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

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

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

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

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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 secluded within live cells may influence the switch between silent corpse removal and inflammatory reactions that stimulate the cellular immune response (Zitvogel et al., 2010). We initially described the crucial importance of an eat-me signal represented by the early translocation of the endoplasmic reticulum resident calreticulin–ERp57 complex to the plasma membrane for the immunogenicity of tumor cell death (Obeid et al., 2007; Panaretakis et al., 2008, 2009). Next, we showed that the nuclear alarmin HMGB1 must be released into the tumor microenvironment to engage TLR4 on host DCs to facilitate antigen processing and presentation (Apetoh et al., 2007). We also reported that ATP released from dying tumor cells could trigger the purinergic P2RX7 receptor on host DC, stimulating the release of IL-1β, which in turn facilitates the priming of $CD8^+$ tumor-specific T cells for IFN-γ production that is indispensable for the success of chemotherapy (Ghiringhelli et al., 2009).

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

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

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

RESULTS

A marked Th1 pattern 8 d after chemotherapy

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

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

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

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

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

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

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

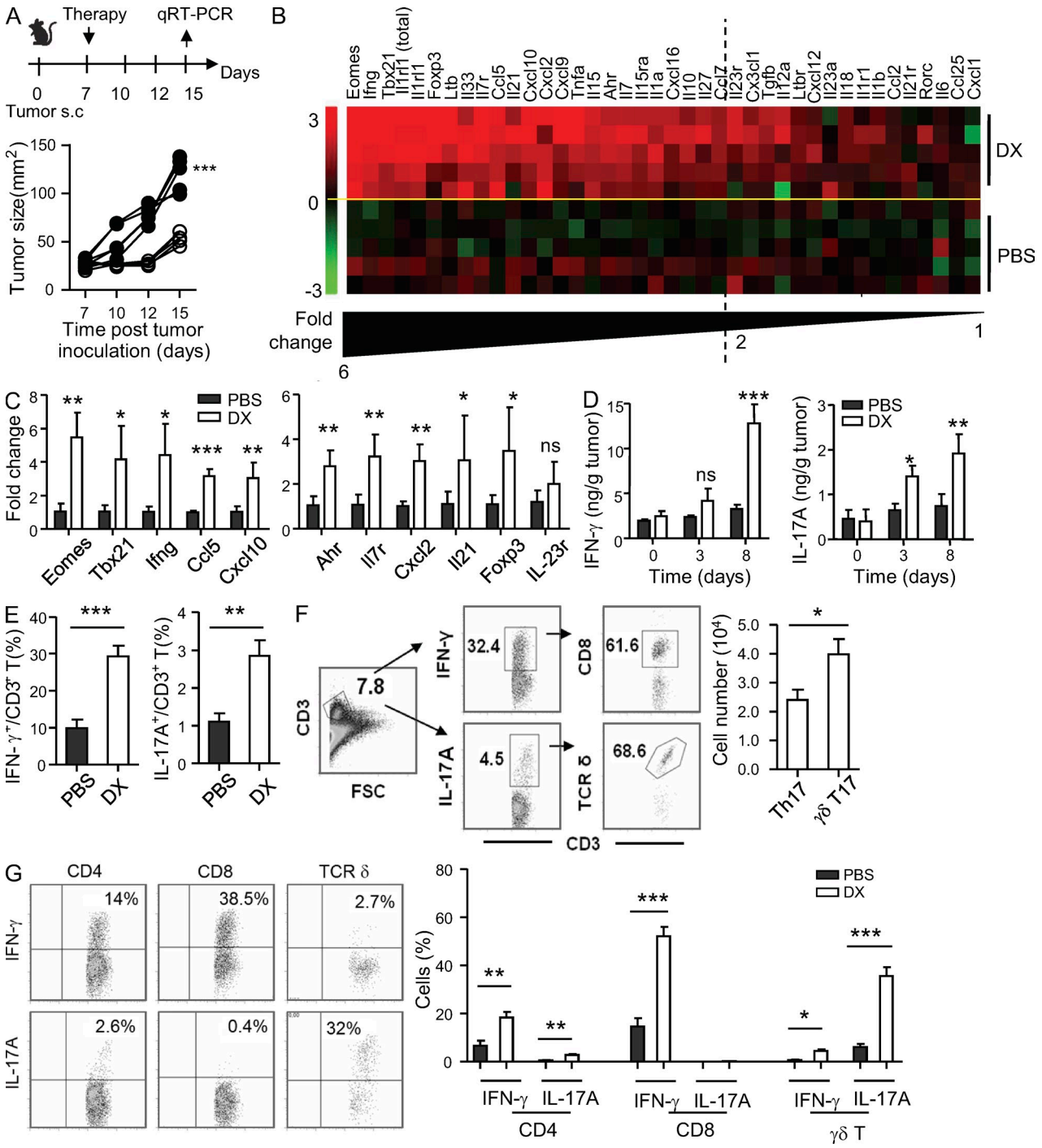

Figure 1. Th1 and Th17 immune response in tumors after chemotherapy. (A) Mice bearing MCA205 tumors were treated with PBS (solid symbols) or DX (open symbols) intratumorally at day 7 after tumor inoculation. Tumor growth was monitored at the indicated time points. (B and C) 8 d after chemotherapy (day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

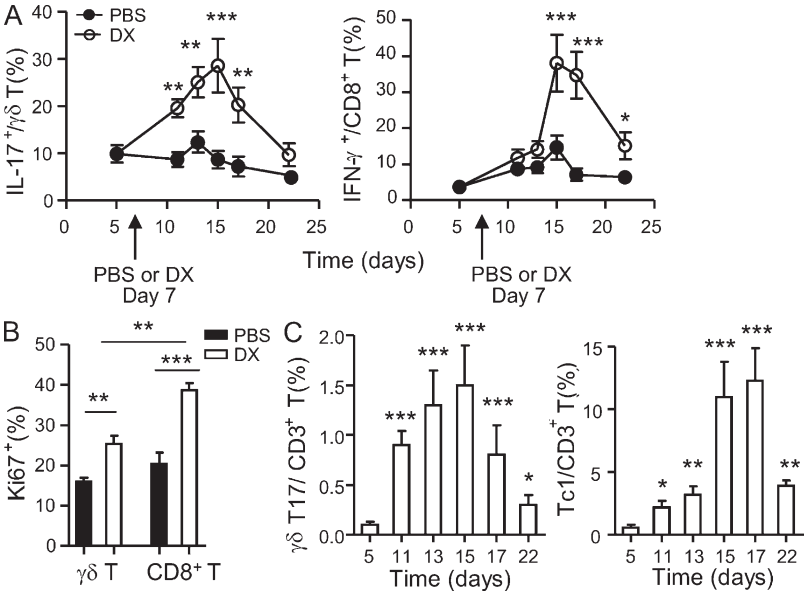

Figure 2. γδ T17 cells preceded Tc1 CTL into tumors after chemotherapy. (A) The percentages of IL-17– and IFN-γ–producing cells among all tumor infiltrating γδ T cells and $CD8^+$ T cells, respectively, are plotted before and at the indicated time points after tumor inoculation. Mice were treated with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

