Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors

Running title – Efficacy of doxorubicin requires anti-tumor immunity

Stephen R. Mattarollo1,2, Sherene Loi3, Helene Duret1, Yuting Ma4, Laurence Zitvogel4,5,6 and Mark J. Smyth1,7.

1Cancer Immunology Program, Peter MacCallum Cancer Centre, East Melbourne, 3002, Victoria, Australia.

2The University of Queensland - Diamantina Institute for Cancer, Immunology and Metabolic Medicine, Princess Alexandra Hospital, Woolloongabba, Queensland, Australia.

3Breast Cancer Translational Research Laboratory JC Heuson, Jules Bordet Institute, 121 Boulevard de Waterloo, Brussels B-1000, Belgium.

4INSERM U1015, Institut Gustave Roussy, 94800 Villejuif, France.

5Center of Clinical Investigations in Biotherapies of Cancer CICBT 507, Institut Gustave Roussy, 94800 Villejuif, France.

6Faculté de Médecine de l'Université Paris-Sud XI, 94270 Le Kremlin-Bicêtre, France.

7Department of Pathology, University of Melbourne, 3010, Victoria, Australia.
Corresponding Author

Name: Mark J. Smyth

Address: Cancer Immunology Program, Peter MacCallum Cancer Centre, Locked Bag 1, A’Beckett Street, East Melbourne, Victoria 8006, Australia.

Telephone: +61-3-96563728

Fax: +61-3-96561411

Email: mark.smyth@petermac.org

Word count: 4036

Number of figures and tables: 6 figures; 1 table; 4 supplementary figures; 1 supplementary table

Keywords: anthracyclines, doxorubicin, breast cancer, sarcoma, CD8 T cells, interferon-γ, interleukin-1β, interleukin-17.

Nonstandard abbreviations: WT: wildtype; DOX: doxorubicin; MCA: 3-methylcholanthrene; OVA: ovalbumin; EV: empty vector; TIL: tumor infiltrating lymphocyte; TDLN: tumor draining lymph node; i.t.: intratumoral; clg: isotype control immunoglobulin; pCR: pathological complete response; ROC: receiver operating characteristic.
**ABSTRACT**

We demonstrate, in a series of established experimental breast adenocarcinomas and fibrosarcomas induced by carcinogen de novo in mice, that the therapeutic efficacy of doxorubicin treatment is dependent on CD8 T cells and IFN-γ production. Doxorubicin treatment enhances tumor antigen-specific proliferation of CD8 T cells in tumor-draining lymph nodes and promotes tumor infiltration of activated, IFN-γ-producing CD8 T cells. Optimal doxorubicin treatment outcome also requires both IL-1β and IL-17 cytokines, as blockade of IL-1β/IL-1R or IL-17A/IL-17RA signaling abrogated the therapeutic effect. IL-23p19 had no observed role. The presence of γδ T cells, but not Jα18+ NKT cells, at the time of doxorubicin treatment was also important. In tumor samples taken from breast cancer patients prior to treatment with anthracycline chemotherapy, a correlation between CD8α, CD8β and IFN-γ gene expression levels and clinical response was observed, supporting their role in the therapeutic efficacy of anthracyclines in humans. Overall, this data strongly supports the pivotal contribution of both innate and adaptive immunity in treatment outcomes of anthracycline chemotherapy.
INTRODUCTION

