. *Cancer Res* 2009, **69**:2000-2009.

6. •• Cao W, Bover L, Cho M, Wen X, Hanabuchi S, Bao M, Rosen DB, Wang YH, Shaw JL, Du Q *et al.*: **Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction**. *J Exp Med* 2009, **206**:1603-1614.
An important description of a molecular pathway affecting type 1 IFN production by pDC in the context of tumor progression.

7. Ahmadi M, Emery DC, Morgan DJ: **Prevention of both direct and cross-priming of antitumor CD8+ T-cell responses following overproduction of prostaglandin E2 by tumor cells in vivo**. *Cancer Res* 2008, **68**:7520-7529.

8. Yang L, Yamagata N, Yadav R, Brandon S, Courtney RL, Morrow JD, Shyr Y, Boothby M, Joyce S, Carbone DP *et al.*: **Cancer-associated immunodeficiency and dendritic cell abnormalities mediated by the prostaglandin EP2 receptor**. *J Clin Invest* 2003, **111**:727-735.

9. von Bergwelt-Baildon MS, Popov A, Saric T, Chemnitz J, Classen S, Stoffel MS, Fiore F, Roth U, Beyer M, Debey S *et al.*: **CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition**. *Blood* 2006, **108**:228-237.

10. •• Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, Corzo A, Cho HI, Celis E, Lennox B *et al.*: **Lipid accumulation and dendritic cell dysfunction in cancer**. *Nat Med* 2010, **16**:880-886.
A novel lipid metabolic pathway affecting DC integrity and contributing to altered T cell priming.

11. •• Villablanca EJ, Raccosta L, Zhou D, Fontana R, Maggioni D, Negro A, Sanvito F, Ponzoni M, Valentinis B, Bregni M *et al.*: **Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses**. *Nat Med* 2010, **16**:98-105.
Interesting report showing that tumor-derived ligands of LXRs can compromise CCR7-dependent dendritic cell homing to tumor-draining lymph nodes.

12. Perego M, Tortoreto M, Tragni G, Mariani L, Deho P, Carbone A, Santinami M, Patuzzo R, Mina PD, Villa A *et al.*: **Heterogeneous phenotype of human melanoma cells with in vitro and in vivo features of tumor-initiating cells**. *J Invest Dermatol* 2010, **130**:1877-1886.

13. Dhodapkar KM, Feldman D, Matthews P, Radfar S, Pickering R, Turkula S, Zebroski H, Dhodapkar MV: **Natural immunity to pluripotency antigen OCT4 in humans**. *Proc Natl Acad Sci U S A* 2010, **107**:8718-8723.

14. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S: **Identification of Tim4 as a phosphatidylserine receptor**. *Nature* 2007, **450**:435-439.

15. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS: **BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module**. *Nature* 2007, **450**:430-434.

16. Jinushi M, Sato M, Kanamoto A, Itoh A, Nagai S, Koyasu S, Dranoff G, Tahara H: **Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms**. *J Exp Med* 2009, **206**:1317-1326.

17. •• A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, Deniz J, Ramirez C, Diaz M, Gallardo G *et al.*: **Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR**. *Immunity* 2009, 31:245-258.
A novel mechanism of immune tolerance to dying cells: Apoptotic cells can activate LXR in macrophage, promoting self-clearance and avoiding inflammation.

18. Gude DR, Alvarez SE, Paugh SW, Mitra P, Yu J, Griffiths R, Barbour SE, Milstien S, Spiegel S: **Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal**. *FASEB J* 2008, **22**:2629-2638.

19. Du W, Takuwa N, Yoshioka K, Okamoto Y, Gonda K, Sugihara K, Fukamizu A, Asano M, Takuwa Y: **S1P(2), the G protein-coupled receptor for sphingosine-1-phosphate, negatively regulates tumor angiogenesis and tumor growth in vivo in mice**. *Cancer Res* 2010, **70**:772-781.

20. Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S *et al.*: **Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal**. *Cell* 2003, **113**:717-730.

21. Peter C, Waibel M, Radu CG, Yang LV, Witte ON, Schulze-Osthoff K, Wesselborg S, Lauber K: **Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A**. *J Biol Chem* 2008, **283**:5296-5305.

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22. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R,
• Traver D, van Rooijen N, Weissman IL: **CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis**. *Cell* 2009, **138**:271-285.
A seminal report showing that myeloid leukemia evade phagocytosis and macrophage killing by upregulating CD47 expression.

23. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD Jr, van Rooijen N, Weissman IL: **CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells**. *Cell* 2009, **138**:286-299.

24. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM: **Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte**. *Cell* 2005, **123**:321-334.

25. Villadangos JA, Schnorrer P: **Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo**. *Nat Rev Immunol* 2007, **7**:543-555.

26. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, Yagita H, Okumura K: **Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation**. *Blood* 2009, **113**:3821-3830.

27. Qiu CH, Miyake Y, Kaise H, Kitamura H, Ohara O, Tanaka M: **Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens**. *J Immunol* 2009, **182**:4127-4136.

28. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L *et al.*: **Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures**. *J Clin Oncol* 2008, **26**:4410-4417.

29. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA: **Induction
• of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21**. *Science* 2010, **328**:749-752.
CCL21 secretion by tumors induces a tolerogenic microenvironment with stromal features resembling those of the LN paracortex.

30. Forster R, Davalos-Misslitz AC, Rot A: **CCR7 and its ligands: balancing immunity and tolerance**. *Nat Rev Immunol* 2008, **8**:362-371.

31. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM *et al.*: **A critical function for type I interferons in cancer immunoediting**. *Nat Immunol* 2005, **6**:722-729.

32. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H,
• Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS *et al.*: **Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity**. *Science* 2008, **322**:1097-1100.
This pioneering study describing a transcription factor controlling the development of CD8α^+ DC and its relevance for tumor antigen cross-presentation.

33. Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, Rosewell I, Reis e Sousa C: **Identification of a dendritic cell receptor that couples sensing of necrosis to immunity**. *Nature* 2009, **458**:899-903.

34. Sancho D, Mourao-Sa D, Joffre OP, Schulz O, Rogers NC,
•• Pennington DJ, Carlyle JR, Reis e Sousa C: **Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin**. *J Clin Invest* 2008, **118**:2098-2110.
These studies unravel a key lectin-type receptor of necrotic cells involved in cross-presentation of cell associated antigens in both mouse CD8α^+DC and human BDCA3high DC.

35. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L,
•• Chen JL, Keller AM, Joffre O, Zelenay S, Nye E *et al.*: **Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells**. *J Exp Med* 2010, **207**:1261-1271.
These studies unravel a key lectin-type receptor of necrotic cells involved in cross-presentation of cell associated antigens in both mouse CD8α^+DC and human BDCA3high DC.

36. Qu C, Nguyen VA, Merad M, Randolph GJ: **MHC class I/peptide
• transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells**. *J Immunol* 2009, **182**:3650-3659.
A comprehensive study analyzing cross-dressing versus cross-presentation by different DC subsets for T cell triggering.

37. Dolan BP, Gibbs KD Jr, Ostrand-Rosenberg S: **Tumor-specific CD4+ T cells are activated by ''cross-dressed'' dendritic cells presenting peptide-MHC class II complexes acquired from cell-based cancer vaccines**. *J Immunol* 2006, **176**:1447-1455.

38. Dolan BP, Gibbs KD Jr, Ostrand-Rosenberg S: **Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells**. *J Immunol* 2006, **177**:6018-6024.

39. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D *et al.*: **Effector memory T cells, early metastasis, and survival in colorectal cancer**. *N Engl J Med* 2005, **353**:2654-2666.

40. Zammit DJ, Cauley LS, Pham QM, Lefrancois L: **Dendritic cells maximize the memory CD8 T cell response to infection**. *Immunity* 2005, **22**:561-570.

41. Belz GT, Bedoui S, Kupresanin F, Carbone FR, Heath WR: **Minimal activation of memory CD8+ T cell by tissue-derived dendritic cells favors the stimulation of naive CD8+ T cells**. *Nat Immunol* 2007, **8**:1060-1066.

42. Terasawa M, Nagata K, Kobayashi Y: **Neutrophils and monocytes transport tumor cell antigens from the peritoneal cavity to secondary lymphoid tissues**. *Biochem Biophys Res Commun* 2008, **377**:589-594.

43. Tomihara K, Guo M, Shin T, Sun X, Ludwig SM, Brumlik MJ,
• Zhang B, Curiel TJ, Shin T: **Antigen-specific immunity and cross-priming by epithelial ovarian carcinoma-induced CD11b(+)Gr-1(+) cells**. *J Immunol* 2010, **184**:6151-6160.
A pioneering report demonstrating that neutrophils can engulf tumor cells and cross-present tumor antigens to T lymphocytes.

44. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H: **Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma**. *J Clin Oncol* 2009, **27**:4709-4717.

45. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, Beutler B, Janssen E, Hoebe K: **NK-cell-mediated killing of target cells triggers robust antigen-specific T-cell-mediated and humoral responses**. *Blood* 2009, **113**:6593-6602.

46. Adam C, King S, Allgeier T, Braumuller H, Luking C, Mysliwietz J, Kriegeskorte A, Busch DH, Rocken M, Mocikat R: **DC-NK cell cross talk as a novel CD4+ T-cell-independent pathway for antitumor CTL induction**. *Blood* 2005, **106**:338-344.

47. Vitale M, Della Chiesa M, Carlomagno S, Pende D, Arico M, Moretta L, Moretta A: **NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor**. *Blood* 2005, **106**:566-571.

48. Andrews DM, Estcourt MJ, Andoniou CE, Wikstrom ME, Khong A, Voigt V, Fleming P, Tabarias H, Hill GR, van der Most RG *et al.*: **Innate immunity defines the capacity of antiviral T cells to limit persistent infection**. *J Exp Med* 2010, **207**:1333-1343.

49. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S: **Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells**. *J Exp Med* 2007, **204**:2641-2653.

50. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, Rossjohn J, Perlmutter P, Cao J, Godfrey DI *et al.*: **Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs**. *Nat Immunol* 2010, **11**:313-320.

51. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, Brenner MB: **CD1-mediated gamma/delta T cell maturation of dendritic cells**. *J Exp Med* 2002, **196**:1575-1584.