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

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

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

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

To rationalize the sequential recruitment of γδ T17 and Tc1 cells into the tumor bed after chemotherapy, we hypothesized that γδ T17 might act as helper cells for Tc1 priming. We previously reported that specific antitumor immune responses rely on Tc1 cells primed by tumor cells undergoing immunogenic cell death by using a system in which IFN-γ production by OVA-specific T cells could be triggered by OX-treated EG7 cells (Ghiringhelli et al., 2009). We used this system to check whether IL-17 is involved in initiating the specific antitumor response, comparing normal WT with IL-$17RA^{-/-}$ mice. In this assay, the absence of IL-17RA fully abolished antigen-specific T cell priming in response to dying cells, yet had no negative effect on T cell priming by OVA holoprotein admixed with CpG oligodeoxynucleotides (Fig. 5 A, left). Consistently, a neutralizing anti–IL-17A antibody, but not the isotype control Ig (CIg), markedly impaired the OVA-specific T cell induced by OX-treated EG7 (Fig. 5 A, right). Because Th1/Tc1 immune responses against dying tumor cells mediate a prophylactic protection against rechallenge with live tumor cells (Apetoh et al., 2007; Ghiringhelli et al., 2009), we addressed the functional relevance of the IL-17A–IL-17RA pathway in this setting. Subcutaneous injection of mitoxantrone (MTX)-treated MCA205 sarcoma cells could protect WT mice, but not athymic nude mice, against rechallenge with live MCA205 tumor cells (Fig. 5 B). The efficacy of this vaccination was attenuated in IL-$17RA^{-/-}$ mice. Because IL-17 was not significantly produced by $CD4^+$ or $CD8^+$ T cells, neither in tumor beds during chemotherapy (Fig. 1 G) nor in the tumor draining LNs (unpublished data), we refrained from investigating Th17 cells and rather focused on γδ T and NKT cells as potential IL-17 producers (Mills, 2008; Pichavant et al., 2008) that might contribute to the anticancer vaccination by dying tumor cells. Although CD1$d^{-/-}$ mice, which lack all NKT population (Godfrey et al., 2010), were undistinguishable from WT controls in their ability to resist live tumor cells rechallenge after a dying tumor cell vaccine, Vγ4/$6^{-/-}$ mice (Sunaga et al., 1997) exhibited a reduced capacity to mount this anticancer immune response (Fig. 5 B). These results suggest that IL-17A, IL-17R, and γδ T17 cells all play a partial role in the afferent phase of the immune response against dying tumor cells, which includes T cell priming for IFN-γ production.

