



# 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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**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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## 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<sup>+</sup> T (Tc1) productrices d'IFN- $\gamma$  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-1 $\beta$  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- $\gamma$  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 $\gamma$ 4<sup>+</sup> et V $\gamma$ 6<sup>+</sup>) 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<sup>+</sup> (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- $\gamma$  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 $\gamma$ 4<sup>+</sup> et V $\gamma$ 6<sup>+</sup>) dans l'effet bénéfique de la chimiothérapie est essentielle puisque les souris V $\gamma$ 4/6<sup>-/-</sup> ont montré une réduction de leur sensibilité à la chimiothérapie et la vaccination

anti-tumorale et l'infiltration tumorale par les cellules  $\gamma\delta$  T17 et Tc1 a été réduite au niveau basal chez ces souris. L'axe IL-1 $\beta$ /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  $\gamma\delta$  T peut rétablir l'efficacité de la chimiothérapie dans le modèle de souris IL-17A<sup>-/-</sup> 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-1 $\beta$ ) → cellules T (IL-17) → Tc1 (IFN- $\gamma$ ), 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<sup>-/-</sup> 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. Nos 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,  $\gamma\delta$  T, IL-17A, IL-1 $\beta$ , 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- $\gamma$  producing CD8<sup>+</sup> T cells (Tc1) are mandatory for the success of chemotherapy to prevent tumor outgrowth. Priming of Tc1 response depends on IL-1 $\beta$  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- $\gamma$  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  $\gamma\delta$  T lymphocytes (V $\gamma$ 4<sup>+</sup> and V $\gamma$ 6<sup>+</sup>) 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  $\gamma\delta$  T ( $\gamma\delta$  T17) and IFN- $\gamma$ -producing CD8<sup>+</sup> TIL (Tc1) was discovered. IL-17A signaling acts as upstream of IFN- $\gamma$  since defect in IL-17RA led to complete loss of antigen specific Tc1 priming. The contribution of  $\gamma\delta$  T17 cells (V $\gamma$ 4<sup>+</sup> and V $\gamma$ 6<sup>+</sup>) to chemotherapy is critical as V $\gamma$ 4/6<sup>-/-</sup> mice showed reduced sensitivity to chemotherapy and vaccination. Also, tumor infiltrating  $\gamma\delta$  T17 and Tc1 cells were reduced to basal level in this strain. IL-1 $\beta$ /IL-1R, but not IL-23/IL-23R, is pivotal for IL-17 production by  $\gamma\delta$  T cells and the success of chemotherapy. Importantly, adoptive transfer of  $\gamma\delta$  T cells could restore the efficacy of chemotherapy in IL-17A<sup>-/-</sup> 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-1 $\beta$ )  $\rightarrow$   $\gamma\delta$  T cells (IL-17)  $\rightarrow$  Tc1 (IFN- $\gamma$ ) 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<sup>-/-</sup> 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,  $\gamma\delta$  T, IL-17A, IL-1 $\beta$ , 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-1 $\alpha$ : hypoxia-inducible factor-1 $\alpha$

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

IRE1 $\alpha$ : 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: oxaliplatin

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: phosphatidylinositol 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- $\alpha$ : 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?***

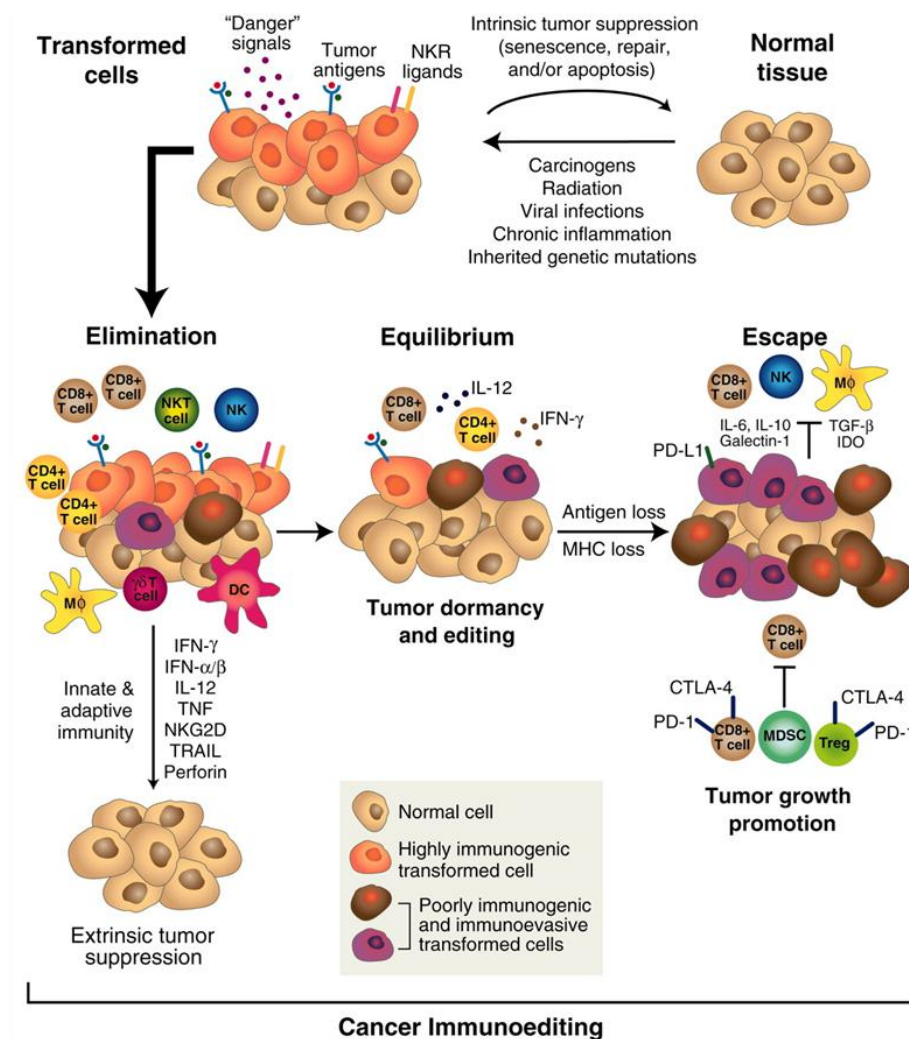
#### **Immunosurveillance versus immunediting**

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- $\gamma$  [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  $\gamma\delta$  T cells [18,19]. The Link between susceptibility to tumorigenesis 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<sup>+</sup>, CD8<sup>+</sup> T cells) and effector molecules (eg. IFN- $\gamma$ , 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<sup>-/-</sup> 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.



R D Schreiber et al. Science 2011;331:1565-1570

**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].

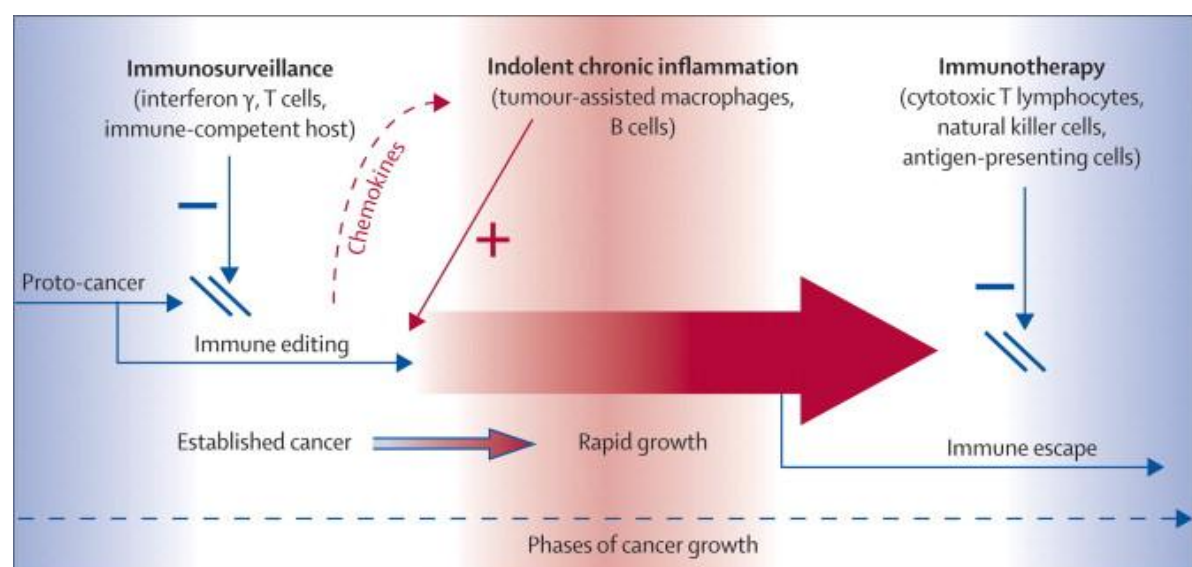
Up to 20% of all cancers arise in association with persistent and unresolving inflammation and most, if not all, solid tumors contain inflammatory infiltrates [31]. Inflammation, cytokines, and signal transducer and activator of transcription 3 (STAT3) have been classified as the “Unholy Trinity” that shapes the pro-tumorigenic microenvironment [32]. In ovarian, lung and gastro-oesophageal cancer patients, elevated IL-1 $\beta$ , 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-1 $\beta$ , IL-23, IL-10 and myeloid differentiation primary response gene (88) (MyD88) [20]. In murine transplantable tumor models, IL-1 $\beta$  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- $\alpha$ ), 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- $\alpha$  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-1 $\alpha$ ) and immune suppression (IL-10, TGF- $\beta$ ) while opposing STAT1- and NF- $\kappa$ B-mediated anti-tumor Th1



response (IL-12, CXCL10, IFN- $\gamma$ , IFN- $\beta$ , 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<sup>+</sup>, CD8<sup>+</sup>, Granzyme B<sup>+</sup>, and CD45RO<sup>+</sup> 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<sup>+</sup> memory cells. CD8<sup>+</sup> plus CD45RO<sup>+</sup> 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 FcR $\gamma$ -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 V $\alpha$ 14J $\alpha$ 18<sup>-</sup> type II NKT cells may be responsible and sufficient for the negative regulation of antitumor immunity, V $\alpha$ 14J $\alpha$ 18<sup>+</sup> 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- $\beta$  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- $\alpha$ -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<sup>+</sup> Gr1<sup>+</sup> 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- $\gamma$  and TNF- $\alpha$  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<sup>+</sup>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<sup>+</sup> 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 secrete GM-CSF

could recruit DC, plasma cells, invariant NKT cells and tumor reactive CD4<sup>+</sup> and CD8<sup>+</sup> 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 mitoxantrone) 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  $\gamma$ -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- $\alpha$  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  $\alpha$  (IRE1 $\alpha$ ) enhances IRE1 $\alpha$ /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-1 $\beta$  secretion which is indispensable 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-1 $\beta$  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- $\beta$ , CXCL8, IL-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 immunosuppressive host-tumor equilibrium is reversed during the course of successful chemotherapies (Figure 1.3).

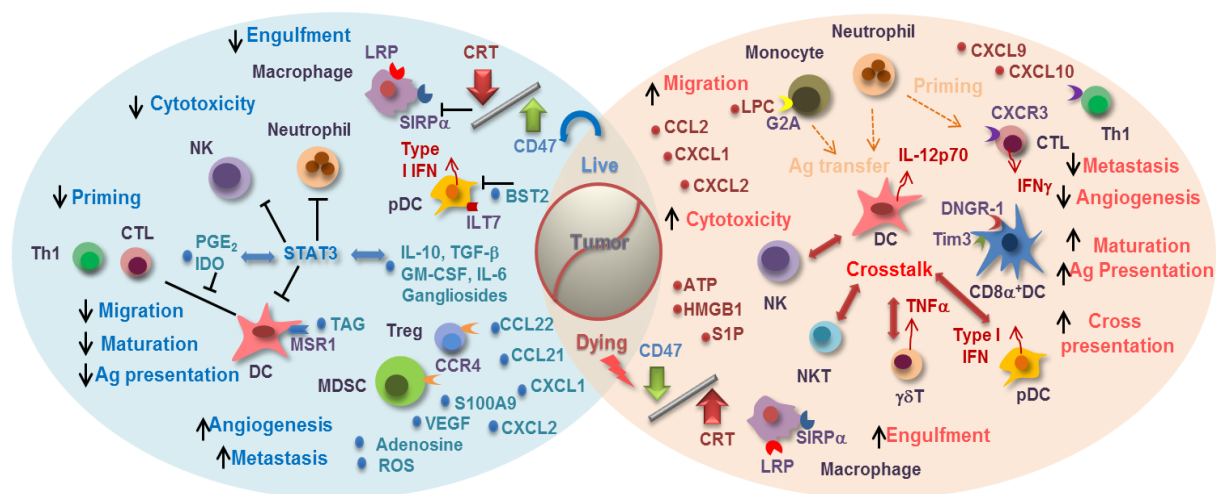
Innate and cognate immune responses elicited by anti-cancer agents are required for an optimal therapeutic outcome. Indeed, tumoricidal activity of oxaliplatin or 10-Gy

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<sup>+</sup> lymphocytes [117]. Using CD11c-DTR transgenic mice in which conventional DC could be depleted by diphtheria 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-1 $\beta$  was shown to be a key cytokine which gears the Tc1 polarization of TCR-triggered CD8<sup>+</sup> T cells and IFN- $\gamma$  is indispensable 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 recruit pDC, CD8<sup>+</sup> DC and CD11b<sup>+</sup> DC and potentially prime local and systemic CD8<sup>+</sup> cytotoxic T lymphocyte (CTL). This tumor vaccine could completely eliminate distant and established melanoma and both pDC and CD8<sup>+</sup> conventional DC (cDC) are necessary for generating this protective antitumor immunity [154]. CD11b<sup>+</sup> stromal cells could capture tumor antigen from surrounding cancer cells, thus eliminating bystander antigen-loss tumor variants [155,156], in the IFN- $\gamma$ - and TNF- $\alpha$ -dependent manner [157]. During this process, the cooperation between CD4<sup>+</sup> and CD8<sup>+</sup> T cells is mandatory because CD4<sup>+</sup> T cells were needed not only for optimal CD8<sup>+</sup> 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<sup>+</sup>/CD4<sup>+</sup> T cell clusters surrounded by CD20<sup>+</sup> B-cell follicles, have been recently described in non-small cell lung cancers endowed with

favorable clinical outcome [159]. Lymphotoxin  $\alpha$ ,  $\beta$  and their receptor (LT $\beta$ R), 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 uptake 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 recruit 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 C57BL/6 (H-2<sup>b</sup>) and BALB/c (H-2<sup>d</sup>) 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<sup>b</sup>) mice were bred at CDTA, Orléans, France through BR and PP (as for TCR  $\delta^{-/-}$ ). IL-23p19 $^{-/-}$  and IL-17A $^{-/-}$  (H-2<sup>b</sup>) were kindly provided by MJS. V $\gamma$ 4 $\gamma$ 6 $^{-/-}$  mice (H-2<sup>b</sup>) were kindly provided by GM and KI. CD1d $^{-/-}$  and CCR6 $^{-/-}$  (H-2<sup>b</sup>) 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<sup>d</sup>) colon cancer, MCA205 (H-2<sup>b</sup>) and MCA2 (H-2<sup>d</sup>) sarcoma, TS/A mamalian cancer (H-2<sup>d</sup>) and EG7 thymoma (H-2<sup>b</sup>), B16-OVA melanoma (H-2<sup>b</sup>) 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<sub>2</sub>. 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  $\mu$ M 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-1 $\beta$ , IL-23, IL-6, TGF- $\beta$  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-1 $\beta$ , 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), CD3 $\epsilon$  (145-2C11 or 17A2), CD4 (GK1.5), CD8 $\alpha$  (53-6.7), TCR  $\delta$  (GL-3), CD69 (H1.2F3), IL-17A (TC11-18H10) or IFN- $\gamma$  (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- $\gamma$  (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- $\beta$  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  $\alpha$ 2b 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.**  $8 \times 10^5$  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  $\mu$ l, i.t) or OX (5 mg/kg body weight, i.p) when tumors reached the size of 25-45mm<sup>2</sup>. 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<sup>2</sup>.

$3 \times 10^5$  B16-OVA or  $6 \times 10^5$  GL26 cells were inoculated s.c. into the left flank of C57BL/6 mice. Vaccines were composed either of CpG ODN 1668 (5  $\mu$ g/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 20 $\mu$ M 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  $\mu$ g/mouse in B16-OVA model and at 500  $\mu$ g/mouse in the GL26 model. MetRantes (10  $\mu$ g/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  $\mu$ g/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  $\mu$ g/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  $\mu$ g 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  $\mu$ M cell strainer. Cells were blocked with 10  $\mu$ g/ml anti-CD16/CD32 (eBioscience) before surface staining (2.5  $\mu$ g/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  $\mu$ g/ml ionomycin and Golgi-stop (BD Pharmingen) for 4hrs, at 37°C in RPMI containing 2% mouse serum (Janvier, France). Cells were then stained with anti-IFN- $\gamma$  and anti-IL-17A using BD Cytfix/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  $\gamma\delta$  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  $\gamma\delta$  T cells purification (TCR $\gamma/\delta^+$  T Cell Isolation Kit) using AutoMACS™ Separator (Miltenyi Biotec) with recommended programs. Purity of this isolation normally reached above 95%. The TCR  $\delta^-$  CD3 $^+$  cell fraction was also collected and used as 'non  $\gamma\delta$  T cells for some experiments. Day 2 after chemotherapy,  $2.5 \times 10^5$  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  $\mu$ g/ml OX overnight or left untreated were washed thoroughly and injected at 1 million/50  $\mu$ l into the foodpad of naïve syngeneic mice. CpG/OVA (5  $\mu$ g CpG+1 mg OVA/mouse) and PBS injection were used as positive and negative controls. In some setting, neutralizing antibody (200  $\mu$ g/mouse) for IL-17A or Clg was injected i.p. 5 days later, the popliteal lymph node cells were harvested, seeded in a 96 well plate at  $3 \times 10^5$ /well and restimulated with 1 mg/ml OVA protein. IFN- $\gamma$  secretion was measured by OptEIA™ Mouse IFN- $\gamma$  ELISA kit (BD Bioscience). MCA205 cells were treated with 2  $\mu$ M 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  $5 \times 10^4$  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  $\mu$ M 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  $\mu$ l/well in U bottom 96 well plates. Tumor cells were treated overnight with 25 $\mu$ M DX or left untreated, washed and used at  $7.5 \times 10^4$ /100  $\mu$ l/well.  $2 \times 10^4$ /50  $\mu$ l  $\gamma\delta$  T cells were added 12 hrs later. Supernatant was collected 36 hrs later.

***In vitro tumor stimulation with type I IFN and TLR3L assays.***  $5 \times 10^4$  B16-OVA or GL26 and  $2 \times 10^5$  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 particles 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  $\pm$  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

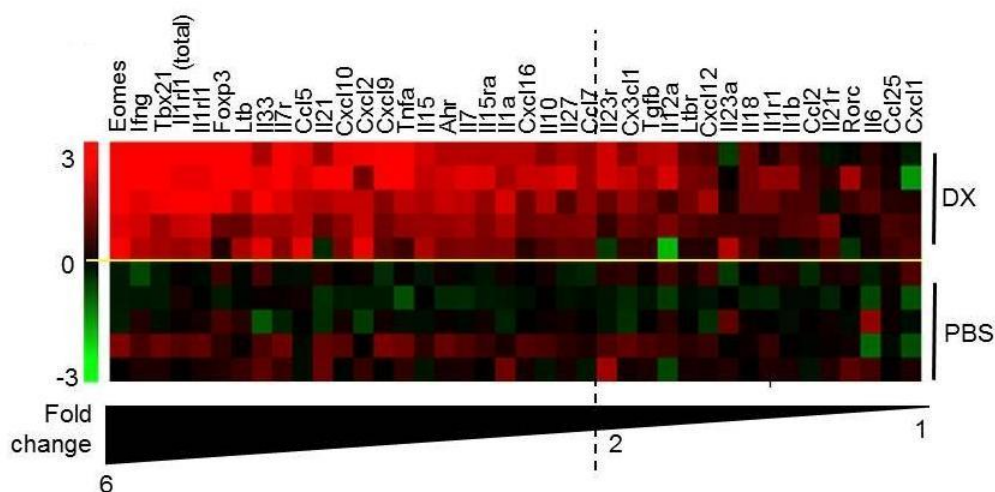
### ***Part I. The contribution of IL-17 producing $\gamma\delta$ T cells during chemotherapy and radiotherapy***

This work 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 quantitative 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- $\gamma$ , 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-1 $\alpha$ , membrane-bound 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- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$ 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 involved 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<sup>+</sup>T [167], Treg [180,181], CD8<sup>+</sup> T [182-184], CD4<sup>+</sup> NKT [185,186],  $\gamma\delta$  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  $\alpha\beta$ -TCR<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> 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<sup>+</sup> CD45<sup>+</sup> TILs rather than from tumor cells. The majority of IL-17A-producing lymphocytes were CD3<sup>bright</sup>, a phenotype indicating that they were most likely to be  $\gamma\delta$  T cells [199]. A reverse gating on all IL-17A<sup>+</sup> cells confirmed that most of them (60-70%) do co-express CD3 and TCR  $\delta$ , but not CD4, CD8 or Gr1. CD8<sup>+</sup> T cells were the major contributor of IFN- $\gamma$ . It is interesting that  $\gamma\delta$  T cells exclusively produce IL-17A but rarely IFN- $\gamma$  and we could not see IFN- $\gamma$  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<sup>+</sup>, it is possible that Th17 and probably  $\alpha\beta$ -TCR<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> T and/or CD4<sup>-</sup> NKT cells constitute this fraction.

### **The contribution of IFN- $\gamma$ and IL-17A during chemotherapy**

Consistently, IFN- $\gamma$  and IL-17 production by tumor-infiltrating CD8<sup>+</sup>T and  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T17 infiltration in tumor bed after chemotherapy or radiotherapy could be a beneficial prognostic factor for therapeutic response.