Chemotherapy treatment in cancer has long been associated with development of systemic immune suppression (1, 2). However, more recent studies have reported augmentation of anti-tumor immune responses by chemotherapy (reviewed in (3)). The immunostimulatory properties of chemotherapy have been associated with increased expression of ‘stress-like’ molecules by damaged or dying tumor cells, such as, NKG2D ligands, Fas and TRAIL death receptors and heat-shock proteins, that render cells receptive to direct attack by cells of the innate immune system; including NK cells, NKT cells and γδ T cells (reviewed in (4)). Until recently, the mechanisms by which chemotherapy treatment facilitated an adaptive, tumor-specific immune response were largely uncharacterised. Certain alarmins or danger-associated molecular patterns (DAMPs) are expressed or released during tumor cell death induced by immunogenic chemotherapeutic agents, including anthracyclines. These include translocation of the endoplasmic reticulum resident calreticulin complex to the plasma membrane, providing an ‘eat-me’ signal (5-7) and release of the nuclear alarmin HMGB1 to engage TLR-4 on dendritic cells (DC) (8, 9). In addition, we showed recently that dying tumor cells release ATP, which then acts on P2X7 purinergic receptors from DCs and triggers the NOD-like receptor family, pyrin domain containing-3 protein (NLRP3)-dependent caspase-1 activation complex (‘inflammasome’), allowing for the secretion of pro-inflammatory IL-1β. Accordingly, anticancer chemotherapy turned out to be inefficient against tumors established in purinergic receptor P2rx7- or Nlrp3- or Casp1-deficient hosts (10).
Anthracyclines have been used in anti-cancer treatment for more than 40 years and now have a major role in the management of leukaemia, lymphoma, uterine, ovarian, sarcoma and breast malignancies. Doxorubicin became the most widely used anthracycline because of lower toxicity and potent anti-tumor activity against solid tumors. Although there were earlier reports evidencing the immune system contribution to doxorubicin-mediated anti-tumor effects (11, 12), Maccubbin et al. 1990, was the first to show that doxorubicin was an effective immunomodulator capable of boosting cytotoxic T lymphocyte (CTL) responses (13). A multitude of studies investigating combination treatment of doxorubicin and immune therapies followed, however the underlying mechanisms of how immunogenic cell death caused by doxorubicin links to induction of a cytotoxic CD8 T cell response was largely unknown.

Using experimental and carcinogen-induced mouse models of breast cancer and fibrosarcoma, respectively, here we demonstrate key cytokines and immune cell populations required for the anti-tumor therapeutic efficacy of doxorubicin. We show that CD8 T cells and IFN-γ are critical effectors of IL-1β- and IL-17-dependent signaling in response to doxorubicin treated breast tumors and sarcomas and corroborating this we also report that CD8α, CD8β and IFN-γ gene expression levels in breast cancer patients treated with anthracycline chemotherapy correlate with treatment response.
MATERIALS AND METHODS

Mice

Inbred wild-type C57BL/6 and BALB/c (WT) mice, and OT-I mice carrying a MHC class I-restricted transgenic TCR for the OVA_{257-264} (SIINFEKL) peptide were obtained from Walter and Eliza Hall Institute (WEHI; Parkville, Australia). C57BL/6 gene-targeted knockout IFN-γ⁻/⁻, IL-1R⁻/⁻, IL-17A⁻/⁻, TCRδ⁻/⁻, Jα18⁻/⁻, IL-23p19⁻/⁻ and IL-12p35⁻/⁻ mice were bred and maintained at the Peter MacCallum Cancer Center (Peter Mac; East Melbourne, Australia) as previously described (14, 15). TCRδ⁻/⁻ mice were obtained from I. Frazer (University of Queensland, Australia) and IL-17A⁻/⁻ mice were kindly provided by Y. Iwakura (University of Tokyo, Japan). Mice 6-14 weeks of age were used in all experiments and all procedures were approved by the Peter Mac Animal Ethics Committee.