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

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

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

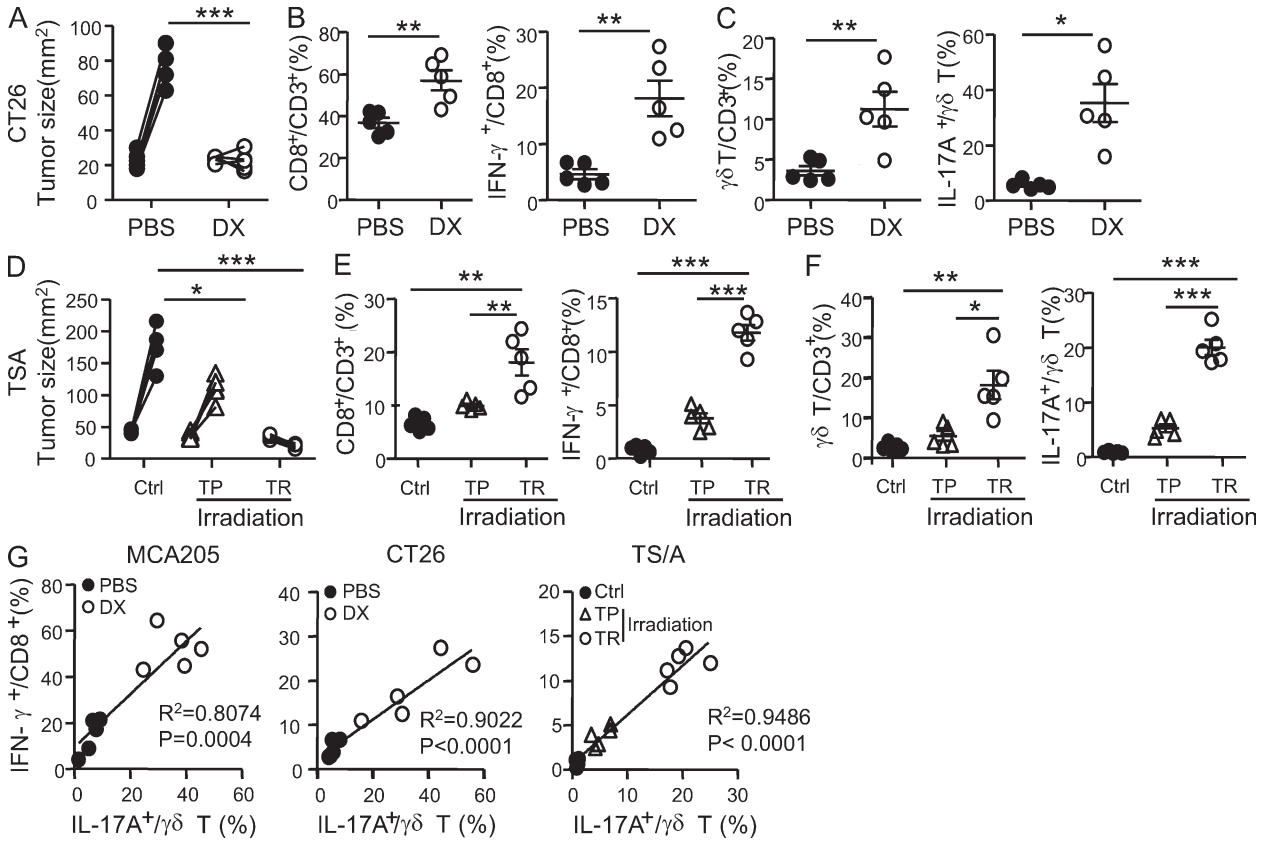

Figure 3. Recruitment of both Tc1 and γδ T17 cells in CT26 and TS/A tumors that correlate with better tumor control. (A–C) CT26 colon cancer treated with anthracyclines. (A) Tumor size before and 8 d after treatment with PBS (filled symbols) or DX (open symbols). (B) The percentage of $CD8^+$ T cells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