IFN- $\gamma$  is widely recognized to be contributive for immunosurveillance and it plays a central role in coordinating anti-tumor immune responses [200,201]. IFN- $\gamma$  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- $\gamma$  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- $\gamma$  or IFN- $\gamma$ R or IFN- $\gamma$  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- $\gamma$  neutralizing antibody (clone XMG1.2, 200  $\mu$ g per injection for total 3 injections, every other day since the day of DX treatment), we confirmed the critical role of IFN- $\gamma$  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<sup>+</sup> T cells (with CD8 $\alpha$ - or CD8 $\beta$ - specific antibody), the cellular source of IFN- $\gamma$ , also resulted in failure of chemotherapy. The IFN- $\gamma$ /CD8<sup>+</sup>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<sub>2</sub>, 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-derived IL-9 acted as a positive feedback loop to strengthen immunosuppression in tumor microenvironment [211]. In B16 melanoma and MB49 bladder carcinoma where CD4<sup>+</sup> 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- $\gamma$  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<sup>+</sup> 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<sup>+</sup> T cells [217]. IL-17-producing CD8<sup>+</sup> T cells could differentiate into long-lasting IFN- $\gamma$  producers and reduce the volume of large established tumors [218]. In contrast, Kwong et al. described a tumor-promoting, IL-17-producing TCR  $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> 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 indispensable 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 (Matarollo SR, Cancer Res. 2011, accepted). We noticed that IFN- $\gamma$ , 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 $\gamma\delta$ T cells and associated cytokines during chemotherapy**

$\gamma\delta$  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  $\gamma\delta$  T cells could mediate antibody-dependent cellular cytotoxicity (ADCC) [223-225]. They produce cytokines like IFN- $\gamma$  and IL-17 rapidly and promote inflammation, partly due to their inherent epigenetic and transcriptional programs [226]. Certain subsets of  $\gamma\delta$  T cells also possess the antigen-presenting ability, such as human V $\gamma$ 9V $\delta$ 2<sup>+</sup> T cells [227-230] and murine dendritic epidermal  $\gamma\delta$  T cells (DETC) [231,232]. Similar with other T cell populations,  $\gamma\delta$  T cells also show regulatory activities. They are involved in inflammation resolution [233-235]. Distinct subsets of  $\gamma\delta$  T cells have non-overlapping

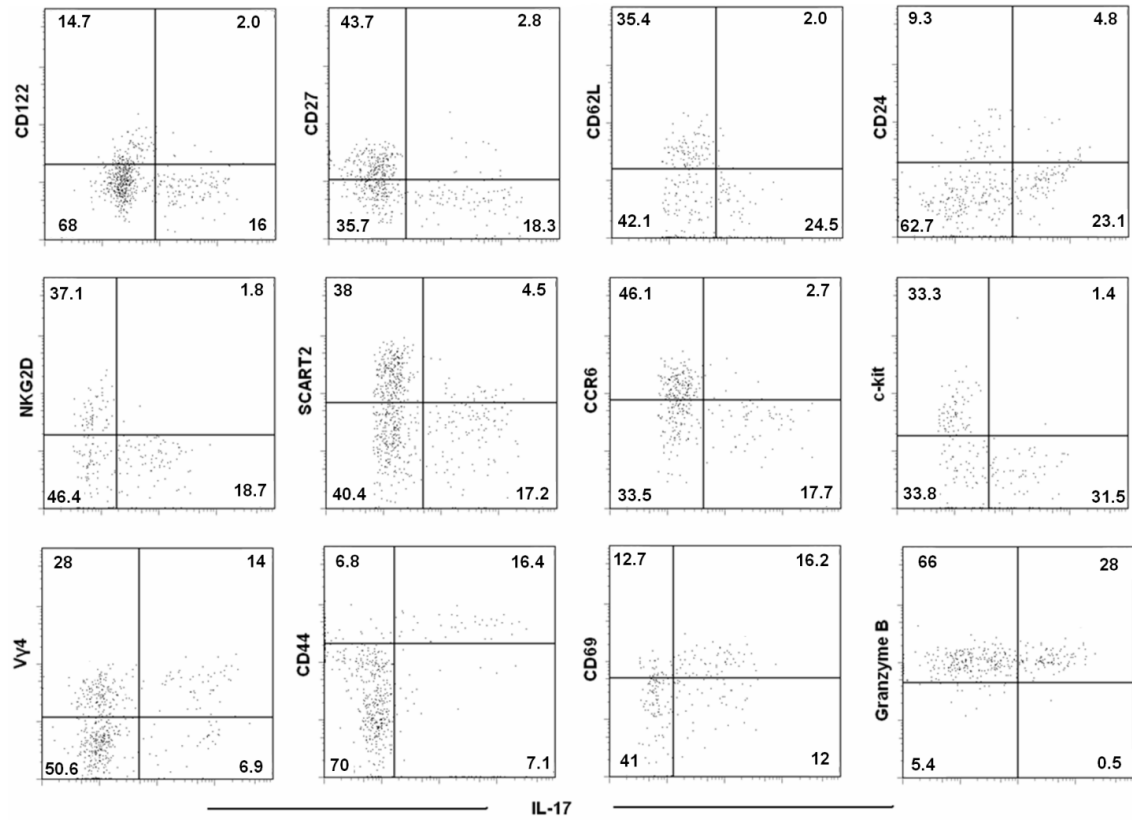
functional potentials and the outcome depends on their functional balance [236]. Interestingly,  $\gamma\delta$  T cells were found as the dominant producers of IL-17 in both ischemic brain injury and bleomycin induced lung injury models while the “same” population exerted completely opposite effects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

The contribution of  $\gamma\delta$  T cells in tumor immunosurveillance is still elusive [238]. In C57BL/6 mice,  $\gamma\delta$  T cells strongly protected the host against chemically induced cutaneous malignancy in a NKG2D-dependent manner [18,239].  $\gamma\delta$  T cells were recruited quickly to the tumor sites and provided an early source of IFN- $\gamma$  which was critical to induce tumor antigen-triggered  $\alpha\beta$  T cell response [19]. Tumor infiltrating IL-17-producing  $\gamma\delta$  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- $\beta$ , TCR and NKG2D contributed to the IL-17 production [241].  $\gamma\delta$  T cells have been suggested as suppressor cells in early tumor formation. They secreted IL-10 and TGF- $\beta$ , but not IL-4 or IFN- $\gamma$ , which attenuated the activity of CTLs and NK cells [242,243]. In human, V $\delta$ 1<sup>+</sup>  $\gamma\delta$  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 $\delta$ 2<sup>+</sup>  $\gamma\delta$  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  $\gamma\delta$  T cells.

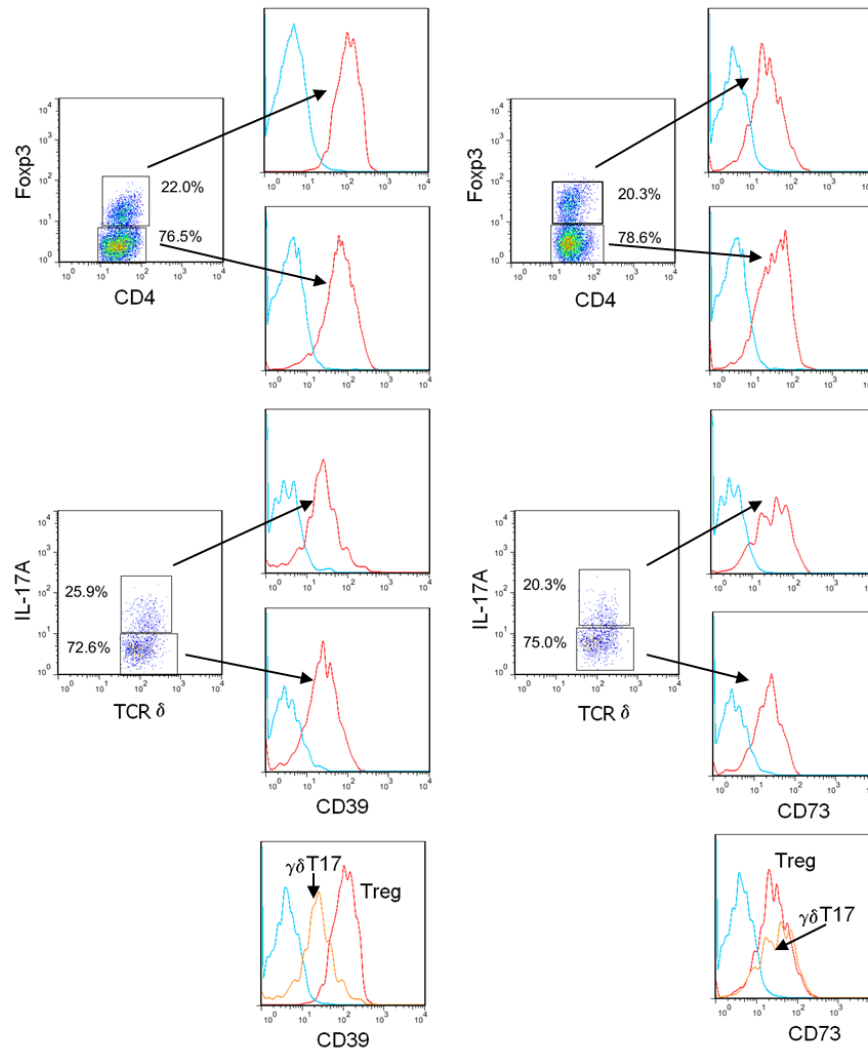
$\gamma\delta$  T17 cells have been reported to share most phenotypic markers with Th17 cells (expressing CCR6, ROR $\gamma$ t, AhR, IL-23R, IL-17A, IL-22) [251]. They are unrestricted by V $\gamma$  usage (although they are mostly V $\gamma$ 4 in the context of mycobacteria [251] and experimental autoimmune encephalitis [252]). Recent work suggests that thymic selection does little to constrain  $\gamma\delta$  T cell antigen specificities, but instead determines their effector

fate. When triggered through the T cell receptor, ligand-experienced cells make IFN- $\gamma$ , strongly self-reactive cells make IL-4 whereas ligand-naïve  $\gamma\delta$  T cells produce IL-17 [188,253,254]. CD27 also regulates  $\gamma\delta$  T cells differentiation. CD27<sup>+</sup>  $\gamma\delta$  thymocytes express LT $\beta$ R and genes associate with a Th1 phenotype, in contrast to CD27<sup>-</sup>  $\gamma\delta$  thymocytes which give rise to IL-17-producing cells [255]. It is reported that IL-17- and IFN- $\gamma$ -producing  $\gamma\delta$  T cells express CD25 (IL-2R $\alpha$ ) and CD122 (IL-2R $\beta$ ) respectively. CD25 expression may be downregulated on  $\gamma\delta$  T cells that have experienced antigen in the thymus, making them less sensitive to endogenous IL-2 production [256]. IFN- $\gamma$ -secreting  $\gamma\delta$  T cells in the periphery also express NK1.1, whereas IL-17-secreting  $\gamma\delta$  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  $\gamma\delta$  T cells homing to the peripheral LN and dermis. SCART2<sup>high</sup> cells are enriched for V $\gamma$ 4 but strong TCR stimulation leads to SCART2 down-regulation [258].

To analyse the functional relevance of IL-17-producing  $\gamma\delta$  T cells (that we termed “ $\gamma\delta$  T17”) in cancer, we performed phenotype of these tumor infiltrating cells after chemotherapy. Most  $\gamma\delta$  T17 TILs showed an effector memory phenotype (preponderantly CD69<sup>+</sup> granzyme B<sup>+</sup> CD44<sup>+</sup> CD62L<sup>-</sup>). 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  $\gamma\delta$  T17 TILs were CD39<sup>low</sup> (Figure. c).



**Figure b. Phenotype of tumor infiltrating  $\gamma\delta$  T cells after DX therapy.** MCA205 tumors were analyzed 8 days post-DX. After gated on live CD45.2<sup>+</sup>CD3<sup>+</sup>TCR  $\delta$ <sup>+</sup> 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  $\gamma\delta$  T17 after DX treatment.** MFI of CD39 (left) and CD73 (right) staining on CD4<sup>+</sup> T and  $\gamma\delta$  T cells were shown, comparing Foxp3<sup>+</sup> vs Foxp3<sup>-</sup>, IL-17A<sup>+</sup> vs IL-17<sup>-</sup> fractions.

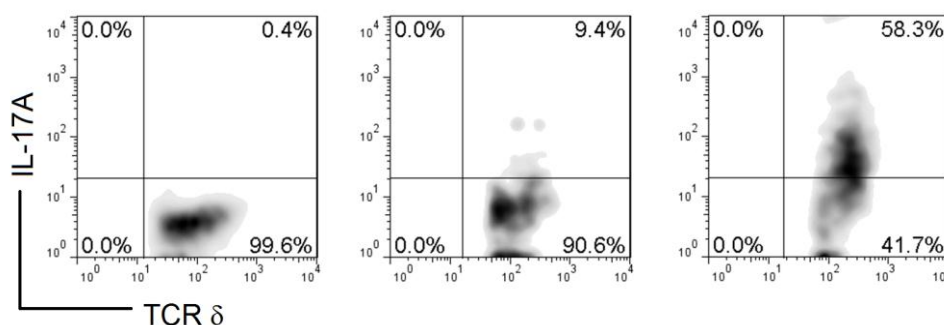
FACS indicated that around 60-75% of  $\gamma\delta$  T17 utilized V $\gamma$ 4 chain according to Heilig and Tonegawa's nomenclature [260] (Figure. b and published Figure S2B) but expression of V $\gamma$ 1 and V $\gamma$ 7 chain was rarely found (published Figure S2B). We then sorted V $\gamma$ 1<sup>-</sup>V $\gamma$ 4<sup>-</sup>V $\gamma$ 7<sup>-</sup>  $\gamma\delta$  T17 TILs and performed single-cell PCRs [261] to examine their V $\gamma$  chain usage. These experiments revealed that 21 out of 23 sorted single cells contained a functional V $\gamma$ 6 rearrangement identical to the one found in fetal  $\gamma\delta$  T cells (shown in published Figure S2C-E), suggesting that most  $\gamma\delta$  T17 TILs express either V $\gamma$ 4 or V $\gamma$ 6. Indeed,  $\gamma\delta$  T cell subsets appear to be biased to carry out particular functions which are somehow

predetermined in the thymus [262].  $V\gamma 4^+$   $\gamma\delta$  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  $V\gamma 6V\delta 1$  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,  $V\gamma 6V\delta 1$  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  $V\gamma 4^+$  and  $V\gamma 6^+$  cells were deficient (shown in published Figure 6A). Consistently, IL-17A production by tumor infiltrating cells was abolished in  $V\gamma 4/6^{-/-}$  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  $J\alpha 18^+$  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  $V\gamma 4/6^{-/-}$  mice but not in NKT cell deficient  $CD1d^{-/-}$  mice (shown in published Figure 5B), indicating that the indispensable role of IL-17A/IL-17R pathway and  $V\gamma 4^+$  and  $V\gamma 6^+$  cells in triggering protective adaptive immunity against tumor.

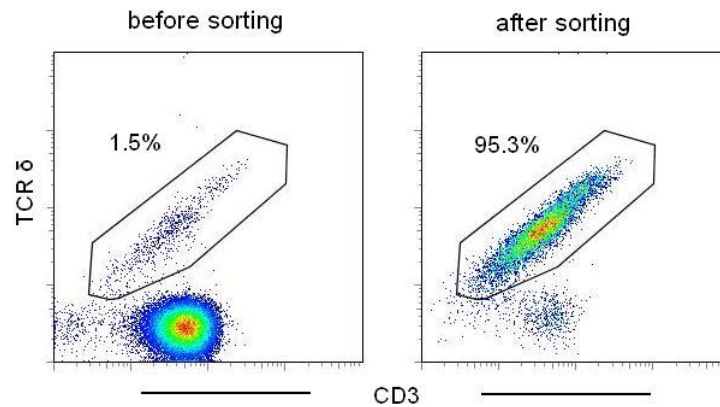
The biological rationale for using adoptively transfer ex vivo-expanded autologous  $\gamma\delta$  T cells in clinical trials of cancer treatment has been shown in mouse prostate tumor model. Tumor-bearing mice treated i.v. with supraphysiological numbers of syngeneic  $\gamma\delta$  T cells developed 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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  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  $\gamma\delta$  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<sup>+</sup> and the majority of them were  $\gamma\delta$  T cells [269]. It would be also interesting to know whether  $\gamma\delta$  T cells from different organs or various subsets of  $\gamma\delta$  T cells express IL-1R at different levels.



**Figure d. IL-17 production by  $\gamma\delta$  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  $\gamma\delta$  T cells with AutoMACS (purity above 90%, shown in Figure e) and adoptively transfer these cells directly into tumor bed 2 days after chemotherapy.  $\gamma\delta$  T cells transfer showed additive effect to control tumor growth provided that chemotherapy (DX) was applied in advance (shown in published Figure 7A). IL-17<sup>-/-</sup>  $\gamma\delta$  T cells adoptive transfer could not improve the efficacy of chemotherapy but IL-17-sufficient wild type  $\gamma\delta$  T cells could restore the chemosensitivity of MCA205 sarcoma. These findings strongly suggest that IL-17 derived from  $\gamma\delta$  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/ $\gamma\delta$ T and IFN- $\gamma$ /CD8<sup>+</sup>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.  $\gamma\delta$  TILs were quickly switched on to proliferate and produce IL-17A within 4 days after DX, resulting in around 9 fold increase of  $\gamma\delta$  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- $\gamma$ <sup>+</sup> Ki-67<sup>+</sup> phenotype) within the following 2 days. PBS treatment could modify neither IFN- $\gamma$  or IL-17A secretion throughout the chosen time points (shown in published Figure 2A, 2C).  $\gamma\delta$  T cells activated by IL-1 $\beta$  and IL-23 could promote IL-17 production by CD4<sup>+</sup> T cells and increase susceptibility to EAE [252]. We noticed that tumor infiltrating  $\gamma\delta$  T17 cells did not sustain long after Th1 response was primed. And we could not find significant increase of Th17 TILs, suggesting that albeit  $\gamma\delta$  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  $\gamma\delta$  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- $\gamma$  production by OVA-specific T cells in the draining LNs could be triggered by footpad injection of oxaliplatin-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<sup>-/-</sup> mice could not mount a protective immunity after dying tumor vaccine (shown in published Figure 5B). We also noticed that tumor infiltrating CD8<sup>+</sup> T cells failed to produce IFN- $\gamma$  in V $\gamma$ 4/6<sup>-/-</sup> 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- $\gamma$ ) 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<sup>+</sup> T cells or upregulated CD121a (IL-1R1) expression on CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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- $\gamma$  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 (Matarollo SR, Cancer Res. 2011, accepted). IL-17A derived from  $\gamma\delta$  T cells is required for the generation of antigen-specific CD8<sup>+</sup> CTL response against primary *Listeria monocytogenes* infection through enhancing DC cross presentation [272]. IL-23-activated  $\gamma\delta$  T cells produce IL-17

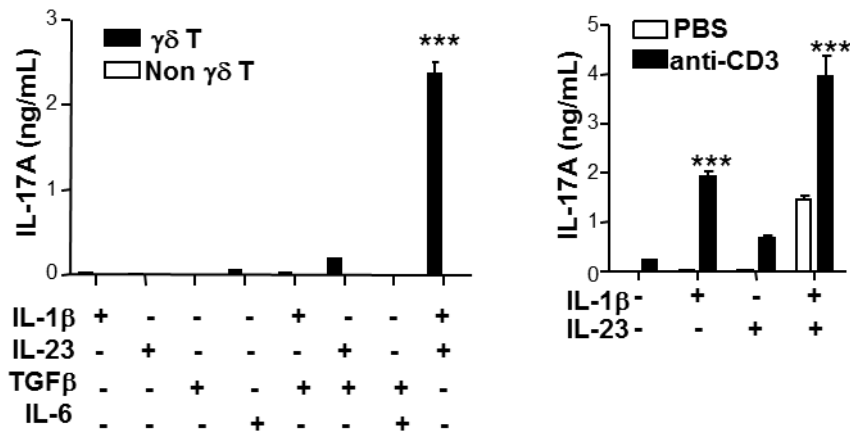
and they could render  $\alpha\beta$  effector T cells refractory to the suppressive activity of Treg cells and also prevent the conversion of conventional T cells into Foxp3<sup>+</sup> Treg cells in vivo [273]. What's more, IL-17 could synergize with IFN- $\gamma$  to stimulate CXCL9 and CXCL10 production and recruit effector T cells to the tumor microenvironment [216]. Interestingly, in experimental lung metastasis B16 and 3LL tumor models, tumor microenvironment derived IL-1 $\beta$  promoted  $\gamma\delta$  T cells recruitment and IL-17 production. IL-1 $\beta$  deficiency resulted in reduced IFN- $\gamma$  production by CD8<sup>+</sup> TILs while IL-17, which acted mainly downstream of IL-1 $\beta$ , did not contribute to DC maturation or Tc1 response and it suppressed IL-12 production by CD11c<sup>+</sup> cells in tumor bed [274]. Thus, coordinated IL-17 production by  $\gamma\delta$  T and IFN- $\gamma$  secretion by CD8<sup>+</sup> T cells predict the beneficial anti-tumor response. The orchestration and interactions of  $\gamma\delta$  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 $\gamma\delta$ T17 activation in the context of chemotherapy induced tumor cell death**

The differentiation factors (TGF- $\beta$  plus IL-6 or IL-21), the growth and stabilization factor (IL-23) and the transcription factors (STAT3, ROR $\gamma$ t, and ROR $\alpha$ ) 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  $\gamma\delta$  T, NKT, memory T cells and human MAIT cells express IL-23R and ROR $\gamma$ t 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  $\gamma\delta$  T cells directly or through inducing IL-1 $\beta$  and/or IL-23 production from antigen presenting cells [251].  $\gamma\delta$  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- $\beta$ ), engagement of NKG2D and TCR has been reported to activate  $\gamma\delta$  T produce IL-17 [241].