52. Takeda K, Kojima Y, Uno T, Hayakawa Y, Teng MW, Yoshizawa H, Yagita H, Gejyo F, Okumura K, Smyth MJ: **Combination therapy of established tumors by antibodies targeting immune**

Please cite this article in press as: Ma Y, et al. The dendritic cell–tumor cross-talk in cancer, Curr Opin Immunol (2010), doi:10.1016/j.coi.2010.09.008

activating and suppressing molecules. *J Immunol* 2010, **184**:5493-5501.

53. Ali OA, Emerich D, Dranoff G, Mooney DJ: **In situ regulation of DC subsets and T cells mediates tumor regression in mice**. *Sci Transl Med* 2009, **1**:8ra19.

•• This study elegantly indicates that all DC subsets should be coordinated and cooperate to elicit a proper microenvironment for vaccine efficacy.

54. Xie Y, Akpinarli A, Maris C, Hipkiss EL, Lane M, Kwon EK, Muranski P, Restifo NP, Antony PA: **Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma**. *J Exp Med* 2010, **207**:651-667.

55. Menke J, Zeller GC, Kikawada E, Means TK, Huang XR, Lan HY, Lu B, Farber J, Luster AD, Kelley VR: **CXCL9, but not CXCL10, promotes CXCR3-dependent immune-mediated kidney disease**. *J Am Soc Nephrol* 2008, **19**:1177-1189.

56. Ma Y, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C, Tesniere A, Martins I, Ly A, Haynes NM *et al.*: **Chemotherapy and radiotherapy: cryptic anticancer vaccines**. *Semin Immunol* 2010, **22**:113-124.

57. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N *et al.*: **Calreticulin exposure dictates the immunogenicity of cancer cell death**. *Nat Med* 2007, **13**:54-61.

58. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P *et al.*: **Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy**. *Nat Med* 2007, **13**:1050-1059.

59. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E *et al.*: **Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors**. *Nat Med* 2009, **15**:1170-1178.

60. Obeid M, Tesniere A, Panaretakis T, Tufi R, Joza N, van Endert P, Ghiringhelli F, Apetoh L, Chaput N, Flament C *et al.*: **Ecto-calreticulin in immunogenic chemotherapy**. *Immunol Rev* 2007, **220**:22-34.

61. Haynes NM, Hawkins ED, Li M, McLaughlin NM, Hammerling GJ, Schwendener R, Winoto A, Wensky A, Yagita H, Takeda K *et al.*: **CD11c(+) dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors**. *J Immunol* 2010, **185**:532-541.

62. Jinushi M, Hodi FS, Dranoff G: **Enhancing the clinical activity of granulocyte-macrophage colony-stimulating factor-secreting tumor cell vaccines**. *Immunol Rev* 2008, **222**:287-298.

Please cite this article in press as: Ma Y, et al. The dendritic cell–tumor cross-talk in cancer, Curr Opin Immunol (2010), doi:10.1016/j.coi.2010.09.008

Cancer Metastasis Rev (2011) 30:71–82
DOI 10.1007/s10555-011-9283-2

How to improve the immunogenicity of chemotherapy and radiotherapy

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Published online: 5 February 2011

Abstract Chemotherapy or radiotherapy could induce various tumor cell death modalities, releasing tumor-derived antigen as well as danger signals that could either be captured for triggering antitumor immune response or ignored. Exploring the interplay among therapeutic drugs, tumor cell death and the immune cells should improve diagnostic, prognostic, predictive, and therapeutic management of tumor. We summarized some of the cell death-derived danger signals and the mechanism for host to sense and response to cell death in the tumor microenvironment. Based on the recent clinical or experimental findings, several strategies have been suggested to improve the immunogenicity of cell death and augment antitumor immunity.

Keywords Cell death · DAMP · Tumor microenvironment · Immune cells

Abbreviations

DC	Dendritic cells
ALK	Anaplastic lymphoma kinase
ALCL	Anaplastic large cell lymphoma
DAMP	Damage-associated molecular patterns
HSP	Heat shock proteins
LysoPC	Lysophosphatidylcholine
HMGB1	High-mobility group box 1 protein
CRT	Calreticulin
ER	Endoplasmic reticulum
Tim	T cell immunoglobulin mucin
Mincle	Macrophage-inducible C-type lectin
SAP130	Spliceosome-associated protein 130
MDSCs	Myeloid-derived suppressor cells
DNAM-1	DNAX accessory molecule-1
SAP130	Spliceosome-associated protein 130
Treg	Regulatory T cells

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MDR	Multidrug resistance
CTX	Cyclophosphamide
TSC	Tumor stem cells
DLN	Draining lymph node
IDO	Indoleamine-pyrrole 2,3-dioxygenase

1 Immunogenicity of chemotherapy and radiotherapy

Chemotherapy and radiotherapy are commonly believed to kill cancer cells by apoptosis. This cell death modality is generally considered as a non-immunogenic. However, massive cell death might saturate the local capacity of silent corps removal, causing the accumulation of late-stage apoptotic cells. What is more important is that accumulating evidence suggest that certain chemotherapeutic agents and ionizing radiation could confer dying tumor cells immunogenic.

Irradiated tumor cells (cell lines or autologous dissociated tumor pieces) engineered to secrete GM-CSF are able to mobilize dendritic cells (DC), plasma cells, invariant NKT cells, and tumor-reactive $CD4^+$ and $CD8^+$ T cells, both in mice and in metastatic cancer patients [1]. This vaccine could promote tumor destruction, necrosis, and fibrosis correlating with humoral immune responses and favorable clinical outcome [2, 3]. In a lung metastasis model (B16F0 melanoma), irradiation of cutaneous melanomas prior to their resection resulted in more than a 20-fold reduction in lung metastases after systemic challenge with untreated melanoma cells. This study suggests that neoadjuvant irradiation of cutaneous melanoma tumors prior to surgical resection can stimulate an endogenous anti-melanoma immune response [4].

Our group has performed screening of anticancer compounds for their ability to induce immunogenic cancer cell death. In the absence of any adjuvant, subcutaneous inoculation of dying CT26 tumor cells pretreated with some chemotherapeutic agents could prevent tumor growth upon live CT26 cell rechallenge in immunocompetent Balb/c mice. Anthracyclines (daunorubicin, idarubicin, mitoxanthrone) were the most potent immunogenic cell death inducers not only in CT26 colon cancer but also in EL4 thymomas and MCA205 sarcomas [5, 6].

Furthermore, chemotherapy and immunotherapy could synergize. DNA-based vaccination targeting an oncogenic protein anaplastic lymphoma kinase (ALK) can immunize mice against anaplastic large cell lymphoma (ALCL) in a $CD8^+$ T cell- and IFN-γ-dependent manner and cure animals bearing advanced ALCL when combined with doxorubicin [7]. In a phase II trial launched in metastatic colon cancer, gemcitabine plus FOLFOX-4 (oxaliplatin, fluorouracil, and folinic acid) polychemotherapy in combination with GM-CSF and IL-2 could elicit tumor antigen-specific immune responses and induce very high objective response and disease control rates [8].

Many chemotherapeutic agents used to treat malignant diseases damage lymphocytes and consequently suppress cell-mediated immunity. More recently, new cancer treatment agents such as tyrosine kinase inhibitors, thalidomide and its derivatives, proteasome inhibitors, and interferons have been found to have diverse immunomodulatory activities blocking immune surveillance of the malignancy and permitting disease recurrence, or, favorably, by reprogramming immunity to increase autologous antitumor effects.

These findings suggest that certain chemotherapy could reset tumor immunogenicity and tumor cell death modalities triggered by specific antitumor response and act as cryptic vaccines [9].

2 Cell death-derived DAMP could induce sterile inflammation

Robust acute inflammation could be triggered by sterile cell death which induces damage-associated molecular patterns (DAMP) exposed on the plasma membrane or secreted extracellularly. These cell-derived DAMP, such as uric acid, DNA (specifically unmethylated CpG-rich regions), HMGB1, SAP130, S100 proteins, and heat shock proteins (HSP) [10–16] could stimulate an IL-1- and inflammasome-dependent response [17]. Interestingly, inflammations triggered by sterile cell death and microbial stimulus differ in their dependency of the IL-1R-Myd88 pathway [18]. Upon chemotherapy or radiotherapy, various cell death modalities occur, including apoptosis, necrosis, autophagy, mitotic catastrophe, and pyroptosis. Here, we will give a brief summary of the DAMP profile derived from dying tumor cells and sensors from host phagocytes, especially focusing on the immunogenic cell death modules.

2.1 “Find me” DAMP

Dying cells could recruit phagocytic cells owing to the release of various “find me” signals. Apoptotic microparticles could transfer chemokine receptors and arachidonic acid between cells, activate complement, promote leukocyte rolling, and stimulate the release of pro-inflammatory mediators [19]. Lysophosphatidylcholine (lysoPC), but none of the lysoPC metabolites or other lysophospholiplids, represents the essential apoptotic attraction signal able to trigger a chemotactic response through phagocyte receptor G2A [20, 21]. The prototypical DAMP high-mobility group box 1 protein (HMGB1) is released with sustained autophagy, late apoptosis, and

necrosis. HMGB1 could act as chemotactic and/or activating factors for macrophages, neutrophils, and DC [22–24]. ATP and UTP released by apoptotic or necrotic cells play a non-redundant role in dying cell clearance by monocytes/macrophages that express the purinergic receptor P2RY2. Forced expression of CD39 (NTPDase-1, an ecto-apyrase responsible for the degradation of NTP) could abrogate the chemoattractant activity of apoptotic cells [25]. Both pan-sphingosine kinase (SphK) inhibitor and chemotherapeutic drug doxorubicin could induce apoptosis and upregulate sphingosine kinase 1 expression, allowing the release of sphingosine-1-phosphate which causes cytoskeletal rearrangements and chemoattraction of macrophages, even at low nanomolar ranges [26]. Despite emerging "find me" signals identified, future research should also dissociate the factors recruiting antitumor *versus* pro-tumor effectors.