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

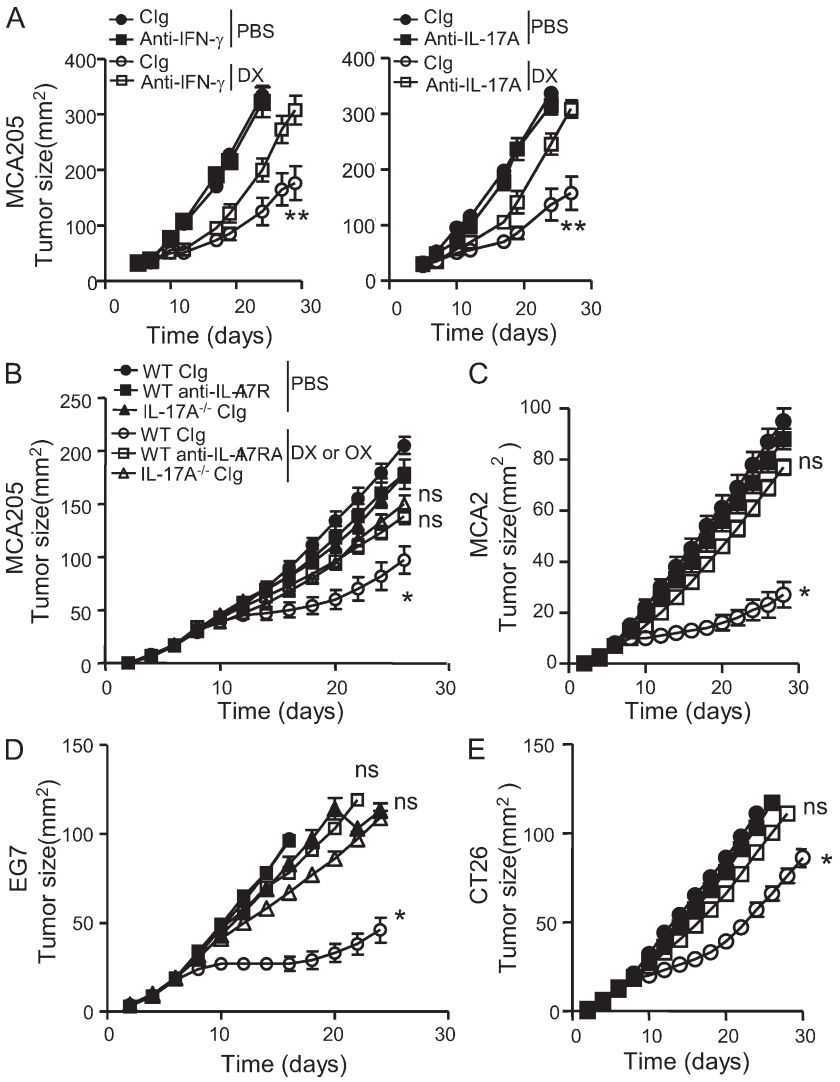

Figure 4. A mandatory role for the IL-17A–IL-17RA pathway in the efficacy of chemotherapy. (A) Mice bearing established MCA205 sarcomas were treated with local PBS (filled symbols) or DX (open symbols) 7 d after tumor inoculation and with systemic neutralizing antibodies against mouse IFN-γ (left), IL-17A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