By in vitro experiment, we confirmed that  $\gamma\delta$  T from peripheral LNs potently produced IL-17A, and IL-22 to a lesser extent, in response to IL-1 $\beta$ +IL-23, CD3 crosslinking and IL-1 $\beta$  or/and IL-23 (Figure f). To mimic the microenvironment after chemotherapy, we cocultured  $\gamma\delta$  T with BMDC and dying/live tumor cells and tested IL-17A, IL-22 and IFN- $\gamma$  in the supernatant. Although DX-treated MCA205 cells failed to directly induce IL-17 or IL-22 secretion by  $\gamma\delta$  T cells, they could do so indirectly through triggering IL-1 $\beta$  production by BMDC. It is noteworthy that these stimuli specifically activated IL-17A, but not IFN- $\gamma$  production by  $\gamma\delta$  T cells. And none of these cytokines were induced by live tumor cells. We and others have described that peripheral LN  $\gamma\delta$  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/ $\gamma\delta$  T cell cross-talk in the presence of dying cells relied on IL-1 $\beta$ /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 moderately regulated IL-17 production by  $\gamma\delta$  T cells. IL-22 production was completely abolished by blocking the IL-1 $\beta$ /IL-1R or IL-23/IL-23R pathways but not affected by IL-18R blockade (shown in published Figure 5C). Although IL-1 $\beta$  production could be readily detected in dying tumor/BMDC coculture, IL-23 production from 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<sup>-/-</sup> mice (shown in published Figure 5E-G). Adoptive transfer of IL-1R<sup>-/-</sup>  $\gamma\delta$  T cells could not improve the efficacy of chemotherapy. These findings emphasize the pivotal contribution of IL-1 $\beta$ , 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-10R $\beta$  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],  $\gamma\delta$  T [251], NK22 [280,281], LTI and ROR $\gamma$ t<sup>+</sup> 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,  $\gamma\delta$  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-1 $\beta$ -activated  $\gamma\delta$  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<sup>+</sup> cells purified from tumor bed. Neither could we obtain positive 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).

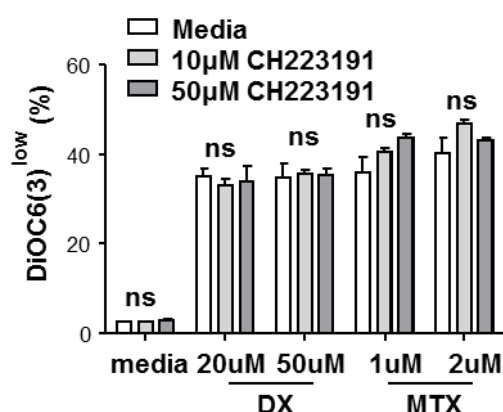
It remains possible that TCR signaling facilitates  $\gamma\delta$  T17 activation in our setting. Possible ligands of  $\gamma\delta$  TCR have been summarized elsewhere [289,290]. Irradiation and chemotherapeutic drugs, especially anthracyclines, could generate high level of oxidative stress on tumor 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  $\gamma\delta$  TCR were found to produce cytokines in response to cardiolipin and structurally related anionic phospholipids. Cardiolipin can be presented by CD1d to subsets of  $\gamma\delta$  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 potentially immunostimulatory. Around 10-20% of normal splenic and lymph node  $\gamma\delta$  T cells have been reported responding to HSP60 [297].  $\gamma\delta$  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  $\gamma\delta$  TCR to facilitate  $\gamma\delta$  T17 activation.

### **Contribution of AhR, CCR6/CCL20, TGF- $\beta$ and IL-6 pathway during chemotherapy**

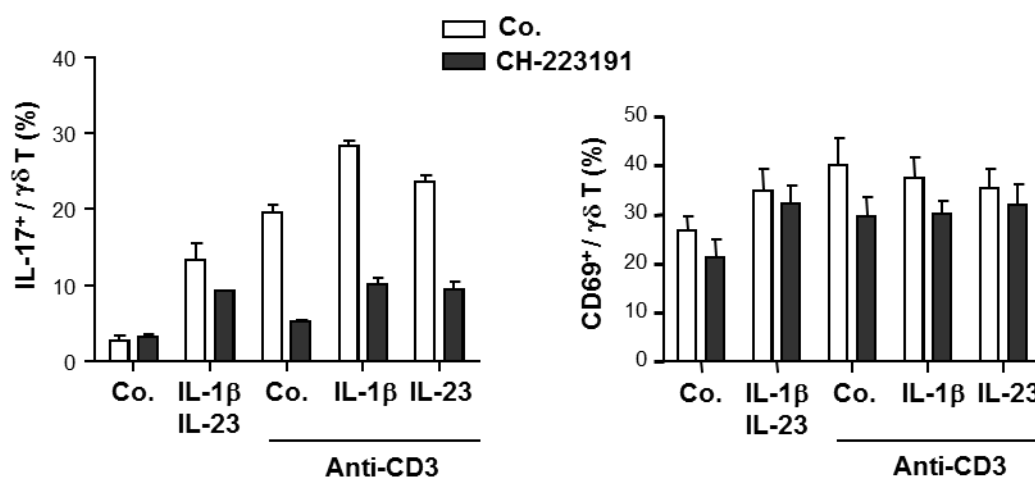
Aryl hydrocarbon receptor (AhR) is highly expressed on Th17 [299-301] and  $\gamma\delta$  T17 cells [251]. This transcriptional factor is closely related to the induction of IL-22. It remains

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 ROR $\gamma$ t to induce maximal amounts of IL-17 and IL-22 production and to inhibit TGF $\beta$ -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- $\kappa$ B [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  $\gamma\delta$  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  $\gamma\delta$  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-inflammatory roles in the same context or at different phases.



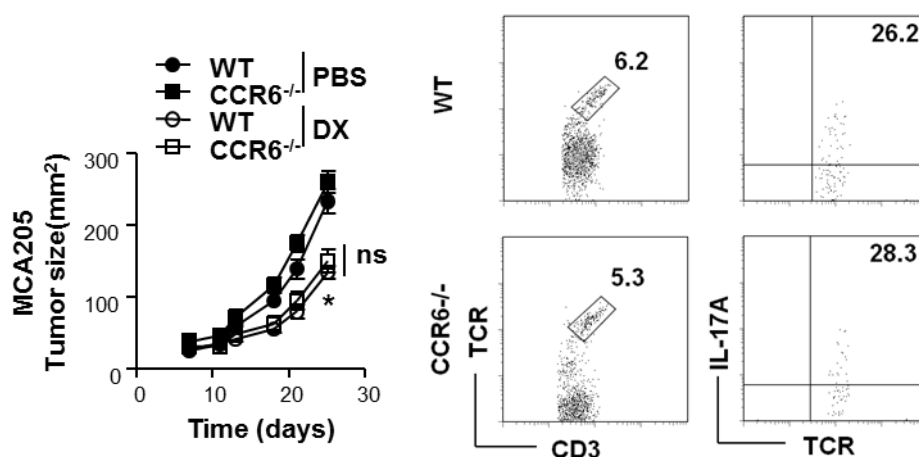
**Figure g. The impact of the AhR-blocking agent, CH-223191, on the proapoptotic effect of DX or MTX against MCA205.** A reduction in mitochondrial membrane potential indicated by decreased DiOC6(3) fluorescence was used to measure imminent apoptosis.



**Figure h. The impact of CH-223191 on the activation and IL-17 production by  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T17 (Figure i), suggesting CCR6/CCL20 are dispensable for the IL-17A/IFN- $\gamma$ -dependent chemotherapy against tumor. IL-6 and TGF- $\beta$  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  $\gamma\delta$  T17 in tumor bed.** Tumor growth was monitored in WT and CCR6<sup>-/-</sup> mice after PBS or DX treatment (left). Flow cytometry analyses of the  $\gamma\delta$  T17 cells in the gate of live, CD45.2<sup>+</sup>, CD3<sup>+</sup> 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 $\gamma\delta$ T cells

Recent advances in the characterization of the functional capabilities of human resident V $\delta$ 1 and circulating V $\delta$ 2 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. V $\delta$ 1 T cell could recognize these tumor-derived ligands through TCR or its activating natural killer receptors while V $\delta$ 2 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- $\gamma$  production of mainly V $\gamma$ 9V $\delta$ 2 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  $\gamma\delta$  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  $\gamma\delta$  T17 cells.

A. Adoptive cell transfer of autologous  $\gamma\delta$  T17 cells expanded in vitro.

Adoptive transfer strategy normally requires supraphysiological numbers of  $\gamma\delta$  T cells which restrain tumor outgrowth through their potent cytotoxicity and IFN- $\gamma$  secretion. Importantly, in vitro expanded V $\gamma$ 9V $\delta$ 2 T cells maintained their antitumor activity in vivo as shown in a xenografted human tumor model [308]. Autologous V $\gamma$ 9V $\delta$ 2 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 V $\gamma$ 9V $\delta$ 2 cells with phosphoantigens and a cocktail of cytokines (IL-1 $\beta$ , TGF- $\beta$ , IL-6 and IL-23) leads to selective expression of the transcription factor ROR $\gamma$ t and polarization towards IL-17 production. These cells shared similar phenotype with IL-17 $^{+}$  V $\gamma$ 9V $\delta$ 2 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 through TRAIL. To expand and polarize  $\gamma\delta$  T17 cells for adoptive transfer, tumor-infiltrating  $\gamma\delta$  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  $\gamma\delta$  T cells (mostly V $\gamma$ 4 $^{+}$ ) expanded with TCR crosslinking (clone UC7-13D5, 5  $\mu$ g/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,  $\gamma\delta$  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  $\gamma\delta$  T17 progenitors (not shown). Another challenging issue is how to guide infused  $\gamma\delta$  T17 towards tumor vicinity. IL-17<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells preferentially express CCR6 while IFN- $\gamma$ <sup>+</sup> V $\gamma$ 9V $\delta$ 2 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  $\gamma\delta$  T cells [311]. Further investigation on tumor-derived chemotactic factors triggered by chemotherapy will shed light on the optimal combination of chemotherapy with  $\gamma\delta$  T17 cells infusion.

#### B. Educating or activating $\gamma\delta$ T17 in vivo.

Another alternative strategy is to create a favorable microenvironment in vivo for  $\gamma\delta$  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  $\gamma\delta$  T17).

$\gamma\delta$  TCR-targeted delivery of pro-Th17 cytokines is a feasible choice. Antibody-based targeted delivery of cytokines (eg. IL-1 $\beta$ , IL-23), which has been termed as “immunocytokines” [312], could enhance the therapeutic index of recombinant cytokines. Cytokines-based fusion proteins-containing antibody targeting  $\gamma\delta$  TCR could be a promising option to activate/polarize  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  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. $\gamma\delta$ T17 activation through DC.

As we have shown, dying tumor cells could induce IL-1 $\beta$  production by DC, which is indispensable for  $\gamma\delta$  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  $\gamma\delta$  T17 and adaptive tumor specific Tc1 response. Several PRR, such as TLR1, TLR2 and dectin-1, are specifically expressed on CCR6<sup>+</sup> IL-17-producing  $\gamma\delta$  T cells [251]. They can be targeted directly to induce IL-17 production by  $\gamma\delta$  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  $\gamma\delta$  T cells or used during autologous DC generation for infusion.

#### D. Tumor-infiltrating or circulating $\gamma\delta$ T17 as a potential beneficial prognostic factor.

We discovered that  $\gamma\delta$  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  $\gamma\delta$  T17 could be an early prognostic factor after chemotherapy and whether correlated  $\gamma\delta$  T17 and Tc1 infiltration predict superior clinical outcome.

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

This work has been published in Cancer Research (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 tumor 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)) mimicking 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<sup>+</sup> 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- $\gamma$  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 wide 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- $\alpha$  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 recurrent 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 oxaliplatin, 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<sup>-/-</sup> 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- $\gamma$ -producing CD8<sup>+</sup>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 thawed 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- $\beta$  could up-regulate TLR3 expression in both murine and human. IFN- $\beta$ -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 phosphatidylinositol 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 immunohistochemistry 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 (oxaliplatin) 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<sup>+</sup> TILs and also CD45<sup>-</sup> 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).

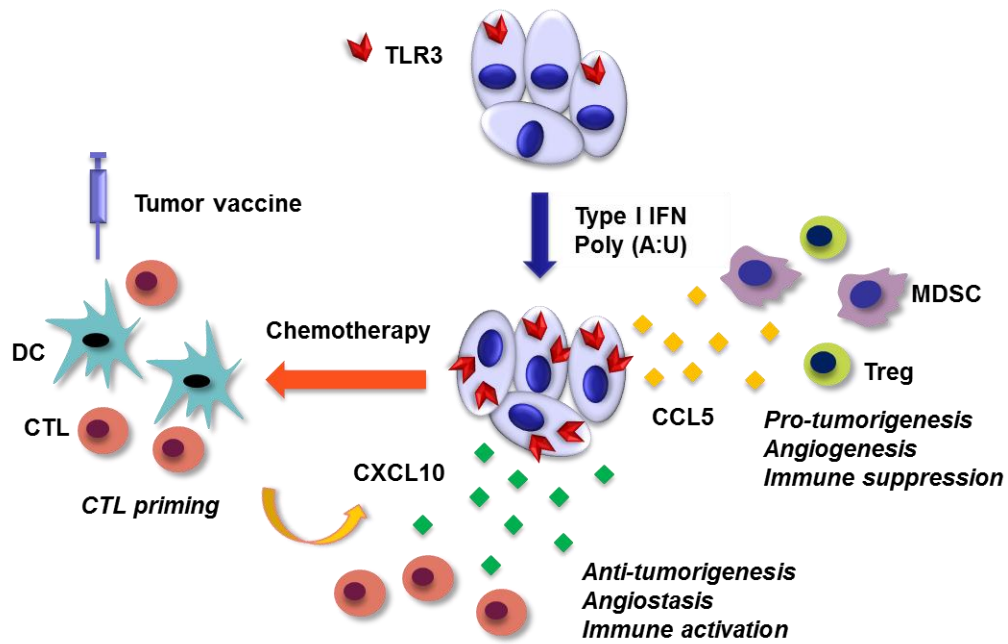
Certain chemokines are involved in forming immune contexture *in situ* within tumors. An improved prognosis is associated with the expression of specific chemoattractants and adhesion molecules. In a system biology study performed with a cohort of 108 CRC patients, the highest prediction score concerned 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<sup>-/-</sup> mice rather than to WT mice. Consistently, when combined with VCT protocol, MetRantes (a pharmacologic inhibitor of CCL5) showed significant additive 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- $\gamma$  production, correlating with enriched IFN- $\gamma$ -producing CXCR3<sup>+</sup> CD8<sup>+</sup> 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<sup>+</sup> CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and CXCR3<sup>+</sup> 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 microenvironment 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?

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## 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 <sub>2</sub> O	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  $\text{NH}_4\text{Cl}$ , 0.037g EDTA, 1g  $\text{KHCO}_3$  in 1L ddH<sub>2</sub>O) and IMDM culture media (10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol complemented with 3% of J558 supernatant (equal to 40ng/ml GM-CSF final), 10 ng/ml rmGM-SCF and 10 ng/ml rmlL-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  $\mu\text{M}$  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  $200 \times g$ , 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  $\text{mm}^2$  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% trypsin 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<sup>high</sup>.

## 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.
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Mignot G, Ullrich E, et al.: **Activation of the NLRP3 inflammasome in dendritic cells induces IL-1 $\beta$ -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/EP2011/055134 filed 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**

# Contribution of IL-17-producing $\gamma\delta$ 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- $\gamma$ -producing CD8 $^+$   $\alpha\beta$  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  $\gamma\delta$  (V $\gamma$ 4 $^+$  and V $\gamma$ 6 $^+$ ) T lymphocytes ( $\gamma\delta$  T17 cells) that precedes the accumulation of Tc1 CTLs within the tumor bed. In T cell receptor  $\delta^{-/-}$  or V $\gamma$ 4/6 $^{-/-}$  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  $\gamma\delta$  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  $\gamma\delta$  T cells restored the efficacy of chemotherapy in IL-17A $^{-/-}$  hosts. The anticancer effect of infused  $\gamma\delta$  T cells was lost when they lacked either IL-1R1 or IL-17A. Conventional helper CD4 $^+$   $\alpha\beta$  T cells failed to produce IL-17 after chemotherapy. We conclude that  $\gamma\delta$  T17 cells play a decisive role in chemotherapy-induced anticancer immune responses.**

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.

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

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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 sequestered 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-1 $\beta$ , which in turn facilitates the priming of CD8 $^{+}$  tumor-specific T cells for IFN- $\gamma$  production that is indispensable for the success of chemotherapy (Ghiringhelli et al., 2009).

Although the contribution of IFN- $\gamma$  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- $\gamma$  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- $\gamma$  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  $\gamma\delta$  T cells in tumor immunosurveillance is still elusive (Hayday, 2009). In humans, V $\delta$ 1 $^{+}$   $\gamma\delta$  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 $\delta$ 2 $^{+}$   $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells (that we termed  $\gamma\delta$  T17) in cancer (Gonçalves-Sousa et al., 2010).  $\gamma\delta$  T17 cells have been reported to share most phenotypic markers with Th17 cells (expressing CCR6, ROR $\gamma$ t, aryl hydrocarbon receptor [AhR], IL-23R, IL-17A, and IL-22; Martin et al., 2009).  $\gamma\delta$  T17 cells depend upon TGF- $\beta$  but not IL-23 or IL-6 for their development and maintenance (Do et al., 2010) and can be activated by IL-1 $\beta$  plus IL-23 (Sutton et al., 2009). They are unrestricted by V $\gamma$  usage (although they are mostly V $\gamma$ 4 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  $\gamma\delta$  T cell antigen specificities, but instead determines their effector fate. When triggered through the TCR, ligand-experienced cells secrete IFN- $\gamma$ , whereas ligand-naïve  $\gamma\delta$  T cells produce IL-17 (Jensen et al., 2008). CD27 $^{+}$   $\gamma\delta$  thymocytes expressed LT $\beta$ R and genes associated with a Th1 phenotype, in contrast to CD27 $^{-}$   $\gamma\delta$  thymocytes which give rise to IL-17–producing  $\gamma\delta$  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  $\gamma\delta$  T cells are required for optimal anticancer responses and that the source of IL-17A is the  $\gamma\delta$  T population during immunogenic chemotherapy and radiotherapy. We show that an early tumor infiltration by  $\gamma\delta$  T17 cells is a prerequisite for optimal tumor colonization of IFN- $\gamma$ –producing CD8 $^{+}$  T cells.  $\gamma\delta$  T cell activation depends on IL-1R1 and IL-1 $\beta$  (but not IL-23) produced by DCs in response to immunogenic dying tumor cells. Finally, the adoptive transfer of WT  $\gamma\delta$  T17 cells can restore the therapeutic efficacy of anticancer chemotherapy that is compromised in IL-17A $^{-/-}$  hosts.

## RESULTS

**A marked Th1 pattern 8 d after chemotherapy**

Anthracyclines induce immune responses that culminate in CD8<sup>+</sup> T cell- and IFN- $\gamma$ /IFN- $\gamma$ R-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- $\gamma$ , were increased by 4–5 fold in DX versus PBS-treated tumors (Fig. 1 C, left). Unsupervised hierarchical clustering indicated that IFN- $\gamma$  production correlated with that of the quintessential Th1 transcription factor, Tbx21. By day 8, the protein levels of IFN- $\gamma$  also increased in DX-treated MCA205 sarcomas (Fig. 1 D, left). Other surrogate markers of Th1 responses (lymphotoxin- $\beta$ , 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  $\mu$ M (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- $\gamma$ - and IL-17-producing tumor-infiltrating lymphocytes (TILs) as tested by flow cytometry (FACS; Fig. 1 E). To identify the cellular source of IFN- $\gamma$  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- $\gamma$  was CD8<sup>+</sup> T cells, whereas that of IL-17 was mostly TCR  $\delta$ <sup>+</sup> T cells rather than CD4<sup>+</sup> Th17 cells 8 d after chemotherapy in MCA205 sarcomas (Fig. 1 F). We further analyzed the IFN- $\gamma$  and IL-17 production by each subset of TILs. CD4<sup>+</sup> T cells could secrete IFN- $\gamma$ , but rarely IL-17.

CD8<sup>+</sup> T and  $\gamma\delta$  T cells were polarized to become potent producers of IFN- $\gamma$  and IL-17, respectively. DX-based chemotherapy substantially enhanced IFN- $\gamma$  production by CD8<sup>+</sup> and CD4<sup>+</sup> TILs, as well as IL-17 production by  $\gamma\delta$  TILs (Fig. 1 G).