2.2 "Eat me" and "don't eat me" signals

Calreticulin (CRT) is a highly conserved Ca^{2+}-binding protein mainly located in the lumen of the endoplasmic reticulum (ER) and also in the nucleus and cytoplasm [27]. Cancer cells treated with UV expose larger amounts of ecto-CRT (CRT on the plasma membrane), which is redistributed in the form of "patches" and pre-dominantly co-localized with phosphatidylserine (PS) [28]. Cytotoxic treatment, such as anthracyclines, oxaliplatin, UVC, and γ-radiation, could induce apoptotic cancer cell death and exposure of ecto-CRT, which became receptive for engulfment by DC [6, 29, 30]. Ecto-CRT exposure was found to be a pre-apoptotic event accompanied by the co-translocation of ERp57. Both Ecto-CRT and ERp57 have been proven to determine the immunogenicity of cell death [31]. ER stress, and more specifically the PERK/eIF2α arm of the unfolded protein response pathway, plays an important role in ecto-CRT/ERp57 translocation [32]. Myeloid leukemia, migrating hematopoietic progenitors, and also solid tumors were found to overexpress CD47, resulting in a reduced uptake by SIRPα-expressing macrophages. Thus, CD47 could act as a "don't eat me" signal that prevents the recognition and removal of apoptotic cells by professional and nonprofessional phagocytes.

Tumors have been shown to overexpress HSP, probably due to the stressful tumor microenvironment [33]. Intracellular overexpression of HSP could inhibit apoptosis and exhibit cytoprotective activity [34], while membrane expression of HSP could be potently immunostimulatory [35]. Ecto-HSP70 and HSP90 could determine the immunogenicity of stressed or dying tumor cells due to their ability to interact with a number of antigen-presenting cells (APC) surface receptors, such as CD91, LOX1, and CD40, and facilitate cross-presentation of tumor antigens [36–38]. Furthermore, HSP could promote DC maturation [39] and activate NK cells [40, 41] and act as an immunoadjuvant. Interestingly, stress tissue-derived HSP are more immunostimulatory than recombinant HSP [42]. Large stress protein (e.g., HSP110 and GRP170) chaperoned protein antigen could induce a superior antitumor response compared with peptide antigen [43]. These findings will provide a rational chaperoning-based antitumor vaccine designing for clinical investigation.

2.3 Sensors of cell death on phagocytes

Several receptors expressed on phagocytes could act as sensors of cell death. Tim (T cell immunoglobulin mucin) family member (Tim4 and Tim1) expression on professional phagocytes, such as resident peritoneal macrophages and splenic dendritic cells, or semiprofessional, non-myeloid phagocytes could specifically bind PS and are critical for the efficient apoptotic cell clearance [44]. Liver X receptor [45], MerTK [46, 47], MFG-E8 [48, 49], and BAI1 [50] are also important phagocyte receptors responsible for apoptotic cell clearance. Blocking these signal pathways may evoke extensive tumor cell apoptosis, revert immunotolerance [51], trigger efficient pro-inflammatory cytokine secretion and cross-presentation of dying tumor cells [48, 52, 53]. Mincle (macrophage-inducible C-type lectin) expression is induced after exposure to various stimuli and stresses on macrophages. It detects dead cell-derived spliceosome-associated protein 130 (SAP130) and acts as the sensor of non-homeostatic cell death for inflammatory response [12, 54].

3 Tumor antigen presentation: APC and mechanisms

APC provide a critical link between tumor cell death and adaptive immunity. Kenneth Murphy's group discovered Batf3 as the transcription factor for CD8α^+ DC, and they proved the critical role of CD8α^+ DC-mediated cross-presentation in tumor rejection using Batf3 knockout mice [55]. By targeting $CD8^+$ and $CD8^-$ DC using chimeric monoclonal antibodies for CD205 and 33D1, respectively, Dudziak et al. [56] showed that these two DC populations are specialized in presenting antigen on major histocompatibility complex (MHC) class I and class II, respectively. As these two populations could capture comparable amounts of soluble and bead-associated antigen, the possible explanation may be that CD8α^+ DC are equipped with a specialized machinery for cross-presentation [57]. Both $CD8^+$ and $CD8^-$ DC are effective at cross-presenting HA tumor antigen, but they may differ in the expression of co-stimulatory receptor, thus contributing to either induction or regulation of tumor-specific responses [58].

Langerin (CD207), a C-type lectin that is sufficient to induce the formation of Birbeck granules, is expressed on Langerhans cell as well as dermal DC [59]. In the skin-draining lymph nodes and spleen, Langerin is also expressed by the resident $CD8^+$ DC at lower levels [60]. The proportion of $CD8^+Langerin^+$ DC in lymphoid tissues varies among inbred mouse strains and is more prominent in Balb/c mice [61]. With genetically engineered antibody targeting extracellular domain of Langerin, Idoyaga et al. [62] showed that OVA targeting Langerin results in efficient presentation to OVA-specific $CD4^+$ and $CD8^+$ transgenic T cells. Intravenous administration of horse cyt c was previously shown to specifically deplete cells capable of shuttling Ag through the cytosol, thereby removing cells capable of cross-presentation [63]. Interestingly, only the $Langerin^+$ compartment of the $CD8\alpha^+$ splenic DC population showed a dramatic dose-dependent reduction in response to horse cyt c, and they are critically involved in the cross-presentation of systemic soluble antigen [64]. Among five distinguishable skin DC subsets, the $CD207^+CD103^+$ dermal DC subset is endowed with the unique capability of cross-presenting antigens expressed by keratinocytes [65].

Splenic marginal metallophilic macrophages (MMM) could efficiently capture and transfer antigen exclusively to splenic $CD8^+$ DC for cross-priming cytotoxic T lymphocytes; thus, tumor antigen targeting to MMM is very effective as an antitumor immunotherapy [66]. After irradiation or chemotherapy, $CD11b^+$ tumor stromal cells (containing immature myeloid cells, macrophages, bone marrow-derived endothelial precursors, and other pro-angiogenic cells such as pericytes) [67] could acquire and cross-present tumor antigen, thus facilitating direct killing of parental tumor cells as well as bystander elimination of antigen loss variants. This process requires the cooperation of $CD4^+$ and $CD8^+$ T cells and can lead to the complete destruction of well-established solid tumors [68–70]. Besides the myeloid lineage, cells of the lymphoid lineage could also act as antigen-presenting cells. Freshly isolated human peripheral blood gammadelta T cells can phagocytose via Ab opsonization and CD16 (FcgammaRIII) [71] and upregulate expression of co-stimulatory MHC class I and II molecules, leading to Ag processing and presentation [72].

Possible mechanisms for the transfer of tumor antigen to DC for cross-presentation include phagocytosis of cell-associated antigens, pinocytosis/endocytosis of soluble antigen, capture of soluble antigen bound to HSP, capture of exosomes, “nibbling” of live tumor cell membranes, and “cross-dressing” whereby DC acquire peptide MHC complexes from contact with dying tumor cells [73]. Interestingly, DC could also receive preprocessed antigenic peptides from tumor directly through gap junction. Infecting both human and murine melanoma cells with *Salmonella* can induce connexin 43 (Cx43) upregulation and facilitate gap junction formation between DC and tumor cells. The Cx43-dependent cross-presentation pathway provides a novel strategy for DC loading [74].

4 Antitumor therapy and tumor microenvironment

4.1 Immune effectors in tumor microenvironment

The tumor microenvironment contains innate immune cells ($\gamma\delta$ T, NK cells, neutrophils, macrophages, mast cells, myeloid-derived suppressor cells (MDSC), and DC) and adaptive immune cells (T and B lymphocytes) in addition to tumor cells and their surrounding stroma (fibroblasts, endothelial cells, pericytes, and mesenchymal cells) [75].

Natural cytotoxicity receptors and DNAX accessory molecule-1 (DNAM-1) are critical for NK cell-mediated innate immunity to melanoma cells [76, 77]. Reduced DNAM-1 expression on bone marrow NK cells is associated with impaired killing of $CD34^+$ blasts in myelodysplastic syndrome [78]. DNA-damaging agents can induce the expression of NKG2D ligands on tumor cells [79]. Myeloma cells treated with low doses of common therapeutic agents, such as doxorubicin, melphalan, and bortezomib, upregulate DNAM-1 and NKG2D ligands [80]. Novel therapeutic drugs such as histone deacetylase inhibitors could induce MICA and MICB [81], poliovirus receptor (CD155), and Nectin-2 (CD112) [82] expression on tumor cells, leading to better NK-mediated killing via NKG2D and DNAM-1. Chemotherapy-induced genotoxic stress could promote MHC-independent NKR-P1B:Clr-b missing self-axis in leukemia cells and enhance cytotoxicity mediated by NKR-P1B(+) NK cells [83]. Immunomodulatory drugs lenalidomide could induce $\gamma\delta$ T cell expansion, improvement of IFN-γ secretion, and enhancement of cytotoxicity as well as inducing expression of CD1c on tumor cells. Proteasome inhibitors bortezomib, the first proteasome inhibitor to be used in the treatment of multiple myeloma, can also sensitize tumor cells to chemotherapeutic drugs or radiation through the improvement of TRAIL-mediated lysis or NK-mediated killing by downregulation of HLA class I molecules and upregulation of DNAM-1 and NKG2D ligands [84]. Azacytidine enhances tumor antigenicity by upregulating MHC class I and tumor antigen expression, increasing the release of pro-inflammatory cytokines and danger signals, and promoting antigen uptake by DC and killing by NK cells.

Infiltration of the primary tumor by memory T cells, particularly of the Th1 and cytotoxic types, is the strongest prognostic factor in terms of disease-free and overall survival at all stages of clinical disease [85]. We and others

have shown the critical role of tumor-specific cytotoxic T cells (CTLs) during chemotherapy and radiotherapy [5, 86–88]. Depending on different models, their antitumor effect relies on IFN-γ or cytolytic machinery (perforin, granzyme, TRAIL, TNF-α, etc.). Introduction of tumor-specific Th1-dominant immunity has been reported to be crucial for inducing tumor-specific CTL. A combined therapy of local radiation with Th1 cell could augment the generation of tumor-specific CTL at the tumor site and might also be effective for the treatment of distant metastases [88].