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

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

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

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

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

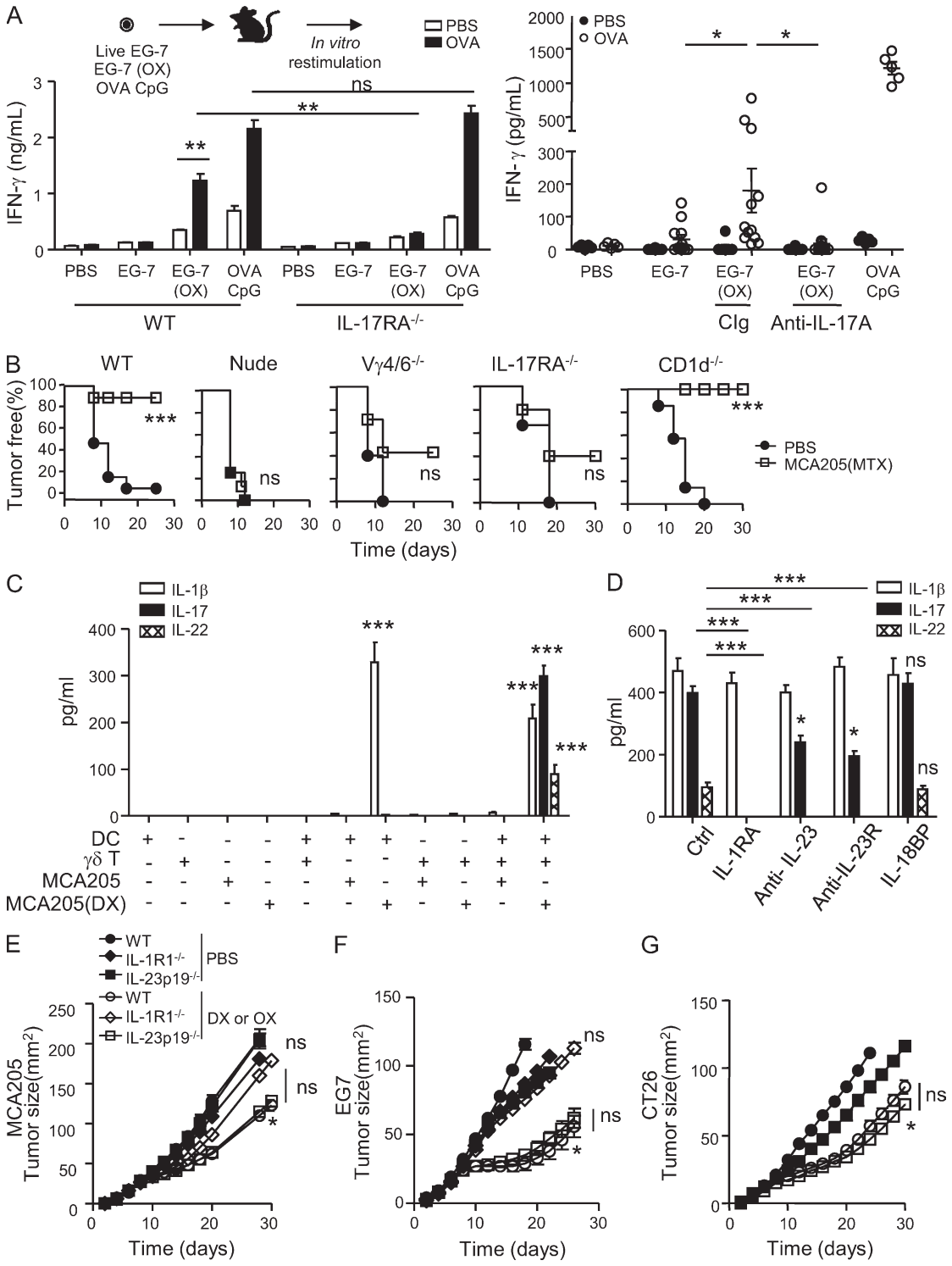

Figure 5. Role of γδ T17 in the priming of T cell responses during an immunogenic cell death and regulation by IL-1β. (A) OX-treated EG-7 cells were inoculated in the footpad of WT versus IL-17RA$^{-/-}$ mice (n = 5; left) along with anti–IL-17A neutralizing antibody (or Clg; right panel). OVA-specific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