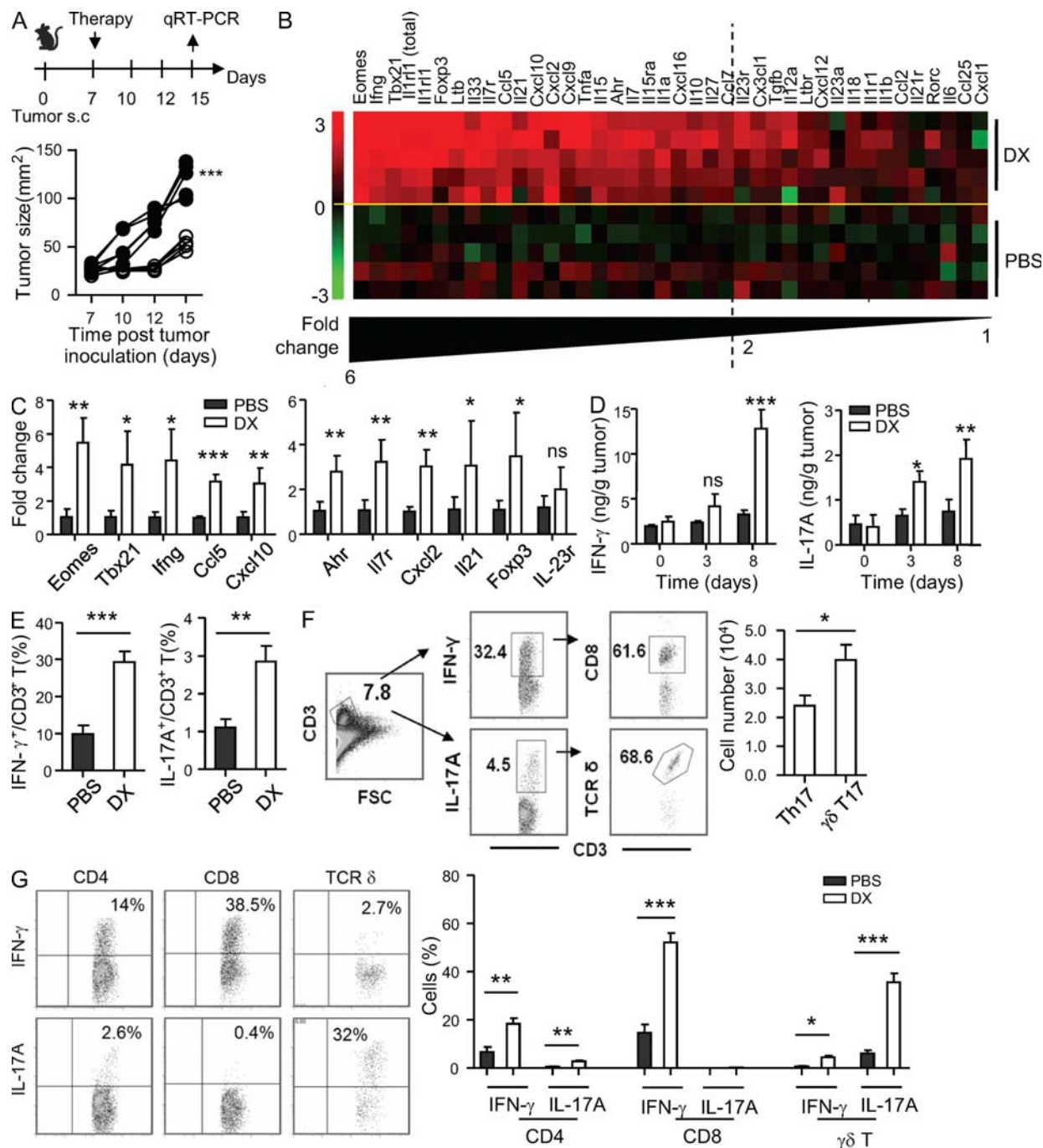
 **$\gamma\delta$  T17 cells preceded and predicted the accumulation of Tc1 CTLs in tumor beds after chemotherapy**

Kinetic experiments revealed that  $\gamma\delta$  TILs invaded MCA205 tumor beds and produced IL-17 shortly after chemotherapy, with significant increases ( $\sim$ 9-fold) over the background 4 d after DX injection (Fig. 2 A, left).  $\gamma\delta$  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  $\gamma\delta$  T cells (Fig. 2 C, left) contrasted with the comparatively late induction of IFN- $\gamma$ -producing CD8<sup>+</sup> 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  $\gamma\delta$  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<sup>+</sup> TILs were CD45<sup>+</sup>CD3<sup>bright</sup>. They failed to express CD4, but were positively stained with anti-TCR  $\delta$ -specific antibodies (Fig. S2 A). Consistently, chemotherapy dramatically increased the frequency of IFN- $\gamma$ -producing CD8<sup>+</sup> T lymphocytes (Tc1; Fig. 3 B) and IL-17A-producing  $\gamma\delta$  T cells ( $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T17 and Tc1 cells (Fig. 3 G).

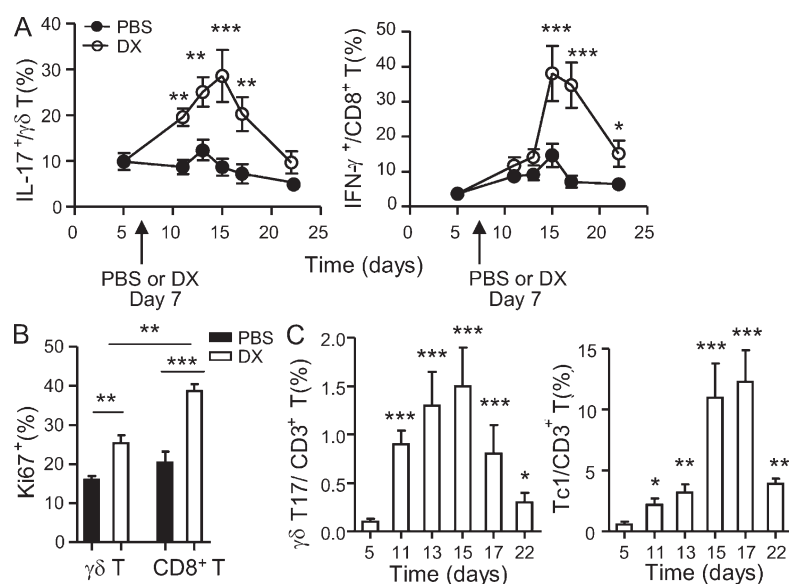
$\gamma\delta$  T17 TILs were preponderantly CD44<sup>+</sup> CD62L<sup>+</sup> CD69<sup>+</sup> and Granzyme B<sup>+</sup>. They did not express CD24, c-kit, NKG2D, CD27 (a thymic determinant for IFN- $\gamma$ -producing  $\gamma\delta$  T cells; Ribot et al., 2009), SCART2 (a specific marker for peripheral IL-17-producing cells which can be down-regulated upon activation; Kisielow et al., 2008), or CD122 (a marker for self antigen-experienced  $\gamma\delta$  T cells with potential to produce IFN- $\gamma$  (Jensen et al., 2008; unpublished data). FACS indicated that  $\sim$ 60% of  $\gamma\delta$  T17 used V $\gamma$ 4 chain (nomenclature of V $\gamma$  genes according to Heilig and Tonegawa [1986]), but expression of V $\gamma$ 1 and V $\gamma$ 7 chain was rarely found (Fig. S2 B). We then sorted V $\gamma$ 1<sup>+</sup>V $\gamma$ 4<sup>+</sup>V $\gamma$ 7<sup>+</sup>  $\gamma\delta$  T17 TILs (Fig. S2 C) and performed single-cell PCRs and sequencing (Pereira and Boucontet, 2004) to examine their V $\gamma$  chain usage. The majority of these cells (21 of 23 clones) contained functional





**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<sup>+</sup> and CD3<sup>+</sup> cells. (F) IFN-γ<sup>+</sup> and IL-17A<sup>+</sup> cells were gated, and the proportions of CD3<sup>+</sup> CD8<sup>+</sup> cells and CD3<sup>+</sup> TCR δ<sup>+</sup> 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<sup>+</sup>, CD8<sup>+</sup>, and TCR δ<sup>+</sup> 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.





**Figure 2.  $\gamma\delta$  T17 cells preceded Tc1 CTL into tumors after chemotherapy.** (A) The percentages of IL-17<sup>+</sup> and IFN- $\gamma$ -producing cells among all tumor infiltrating  $\gamma\delta$  T cells and CD8<sup>+</sup> 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  $\gamma\delta$  T and CD8<sup>+</sup> TILs 8 d after treatment. (C) The percentages of  $\gamma\delta$  T17 and Tc1 among all CD3<sup>+</sup> 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. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

V $\gamma$ 6 rearrangements identical to those found in fetal  $\gamma\delta$  T cells (Lafaille et al., 1989). These experiments show that most  $\gamma\delta$  T17 TILs express V $\gamma$ 4 or V $\gamma$ 6 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  $\gamma\delta$  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  $\gamma\delta$  T17 cells accumulated within tumors after chemotherapy or radiotherapy in a coordinated fashion, we determined whether neutralizing their signature cytokines IFN- $\gamma$  and IL-17A could mitigate the efficacy of anticancer therapies. Antibody-mediated neutralization of either IFN- $\gamma$  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<sup>−/−</sup> 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).

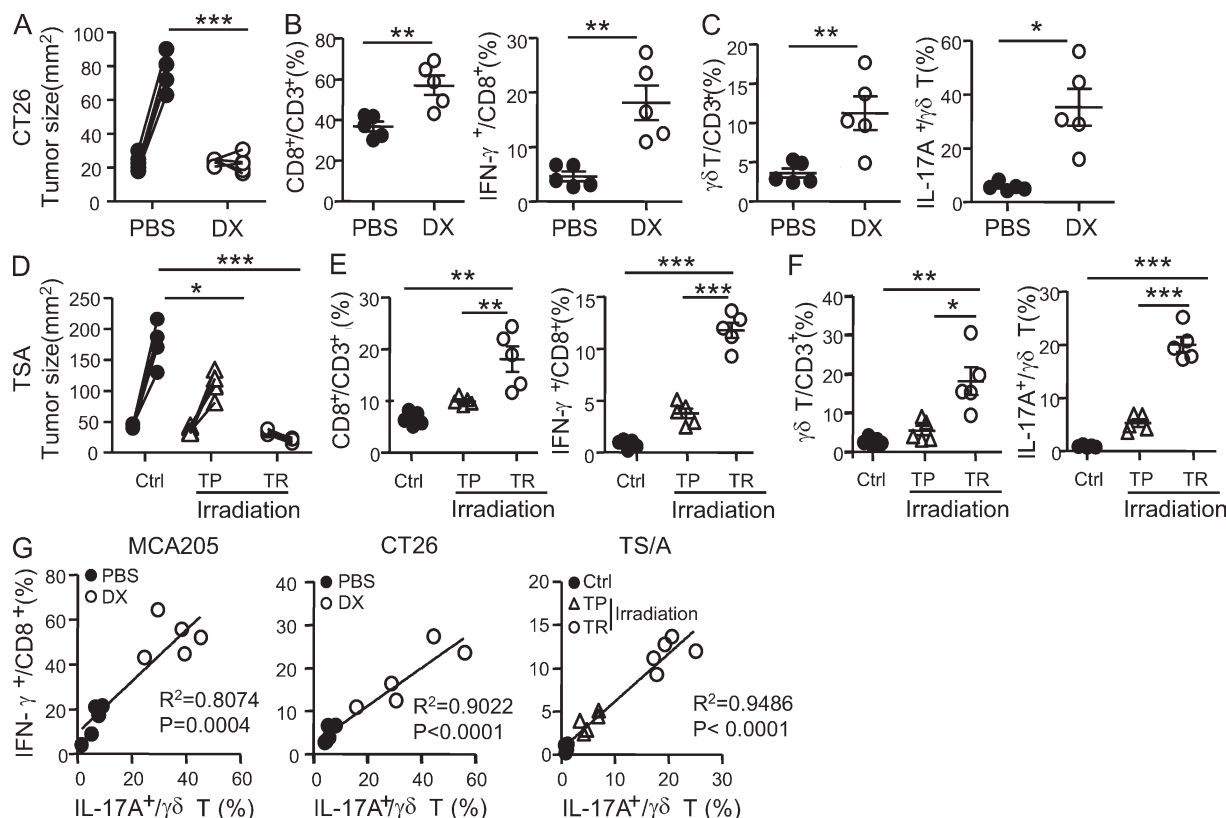
To rationalize the sequential recruitment of  $\gamma\delta$  T17 and Tc1 cells into the tumor bed after chemotherapy, we hypothesized that  $\gamma\delta$  T17 might act as helper cells for Tc1 priming. We previously reported that specific antitumor immune responses rely on Tc1 cells primed by tumor cells undergoing immunogenic cell death by using a system in which IFN- $\gamma$  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<sup>−/−</sup> 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<sup>−/−</sup> mice. Because IL-17 was not significantly produced by CD4<sup>+</sup> or CD8<sup>+</sup> 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  $\gamma\delta$  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<sup>−/−</sup> 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, V $\gamma$ 4/6<sup>−/−</sup> 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  $\gamma\delta$  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- $\gamma$  production.

### IL-1 $\beta$ -dependent, but not IL-23-dependent, activation of $\gamma\delta$ T lymphocytes

The IL-1 $\beta$ –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  $\gamma\delta$  T17 cells.

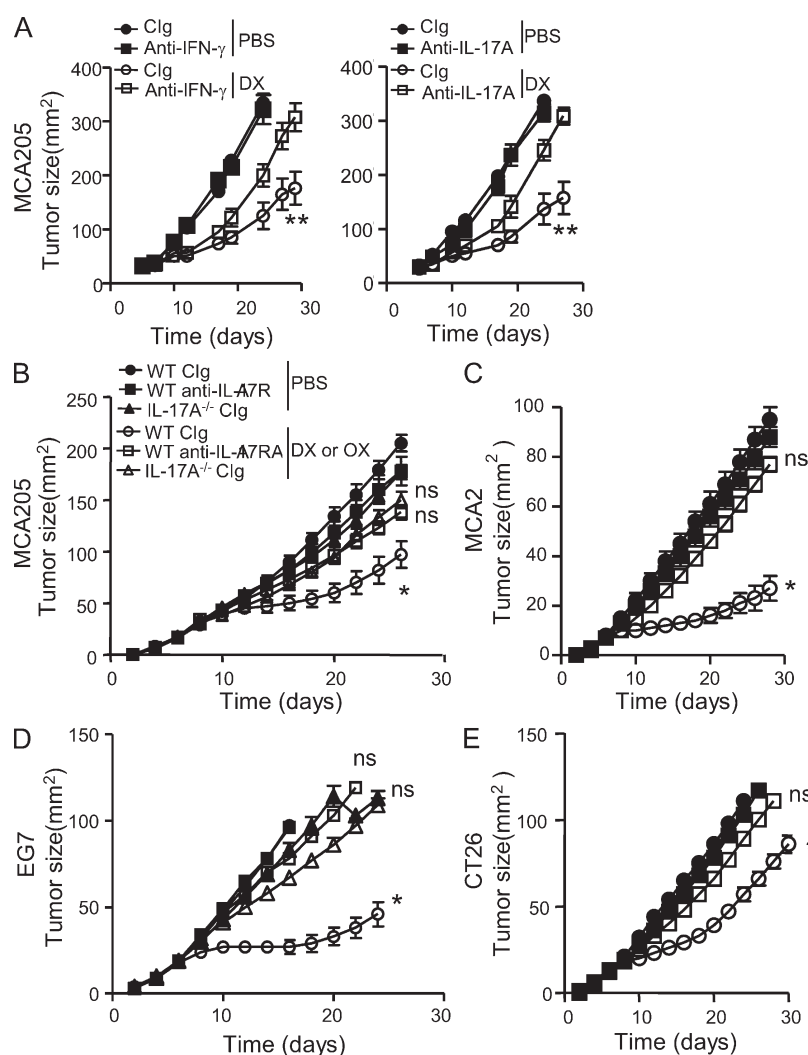
To explore the molecular requirements for  $\gamma\delta$  T17 activation in situ, we sorted  $\gamma\delta$  T cells from the skin-draining LNs



**Figure 3. Recruitment of both Tc1 and  $\gamma\delta$  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<sup>+</sup> T cells among CD3<sup>+</sup> cells and of IFN- $\gamma$ -producing cells among CD8<sup>+</sup> T cells. (C) The percentage of  $\gamma\delta$  T cells among CD3<sup>+</sup> cells and of IL-17A-producing cells among CD3<sup>+</sup>  $\gamma\delta$  T cells. Data are presented as mean  $\pm$  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<sup>+</sup> T cells among CD3<sup>+</sup> cells and of IFN- $\gamma$ -producing cells among CD8<sup>+</sup> T cells; (F) The percentage of  $\gamma\delta$  T cells among CD3<sup>+</sup> cells and of IL-17A-producing cells among CD3<sup>+</sup>  $\gamma\delta$  T cells are indicated as mean  $\pm$  SEM. (G) The correlation between the percentages of  $\gamma\delta$  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  $\gamma\delta$  T cells,  $\sim 70\%$  harbored the V $\gamma 4$  TCR. Moreover, these cells vigorously produced IL-17A (but not IFN- $\gamma$ ) upon stimulation with PMA/ionomycin (Fig. S2 F; Do et al., 2010). In contrast to Th17 cells (Ivanov et al., 2006), LN-resident  $\gamma\delta$  T cells failed to produce IL-17 in response to TGF- $\beta$  or IL-6 alone, or in combination with IL-1 $\beta$ . However, they potently secreted IL-17 and IL-22 in response to the combined stimulation of IL-1 $\beta$  plus IL-23 (unpublished data; Sutton et al., 2009). TCR engagement also synergized with IL-1 $\beta$  (and to a lesser extent with IL-23) to trigger IL-17 and IL-22 secretion by LN-resident  $\gamma\delta$  T cells (unpublished data). It is noteworthy that these stimuli specifically activated IL-17A, but not IFN- $\gamma$  production by  $\gamma\delta$  T cells. Because  $\gamma\delta$  T17 cells were activated (as indicated by their Ki67<sup>+</sup>, GzB<sup>+</sup>, CD69<sup>+</sup>, and IL-17<sup>+</sup> phenotype) after chemotherapy, we addressed whether dying tumor cells could directly promote the activation of  $\gamma\delta$  T17. Although DX-treated MCA205 cells failed to directly induce IL-17 secretion by  $\gamma\delta$  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-1 $\beta$  and markedly stimulated the release of IL-17 and IL-22 by  $\gamma\delta$  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<sup>+</sup>MHCII<sup>+</sup>) rather than macrophages ( $>70\%$  CD11b<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>−</sup>) could activate  $\gamma\delta$  T cells for IL-17A production when they encountered DX-treated tumor cells. CD11b<sup>+</sup>Gr1<sup>+</sup> neutrophils reportedly produce IL-17 and promote downstream IL-12/IFN- $\gamma$  contributing to reperfusion injury (Li et al., 2010). Interestingly, CD11b<sup>+</sup>Gr1<sup>+</sup> cells sorted from DX-treated tumor beds bearing the IL-1 $\beta$  messenger RNA failed to secrete IL-1 $\beta$  or IL-17A protein and failed to activate  $\gamma\delta$  T cells for IL-17A production in vitro (unpublished data). IL-17 production by  $\gamma\delta$  T cells was dependent on IL-1 $\beta$  because the IL-1R1 antagonist IL-1RA entirely abrogated the DC/ $\gamma\delta$  T cell cross talk in the presence



**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- $\gamma$  (left), IL-17A (right), or control Ig (Clg) i.p. every 2 d (3 injections, 200  $\mu$ g/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-17A (squares) or Clg. Tumor sizes are plotted as mean  $\pm$  SEM for 5–15 mice/group, and each experiment was repeated at least 2 times, yielding similar results. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

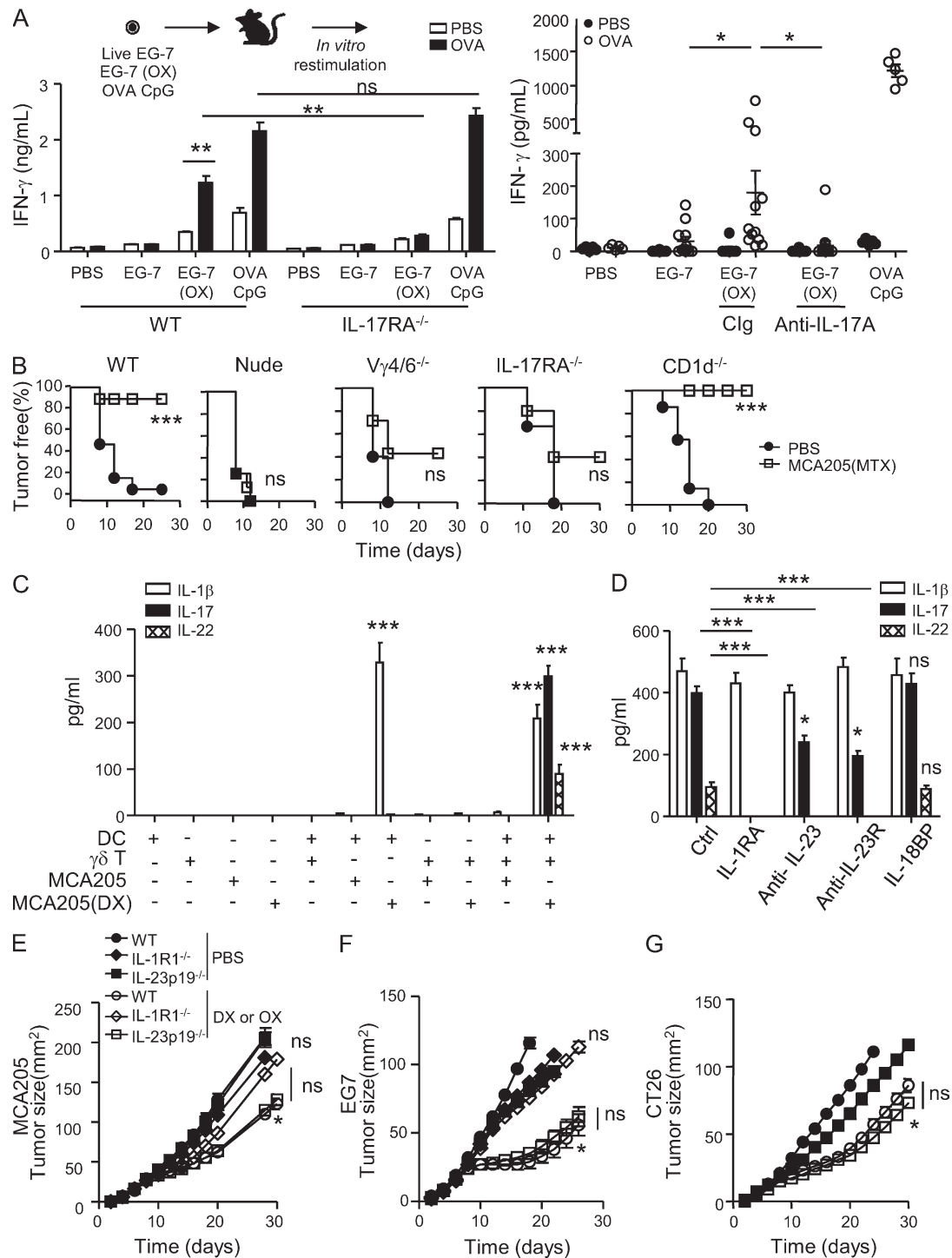
#### $\gamma\delta$ T lymphocytes are indispensable for the immune-dependent effects of chemotherapy

To further evaluate the contribution of  $\gamma\delta$  T cells to the therapeutic action of DX on established MCA205 sarcomas, such tumors were implanted into age- and sex-matched WT, TCR  $\delta^{-/-}$ , V $\gamma$ 4/6 $^{-/-}$  mice, and then subjected to chemotherapy. As compared with WT controls, the absence of the TCR  $\delta$  chain, as well as that of V $\gamma$ 4 and V $\gamma$ 6  $\gamma\delta$  T cells, greatly reduced the efficacy of chemotherapy (Fig. 6 A). At day 8 after chemotherapy, when  $\gamma\delta$  T17 and Tc1 massively infiltrated tumor beds in WT mice, these cytokine-producing TILs were either absent or greatly reduced in V $\gamma$ 4/6 $^{-/-}$  mice (Fig. 6 B), suggesting that the presence of V $\gamma$ 4 and V $\gamma$ 6  $\gamma\delta$  T cells are critical for the optimal Tc1 response in tumor beds.