Tumor-infiltrating immune cells could also favor pro-tumor immunity [89] and act as mediators of solid tumor metastasis [90]. MDSCs are $CD11b^{+}Gr1^{+}$ cells which accumulate in peripheral blood of cancer patients as well as in tumors and lymphoid organs [91–93]. It is a phenotypically heterogeneous cell population that includes myeloid progenitor cells as well as immature myeloid cells [94]. The suppressive activity of MDSCs is associated with the intracellular metabolism of L-arginine, which serves as a substrate for inducible nitric oxide synthase (iNOS/NOS2) that generates NO and arginase 1 (ARG1) which converts L-arginine into urea and L-ornithine. Tumor-derived exosome-associated Hsp72 could trigger Stat3 activation in MDSCs and determine their suppressive activity in a TLR2/MyD88-dependent manner [95]. MDSC could suppress the activation of $CD4^{+}$ and $CD8^{+}$ T cells through sequestration of cysteine, and perturb T cell trafficking through down-regulating L-selectin [96]. MDSCs caused dissociation between TCR and CD3zeta molecules, disrupting TCR complexes on T cells [97].

Increasing evidence suggests that regulatory T cells (Treg) accumulate in peripheral blood, ascites, tumors, and tumor-draining lymph nodes in a variety of solid cancers such as lung, head and neck, gastrointestinal, and ovarian. They can be preferentially attracted by the CCR4/CCL22 axis to tumor and lymphoid aggregates and correlate with adverse clinical outcome [98, 99]. More recently, human and murine pancreatic adenocarcinomas have been shown to secrete CCL5, which preferentially attracts Treg through CCR5 [100]. Possible mechanisms of their suppressive activity include cell contact-dependent factors such as membrane TGF-β, CTLA-4, perforin/granzyme, extracellular adenosine, gap junction formation infusing cAMP, as well as soluble immunosuppressive cytokines such as IL-10, TGF-β, and IL-35.

4.2 Role of chemotherapeutic drugs on immune cells

Besides acting on tumor cells, chemotherapeutic drugs also regulate the immune effectors. Paclitaxel, cisplatin, and doxorubicin could upregulate mannose-6-phosphate receptors on tumor cells of mouse and human origin and increase their permeability to granzyme B to facilitate NK and CTL killing [101]. Paclitaxel and cyclophosphamide (CTX) appear to amplify the antigen-specific Th1 response [102] and CTX could reduce Treg in both mice and in end-stage cancer patients [103–105], while tyrosine kinase inhibitors such as imatinib and dasatinib are immunosuppressive, blocking T cell function but sparing $CD4^{+}$ $CD25^{+}$ $FoxP3^{+}$ Treg. Vinblastine, chlorambucil, and docetaxel could inhibit cytotoxic T cell- and NK cell-mediated killing of target cells, while asparaginase, bleomycin, and doxorubicin could enhance it [106]. STAT3 inhibitors and TK inhibitors such as sunitinib could inhibit MDSC maturation, and gemcitabine can block MDSC accumulation [107].

Targeting the tumor microenvironment with more sophisticated and selective tumoricidal drugs could differentially regulate tumor-promoting or tumor-eliminating immune cells and improve the therapeutic outcome [108].

5 Strategies to improve immunogenicity of tumor cell death during cytotoxic therapy

5.1 At the level of tumor

Decreased intracellular drug concentration mediated by multidrug resistance (MDR) gene products, alterations in drug activity, and half-life enhanced DNA repair and defects in cell death pathways could account for chemoresistance, which remains one of the most significant obstacles to the progress of chemotherapy [109]. Possible chemosensitization strategies include cocktail therapy, blocking drug resistance gene expression or activity as well as modulation of the cell death pathways.

Mutated N-Ras oncogene has recently been implicated in melanoma resistance to cisplatin, both *in vitro* and *in vivo* [110]. Overexpression of Bcl-2, Bcl-xL, and Mcl-1 and mutation of p53 in malignant cells contribute to the anti-apoptotic axis [111]. Therapeutics designed to reboot the pro-apoptotic axis will make chemotherapy-induced death the only option. Secretory clusterin could stabilize Ku70-Bax complexes as a protein chaperone, retenting Bax in the cytosol to prevent cytochrome c release which triggers cell apoptosis [112]. Its expression leads to broad-based resistance to chemotherapeutic agents such as doxorubicin, cisplatin, etoposide, camphothecin, and death-inducing molecules (TNF-α, Fas and TRAIL, or histone deacetylase inhibitors) [113]. Mer (MerTK) and Axl receptor tyrosine kinases are expressed at abnormally high levels in a variety of malignancies. Inhibiting their activity or knockdown of their expression could increase apoptosis and perhaps also reduce the silent clearance of tumor cells by phagocytes [114]. Interestingly, depending on its redox status, reducible HMGB1 induces Beclin 1-

dependent autophagy and promotes tumor resistance to chemotherapeutic agents or ionizing radiation, while oxidized HMGB1 increases the cytotoxicity of these agents and induces apoptosis via the mitochondrial pathway [115]. Galectin-3, a beta-galactoside-binding protein with anti-apoptotic activity, protects papillary thyroid cancer against both TRAIL- and doxorubicin-induced apoptosis, at least partially through he PI3K-Akt axis [116].

Both ER stress and autophagy follow a "yin–yang" principle by which their low to moderate activity is cell protective and supports chemoresistance ("yin"), but where severe conditions will aggravate these mechanisms to the point where they abandon their protective efforts and instead will trigger cell death ("yang") [117].

Some tumor cells survive and grow again after cytotoxic therapy, probably arising from the residual self-renewing tumor stem cells (TSC) which possess MDR properties. Preclinical studies suggested targeting leukemia stem cell surface molecules using antibody to enhance leukemia therapy [118]. Identification of components in the tumor microenvironment required for maintaining self-renewal, differentiation, and quiescence of TSC in the face of cytotoxic therapeutic regimens could also help in targeting TSC niches to prevent ultimate recurrence [119].

5.2 At the level of immune system

5.2.1 Antitumor vaccination

Each of the components of tumor vaccine (antigens, adjuvants, delivery systems) contributes specifically to induction and maintenance of T cell responses. Tumor-specific antigens (MAGE-A, NY-ESO-1, etc.), oncogenic proteins which are overexpressed in tumors (WT1 protein), as well as antigens selectively expressed by tumor-initiating cells or cancer stem cells are ideal targets for vaccine designing [120]. Depending on the delivery vehicles (such as liposomes, virosomes, DC, etc.), antitumor vaccines differ in their ability to induce various immune response as well as the intensity of immune response [121]. The first generation of DC-based tumor vaccine proves that this strategy is feasible to induce, regulate, and maintain T cell immunity [122, 123], and further improvement should be made to generate quantitative and qualitative CTLs and T helper cells as well as to break the immunosuppressive microenvironment. Cytokines (GM-CSF, IL-2, IFN, Flt-3 ligand), saponins, bacterial exotoxins, and, most importantly, TLR/NLR/RLR ligands are commonly used immunoadjuvants for antitumor vaccine [121]. CpG+OVA-liposome administered near the draining lymph node (DLN) of the tumor mass plus radiation-augmented induction of OVA-specific CTLs in DLN of tumor-bearing mice greatly inhibited tumor growth, and approximately 60% of the mice treated were completely cured [124]. Our group developed a combined therapy using vaccination, chemotherapy, and TLR3 agonist ploy(A:U) (VCT) against B16OVA melanoma and GL-26 glioma. Type I IFN and poly(A:U) could induce tumor cells and produce large amounts of CCL5 and CXCL10. Interestingly, VCT therapy relies on CXCR3-expressing CTLs and could be further improved when CCL5 derived from tumor or CCR5 expression on the host is blocked. Combination of chemotherapy and TLR3 agonist could not control tumor growth unless vaccination is given in advance [125]. TLR7

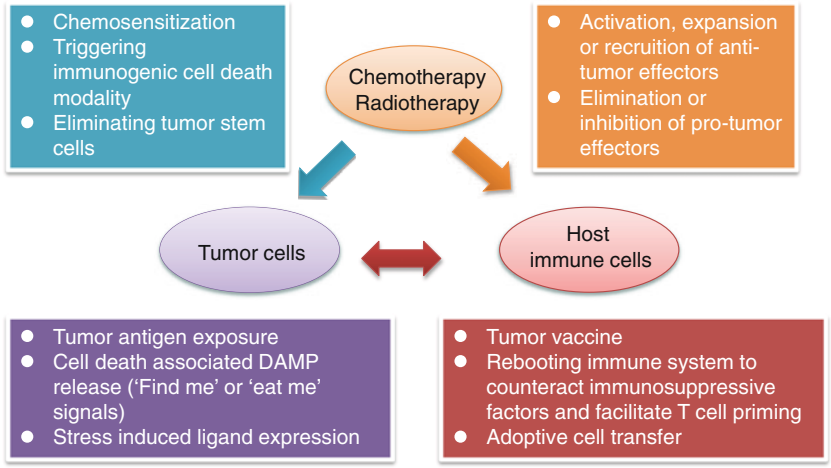

Fig. 1 Strategies to improve the immunogenicity of chemotherapy and radiotherapy. The interplay among chemotherapy and radiotherapy, tumor cells, and host immune system determines the therapeutic outcome. Strategies aiming at triggering sufficient immunogenic cell death and resetting tumor microenvironment at the level of therapy, releasing tumor-derived antigen, danger signals, and stress-induced molecules at the level of tumor are listed. In addition, the efficacy of chemotherapy and radiotherapy could further be improved in combination with tumor vaccine, immunomodulation, and adoptive cell transfer

and TLR8 ligands could also trigger pro-inflammatory cytokines, chemokines, and type I interferon production and upregulate co-stimulatory molecule expression [121].

5.2.2 Blocking immunosuppressive factors

A small population of plasmacytoid DCs in mouse tumor-draining LNs can express immunosuppressive enzyme, indoleamine-pyrrole 2,3-dioxygenase (IDO), which directly activates resting Treg for potent suppressor activity [126]. Inhibiting IDO with 1-methyl-tryptophan could enhance the antitumor efficacy elicited by DC-based vaccine [127]. Stat3 is the key transcription factor mediating immunosuppression. Silencing Stat3 combined with CpG greatly increases killing activity and tumor infiltration of transferred T cells [128]. TGF-β expression in the tumor microenvironment modulates a complex web of intercellular interactions that aggregately promote metastasis and progression. TGF-beta antibodies could reverse this effect [129]. IL-6 is a key molecule involved in malignancies and could activate Stat3 signaling [130]. Targeting IL-6R using antibody could significantly reduce tumor growth and suppress tumor angiogenesis [131, 132].