Tumor Models

Experimental. C57BL/6-derived AT3 (kindly provided by Trina Stewart, Peter Mac) and E0771 (16), and BALB/c-derived H2N100 mammary adenocarcinoma (17) lines; BALB/c-derived MCA2 (derived in house from mice with a MCA-induced tumor) and C57BL/6-derived MCA205 (provided by LZ) fibrosarcoma lines; were all maintained in RPMI 1640 supplemented with 10% FCS. The ovalbumin (OVA)-expressing AT3 line (AT3^{OVA}) was generated by retrovirally infecting the AT3 parental line with pMIG/MSCV-IRES-eGFP plasmid encoding membrane-bound OVA. To examine subcutaneous (s.c) tumor growth, WT or gene-targeted mice were inoculated s.c. on the hind flank with the indicated number of cells and tumor size...
monitored. Once tumors were established (0.2-0.5 cm²) mice received a single intratumoral (i.t.) or intravenous (i.v) treatment of doxorubicin hydrochloride (DOX; Sandoz, Pyrmont, Australia) or equivalent volume of PBS. Some mice received control Ig (cIg; Mac-4 or agp3), anti-IFN-γ (R4-6A2), anti-CD8α (53.6.72), anti-CD8β (53.5.8), anti-IL-1β (B122.10), anti-IL-17A (M210), anti-IL-17Rα (M751) or anti-IL-23p19 (16E5) monoclonal antibody (mAb) at the time of PBS/DOX treatment, as indicated in the legends to neutralize or deplete cell subsets. Anti-agp3, anti-IL-23p19, anti-IL-17A and anti-IL-17Rα mAb were kindly provided by AMGEN Inc. (Seattle, WA, USA).

3-Methylcholanthrene-induced carcinogenesis. Groups of male BALB/c WT mice were inoculated s.c. in the hind flank with 400 μg of 3-methylcholanthrene (MCA; Sigma-Aldrich in 0.1 mL of corn oil as described (18). When sarcomas had established (palpable tumor – 0.2-0.45 cm²) mice received i.t. DOX or PBS once a week for two weeks. Some mice received cIg, anti-CD8α, anti-IFN-γ, anti-IL-1β, anti-IL-23p19 or anti-IL-17Rα on day -1, 0 and weekly thereafter for 6 weeks relative to initial PBS/DOX treatment. Development of fibrosarcomas was monitored weekly over the course of 250 days.

In vivo CD8 T cell assays and flow cytometry

OT-I T cells were purified from spleens and lymph nodes of OT-I transgenic mice, with positive selection using a CD8 T cell isolation kit (Miltenyi Biotec, North Ryde, Australia). CD8 T cell purity was always found to be greater than 90%. Purified OT-I cells were labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) and injected i.v. (2 x 10⁶) into the tail vein of
C57BL/6 mice harboring established, PBS/DOX-treated AT3<sup>OVA</sup> or AT3-empty vector (AT3<sup>EV</sup>) tumors. Five days later, tumor-draining lymph nodes (TDLN) were harvested and CFSE dilution in CD8<sup>+</sup>/KbOVA<sub>257-264</sub>tetramer<sup>+</sup> cells was assessed by flow cytometry. Indices of proliferation were generated using FlowJo software (Tree Star, OR, USA). For tumor infiltrating lymphocyte (TIL) analysis, AT3-OVA tumors were excised and single cell suspensions created by a combination of collagenase type 4 (Worthington Biochemical Corp.) and DNase I (Roche) enzymatic digestion at 37°C, and mechanical disruption. OT-I T cells were identified by antibody staining with anti-CD45.2 (104; eBioscience), anti-CD8α (Ly-2; eBioscience) and KbOVA<sub>257-264</sub>tetramer (provided by A. Brooks, Melbourne, Australia). Dead cells were excluded by addition of 1 μg/ml of fluorogold (Sigma-Aldrich) in the final wash. Cells were acquired on an LSR-II (BD) or Canto II (BD) flow cytometer and analysis was performed using FlowJo software. For intracellular IFN-γ analysis, tumor cell suspensions were cultured overnight in RPMI 1640 supplemented with 10% FCS in the presence of 0.1 μM OVA<sub>257-264</sub> (SIINFEKL) peptide (Auspep Pty, Melbourne, Australia) and 5 ng/ml recombinant mouse IL-2 (BD Biosciences). Monensin (BioLegend, San Diego, CA) was added to the cells in the final 4 hrs of culture to inhibit cytokine release from the Golgi/ER complex. Permeabilization and fixation of cells was conducted using the BD Cytofix/Cytoperm kit according to the manufacturers’ instructions (BD Biosciences) prior to staining with anti-IFN-γ mAb (XMG1.2; eBioscience).