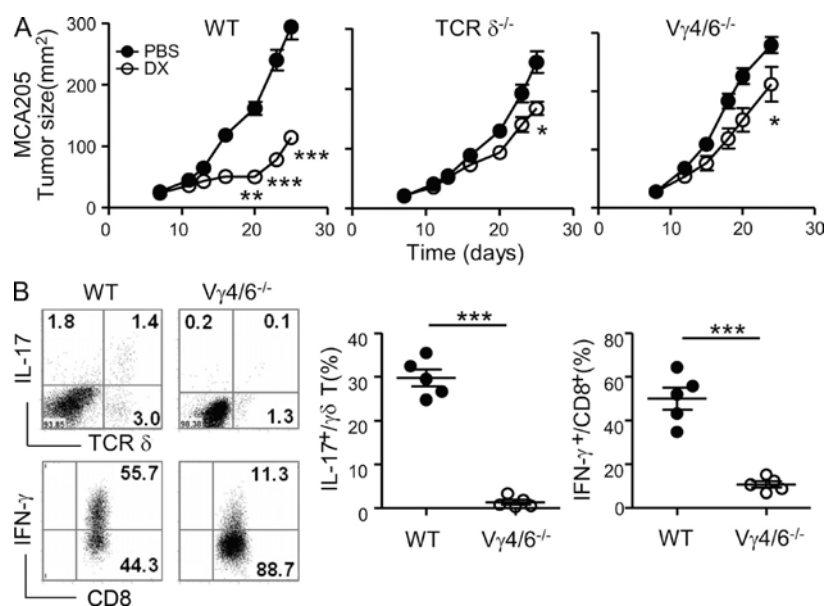
Expression of CCR6 is a phenotypic and functional hallmark of Th17 cells (Reboldi et al., 2009) during some inflammatory processes. We therefore analyzed the role of CCR6 in the efficacy of chemotherapy. Because CCL20 was detectable in tumor tissues before and after chemotherapy (unpublished data), we assessed whether  $\gamma\delta$  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  $\gamma\delta$  T17 (unpublished data). Therefore, V $\gamma$ 4 and V $\gamma$ 6  $\gamma\delta$  T cells contribute to the immune-mediated action of anticancer agents in a CCR6-independent fashion.

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

of dying cells. The neutralization of IL-18R, IL-23, or IL-23R failed to abolish IL-17 production by  $\gamma\delta$  T cells co-cultured with DCs (Fig. 5 D). IL-22 production was completely abolished by blocking the IL-1 $\beta$ -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-1 $\beta$ -activated  $\gamma\delta$  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-1 $\beta$  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.



**Figure 5. Role of  $\gamma\delta$  T17 in the priming of T cell responses during an immunogenic cell death and regulation by IL-1 $\beta$ .** (A) OX-treated EG-7 cells were inoculated in the footpad of WT versus IL-17RA<sup>-/-</sup> mice ( $n = 5$ ; left) along with anti-IL-17A neutralizing antibody (or Clg; right panel). OVA-specific IFN- $\gamma$  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 C57Bl/6 ( $n = 10$ ), nude ( $n = 10$ ), V $\gamma$ 4/6<sup>-/-</sup> ( $n = 15$ ), IL-17RA<sup>-/-</sup> ( $n = 8$ ), and CD1d<sup>-/-</sup> ( $n = 6$ ) 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-1 $\beta$ , IL-17A, and IL-22 from mixed co-cultures of LN-derived  $\gamma\delta$  T cells and/or BMDCs loaded or not loaded with live or DX-treated MCA205 was monitored by ELISA. Data are shown as mean  $\pm$  SEM (D) Co-cultures of DX-treated MCA205/BMDC/ $\gamma\delta$  T were performed in the presence of 20  $\mu$ g/ml IL-1RA (Amgen), anti-IL-23, or



**Figure 6. The therapeutic activity of anthracyclines and tumor colonization of Tc1 depend upon V $\gamma$ 4 V $\gamma$ 6  $\gamma\delta$  T cells.** (A) WT, TCR  $\delta^{-/-}$ , or  $V\gamma 4/6^{-/-}$  mice with established MCA205 tumors were injected intratumorally with PBS or DX. Tumor size was measured at the indicated time and plotted as mean  $\pm$  SEM ( $n = 8/\text{group}$ ). (B) Percentage of IL-17A<sup>+</sup> or IFN- $\gamma$ -expressing cells within CD3<sup>+</sup> TCR $\delta$ <sup>+</sup> and CD3<sup>+</sup> CD8<sup>+</sup> TILs, respectively, in WT or  $V\gamma 4/6^{-/-}$  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  $\gamma\delta$  T cells were lost when the  $\gamma\delta$  T cells were obtained from IL-17A<sup>-/-</sup> or IL-1R1<sup>-/-</sup> donors (Fig. 7, B and C), emphasizing the role of IL-1 $\beta$  responses and IL-17 production in the function of  $\gamma\delta$  T cells. Moreover, the adoptive transfer of WT  $\gamma\delta$  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  $\gamma\delta$  T17 cells to the immune-dependent effects of anticancer chemotherapy.

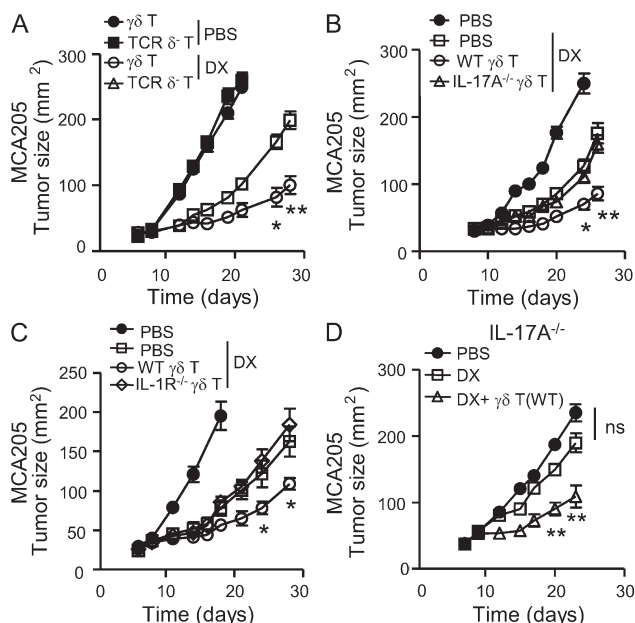
## DISCUSSION

Our results highlight a role of  $\gamma\delta$  T cells, particularly the V $\gamma$ 4<sup>+</sup> and V $\gamma$ 6<sup>+</sup>-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- $\gamma$ -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<sup>+</sup> T cells are depleted (Casares et al., 2005), and chemotherapy fails to work when the IFN- $\gamma$ –IFN- $\gamma$ R 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  $\gamma\delta$  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  $\gamma\delta$  T markers. Second, antigen-specific CD4<sup>+</sup> T cells in LNs draining the dying tumor cells showed a Th1 (IL-2 and IFN- $\gamma$ ) instead of a Th17 cytokine pattern (Ghiringhelli et al., 2009). CD4<sup>+</sup> and CD8<sup>+</sup> TILs were polarized to produce IFN- $\gamma$  instead of IL-17. Also, IL-6 and TGF- $\beta$ , 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-CD3 $\epsilon$  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  $\gamma\delta$  T cells (Sutton et al., 2009). Fourthly, the subset of NKT cells capable of producing IL-17 in LNs (CD103<sup>+</sup>CD4<sup>+</sup>NK1.1<sup>+</sup>CCR6<sup>+</sup>CD1d tetramer<sup>+</sup>; Doisne et al., 2009) did not appear to be specifically triggered by dying cells in vivo (unpublished data). Moreover, CD1d<sup>-/-</sup> mice, which lack NKT cells, were indistinguishable from WT mice when the efficacy of chemotherapy was assessed in prophylactic vaccination settings. Fifthly, knockout of V $\gamma$ 4/6 or TCR  $\delta$  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  $\gamma\delta$  T cells into IL-17A<sup>-/-</sup> hosts could restore the clinical response to chemotherapy and improve

IL-23R neutralizing antibodies, or 10  $\mu\text{g}/\text{ml}$  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<sup>-/-</sup> (diamonds), and IL-23p19<sup>-/-</sup> (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. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .





**Figure 7. Role of  $\gamma\delta$  T cell-derived IL-17A during chemotherapy.** CD3<sup>+</sup> TCR  $\delta^+$  or CD3<sup>+</sup> TCR  $\delta^-$  T cells from WT mice (A), CD3<sup>+</sup> TCR  $\delta^+$  T cells from IL-17A<sup>-/-</sup> (B), or IL-1R1<sup>-/-</sup> (C) mice were injected intratumorally into MCA205-bearing WT mice (A–C) or IL-17A<sup>-/-</sup> mice (D) 2 d after PBS or DX treatment. Tumor sizes are plotted as mean  $\pm$  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  $\gamma\delta$  T cells from IL-17A<sup>-/-</sup> (rather than WT) donors were used.

In the context of immunogenic chemotherapy, it appears clear that IL-1 $\beta$  plays a major role in stimulating IL-17 production and the anticancer function of  $\gamma\delta$  T cells. The key role of IL-1 $\beta$  in regulating  $\gamma\delta$  T cells function was shown by using IL-1RA in co-cultures of DCs/ $\gamma\delta$  T cells in the presence of dying tumor cells. Also,  $\gamma\delta$  T cells that lack IL-1R1 lose the capacity to amplify the tumoricidal action of anthracyclines. Interestingly, inflammasome-dependent IL-1 $\beta$  secretion from DCs was also found to be mandatory for the polarization of CD8<sup>+</sup> T cells toward a Tc1 pattern (Ghiringhelli et al., 2009), suggesting that a connection between DCs,  $\gamma\delta$  T17 cells, and Tc1 cells might be important for optimal anticancer immune responses. We noticed a strong correlation between  $\gamma\delta$  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- $\gamma$  by TILs. It is well possible that besides helping the development of Tc1 response,  $\gamma\delta$  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  $\gamma\delta$  T17 associated with Th1 responses exert protective immune response (Umemura et al., 2007). As IL-17 could not directly induce IFN- $\gamma$  production or enhance proliferation of CD8<sup>+</sup> T cells (unpublished data), our

results imply a causal relationship between the presence of  $\gamma\delta$  T17 cells and the recruitment of antitumor effector Tc1 cells into tumor beds.

$\gamma\delta$  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).  $\gamma\delta$  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).  $\gamma\delta$  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  $\gamma\delta$  T17 cells, our data suggest that IL-1 $\beta$  acts as a major trigger. One previous report demonstrated the pivotal function of IL-1 $\beta$  in regulating  $\gamma\delta$  T17 cells in experimental autoimmune encephalomyelitis (EAE; Sutton et al., 2009). In that model, IL-1 $\beta$  synergized with IL-23 to promote IL-17 production by  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T17 cells also be recruited into human tumor beds after chemotherapy, it would be of the utmost importance to determine their TCR V $\delta$  usage to propose combination therapy of phosphoantigens (for V $\delta$ 2<sup>+</sup>) or other ligands or innate cytokines (for V $\delta$ 2<sup>-</sup>) and anthracyclines to increase therapeutic benefit in neoadjuvant settings or prevent metastases.

## MATERIALS AND METHODS

**Mice.** WT C57BL/6 (H-2<sup>b</sup>) and BALB/c (H-2<sup>d</sup>) 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<sup>-/-</sup>, and IL-17RA<sup>-/-</sup> (H-2<sup>b</sup>) 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<sup>-/-</sup> and IL-17A<sup>-/-</sup> (H-2<sup>b</sup>) were provided by M.J. Smyth (Peter MacCallum Cancer Centre, Victoria, Australia). V $\gamma$ 4 $\gamma$ 6<sup>-/-</sup> mice (H-2<sup>b</sup>) were provided by G. Matsuzaki (University of the Ryukyus, Okinawa, Japan) and K. Ikuta (Kyoto University, Kyoto, Japan). CD1d<sup>-/-</sup> and CCR6<sup>-/-</sup> (H-2<sup>b</sup>) 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<sup>d</sup>) colon cancer, MCA205 (H-2<sup>b</sup>) and MCA2 (H-2<sup>d</sup>) sarcoma, TS/A mammary cancer (H-2<sup>d</sup>), and EG7 thymoma (H-2<sup>b</sup>) were cultured in RPMI 1640 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<sub>2</sub>. All media were purchased from Invitrogen. Recombinant mouse IL-1 $\beta$ , IL-23, IL-6, TGF- $\beta$ , 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<sub>6</sub>(3) were obtained from Sigma-Aldrich. Mouse IL-17A, IL-1 $\beta$ , 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), CD3 $\epsilon$  (145-2C11), CD4 (GK1.5), CD8 $\alpha$  (53-6.7), TCR  $\delta$  (GL-3), CD69 (H1.2F3), IL-17A (TC11-18H10), or IFN- $\gamma$  (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- $\gamma$  (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- $\beta$  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.**  $8 \times 10^5$  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  $\mu$ l) or OX (5 mg/kg body weight, i.p.) when tumors reached 25–45 mm<sup>2</sup>. 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<sup>2</sup>.

**Gene expression assays.** Whole RNA was extracted using RNeasy Mini kit (QIAGEN) from tumor homogenates. 5  $\mu$ g 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- $\mu$ m cell strainer. Cells were blocked with 10  $\mu$ g/ml anti-CD16/CD32 (eBioscience) before surface staining (2.5  $\mu$ g/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  $\mu$ g/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- $\gamma$  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  $\gamma\delta$  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  $\gamma\delta$  T cell purification (TCR $\gamma$ / $\delta^+$  T Cell Isolation kit) using AutoMACS Separator (Miltenyi Biotec) with recommended programs. Purity of this isolation normally reached >95%. The TCR  $\delta^-$  CD3<sup>+</sup> cell fraction was also collected and used as control for some experiments. Day 2 after chemotherapy,  $2.5 \times 10^5$  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  $\mu$ g/ml OX overnight or left untreated were washed thoroughly and injected at 1 million/50  $\mu$ l into the foodpad of naive syngeneic mice. CpG/OVA (5  $\mu$ g CpG+1 mg OVA/mouse) and PBS injection were used as positive and negative controls. In some setting, neutralizing antibody (200  $\mu$ g/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  $3 \times 10^5$ /well and restimulated with 1 mg/ml OVA protein. IFN- $\gamma$  secretion was measured by OptEIA Mouse IFN- $\gamma$  ELISA kit (BD). MCA205 cells were treated with 2  $\mu$ M MTX overnight, washed thoroughly, and injected into left flank s.c. at  $3 \times 10^5$ /mouse. PBS was used as control. Mice were rechallenged with  $5 \times 10^4$  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 Iscove's medium (Sigma-Aldrich) with J558 supernatant (40 ng/ml GM-CSF), 10% FCS, 100 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 50  $\mu$ M 2-mercaptoethanol (Sigma-Aldrich) and used between day 8 and 12 when the proportion of CD11c/MHC class II<sup>+</sup> cells was >80%. In mixed co-cultures, DCs were seeded at  $10^5$ /100  $\mu$ l/well in U-bottom 96-well plates. Tumor cells were treated overnight with 25  $\mu$ M DX or left untreated, washed, and used at  $7.5 \times 10^4$ /100  $\mu$ l/well.  $2 \times 10^4$ /50  $\mu$ l  $\gamma\delta$  T cells were added 12 h later. Supernatant was collected 36 h later.

**Statistical analyses of experimental data.** All results are expressed as mean  $\pm$  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 V $\gamma$  chain usage of tumor-infiltrating  $\gamma\delta$  T17 and  $\gamma\delta$  T cells in the LNs of naive mice. Fig. S3 shows the effect of neutralizing IL-22, CCL20, IL-6, or blocking TGF- $\beta$  on the efficacy of chemotherapy or vaccine. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100269/DC1>.

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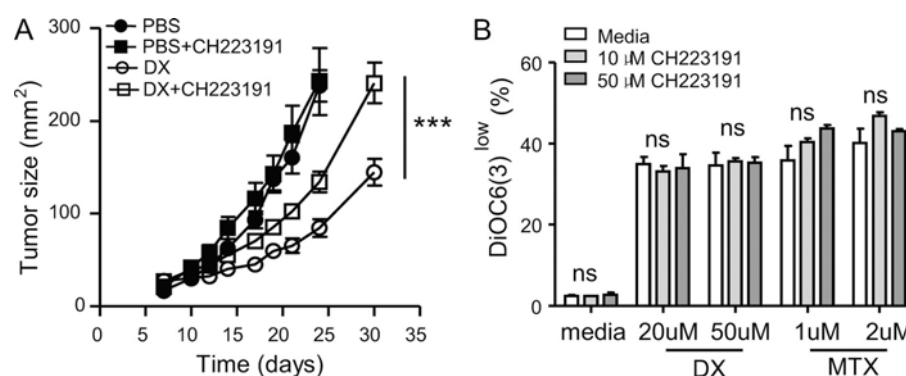
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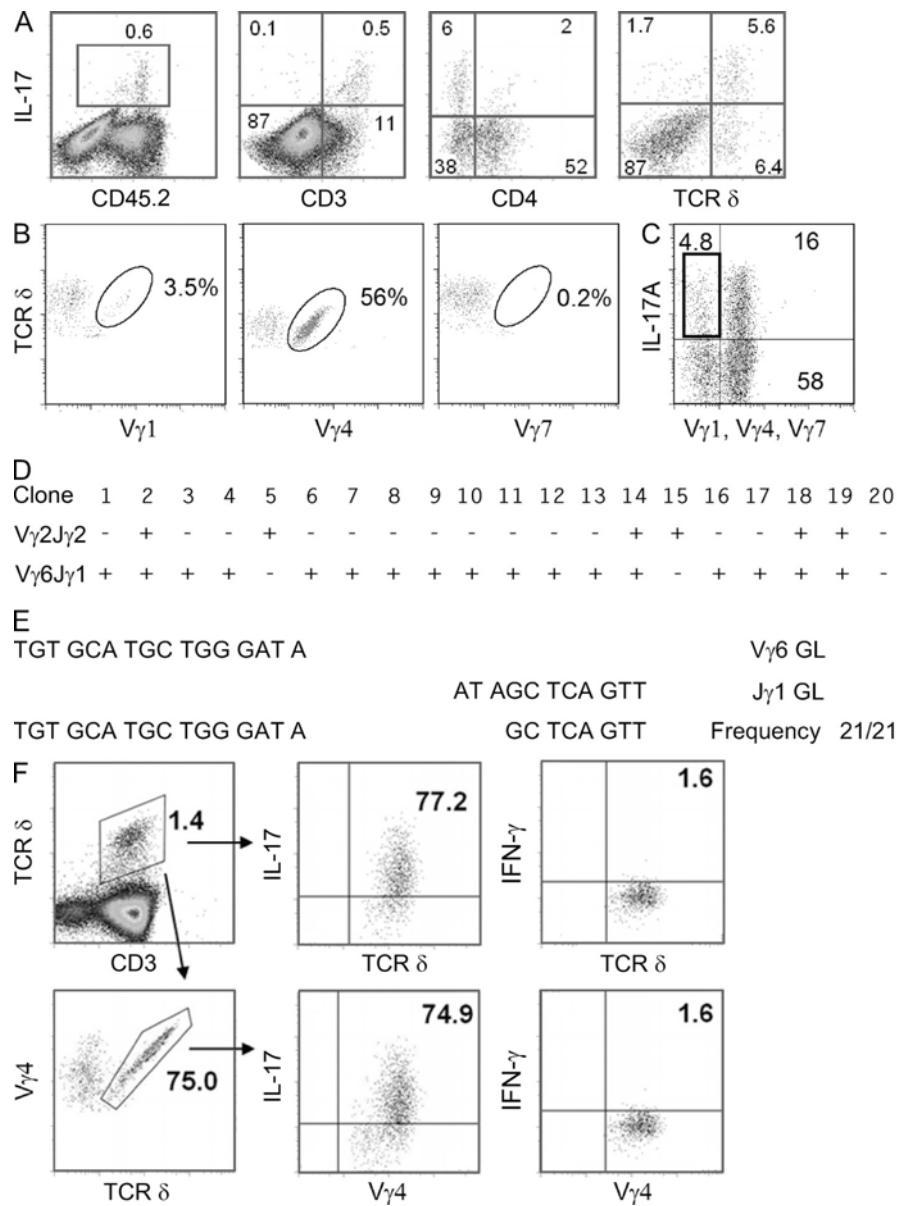
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SUPPLEMENTAL MATERIAL