5.2.3 Enhancing T cell priming

Co-expression of inhibitory molecules LAG-3 and PD-1, Tim-3 and PD-1 on $CD8^+$ T cells is associated with impaired in IFN-γ/TNF-alpha production (T cell anergy or exhaustion) [133–136]. B and T lymphocyte attenuator was identified as a novel inhibitory receptor with structural and functional similarities to CTLA-4 and PD-1 [137]. Antibody targeting these inhibitory receptors could reverse T cell anergy and prolong and sustain T cell activation and proliferation [138–141]. Combination of three agonist antibodies consisting of anti-DR5, anti-CD40, and anti-CD137 could eradicate a large proportion of subcutaneous renal cell carcinoma tumors (75% long-term survival) and orthotopic tumors (55% survival) in combination with IL-2 [142].

5.2.4 Adoptive cellular therapy

Adoptive transfer of autologous tumor-infiltrating T cells expanded *in vitro* leads to potent antitumor responses in patients with refractory metastatic melanoma after lymphodepletion [143]. Without the need for *in vitro* expansion, small numbers of naive tumor-reactive $CD4^+$ T cells transfer into lymphopenic recipients in combination with CTLA-4 blockade and could eradiate poorly immunogenic established B16 melanoma and spontaneous mouse melanoma [144]. After expansion, *in vitro*, polarized tumor-reactive Th17 and Tc17 are capable of rejecting established melanoma [145, 146]. Adoptive transferred haploidentical NK cells can persist and expand *in vivo* and help in the treatment of poor prognosis acute myeloid leukemia [147]. Transfusion of gene-modifying primary mouse NK cells expressing specific receptor for tumor-associated antigen could inhibit tumor progression [148].

6 Conclusions

Effective antitumor therapy should induce sufficient tumor cell death in order to release tumor antigen as well as danger signals attracting phagocytes to uptake and present tumor antigen for specific adaptive immunity. Proper cell death modality should be triggered in both tumor cells, tumor stem cell, and stromal cells. Combining cocktail regimen of chemotherapy and radiotherapy with tumor-specific vaccine using proper immunoadjuvant as well as counteracting the immunosuppressive factors in tumor microenvironment will harness the maximum antitumor response following tumor cell death (Fig. 1).

Acknowledgment The authors thank INFLA-CARE FP7 EU grant, INCa, Fondation pourla Recherche Médicale, and Fondation de France. YM was supported by China Scholarship Council.

References

1. Hodi, F. S., & Dranoff, G. (2006). Combinatorial cancer immunotherapy. *Advances in Immunology, 90*, 341–368. doi:10.1016/S0065-2776(06)90009-1.
2. Soiffer, R., Hodi, F. S., Haluska, F., Jung, K., Gillessen, S., Singer, S., et al. (2003). Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte–macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. *Journal of Clinical Oncology, 21*(17), 3343–3350. doi:10.1200/JCO.2003.07.005.
3. Salgia, R., Lynch, T., Skarin, A., Lucca, J., Lynch, C., Jung, K., et al. (2003). Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte–macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. *Journal of Clinical Oncology, 21*(4), 624–630.
4. Perez, C. A., Fu, A., Onishko, H., Hallahan, D. E., & Geng, L. (2009). Radiation induces an antitumour immune response to mouse melanoma. *International Journal of Radiation Biology, 85*(12), 1126–1136. doi:10.3109/09553000903242099.
5. Casares, N., Pequignot, M. O., Tesniere, A., Ghiringhelli, F., Roux, S., Chaput, N., et al. (2005). Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *The Journal of Experimental Medicine, 202*(12), 1691–1701. doi:10.1084/jem.20050915.
6. Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J. L., et al. (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Natural Medicines, 13*(1), 54–61. doi:10.1038/nm1523.

7. Chiarle, R., Martinengo, C., Mastini, C., Ambrogio, C., D'Escamard, V., Forni, G., et al. (2008). The anaplastic lymphoma kinase is an effective oncoantigen for lymphoma vaccination. *Natural Medicines, 14*(6), 676–680. doi:10.1038/nm1769.
8. Correale, P., Cusi, M. G., Tsang, K. Y., Del Vecchio, M. T., Marsili, S., Placa, M. L., et al. (2005). Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *Journal of Clinical Oncology, 23*(35), 8950–8958. doi:10.1200/JCO.2005.12.147.
9. Ma, Y., Kepp, O., Ghiringhelli, F., Apetoh, L., Aymeric, L., Locher, C., et al. (2010). Chemotherapy and radiotherapy: Cryptic anticancer vaccines. *Seminars in Immunology, 22*(3), 113–124. doi:10.1016/j.smim.2010.03.001.
10. Imaeda, A. B., Watanabe, A., Sohail, M. A., Mahmood, S., Mohamadnejad, M., Sutterwala, F. S., et al. (2009). Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *Journal of Clinical Investigation, 119*(2), 305–314. doi:10.1172/JCI35958.
11. Scaffidi, P., Misteli, T., & Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature, 418*(6894), 191–195. doi:10.1038/nature00858.
12. Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., & Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nature Immunology, 9*(10), 1179–1188. doi:10.1038/ni.1651.
13. Foell, D., Wittkowski, H., Vogl, T., & Roth, J. (2007). S100 proteins expressed in phagocytes: A novel group of damage-associated molecular pattern molecules. *Journal of Leukocyte Biology, 81*(1), 28–37. doi:10.1189/jlb.0306170.
14. Barrat, F. J., Meeker, T., Gregorio, J., Chan, J. H., Uematsu, S., Akira, S., et al. (2005). Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *The Journal of Experimental Medicine, 202*(8), 1131–1139. doi:10.1084/jem.20050914.
15. Osterloh, A., Veit, A., Gessner, A., Fleischer, B., & Breloer, M. (2008). Hsp60-mediated T cell stimulation is independent of TLR4 and IL-12. *International Immunology, 20*(3), 433–443. doi:10.1093/intimm/dxn003.
16. Basu, S., Binder, R. J., Ramalingam, T., & Srivastava, P. K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity, 14*(3), 303–313.
17. Rock, K. L., Latz, E., Ontiveros, F., & Kono, H. (2010). The sterile inflammatory response. *Annual Review of Immunology, 28*, 321–342. doi:10.1146/annurev-immunol-030409-101311.
18. Chen, C. J., Kono, H., Golenbock, D., Reed, G., Akira, S., & Rock, K. L. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Natural Medicines, 13*(7), 851–856. doi:10.1038/nm1603.
19. Distler, J. H., Huber, L. C., Gay, S., Distler, O., & Pisetsky, D. S. (2006). Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity, 39*(8), 683–690. doi:10.1080/08916930601061538.
20. Peter, C., Waibel, M., Radu, C. G., Yang, L. V., Witte, O. N., Schulze-Osthoff, K., et al. (2008). Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *The Journal of Biological Chemistry, 283*(9), 5296–5305. doi:10.1074/jbc.M706586200.
21. Lauber, K., Bohn, E., Krober, S. M., Xiao, Y. J., Blumenthal, S. G., Lindemann, R. K., et al. (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell, 113*(6), 717–730.
22. Yang, D., Chen, Q., Yang, H., Tracey, K. J., Bustin, M., & Oppenheim, J. J. (2007). High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *Journal of Leukocyte Biology, 81*(1), 59–66. doi:10.1189/jlb.0306180.
23. Orlova, V. V., Choi, E. Y., Xie, C., Chavakis, E., Bierhaus, A., Ihanus, E., et al. (2007). A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. *The EMBO Journal, 26*(4), 1129–1139. doi:10.1038/sj.emboj.7601552.
24. Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., et al. (2007). Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Natural Medicines, 13*(9), 1050–1059. doi:10.1038/nm1622.
25. Elliott, M. R., Chekeni, F. B., Trampont, P. C., Lazarowski, E. R., Kadl, A., Walk, S. F., et al. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature, 461*(7261), 282–286. doi:10.1038/nature08296.
26. Gude, D. R., Alvarez, S. E., Paugh, S. W., Mitra, P., Yu, J., Griffiths, R., et al. (2008). Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *The FASEB Journal, 22*(8), 2629–2638. doi:10.1096/fj.08-107169.
27. Mesaeli, N., & Phillipson, C. (2004). Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. *Molecular Biology of the Cell, 15*(4), 1862–1870. doi:10.1091/mbc.E03-04-0251.
28. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., et al. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell, 123*(2), 321–334. doi:10.1016/j.cell.2005.08.032.
29. Obeid, M., Tesniere, A., Panaretakis, T., Tufi, R., Joza, N., van Endert, P., et al. (2007). Ecto-calreticulin in immunogenic chemotherapy. *Immunological Reviews, 220*, 22–34. doi:10.1111/j.1600-065X.2007.00567.x.
30. Obeid, M., Panaretakis, T., Joza, N., Tufi, R., Tesniere, A., van Endert, P., et al. (2007). Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. *Cell Death and Differentiation, 14*(10), 1848–1850. doi:10.1038/sj.cdd.4402201.
31. Panaretakis, T., Joza, N., Modjtahedi, N., Tesniere, A., Vitale, I., Durchschlag, M., et al. (2008). The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death and Differentiation, 15*(9), 1499–1509. doi:10.1038/cdd.2008.67.
32. Panaretakis, T., Kepp, O., Brockmeier, U., Tesniere, A., Bjorklund, A. C., Chapman, D. C., et al. (2009). Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *The EMBO Journal, 28*(5), 578–590. doi:10.1038/emboj.2009.1.
33. Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., & Kroemer, G. (2006). Heat shock proteins 27 and 70: Anti-apoptotic proteins with tumorigenic properties. *Cell Cycle, 5*(22), 2592–2601.
34. Lanneau, D., Brunet, M., Frisan, E., Solary, E., Fontenay, M., & Garrido, C. (2008). Heat shock proteins: Essential proteins for apoptosis regulation. *Journal of Cellular and Molecular Medicine, 12*(3), 743–761. doi:10.1111/j.1582-4934.2008.00273.x.
35. Spisek, R., & Dhodapkar, M. V. (2007). Towards a better way to die with chemotherapy: Role of heat shock protein exposure on dying tumor cells. *Cell Cycle, 6*(16), 1962–1965.
36. Tesniere, A., Panaretakis, T., Kepp, O., Apetoh, L., Ghiringhelli, F., Zitvogel, L., et al. (2008). Molecular characteristics of