**Gene expression analysis**

Two cohorts of breast cancer gene expression datasets were used for these analyses. Complete clinical data is included in the supplementary information (Supp Table 1). The first comprised of
early stage breast cancer patients who had received no systemic treatment in order to determine the association of the genes with clinical outcome without anthracycline (i.e. association with prognosis). The four datasets used (NKI (19), VDX (20), MAINZ (21) and TRANSBIG (22)) have been previously described. Clinical endpoint used for the analyses was the first distant metastatic event. Tumor biopsies taken for gene expression profiling were collected at surgery. Gene expression datasets were retrieved from public databases or authors’ websites. We used normalized data ($\log_2$ intensity in single-channel platforms or $\log_2$ ratio in dual-channel platforms). Hybridization probes were mapped to the Entrez GeneID as in Shi et al., (23) using RefSeq and Entrez database version 2007.01.21. When multiple probes were mapped to the same GeneID, the one with the highest variance in a particular dataset was selected to represent the GeneID. Each gene analysed was then scaled such that quartiles 2.5% and 97.5% are equal to -1 and +1 respectively. This scaling is robust to outliers and hence allows combination of the microarray data from each dataset. This meta-analytical technique has been previously described (24-26). Univariate Cox regression analysis was used to determine prognostic significance with clinical outcome of the genes as a continuous measurement and were calculated stratified by dataset.

The second cohort of breast cancer patients was treated with anthracycline chemotherapy to determine the association of immune genes with clinical response to doxorubicin. These women received epirubicin as single agent chemotherapy (100 mg/m²) for four cycles prior to surgery in the setting of a neoadjuvant clinical trial (clinicaltrials.gov NCT00162812) (27). Epirubicin is an anthracycline chemotherapy favoured in clinical practise due to its better toxicity profile
compared with doxorubicin. Tumor biopsies were taken using a core biopsy 14-16 G needle prior to chemotherapy. Clinical endpoint used was pathological complete response (pCR) documented at surgery, which is an accepted surrogate for disease-free and overall survival in breast cancer. Genes were correlated with pCR as a continuous variable (i.e. to determine if higher expression correlated with a higher chance of obtained pCR) using a receiver operating characteristic (ROC) and measured using an area under the curve (AUC). Microarray analysis was performed using Affymetrix GeneChips.

**Statistical analysis**

*Mouse.* Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). Significant differences between groups were assessed by a two-tailed $t$ test or Mann-Whitney $U$ test, as indicated. Values of $P < 0.05$ were considered significant.

*Human.* Statistical analysis using gene expression data was performed with R version 2.5.1 and BioConductor version 1.8. R code used is available as “genefu” available from the comprehensive R archive network. The remaining statistical analyses were performed on SPSS 18.0 (Chicago, IL). Results were not corrected for multiple testing as the analyses were considered hypothesis generating.
RESULTS

The efficacy of doxorubicin therapy in established subcutaneous tumors requires CD8+ cells and IFN-γ.