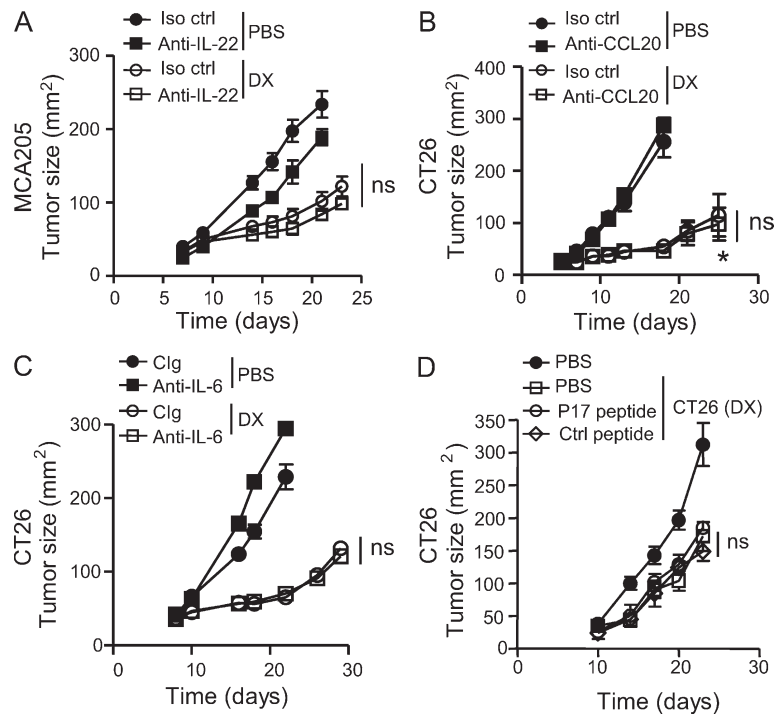
Ma et al., <http://www.jem.org/cgi/content/full/jem.20100269/DC1>



**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.



**Figure S2. V $\gamma$  chain usage by  $\gamma\delta$  T17 cells in tumor bed and skin draining LNs of naive mice.** (A) CD45, CD3, CD4, and TCR  $\delta$  expression by IL-17A-producing cells from tumor beds of mice 8 d after chemotherapy. (B) V $\gamma$  usage in live CD45<sup>+</sup> CD3<sup>+</sup> TCR  $\delta$ <sup>+</sup> IL-17<sup>+</sup> cells in tumor beds after DX. Numbers represent the percentage of V $\gamma$ 1<sup>+</sup>, V $\gamma$ 4<sup>+</sup>, or V $\gamma$ 7<sup>+</sup> cells among TCR  $\delta$ <sup>+</sup> IL-17<sup>+</sup> cells as indicated. One experiment representative of three is shown. (C–E) Individual V $\gamma$ 1–V $\gamma$ 4–V $\gamma$ 7– $\gamma\delta$  T17 TILs as gated in (C) were sorted in PCR plates, and DNA was amplified with primers specific for V $\gamma$ 2–J $\gamma$ 2 or V $\gamma$ 6–J $\gamma$ 1 rearrangements. (D) Presence (+) or absence (–) of specific amplification bands in 24 clones analyzed. (E) Junctional sequences of the V $\gamma$ 6–J $\gamma$ 1 amplifications present in 21 clones in D. GL denotes the germline sequences of the V $\gamma$ 6 and J $\gamma$ 1 ends as indicated. (F) LN cells from naive mice were stimulated with PMA/ionomycin, and total  $\gamma\delta$  T cells (top) or V $\gamma$ 4<sup>+</sup> cells (bottom) were gated and analyzed for intracellular IL-17 (middle) or IFN- $\gamma$  (right) by FACS. Numbers indicate the percentage of cells inside the gates. One experiment representative of four is shown.



**Figure S3. Dispensable roles of IL-22, CCL20, IL-6, and TGF- $\beta$  for the efficacy of chemotherapy.** (A and B) Neutralizing antibodies against IL-22 (50  $\mu$ g/mouse; A), CCL20 (200  $\mu$ g/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  $\mu$ g/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- $\beta$  or a control peptide (100  $\mu$ g/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.

# 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<sup>+</sup> T-cell responses (5, 6), promotes antigen cross-presentation by dendritic cells (7), and directly acts on effector CD8<sup>+</sup> T and natural killer (NK) cells to augment IFN- $\gamma$  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- $\gamma$  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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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  $\alpha$ 2b 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  $\mu$ mol/L  $\beta$ -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*<sup>-/-</sup>, *Cxcr3*<sup>-/-</sup>, and *Ccr5*<sup>-/-</sup> 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;  $5 \times 10^4$ ) or primary human breast cancer cells ( $2 \times 10^5$ ) 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 ( $3 \times 10^5$  or  $6 \times 10^5$ ) and GL26 ( $6 \times 10^5$ ) 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  $\mu$ g/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  $\mu$ mol/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  $\mu$ g per mouse in B16-OVA model and at 500  $\mu$ g per mouse in the GL26 model. MetRantes (10  $\mu$ g/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  $\mu$ g 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'-CGCGACGTCAAGGAGTATTTCTATTCAAGAGATAGAAATACTCCTTGACGTTTTTTTGA-3' and the reverse primer 3'-TGCAGTTCTCATAAAGATAAGTTCTCTATCTTTATGAGGAAGTGCAGAAAAA-5' were annealed and ligated into vector [pLV-H1-EF1-PURO-IRES-GFP (pV2.3.127)] by cohesive *MluI/NsiI* 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- $\mu$ m cell strainer. CD3 $\epsilon$ -PerCP, CD8-FITC (BD Pharmingen), CXCR3-PE (R&D System), NK1.1-Pacific Blue (eBioscience), and isotype control antibodies (2.5  $\mu$ g/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  $\mu$ g/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- $\gamma$ -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  $10,000 \times g$  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<sup>+</sup> and CD45<sup>-</sup> fractions, and whole-cell protein was extracted using lysis buffer ( $1 \times 10^6$  cells/100  $\mu$ L buffer).

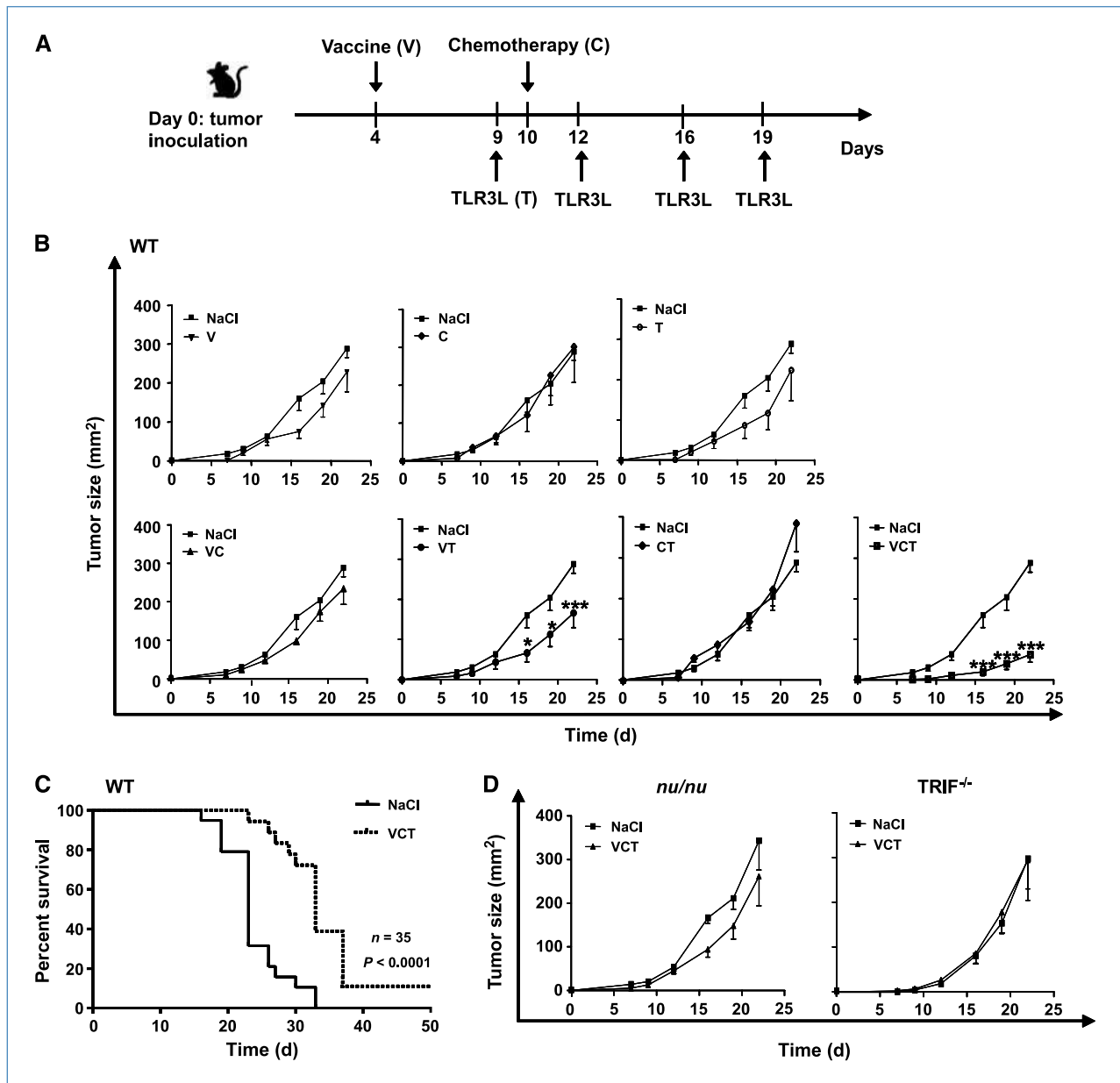
**Statistical analyses.** Comparison of continuous data and categorical data were achieved by the Mann-Whitney *U* test and by  $\chi^2$  as appropriate. The log-rank test was used for analysis of Kaplan-Meier survival curves. Statistical analyses were performed using GraphPad Prism 5.0. All *P* values 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 cyto-

static 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. 1B), following the protocol detailed in Fig. 1A. 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. 1B) and prolonged the survival of tumor-bearing C57BL/6 mice (Fig. 1C). This vaccine, when applied in the



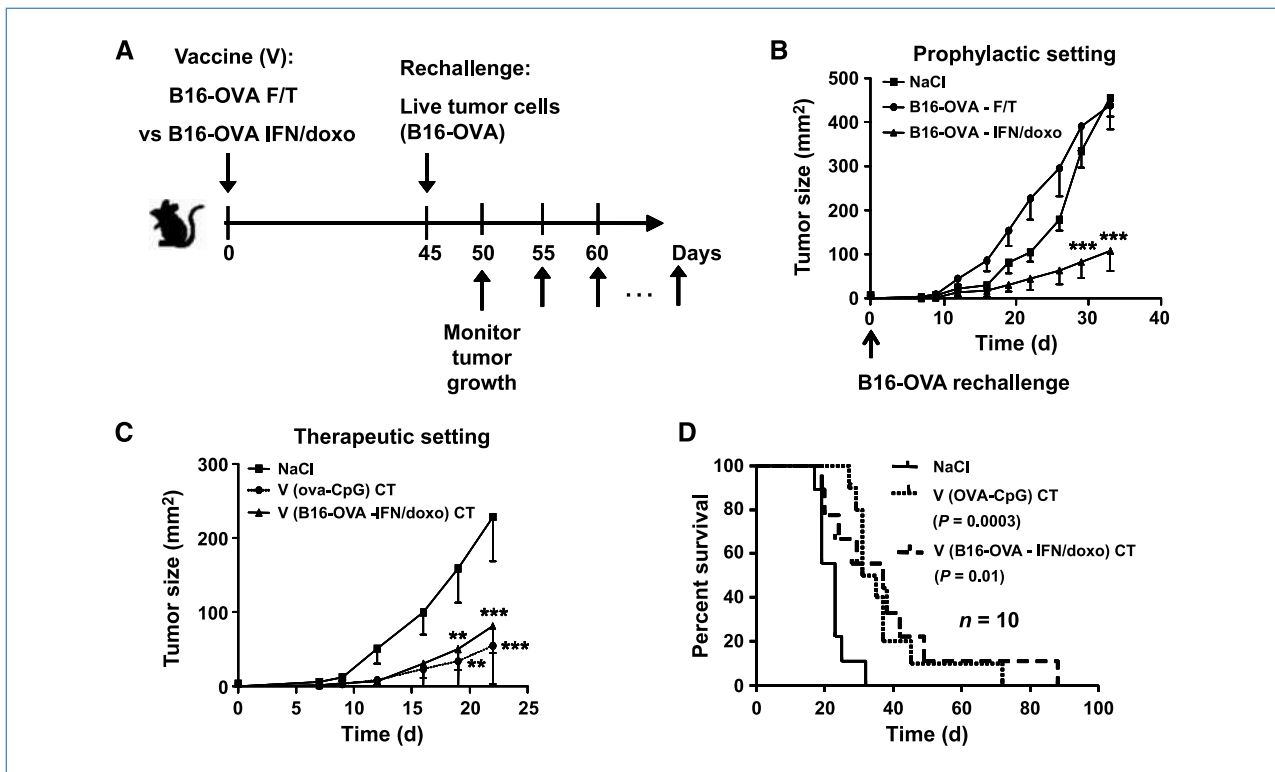
**Figure 1.** Sequential immunochemotherapy is efficient against established melanoma. **A**, therapeutic setting of VCT treatment is shown as a scheme. **B** and **C**, B16OVA tumor growth was monitored in WT mice receiving single-agent therapy (V, C, or T), two agent-based therapy (VC, VT, or CT), 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*<sup>-/-</sup> (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*<sup>-/-</sup> mice (Fig. 1D), 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. 1C), 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

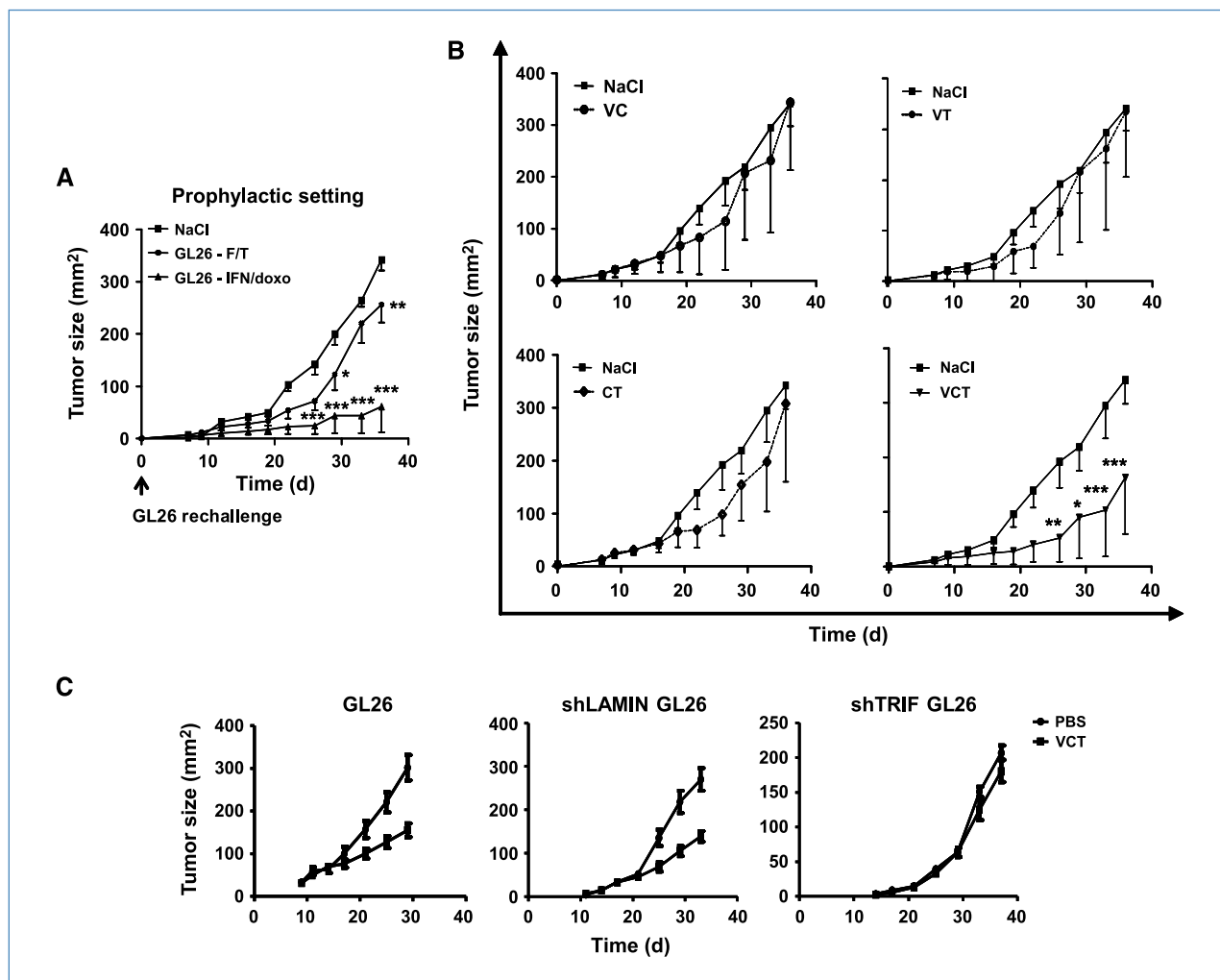
(Fig. 2A and B). This cell-based vaccine boosted the antitumor activity of the combination of oxaliplatin plus poly(A:U) (Fig. 2C) and enhanced survival (Fig. 2D) when used in a therapeutic setting after the implantation of tumors. Very similar results were obtained when B16-OVA melanoma cells were replaced 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. 3A) 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. 1A (Fig. 3B). 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. 3C).

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. 1A 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**).





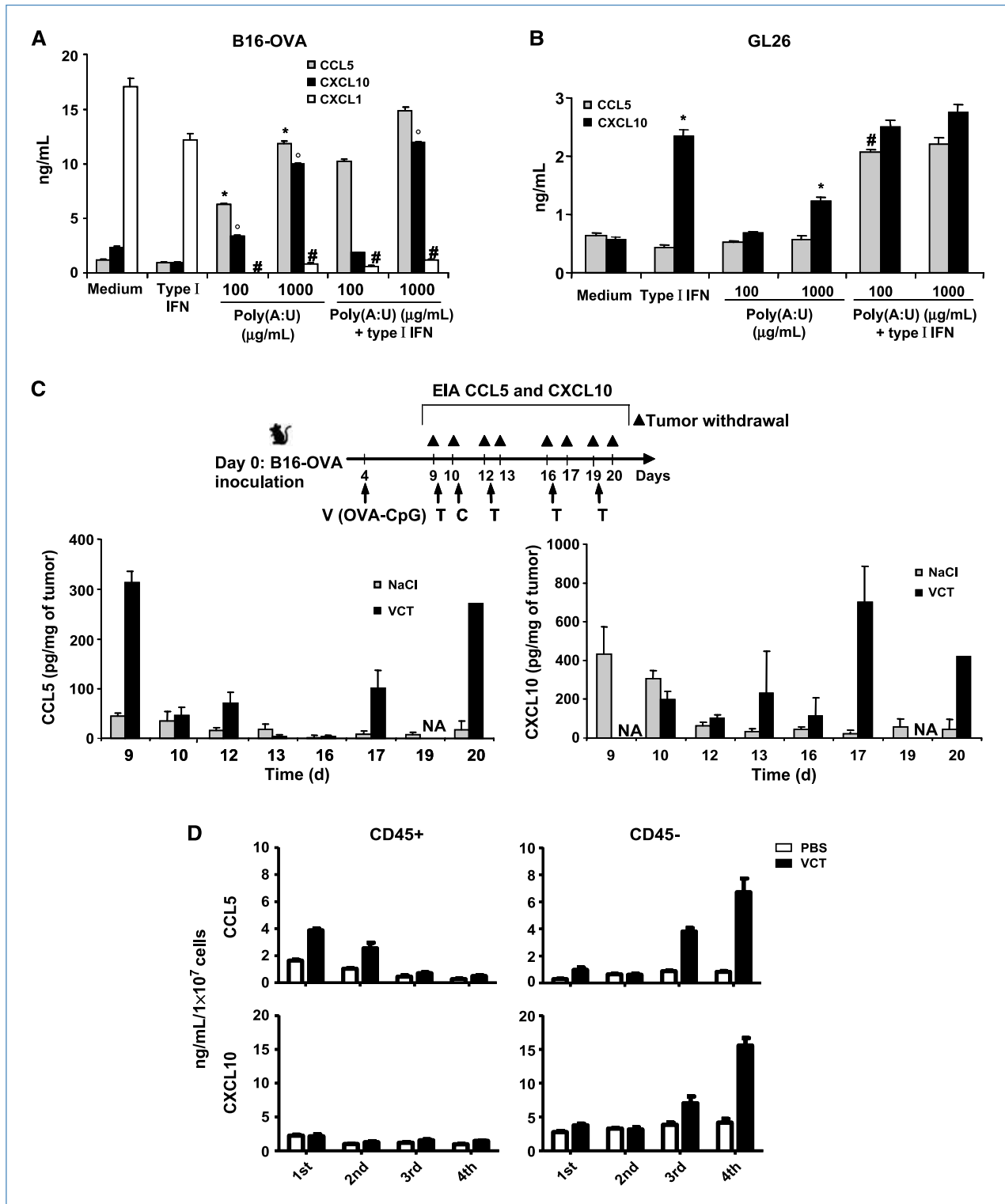
**Figure 3.** Immunochemotherapy is efficient against established glioblastoma. *A*, naïve C57bl/6 mice were vaccinated with GL26 tumor cells pretreated with type I IFN plus doxorubicin and rechallenged with live syngeneic tumor cells 7 d later. The kinetics of tumor outgrowth are monitored. *B*, the 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-1 $\alpha$ , 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<sup>+</sup> versus CD45<sup>-</sup> cells from dissociated tumor beds after each poly(A:U) injection and observed that