immunogenic cancer cell death. *Cell Death and Differentiation, 15*(1), 3–12. doi:10.1038/sj.cdd.4402269.
37. Doody, A. D., Kovalchin, J. T., Mihalyo, M. A., Hagymasi, A. T., Drake, C. G., & Adler, A. J. (2004). Glycoprotein 96 can chaperone both MHC class I- and class II-restricted epitopes for *in vivo* presentation, but selectively primes CD8$^+$ T cell effector function. *Journal of Immunology, 172*(10), 6087–6092.
38. Murshid, A., Gong, J., & Calderwood, S. K. (2010). Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. *Journal of Immunology, 185*(5), 2903–2917. doi:10.4049/jimmunol.0903635.
39. Chan, T., Chen, Z., Hao, S., Xu, S., Yuan, J., Saxena, A., et al. (2007). Enhanced T-cell immunity induced by dendritic cells with phagocytosis of heat shock protein 70 gene-transfected tumor cells in early phase of apoptosis. *Cancer Gene Therapy, 14*(4), 409–420. doi:10.1038/sj.cgt.7701025.
40. Elsner, L., Flugge, P. F., Lozano, J., Muppala, V., Eiz-Vesper, B., Demiroglu, S. Y., et al. (2010). The endogenous danger signals HSP70 and MICA cooperate in the activation of cytotoxic effector functions of NK cells. *Journal of Cellular and Molecular Medicine, 14*(4), 992–1002. doi:10.1111/j.1582-4934.2009.00677.x.
41. Fionda, C., Soriani, A., Malgarini, G., Iannitto, M. L., Santoni, A., & Cippitelli, M. (2009). Heat shock protein-90 inhibitors increase MHC class I-related chain A and B ligand expression on multiple myeloma cells and their ability to trigger NK cell degranulation. *Journal of Immunology, 183*(7), 4385–4394. doi:10.4049/jimmunol.0901797.
42. Dhodapkar, M. V., Dhodapkar, K. M., & Li, Z. (2008). Role of chaperones and FcgammaR in immunogenic death. *Current Opinion in Immunology, 20*(5), 512–517. doi:10.1016/j.coi.2008.05.002.
43. Wang, X. Y., Sun, X., Chen, X., Facciponte, J., Repasky, E. A., Kane, J., et al. (2010). Superior antitumor response induced by large stress protein chaperoned protein antigen compared with peptide antigen. *Journal of Immunology, 184*(11), 6309–6319. doi:10.4049/jimmunol.0903891.
44. Kobayashi, N., Karisola, P., Pena-Cruz, V., Dorfman, D. M., Jinushi, M., Umetsu, S. E., et al. (2007). TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity, 27*(6), 927–940. doi:10.1016/j.immuni.2007.11.011.
45. Gonzalez, N., Bensinger, S. J., Hong, C., Beceiro, S., Bradley, M. N., Zelcer, N., et al. (2009). Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity, 31*(2), 245–258. doi:10.1016/j.immuni.2009.06.018.
46. Shao, W. H., Zhen, Y., Eisenberg, R. A., & Cohen, P. L. (2009). The Mer receptor tyrosine kinase is expressed on discrete macrophage subpopulations and mainly uses Gas6 as its ligand for uptake of apoptotic cells. *Clinical Immunology, 133*(1), 138–144. doi:10.1016/j.clim.2009.06.002.
47. Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., et al. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature, 411*(6834), 207–211. doi:10.1038/35075603.
48. Jinushi, M., Sato, M., Kanamoto, A., Itoh, A., Nagai, S., Koyasu, S., et al. (2009). Milk fat globule epidermal growth factor-8 blockade triggers tumor destruction through coordinated cell-autonomous and immune-mediated mechanisms. *The Journal of Experimental Medicine, 206*(6), 1317–1326. doi:10.1084/jem.20082614.
49. Asano, K., Miwa, M., Miwa, K., Hanayama, R., Nagase, H., Nagata, S., et al. (2004). Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. *The Journal of Experimental Medicine, 200*(4), 459–467. doi:10.1084/jem.20040342.
50. Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., Haney, L. B., Ma, Z., et al. (2007). BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature, 450*(7168), 430–434. doi:10.1038/nature06329.
51. Jinushi, M., Nakazaki, Y., Dougan, M., Carrasco, D. R., Mihm, M., & Dranoff, G. (2007). MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF. *Journal of Clinical Investigation, 117*(7), 1902–1913. doi:10.1172/JCI30966.
52. Linger, R. M., Keating, A. K., Earp, H. S., & Graham, D. K. (2008). TAM receptor tyrosine kinases: Biologic functions, signaling, and potential therapeutic targeting in human cancer. *Advances in Cancer Research, 100*, 35–83. doi:10.1016/S0065-230X(08)00002-X.
53. Sen, P., Wallet, M. A., Yi, Z., Huang, Y., Henderson, M., Mathews, C. E., et al. (2007). Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood, 109*(2), 653–660. doi:10.1182/blood-2006-04-017368.
54. Brown, G. D. (2008). Sensing necrosis with Mincle. *Nature Immunology, 9*(10), 1099–1100. doi:10.1038/ni1008-1099.
55. Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., et al. (2008). Batf3 deficiency reveals a critical role for CD8alpha$^+$ dendritic cells in cytotoxic T cell immunity. *Science, 322*(5904), 1097–1100. doi:10.1126/science.1164206.
56. Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., et al. (2007). Differential antigen processing by dendritic cell subsets *in vivo*. *Science, 315*(5808), 107–111. doi:10.1126/science.1136080.
57. Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., et al. (2006). The dominant role of CD8$^+$ dendritic cells in cross-presentation is not dictated by antigen capture. *Proceedings of the National Academy of Sciences of the United States of America, 103*(28), 10729–10734. doi:10.1073/pnas.0601956103.
58. McDonnell, A. M., Prosser, A. C., van Bruggen, I., Robinson, B. W., & Currie, A. J. (2010). CD8alpha$^+$ DC are not the sole subset cross-presenting cell-associated tumor antigens from a solid tumor. *European Journal of Immunology, 40*(6), 1617–1627. doi:10.1002/eji.200940153.
59. Merad, M., Ginhoux, F., & Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other Langerin-expressing dendritic cells. *Nature Reviews. Immunology, 8*(12), 935–947. doi:10.1038/nri2455.
60. Idoyaga, J., Suda, N., Suda, K., Park, C. G., & Steinman, R. M. (2009). Antibody to Langerin/CD207 localizes large numbers of CD8alpha$^+$ dendritic cells to the marginal zone of mouse spleen. *Proceedings of the National Academy of Sciences of the United States of America, 106*(5), 1524–1529. doi:10.1073/pnas.0812247106.
61. Flacher, V., Douillard, P., Ait-Yahia, S., Stoitzner, P., Clair-Moninot, V., Romani, N., et al. (2008). Expression of Langerin/CD207 reveals dendritic cell heterogeneity between inbred mouse strains. *Immunology, 123*(3), 339–347. doi:10.1111/j.1365-2567.2007.02785.x.
62. Idoyaga, J., Cheong, C., Suda, K., Suda, N., Kim, J. Y., Lee, H., et al. (2008). Cutting edge: Langerin/CD207 receptor on dendritic cells mediates efficient antigen presentation on MHC I and II products *in vivo*. *Journal of Immunology, 180*(6), 3647–3650.
63. Lin, M. L., Zhan, Y., Proietto, A. I., Prato, S., Wu, L., Heath, W. R., et al. (2008). Selective suicide of cross-presenting CD8$^+$ dendritic cells by cytochrome c injection shows functional