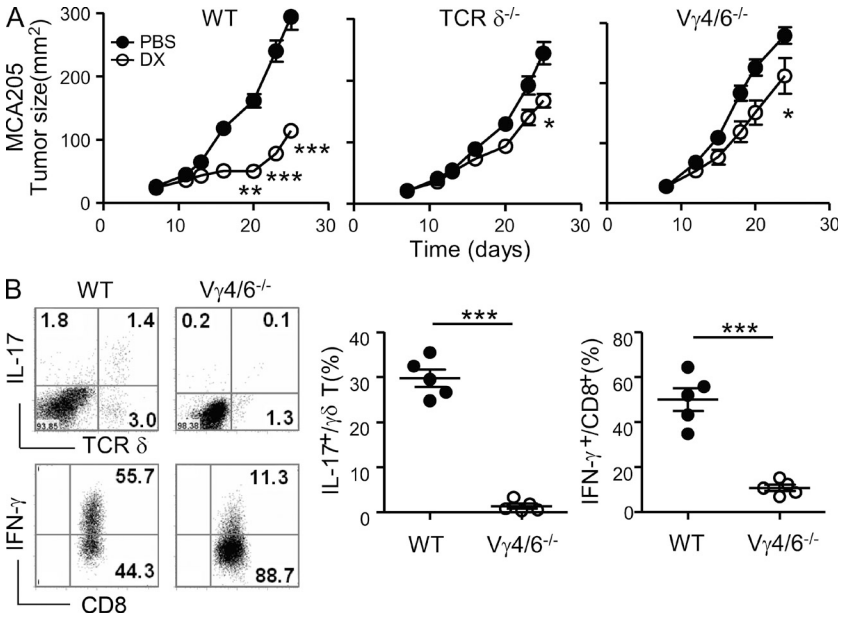

Figure 6. The therapeutic activity of anthracyclines and tumor colonization of Tc1 depend upon Vγ4 Vγ6 γδ T cells. (A) WT, TCR $\delta^{-/-}$, or Vγ4/6$^{-/-}$ mice with established MCA205 tumors were injected intratumorally with PBS or DX. Tumor size was measured at the indicated time and plotted as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

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

DISCUSSION

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

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

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

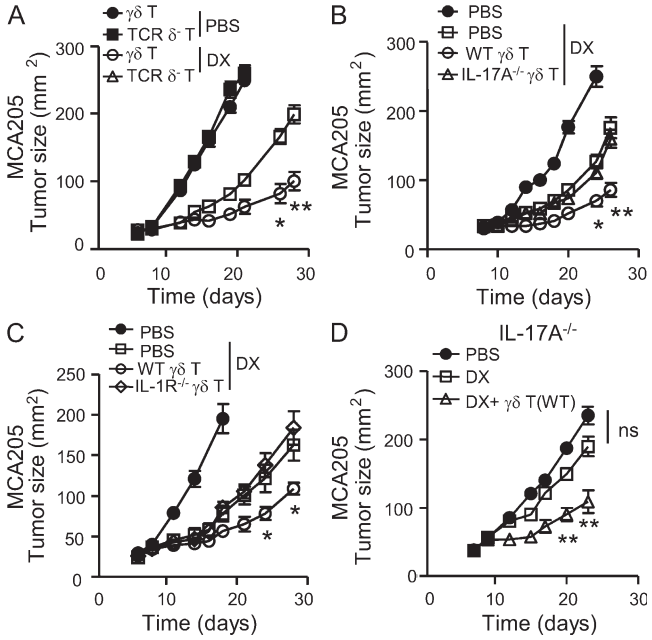

Figure 7. Role of γδ T cell–derived IL-17A during chemotherapy. $CD3^+$ TCR δ^+ or $CD3^+$ TCR δ^- T cells from WT mice (A), $CD3^+$ TCR δ^+ T cells from IL-$17A^{-/-}$ (B), or IL-$1R1^{-/-}$ (C) mice were injected intratumorally into MCA205-bearing WT mice (A–C) or IL-$17A^{-/-}$ mice (D) 2 d after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

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

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

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

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

MATERIALS AND METHODS

Mice. WT C57BL/6 (H-2^b) and BALB/c (H-2^d) mice aged between 7 and 12 wk were purchased from Harlan. Nude mice were bred in the animal facility of Institut Gustave Roussy. TCR $\delta^{-/-}$, IL-$1R1^{-/-}$, and IL-$17RA^{-/-}$ (H-2^b) mice were bred at Cryopréservation, Distribution, Typage, et Archivage Animal (Orléans, France) by B. Ryffel (CNRS, Orleans, France) and P. Pereira (Institut Pasteur, Paris, France; TCR $\delta^{-/-}$ was bred in the same manner). IL-$23p19^{-/-}$ and IL-$17A^{-/-}$ (H-2^b) were provided by M.J. Smyth (Peter MacCallum Cancer Centre, Victoria, Australia). $V\gamma4\gamma6^{-/-}$ mice (H-2^b) were provided by G. Matsuzaki (University of the Ryukyus, Okinawa, Japan) and K. Ikuta (Kyoto University, Kyoto, Japan). CD$1d^{-/-}$ and $CCR6^{-/-}$ (H-2^b) mice were bred at St. Vincent de Paul Hospital AP-HP (Paris, France) and provided by K. Benlagha. The experimental protocols were approved by the Animal Care and Use Committee in the animal facility of Institut Gustave Roussy.

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

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

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

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

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

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

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

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

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

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

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

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