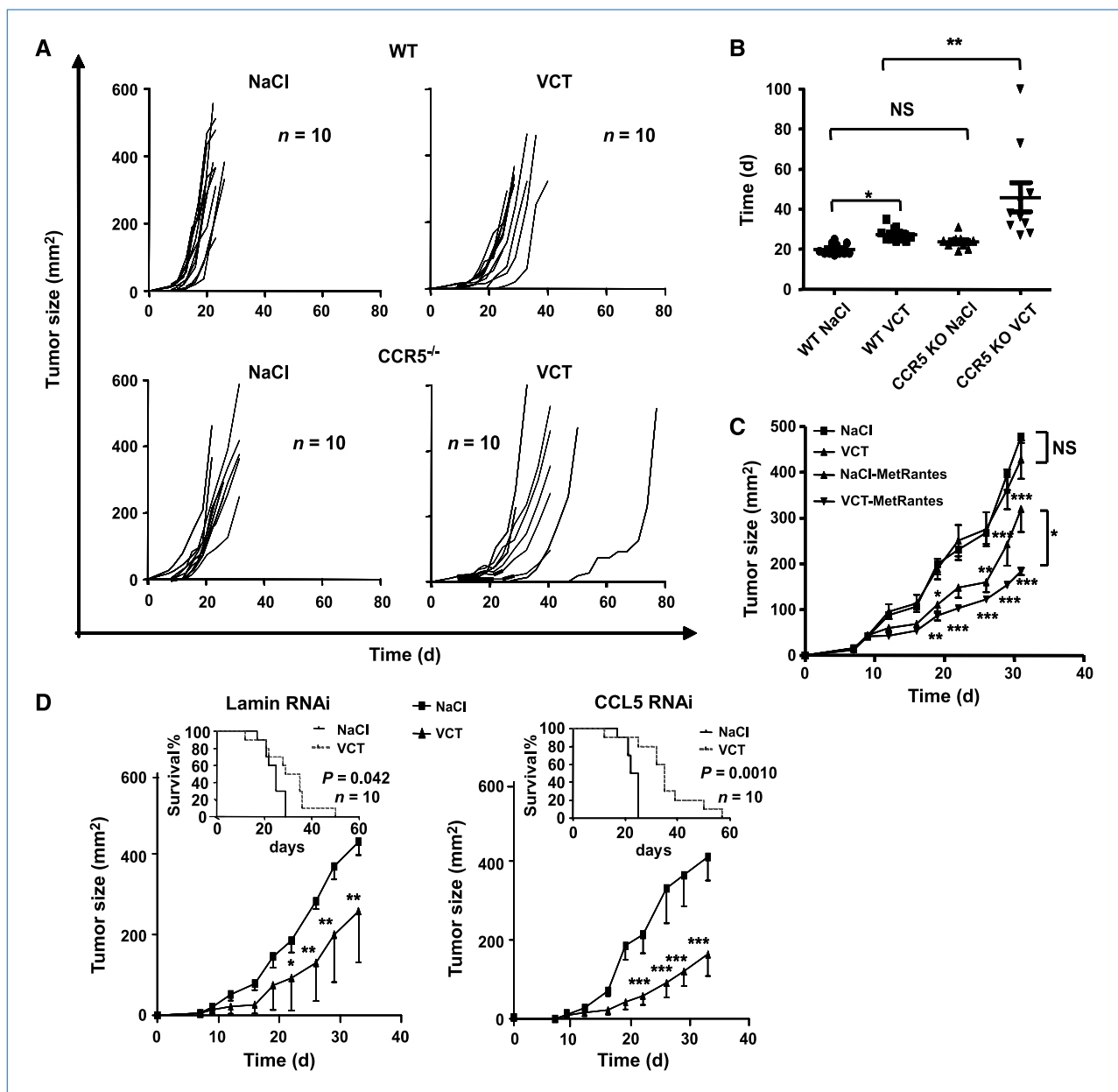
**Figure 4.** CXCL10 and CCL5 release upon stimulation with poly(A:U). B16-OVA (A) and GL26 (B) were treated with type I IFN and poly(A:U) and the supernatants were harvested to dose the chemokine secretion. Columns, mean of two triplicated experiments (#,  $P < 0.05$ ; ##,  $P < 0.01$ ; and ###,  $P < 0.001$ ); 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  $1 \times 10^7$  cells (D); bars, SEM.

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

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*<sup>-/-</sup> mice rather than to WT mice (Fig. 5A



**Figure 5.** CCR5 signaling antagonized the efficacy of immunochemotherapy. B16-OVA tumor growth was compared in WT versus *Ccr5*<sup>-/-</sup> 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<sup>2</sup> was shown for each group (B). C,  $0.6 \times 10^6$  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. 5C). 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. 5D), 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- $\gamma$ -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. 1D), suggesting that IFN- $\gamma$ -producing T lymphocytes are required for the antitumor effects. Knowing that IFN- $\gamma$ -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*<sup>-/-</sup> 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*<sup>-/-</sup> mice (Fig. 6A). 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<sup>+</sup> CXCR3<sup>+</sup> T lymphocytes in the vaccine DLN but not in the tumor DLN (data not shown). These lymphocytes were able to produce IFN- $\gamma$  upon restimulation with OVA (Supplementary Fig. S3; Fig. 6B). NK cells did not express CXCR3 in these settings (data not shown). Importantly, the percentage of CD8<sup>+</sup> CXCR3<sup>+</sup> T cells increased among tumor-infiltrating lymphocytes (TIL) after immunochemotherapy (Fig. 6B), 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. 6C). This gain of tu-

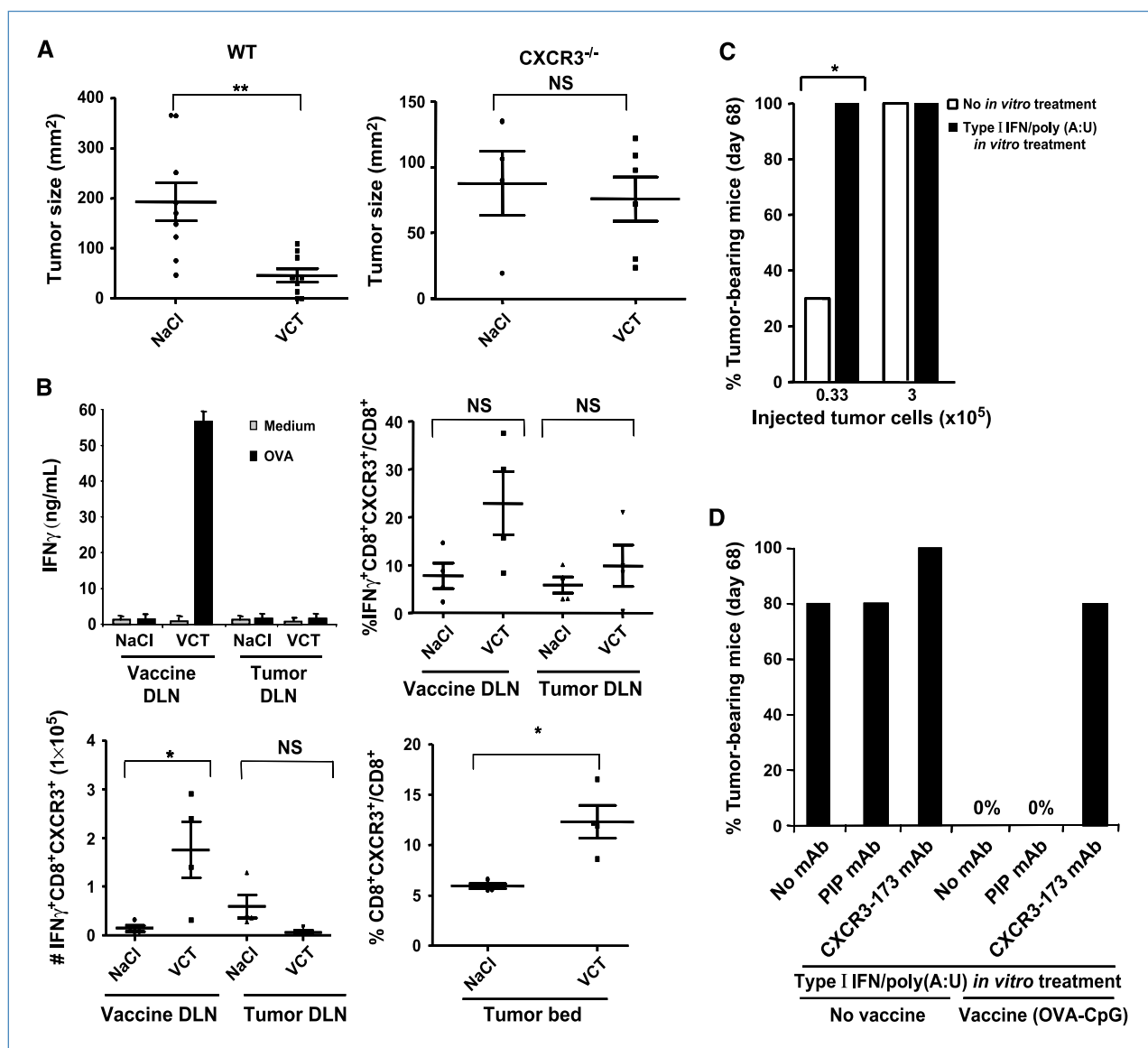
morigenicity was lost when the animals were immunized with the OVA-CpG vaccine (Fig. 6D), indicating that the direct effect of poly(A:U) stimulation of the tumor cells is beneficial only when the host has been immunized (when specific CTL against tumoral antigen are present within the host). The beneficial effect of prophylactic immunization with OVA-CpG was abrogated if the tumor cells were injected together with an anti-CXCR3 neutralizing antibody (Fig. 6D). 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. 1D and 6A 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<sup>+</sup> T cells, only TLR3 was mandatory to generate IFN- $\gamma$ -producing CD8<sup>+</sup> 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. 2B and 3A) and IFN- $\gamma$ -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<sup>+</sup>



**Figure 6.** CXCR3-dependent antitumor effects mediated by VCT therapy associated with CXCR3<sup>+</sup> TILs. **A**, the mean tumor size at day 19 in VCT or control group is compared between *Cxcr3*<sup>-/-</sup> 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<sup>+</sup>CXCR3<sup>+</sup> 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<sup>+</sup> cells among CD8<sup>+</sup> 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 ( $0.33 \times 10^5$ ) 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. 4C). 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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- $\gamma$ -producing T cells was enhanced, which facilitates their trafficking to the tumor beds (Fig. 6B), whereas in *Ccr5*<sup>-/-</sup> mice, we failed to observe an exaggerated accumulation of Tc1 cells (data not shown). Although concanavalin A-treated *Ccr5*<sup>-/-</sup> mice suffered from severe hepatitis related to pronounced recruitment and activation of IFN- $\gamma$ -producing NK cells into the liver (46), we failed to monitor an enhanced proportion of CXCR3<sup>+</sup> 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- $\gamma$ -producing CD8<sup>+</sup> 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 im-

mune 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<sup>+</sup>-immunosuppressive subsets to be identified.

Our results support two important conclusions. First, TLR3 agonists can promote TLR3<sup>+</sup> 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.

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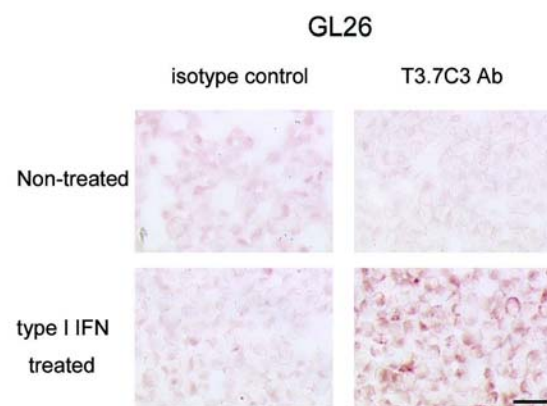
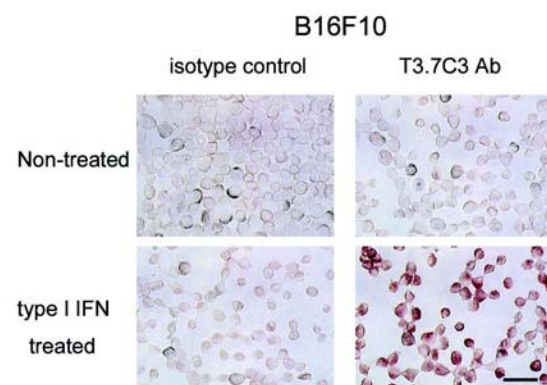
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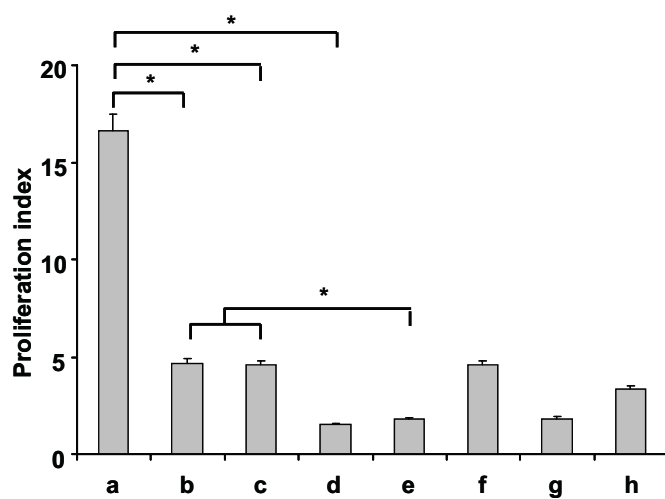




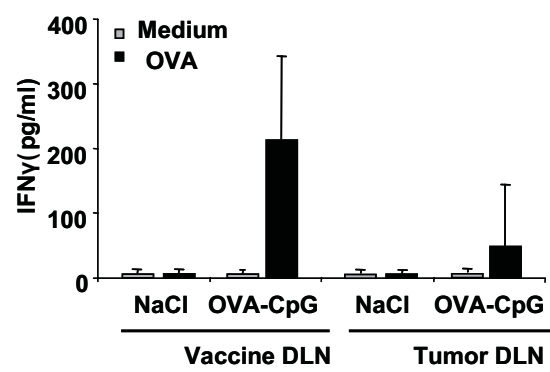
Supplemental Figure 2

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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

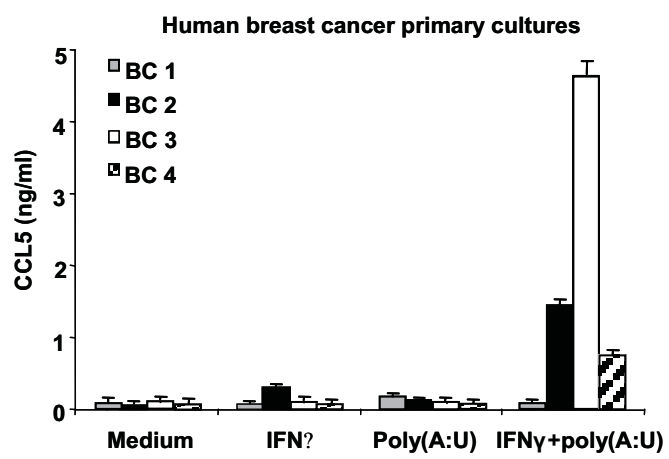


**Supplemental Figure 3**



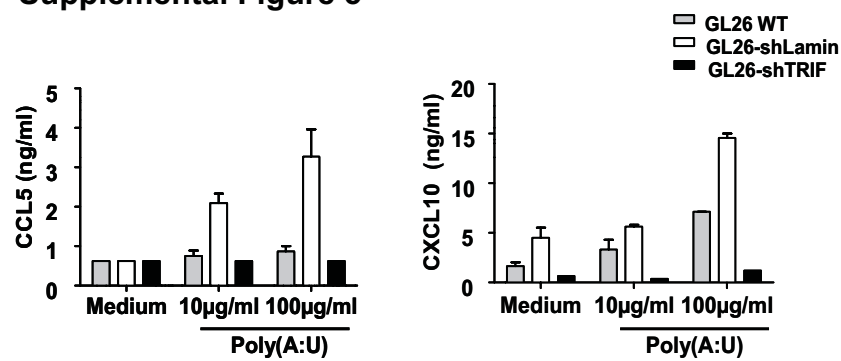
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## Supplemental Figure 4



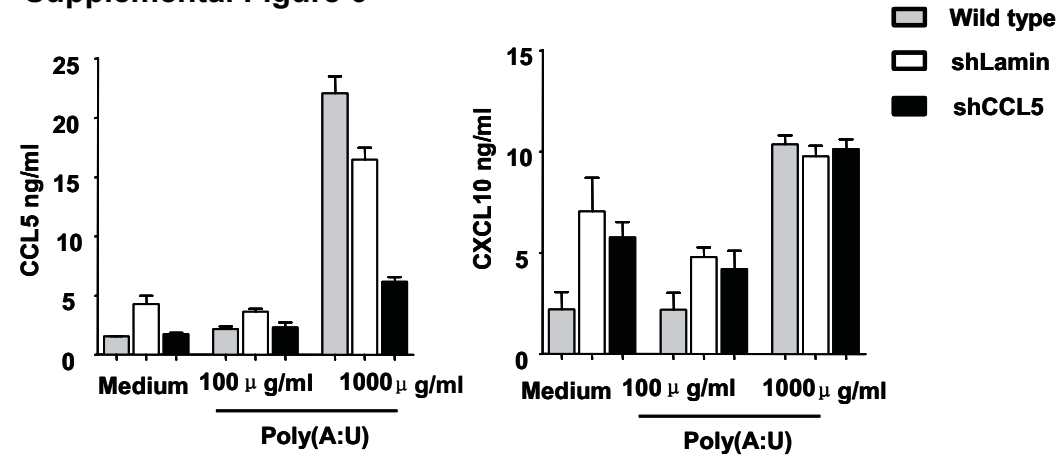
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Supplemental Figure 5



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Supplemental Figure 6



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## Review

## Chemotherapy and radiotherapy: Cryptic anticancer vaccines

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## 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.

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## 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<sup>+</sup> and CD8<sup>+</sup> 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 catastrophe, 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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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- $\gamma$ -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<sup>+</sup> 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<sup>+</sup> and IFN- $\gamma$ -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 + oxaliplatin) 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.

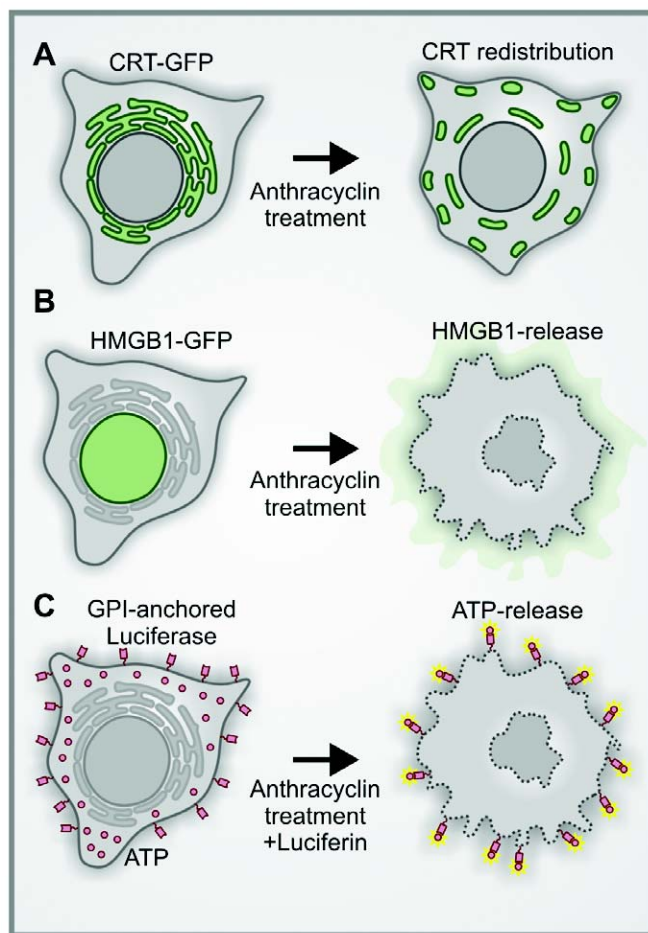
### 3. Checkpoints for tumor immunogenicity at the drug level

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-1 $\beta$  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-1 $\beta$ , IL-6 and IL-12p40. At low dosages (0.1–1  $\mu$ M), 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.