heterogeneity within this subset. *Proceedings of the National Academy of Sciences of the United States of America, 105*(8), 3029–3034. doi:10.1073/pnas.0712394105.
64. Farrand, K. J., Dickgreber, N., Stoitzner, P., Ronchese, F., Petersen, T. R., & Hermans, I. F. (2009). Langerin$^+$ CD8alpha$^+$ dendritic cells are critical for cross-priming and IL-12 production in response to systemic antigens. *Journal of Immunology, 183*(12), 7732–7742. doi:10.4049/jimmunol.0902707.
65. Henri, S., Poulin, L. F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., et al. (2010). CD207$^+$ CD103$^+$ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *The Journal of Experimental Medicine, 207*(1), 189–206. doi:10.1084/jem.20091964.
66. Backer, R., Schwandt, T., Greuter, M., Oosting, M., Jungerkes, F., Tuting, T., et al. (2010). Effective collaboration between marginal metallophilic macrophages and CD8$^+$ dendritic cells in the generation of cytotoxic T cells. *Proceedings of the National Academy of Sciences of the United States of America, 107*(1), 216–221. doi:10.1073/pnas.0909541107.
67. Zhang, B. (2008). Targeting the stroma by T cells to limit tumor growth. *Cancer Research, 68*(23), 9570–9573. doi:10.1158/0008-5472.CAN-08-2414.
68. Zhang, B., Bowerman, N. A., Salama, J. K., Schmidt, H., Spiotto, M. T., Schietinger, A., et al. (2007). Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. *The Journal of Experimental Medicine, 204*(1), 49–55. doi:10.1084/jem.20062056.
69. Schietinger, A., Philip, M., Liu, R. B., Schreiber, K., & Schreiber, H. (2010). Bystander killing of cancer requires the cooperation of CD4$^+$ and CD8$^+$ T cells during the effector phase. *Journal of Experimental Medicine, 207*, 2469–2477. doi:10.1084/jem.20092450.
70. Spiotto, M. T., Rowley, D. A., & Schreiber, H. (2004). Bystander elimination of antigen loss variants in established tumors. *Natural Medicines, 10*(3), 294–298. doi:10.1038/nm999.
71. Wu, Y., Wu, W., Wong, W. M., Ward, E., Thrasher, A. J., Goldblatt, D., et al. (2009). Human gamma delta T cells: A lymphoid lineage cell capable of professional phagocytosis. *Journal of Immunology, 183*(9), 5622–5629. doi:10.4049/jimmunol.0901772.
72. Brandes, M., Willimann, K., Bioley, G., Levy, N., Eberl, M., Luo, M., et al. (2009). Cross-presenting human gammadelta T cells induce robust CD8$^+$ alphabeta T cell responses. *Proceedings of the National Academy of Sciences of the United States of America, 106*(7), 2307–2312. doi:10.1073/pnas.0810059106.
73. Melief, C. J. (2008). Cancer immunotherapy by dendritic cells. *Immunity, 29*(3), 372–383. doi:10.1016/j.immuni.2008.08.004.
74. Saccheri, F., Pozzi, C., Avogadri, F., Barozzi, S., Faretta, M., Fusi, P., et al. (2010). Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity. *Science Translational Medicine, 2*(44), 4457. doi:10.1126/scitranslmed.3000739.
75. de Visser, K. E., Eichten, A., & Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer, 6*(1), 24–37. doi:10.1038/nrc1782.
76. Lakshmikanth, T., Burke, S., Ali, T. H., Kimpfler, S., Ursini, F., Ruggeri, L., et al. (2009). NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines *in vitro* and *in vivo*. *Journal of Clinical Investigation, 119*(5), 1251–1263. doi:10.1172/JCI36022.
77. Chan, C. J., Andrews, D. M., McLaughlin, N. M., Yagita, H., Gilfillan, S., Colonna, M., et al. (2010). DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases. *Journal of Immunology, 184*(2), 902–911. doi:10.4049/jimmunol.0903225.
78. Carlsten, M., Baumann, B. C., Simonsson, M., Jadersten, M., Forsblom, A. M., Hammarstedt, C., et al. (2010). Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of CD34$^+$ blasts in myelodysplastic syndrome. *Leukemia, 24*(9), 1607–1616. doi:10.1038/leu.2010.149.
79. Diefenbach, A., Jensen, E. R., Jamieson, A. M., & Raulet, D. H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature, 413*(6852), 165–171. doi:10.1038/35093109.
80. Soriani, A., Zingoni, A., Cerboni, C., Iannitto, M. L., Ricciardi, M. R., Di Gialleonardo, V., et al. (2009). ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood, 113*(15), 3503–3511. doi:10.1182/blood-2008-08-173914.
81. Kato, N., Tanaka, J., Sugita, J., Toubai, T., Miura, Y., Ibata, M., et al. (2007). Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells. *Leukemia, 21*(10), 2103–2108. doi:10.1038/sj.leu.2404862.
82. Schmudde, M., Braun, A., Pende, D., Sonnemann, J., Klier, U., Beck, J. F., et al. (2008). Histone deacetylase inhibitors sensitize tumour cells for cytotoxic effects of natural killer cells. *Cancer Letters, 272*(1), 110–121. doi:10.1016/j.canlet.2008.06.027.
83. Fine, J. H., Chen, P., Mesci, A., Allan, D. S., Gasser, S., Raulet, D. H., et al. (2010). Chemotherapy-induced genotoxic stress promotes sensitivity to natural killer cell cytotoxicity by enabling missing-self recognition. *Cancer Research, 70*(18), 7102–7113. doi:10.1158/0008-5472.CAN-10-1316.
84. Rey, J., Veuillen, C., Vey, N., Bouabdallah, R., & Olive, D. (2009). Natural killer and gammadelta T cells in haematological malignancies: Enhancing the immune effectors. *Trends in Molecular Medicine, 15*(6), 275–284. doi:10.1016/j.molmed.2009.04.005.
85. Pages, F., Galon, J., Dieu-Nosjean, M. C., Tartour, E., Sautes-Fridman, C., & Fridman, W. H. (2010). Immune infiltration in human tumors: A prognostic factor that should not be ignored. *Oncogene, 29*(8), 1093–1102. doi:10.1038/onc.2009.416.
86. Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., Ortiz, C., et al. (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Natural Medicines, 15*(10), 1170–1178. doi:10.1038/nm.2028.
87. Lee, Y., Auh, S. L., Wang, Y., Burnette, B., Meng, Y., Beckett, M., et al. (2009). Therapeutic effects of ablative radiation on local tumor require CD8$^+$ T cells: Changing strategies for cancer treatment. *Blood, 114*(3), 589–595. doi:10.1182/blood-2009-02-206870.
88. Takeshima, T., Chamoto, K., Wakita, D., Ohkuri, T., Togashi, Y., Shirato, H., et al. (2010). Local radiation therapy inhibits tumor growth through the generation of tumor-specific CTL: Its potentiation by combination with Th1 cell therapy. *Cancer Research, 70*(7), 2697–2706. doi:10.1158/0008-5472.CAN-09-2982.
89. Johansson, M., Denardo, D. G., & Coussens, L. M. (2008). Polarized immune responses differentially regulate cancer development. *Immunological Reviews, 222*, 145–154. doi:10.1111/j.1600-065X.2008.00600.x.
90. DeNardo, D. G., Johansson, M., & Coussens, L. M. (2008). Immune cells as mediators of solid tumor metastasis. *Cancer and Metastasis Reviews, 27*(1), 11–18. doi:10.1007/s10555-007-9100-0.
91. Zou, W. (2005). Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nature Reviews Cancer, 5*(4), 263–274. doi:10.1038/nrc1586.

92. Zea, A. H., Rodriguez, P. C., Atkins, M. B., Hernandez, C., Signoretti, S., Zabaleta, J., et al. (2005). Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: A mechanism of tumor evasion. *Cancer Research, 65*(8), 3044–3048. doi:10.1158/0008-5472.CAN-04-4505.
93. Serafini, P., De Santo, C., Marigo, I., Cingarlini, S., Dolcetti, L., Gallina, G., et al. (2004). Derangement of immune responses by myeloid suppressor cells. *Cancer Immunology, Immunotherapy, 53*(2), 64–72. doi:10.1007/s00262-003-0443-2.
94. Gabrilovich, D. I., & Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nature Reviews Immunology, 9*(3), 162–174. doi:10.1038/nri2506.
95. Chalmin, F., Ladoire, S., Mignot, G., Vincent, J., Bruchard, M., Remy-Martin, J. P., et al. (2010). Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *Journal of Clinical Investigation, 120*(2), 457–471. doi:10.1172/JCI40483.
96. Ostrand-Rosenberg, S. (2010). Myeloid-derived suppressor cells: More mechanisms for inhibiting antitumor immunity. *Cancer Immunology Immunotherapy, 59*(10), 1593–1600. doi:10.1007/s00262-010-0855-8.
97. Nagaraj, S., Schrum, A. G., Cho, H. I., Celis, E., & Gabrilovich, D. I. (2010). Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *Journal of Immunology, 184*(6), 3106–3116. doi:10.4049/jimmunol.0902661.
98. Gobert, M., Treilleux, I., Bendriss-Vermare, N., Bachelot, T., Goddard-Leon, S., Arfi, V., et al. (2009). Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Research, 69*(5), 2000–2009. doi:10.1158/0008-5472.CAN-08-2360.
99. Menetrier-Caux, C., Gobert, M., & Caux, C. (2009). Differences in tumor regulatory T-cell localization and activation status impact patient outcome. *Cancer Research, 69*(20), 7895–7898. doi:10.1158/0008-5472.CAN-09-1642.
100. Tan, M. C., Goedegebuure, P. S., Belt, B. A., Flaherty, B., Sankpal, N., Gillanders, W. E., et al. (2009). Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer. *Journal of Immunology, 182*(3), 1746–1755.
101. Ramakrishnan, R., Assudani, D., Nagaraj, S., Hunter, T., Cho, H. I., Antonia, S., et al. (2010). Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *Journal of Clinical Investigation, 120*(4), 1111–1124. doi:10.1172/JCI40269.
102. Machiels, J. P., Reilly, R. T., Emens, L. A., Ercolini, A. M., Lei, R. Y., Weintraub, D., et al. (2001). Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Research, 61*(9), 3689–3697.
103. Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., et al. (2004). $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *European Journal of Immunology, 34*(2), 336–344. doi:10.1002/eji.200324181.
104. Roux, S., Apetoh, L., Chalmin, F., Ladoire, S., Mignot, G., Puig, P. E., et al. (2008). $CD4^+CD25^+$ Tregs control the TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in rodent models of colon cancer. *Journal of Clinical Investigation, 118*(11), 3751–3761. doi:10.1172/JCI35890.
105. Ghiringhelli, F., Menard, C., Puig, P. E., Ladoire, S., Roux, S., Martin, F., et al. (2007). Metronomic cyclophosphamide regimen selectively depletes $CD4^+CD25^+$ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunology, Immunotherapy, 56*(5), 641–648. doi:10.1007/s00262-006-0225-8.
106. Markasz, L., Skribek, H., Uhlin, M., Otvos, R., Flaberg, E., Eksborg, S., et al. (2008). Effect of frequently used chemotherapeutic drugs on cytotoxic activity of human cytotoxic T-lymphocytes. *Journal of Immunotherapy, 31*(3), 283–293. doi:10.1097/CJI.0b013e3181628b76.
107. Ugel, S., Delpozzo, F., Desantis, G., Papalini, F., Simonato, F., Sonda, N., et al. (2009). Therapeutic targeting of myeloid-derived suppressor cells. *Current Opinion in Pharmacology, 9*(4), 470–481. doi:10.1016/j.coph.2009.06.014.
108. Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell, 140*(6), 883–899. doi:10.1016/j.cell.2010.01.025.
109. Luqmani, Y. A. (2005). Mechanisms of drug resistance in cancer chemotherapy. *Medical Principles and Practice, 14*(Suppl 1), 35–48. doi:10.1159/000086183.
110. Serrone, L., & Hersey, P. (1999). The chemoresistance of human malignant melanoma: An update. *Melanoma Research, 9*(1), 51–58.
111. Plati, J., Bucur, O., & Khosravi-Far, R. (2008). Dysregulation of apoptotic signaling in cancer: Molecular mechanisms and therapeutic opportunities. *Journal of Cellular Biochemistry, 104*(4), 1124–1149. doi:10.1002/jcb.21707.
112. Trougakos, I. P., Lourda, M., Antonelou, M. H., Kletsas, D., Gorgoulis, V. G., Papassideri, I. S., et al. (2009). Intracellular clusterin inhibits mitochondrial apoptosis by suppressing p53-activating stress signals and stabilizing the cytosolic Ku70-Bax protein complex. *Clinical Cancer Research, 15*(1), 48–59. doi:10.1158/1078-0432.CCR-08-1805.
113. Djeu, J. Y., & Wei, S. (2009). Clusterin and chemoresistance. *Advances in Cancer Research, 105*, 77–92. doi:10.1016/S0065-230X(09)05005-2.
114. Keating, A. K., Kim, G. K., Jones, A. E., Donson, A. M., Ware, K., Mulcahy, J. M., et al. (2010). Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. *Molecular Cancer Therapeutics, 9*(5), 1298–1307. doi:10.1158/1535-7163.MCT-09-0707.
115. Tang, D., Lotze, M. T., Zeh, H. J., & Kang, R. (2010). The redox protein HMGB1 regulates cell death and survival in cancer treatment. *Autophagy, 6*(8), 1181–1183. doi:10.4161/auto.6.8.13367.
116. Lin, C. I., Whang, E. E., Abramson, M. A., Donner, D. B., Bertagnolli, M. M., Moore, F. D., Jr., et al. (2009). Galectin-3 regulates apoptosis and doxorubicin chemoresistance in papillary thyroid cancer cells. *Biochemical and Biophysical Research Communications, 379*(2), 626–631. doi:10.1016/j.bbrc.2008.12.153.
117. Schonthal, A. H. (2009). Endoplasmic reticulum stress and autophagy as targets for cancer therapy. *Cancer Letters, 275*(2), 163–169. doi:10.1016/j.canlet.2008.07.005.
118. Jordan, C. T. (2010). Targeting myeloid leukemia stem cells. *Science Translational Medicine, 2*(31), 31ps21. doi:10.1126/scitranslmed.3000914.
119. LaBarge, M. A. (2010). The difficulty of targeting cancer stem cell niches. *Clinical Cancer Research, 16*(12), 3121–3129. doi:10.1158/1078-0432.CCR-09-2933.
120. Speiser, D. E., & Romero, P. (2010). Molecularly defined vaccines for cancer immunotherapy, and protective T cell immunity. *Seminars in Immunology, 22*(3), 144–154. doi:10.1016/j.smim.2010.03.004.
121. Dubensky, T. W., Jr., & Reed, S. G. (2010). Adjuvants for cancer vaccines. *Seminars in Immunology, 22*(3), 155–161. doi:10.1016/j.smim.2010.04.007.

122. Palucka, K., Ueno, H., Zurawski, G., Fay, J., & Banchereau, J. (2010). Building on dendritic cell subsets to improve cancer vaccines. *Current Opinion in Immunology, 22*(2), 258–263. doi:10.1016/j.coi.2010.02.010.
123. Palucka, A. K., Ueno, H., Fay, J. W., & Banchereau, J. (2007). Taming cancer by inducing immunity via dendritic cells. *Immunological Reviews, 220*, 129–150. doi:10.1111/j.1600-065X.2007.00575.x.
124. Chamoto, K., Takeshima, T., Wakita, D., Ohkuri, T., Ashino, S., Omatsu, T., et al. (2009). Combination immunotherapy with radiation and CpG-based tumor vaccination for the eradication of radio- and immuno-resistant lung carcinoma cells. *Cancer Science, 100*(5), 934–939. doi:10.1111/j.1349-7006.2009.01114.x.
125. Conforti, R., Ma, Y., Morel, Y., Paturel, C., Terme, M., Viaud, S., et al. (2010). Opposing effects of Toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. *Cancer Research, 70*(2), 490–500. doi:10.1158/0008-5472.CAN-09-1890.
126. Sharma, M. D., Baban, B., Chandler, P., Hou, D. Y., Singh, N., Yagita, H., et al. (2007). Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *Journal of Clinical Investigation, 117*(9), 2570–2582. doi:10.1172/JCI31911.
127. Ou, X., Cai, S., Liu, P., Zeng, J., He, Y., Wu, X., et al. (2008). Enhancement of dendritic cell-tumor fusion vaccine potency by indoleamine-pyrrole 2,3-dioxygenase inhibitor, 1-MT. *Journal of Cancer Research and Clinical Oncology, 134*(5), 525–533. doi:10.1007/s00432-007-0315-9.
128. Herrmann, A., Kortylewski, M., Kujawski, M., Zhang, C., Reckamp, K., Armstrong, B., et al. (2010). Targeting Stat3 in the myeloid compartment drastically improves the *in vivo* antitumor functions of adoptively transferred T cells. *Cancer Research, 70*(19), 7455–7464. doi:10.1158/0008-5472.CAN-10-0736.
129. Nam, J. S., Terabe, M., Mamura, M., Kang, M. J., Chae, H., Stuelten, C., et al. (2008). An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments. *Cancer Research, 68*(10), 3835–3843. doi:10.1158/0008-5472.CAN-08-0215.
130. Wang, L., Yi, T., Kortylewski, M., Pardoll, D. M., Zeng, D., & Yu, H. (2009). IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *The Journal of Experimental Medicine, 206*(7), 1457–1464. doi:10.1084/jem.20090207.
131. Yoshio-Hoshino, N., Adachi, Y., Aoki, C., Pereboev, A., Curiel, D. T., & Nishimoto, N. (2007). Establishment of a new interleukin-6 (IL-6) receptor inhibitor applicable to the gene therapy for IL-6-dependent tumor. *Cancer Research, 67*(3), 871–875. doi:10.1158/0008-5472.CAN-06-3641.
132. Shinriki, S., Jono, H., Ota, K., Ueda, M., Kudo, M., Ota, T., et al. (2009). Humanized anti-interleukin-6 receptor antibody suppresses tumor angiogenesis and *in vivo* growth of human oral squamous cell carcinoma. *Clinical Cancer Research, 15*(17), 5426–5434. doi:10.1158/1078-0432.CCR-09-0287.
133. Matsuzaki, J., Gnjatic, S., Mhawech-Fauceglia, P., Beck, A., Miller, A., Tsuji, T., et al. (2010). Tumor-infiltrating NY-ESO-1-specific $CD8^+$ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proceedings of the National Academy of Sciences of the United States of America, 107*(17), 7875–7880. doi:10.1073/pnas.1003345107.
134. Jin, H. T., Anderson, A. C., Tan, W. G., West, E. E., Ha, S. J., Araki, K., et al. (2010). Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America, 107*(33), 14733–14738. doi:10.1073/pnas.1009731107.
135. Fourcade, J., Sun, Z., Benallaoua, M., Guillaume, P., Luescher, I. F., Sander, C., et al. (2010). Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific $CD8^+$ T cell dysfunction in melanoma patients. *The Journal of Experimental Medicine, 207*(10), 2175–2186. doi:10.1084/jem.20100637.
136. Sakuishi, K., Apetoh, L., Sullivan, J. M., Blazar, B. R., Kuchroo, V. K., & Anderson, A. C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *The Journal of Experimental Medicine, 207*(10), 2187–2194. doi:10.1084/jem.20100643.
137. Derre, L., Rivals, J. P., Jandus, C., Pastor, S., Rimoldi, D., Romero, P., et al. (2010). BTLA mediates inhibition of human tumor-specific $CD8^+$ T cells that can be partially reversed by vaccination. *Journal of Clinical Investigation, 120*(1), 157–167. doi:10.1172/JCI40070.
138. Robert, C., & Ghiringhelli, F. (2009). What is the role of cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma? *The Oncologist, 14*(8), 848–861. doi:10.1634/theoncologist.2009-0028.
139. Peggs, K. S., Quezada, S. A., Chambers, C. A., Korman, A. J., & Allison, J. P. (2009). Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *The Journal of Experimental Medicine, 206*(8), 1717–1725. doi:10.1084/jem.20082492.
140. Chen, H., Liakou, C. I., Kamat, A., Pettaway, C., Ward, J. F., Tang, D. N., et al. (2009). Anti-CTLA-4 therapy results in higher $CD4^+$ICOShi T cell frequency and IFN-gamma levels in both nonmalignant and malignant prostate tissues. *Proceedings of the National Academy of Sciences of the United States of America, 106*(8), 2729–2734. doi:10.1073/pnas.0813175106.
141. Curran, M. A., Montalvo, W., Yagita, H., & Allison, J. P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proceedings of the National Academy of Sciences of the United States of America, 107*(9), 4275–4280. doi:10.1073/pnas.0915174107.
142. Westwood, J. A., Darcy, P. K., Guru, P. M., Sharkey, J., Pegram, H. J., Amos, S. M., et al. (2010). Three agonist antibodies in combination with high-dose IL-2 eradicate orthotopic kidney cancer in mice. *Journal of Translational Medicine, 8*, 42. doi:10.1186/1479-5876-8-42.
143. Dudley, M. E., Yang, J. C., Sherry, R., Hughes, M. S., Royal, R., Kammula, U., et al. (2008). Adoptive cell therapy for patients with metastatic melanoma: Evaluation of intensive myeloablative chemoradiation preparative regimens. *Journal of Clinical Oncology, 26*(32), 5233–5239. doi:10.1200/JCO.2008.16.5449.
144. Quezada, S. A., Simpson, T. R., Peggs, K. S., Merghoub, T., Vider, J., Fan, X., et al. (2010). Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *The Journal of Experimental Medicine, 207*(3), 637–650. doi:10.1084/jem.20091918.
145. Garcia-Hernandez Mde, L., Hamada, H., Reome, J. B., Misra, S. K., Tighe, M. P., & Dutton, R. W. (2010). Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. *Journal of Immunology, 184*(8), 4215–4227. doi:10.4049/jimmunol.0902995.
146. Muranski, P., Boni, A., Antony, P. A., Cassard, L., Irvine, K. R., Kaiser, A., et al. (2008). Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood, 112*(2), 362–373. doi:10.1182/blood-2007-11-120998.
147. Miller, J. S., Soignier, Y., Panoskaltsis-Mortari, A., McNearney, S. A., Yun, G. H., Fautsch, S. K., et al. (2005). Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood, 105*(8), 3051–3057. doi:10.1182/blood-2004-07-2974.
148. Pegram, H. J., Jackson, J. T., Smyth, M. J., Kershaw, M. H., & Darcy, P. K. (2008). Adoptive transfer of gene-modified primary NK cells can specifically inhibit tumor progression *in vivo*. *Journal of Immunology, 181*(5), 3449–3455.