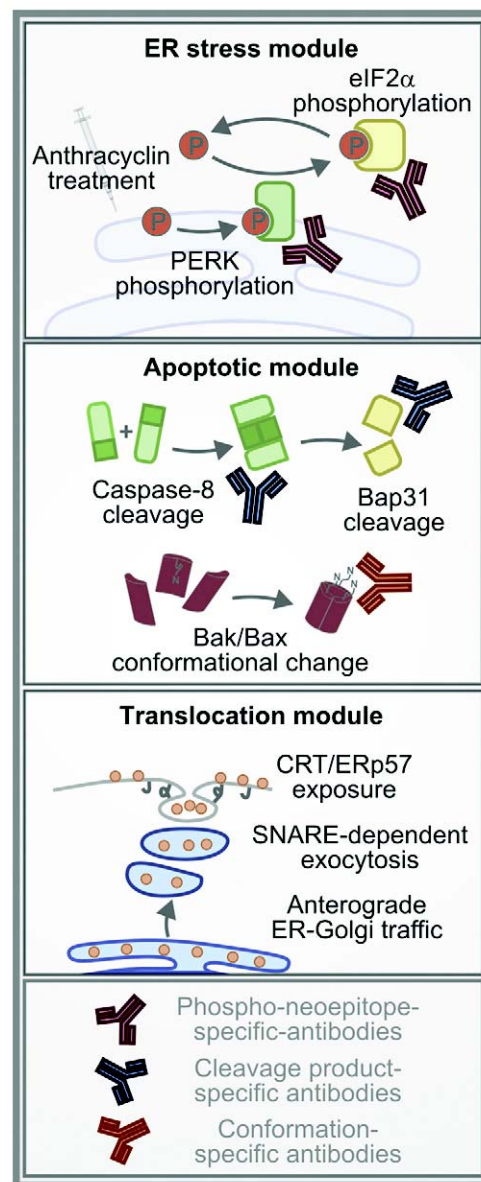
Our groups also performed a systematic screening of anticancer compounds for their ability to induce immunogenic cancer cell death. For this study, CT26 colon cancer cells were treated with a panel of chemotherapeutic agents that all induced  $70 \pm 10\%$  of apoptosis (assessed by staining with and 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, mitoxantrone) 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 2 $\alpha$  (eIF2 $\alpha$ ), through the activation of the eIF2 $\alpha$  kinase PERK [32] and the dissociation of the eIF2 $\alpha$  phosphatase complex composed by PP1 and GADD34 [33]. In general, it appears that chemotherapeutic agents that stimulate eIF2 $\alpha$  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 localization 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) Redistributing 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.

### 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<sup>+</sup>CD25<sup>+</sup> regulatory T (T<sub>reg</sub>) cells [35,36]. The cyclophosphamide-stimulated IFN $\alpha$  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<sup>+</sup>2H4<sup>+</sup> (CD45) suppressor T cells [43] and prolong the survival of metastatic cancer patients [44]. This contrasts with recent trials using intravenous 300 mg/m<sup>2</sup>/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-



**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-phosphorylated eIF2 $\alpha$ , 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.

stage cancer patients significantly reduced peripheral T<sub>reg</sub> numbers and inhibited the suppressive action of T<sub>reg</sub> cells on both T and NK cells [46]. Interestingly, another alkylating agent, dacarbazine, was shown to enhance memory CD8<sup>+</sup> 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- $\gamma$  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<sub>reg</sub> [57,58] and myeloid derived suppressor cells, restored the production of IFN- $\gamma$  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 eIF2 $\alpha$  (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 anthracyclines *in vitro*. Hence, the failure to emit immunogenic signals can result in ineffective chemotherapy responses. We suspect that the expression levels and phosphorylation status of key players of the CRT exposure pathway (such as PERK, eIF2 $\alpha$ , 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- $\alpha$  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<sup>hi</sup> human acute lymphoblastic leukemia cells [67]. On theoretical grounds, preventing the interaction between CD47 and SIRP- $\alpha$ , 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  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrins to promote the uptake of apoptotic cells and the secretion of TGF- $\beta$  and CCL22 by myeloid cells, all contributing to the maintenance of Foxp3<sup>+</sup> T<sub>reg</sub>. 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 Fc $\gamma$ R-mediated uptake of dying cells by DC, thereby

promoting cross-presentation of tumor antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells [69]. Third, they may evoke Th<sub>1</sub> reactivities and enhance tumor infiltration by CTL by virtue of their capacity to suppress the accumulation of Foxp3<sup>+</sup> T<sub>reg</sub> 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 *hmgbl-1*<sup>-/-</sup> 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-1 $\beta$ , IL-6, TNF $\alpha$ , 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<sup>+</sup> 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-1 $\beta$

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 correlate 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<sup>+</sup>CD8 $\alpha$ <sup>+</sup> 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<sup>+</sup>CD8 $\alpha$ <sup>+</sup> 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, mesenchymal 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> lymphocytes also abrogated the immune response against dying tumor cells [30]. Using CD11c-DTR transgenic mice in which diphtheria 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 $\alpha$ <sup>+</sup> 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 $\alpha$ <sup>+</sup> DC take advantage of one of their surface molecules, DC/NK lectin group receptor-1 (DNCR-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.

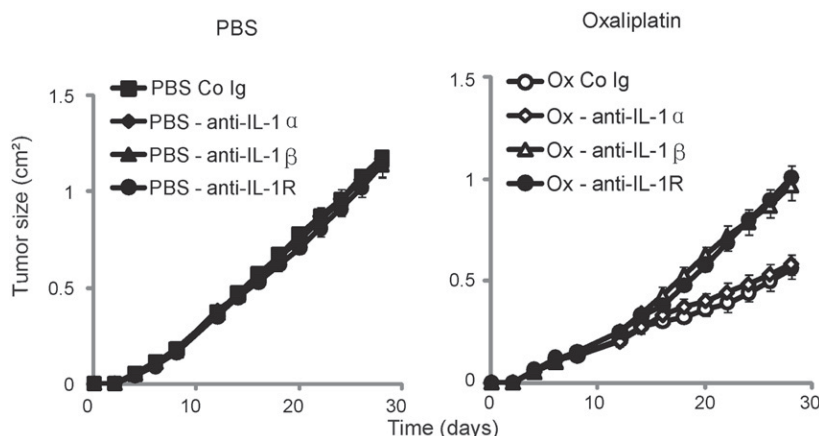
### 5.2. The coordinated action of TLR4 and P2RX7

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<sup>-/-</sup> 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-1 $\beta$  in DC [84]. Indeed, DC loaded with oxaliplatin-treated tumor cells secreted IL-1 $\beta$  in an Nlrp3-, ASC- and caspase-1-dependent fashion, and IL-1 $\beta$  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  $\rightarrow$  ASC  $\rightarrow$  Casp-1  $\rightarrow$  IL-1 $\beta$   $\rightarrow$  IL-1R1 axis and mice treated with neutralizing anti-IL-1 $\beta$  antibodies (Fig. 3). In contrast, IL-1 $\alpha$  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 doxorubicin-treated CT26 against rechallenge with live cells [84].

Why is IL-1 $\beta$  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<sup>+</sup> T cells that produce IFN- $\gamma$  in an TLR4-, caspase-1- and IL-1R1-dependent manner [84]. However, these IFN- $\gamma$  producing T cells did not exhibit potent cytolytic activities against tumor cells. Accordingly, we found that signaling through IFN- $\gamma$ R and IFN- $\gamma$  was mandatory for the efficacy of chemotherapy against a variety of different tumors, while IL-12R $\beta$ 2, perforin or TRAIL were dispensable [84]. Finally, IL-1 $\beta$  was shown to be a key cytokine gearing the polarization of TCR-triggered CD8<sup>+</sup> (but not CD4<sup>+</sup>) T lymphocytes *in vitro*, yet lack-





**Fig. 3.** IL-1 $\beta$ -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-1 $\alpha$ , anti-IL-1 $\beta$ , or anti-IL-1R antibodies (250  $\mu$ g 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  $\pm$  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 spatiotemporal 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- $\kappa$ B ([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].

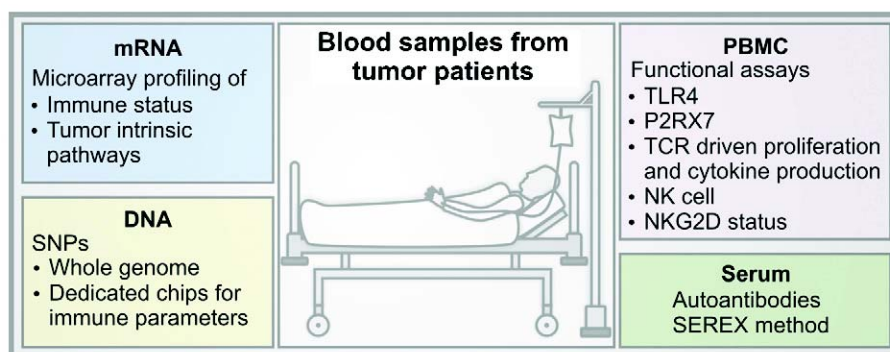
More recently, we investigated the prognostic value of a single-nucleotide polymorphism in the ligand-gated cation channel P2RX7 at nucleotide position 1513 (1513A>C) changing a glutamic acid to alanine at aa 496 (Glu496Ala) which abrogates the ATP-induced Ca<sup>2+</sup> and ethidium influx (and the K<sup>+</sup> efflux) and severely retards the ATP-dependent IL-1 $\beta$  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-1 $\beta$  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-1 $\beta$  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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cell-dependent manner [118].

In genetically engineered Hgf-Cdk4<sup>R24C</sup> 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<sup>+</sup> 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- $\gamma$  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<sub>reg</sub> 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.

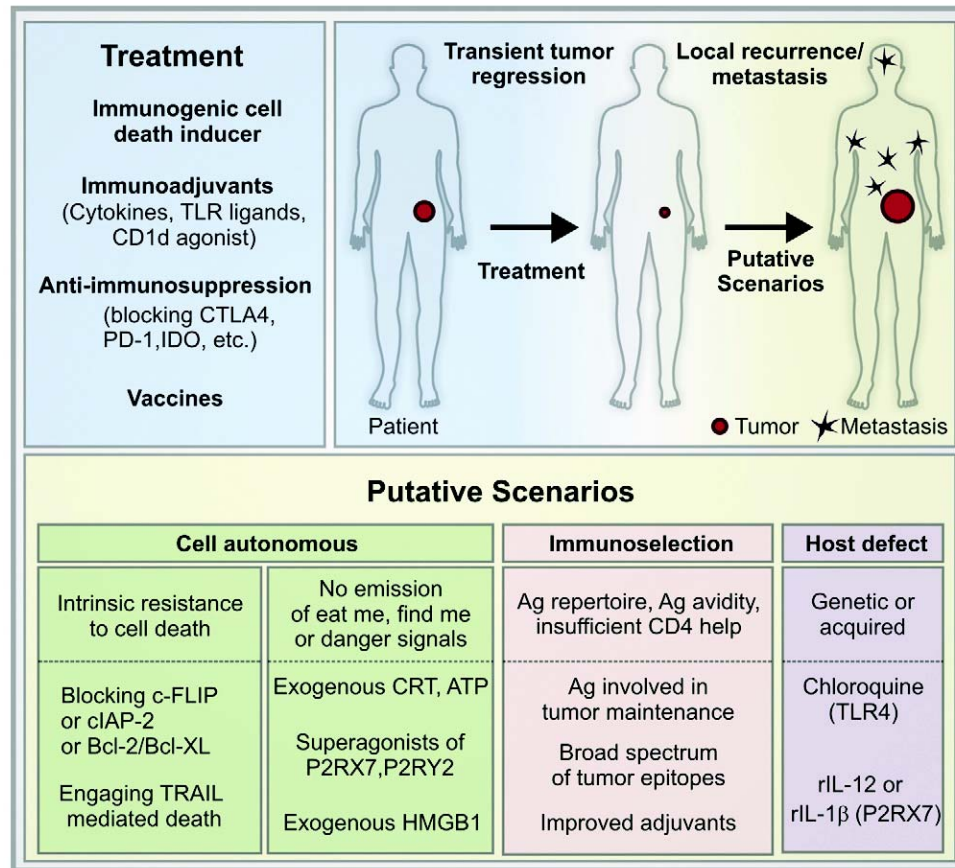
## 7.2. Compensating defects at the level of the tumor and of the host

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  $\rightarrow$  eIF2 $\alpha$  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-1 $\beta$ , we could show that exogenous recombinant IL-1 $\beta$  or rIL-12 restored the Tc1 immune response triggered by cell death inducers [84]. However, exogenous IL-1 $\beta$  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 devise algorithms predictive of clinical responses, (ii) to personalize therapy with cell death inducers, and (iii) to re-orient immunization strategies according to the pre-existing immune status of the patients (Fig. 5).

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# 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.

## Addresses

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## 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

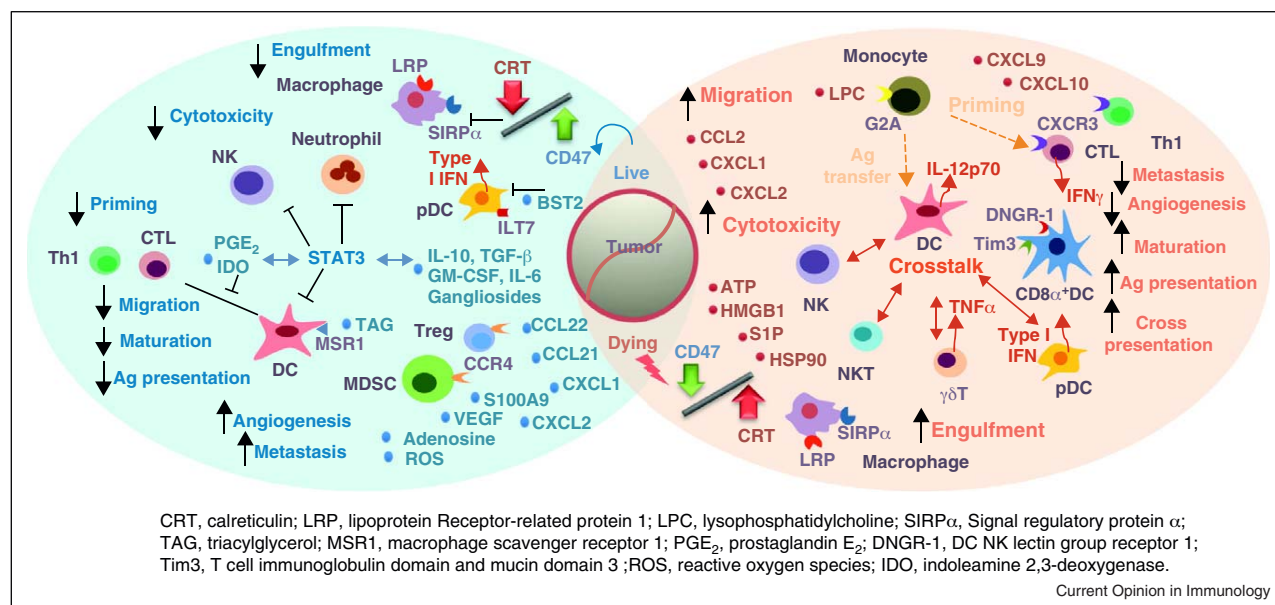
(Figure 1). The mechanisms of inadequate DC functions causing and/or resulting from tumor escape have been reviewed elsewhere [1,2]. It is noteworthy that the regulatory capacity of tumor infiltrating DC is not related to a defined DC subset but rather results from the influence of tumor microenvironment. 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- $\beta$ , 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<sup>+</sup> 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- $\alpha$  and TNF- $\alpha$  significantly increased BST2 secretion, suggesting that the inflammatory status of tumor microenvironment support this immunoregulatory pathway [6•].

Moreover, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 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 PGE<sub>2</sub> compromises CCR7-dependent DC migration in LN promoting abortive CD8<sup>+</sup> T cell responses [7]. These data extended the pioneering finding that tumor growing in PGE<sub>2</sub> 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<sub>2</sub> 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



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 reinforce 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<sup>\*\*</sup>]. Patients bearing head and neck tumors also presented with lipid accumulation in their circulating, tumoral and lymph node Lin<sup>+</sup>CD4<sup>+</sup> DC [10<sup>\*\*</sup>]. 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<sup>\*\*</sup>]. 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<sup>\*\*</sup>]. 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<sup>+</sup>Gr1<sup>+</sup>CD34<sup>+</sup> bone marrow cells including F4/80<sup>+</sup> macrophages in a high VEGF, TGF- $\beta$ 1, basic FGF and IL-1 $\beta$  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

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 (SIRP $\alpha$ ) 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 CD8 $\alpha^+$  DC [25]. This subset may not use CD36, DEC205,  $\alpha\text{v}\beta 3/\alpha\text{v}\beta 5$  integrins for the clearance of dying self. Using UV-irradiated thymocytes, Nakayama *et al.* demonstrated that Tim3 expressed on CD8 $\alpha^+$  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 CD8 $\alpha^+$ 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 ROR $\gamma\text{t}$ , LT $\beta$ , 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 CD8 $\alpha^+$ DC: tolerance or immunity?

CD8 $\alpha^+$  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 anti-tumor 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 CD8 $\alpha^+$  DC that provoked tumor outgrowth of very immunogenic tumor variants [32\*]. Importantly, Reis e Sousa and co-workers identified DC NK lectin group receptor 1 (DNDR-1), a SYK-coupled C type lectin receptor, selectively expressed by CD8 $\alpha^+$  DC and involved in sensing and cross-presenting antigens from necrotic cells [33]. Despite the fact that DNDR-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 DNDR-1-SYK pathway does not activate DC in response to dead cells and targeting of DNDR-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 $^{\text{high}}$ DNDR-1 $^+$  Lin $^-$ DR $^+$  DC, constituting 0.1% of human spleen cellularity, express most of the mouse CD8 $\alpha^+$  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 CD8 $\alpha^+$  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 CD8 $\alpha^+$  versus CD8 $\alpha^-$  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<sup>+</sup> 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<sup>\*</sup>]. 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<sup>+</sup> T cells in a CCL17/CCR4 dependent manner [50]. V $\delta$ 1  $\gamma\delta$ T cells can trigger DC maturation in a TNF $\alpha$  and CD1c-dependent manner, and synergize with LPS for the induction of naïve CD4<sup>+</sup> 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<sup>+</sup> T cell responses and tumor eradication [53<sup>\*\*</sup>]. Such matrices could recruit up to  $1.2 \times 10^6$  pDC,  $6 \times 10^5$  CD8 $\alpha^+$  DC and  $3 \times 10^6$  CD11b<sup>+</sup>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- $\beta$  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<sup>+</sup> T cell help?

Naïve CD4<sup>+</sup> T cells could become cytotoxic and highly contribute to tumor rejection. Adoptively transferred naïve CD4<sup>+</sup> 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<sup>+</sup>T or NK or NKT lymphocytes but strongly relied upon common IL-2R  $\gamma$  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<sup>+</sup> T cell based-ACT. Low IFN- $\gamma$  may also contribute to reduced CXCL9 production by the tumor milieu causing a poor recruitment of CXCR3 expressing Th1 CD4<sup>+</sup> 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<sup>+</sup> 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



bodies by DC. In addition, dying tumor cells must release ATP to engage P2RX7 on host DC, triggering the activation of the inflammasome platform Nlrp3, culminating in the release of IL-1 $\beta$ , which in turn elicits tumor-specific IFN- $\gamma$  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.

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# How to improve the immunogenicity of chemotherapy and radiotherapy

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**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<sup>+</sup> and CD8<sup>+</sup> 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, mitoxantrone) 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<sup>+</sup> T cell- and IFN- $\gamma$ -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 combi-

nation 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 lysophospholipids, 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  $\text{Ca}^{2+}$ -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 colocalized with phosphatidylserine (PS) [28]. Cytotoxic treatment, such as anthracyclines, oxaliplatin, UVC, and  $\gamma$ -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/eIF2 $\alpha$  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 SIRP $\alpha$ -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 potentially 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 CD8 $\alpha^+$  DC, and they proved the critical role of CD8 $\alpha^+$  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 CD8 $\alpha^+$  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<sup>+</sup> DC at lower levels [60]. The proportion of CD8<sup>+</sup>Langerin<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> compartment of the CD8 $\alpha$ <sup>+</sup> 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<sup>+</sup>CD103<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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 (Fc $\gamma$ RIII) [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. Infect-

ing 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 DNAM-1 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<sup>+</sup> 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- $\gamma$  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- $\gamma$  or cytolytic machinery (perforin, granzyme, TRAIL, TNF- $\alpha$ , 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<sup>+</sup>Gr1<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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- $\beta$ , CTLA-4, perforin/granzyme, extracellular adenosine, gap junction formation infusing cAMP, as well as soluble immunosuppressive cytokines such as IL-10, TGF- $\beta$ , 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<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> 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, retentive 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, camptothecin, and death-inducing molecules (TNF- $\alpha$ , 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 the 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].

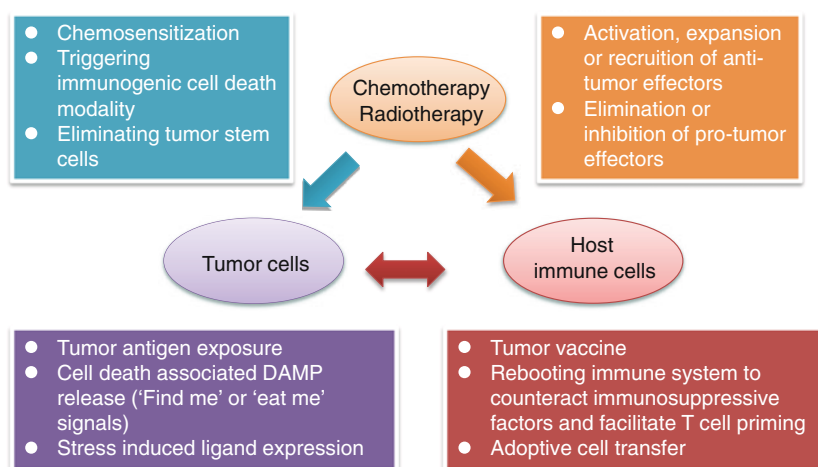
Some tumor cells survive and grow again after cytotoxic therapy, probably arising from the residual self-renewing tumor stem cells (TSC) which possess MDR properties. Preclinical studies suggested targeting leukemia stem cell surface molecules using antibody to enhance leukemia therapy [118]. Identification 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- $\beta$  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<sup>+</sup> T cells is associated with impaired IFN- $\gamma$ /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<sup>+</sup> T cells transfer into lymphopenic recipients in combination with CTLA-4 blockade and could eradicate 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).

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