Both CD8 T cells and IFN-γ are important in immune surveillance of a range of malignancies (28-31). More recently adaptive immunity and IFN-γ have been implicated in the immunogenic effects of chemotherapy (10, 32), but CD8+ T cells have not previously been directly demonstrated to contribute to the anti-tumor activity of doxorubicin. Despite the fact that anthracyclines are commonly used in the clinical management of human breast cancer, very little data concerning the mechanism of action of doxorubicin against mammary carcinomas has been obtained in experimental models or from clinical samples. We assessed the requirement for CD8+ T cells and IFN-γ in the effectiveness of doxorubicin chemotherapy treatment against a variety of established breast cancers and fibrosarcomas. In wildtype mice, one localized (intratumoral) treatment of doxorubicin was sufficient to suppress subcutaneous growth of AT3, H2N100 and EO771 mammary tumors, and MCA205 fibrosarcomas (Fig. 1A-D). Depletion of CD8α+ cells or neutralisation of IFN-γ with mAb administration immediately prior to doxorubicin treatment severely abrogated the anti-tumor effect of the drug in the established tumor setting with significant loss of tumor growth suppression (Fig. 1A-D). Abrogation of doxorubicin efficacy was also observed in IFNγ−/− mice (Fig. 1C). To confirm that the loss of therapeutic effect of doxorubicin following anti-CD8α mAb treatment was specifically due to depletion of CD8+ T cells, we assessed an additional group of mice receiving anti-CD8β mAb prior to doxorubicin treatment of established MCA2 fibrosarcoma tumors. Depletion of CD8 T
cells alone was sufficient to abrogate the anti-tumor effect of doxorubicin (Fig. 1E). In addition, mice challenged with MCA2 tumors were treated with intravenous delivery of doxorubicin to establish that systemic administration of doxorubicin was comparable to localized treatment at inducing CD8 T cell and IFN-γ mediated anti-tumor activity. For the first time these studies validate across a series of experimental tumors the critical role of CD8+ T cells and IFN-γ in vivo in the mechanism of anti-tumor activity of doxorubicin.

Carcinogen-induced tumors respond to doxorubicin in a CD8 T cell and IFN-γ-dependent manner.

The activity and mechanism of action of most cancer therapies, including doxorubicin, have rarely if ever been assessed in mouse models of de novo tumorigenesis. This type of model is more practical in assessing any agent’s action in the context of a developing and progressing tumor, as opposed to a transplant setting. Recently we have employed established fibrosarcomas induced by MCA in mice as a therapeutic model for various immune targets (33, 34) and to determine the importance of host IL-1β in the mechanism of action of doxorubicin (10). To date however the importance of many other host immune elements in doxorubicin anti-tumor activity have not been examined in a mouse model where the tumor has arisen de novo in the host. Depletion of CD8 T cells following establishment of palpable sarcomas (0.20-0.45cm²) did not significantly alter the growth kinetics of the established tumor (Fig 2, left panels; Supplementary Fig. 4). Intratumoral doxorubicin treatment of established MCA-sarcomas achieved modest tumor growth suppression in most mice and 4 of 30 (13%) showed a significant period of retarded tumor growth, but eventually succumbed (Fig. 2A, Supplementary Fig. 4). This variable...
response was consistent with the vast heterogeneity between individual MCA-induced fibrosarcomas. Importantly, the significant therapeutic effect of doxorubicin in inhibiting fibrosarcoma growth across the whole cohort of control mAb-treated mice (p <0.0001) (Supplementary Fig. 4) was abolished if mice received anti-CD8α mAb or anti-IFN-γ mAb during doxorubicin therapy (Fig. 2B,C; Supplementary Fig 4), indicating that both CD8+ T cells and IFN-γ were critical for the anti-tumor effects of doxorubicin.

**Doxorubicin treatment enhances antigen-specific CD8 T cell proliferation in vivo.**

We next investigated in more detail the antigen-driven responses of CD8 T cells following chemotherapy in breast cancer. Immunocompetent B6 mice showed considerable resistance to challenge with subcutaneous AT3OVA tumors, in comparison to AT3-empty vector (AT3EV) control tumors (Supplementary Fig. 1). Next, we adoptively transferred naive OVA-specific CD8 T cells (OT-I cells) one day after mice harboring established AT3OVA or AT3EV were treated with doxorubicin. Not surprisingly, the proportion of OT-I cells recovered from TDLN after 5 days was significantly higher in mice with AT3OVA tumors compared with those inoculated with AT3EV tumors. More importantly, the proportion of OT-I cells in TDLN was enhanced following doxorubicin treatment, however only in the context of cognate antigen recognition (AT3OVA setting) (Fig. 3A). Furthermore, in vivo proliferation of OT-I cells in the presence of OVA, as measured by CFSE dilution, was significantly increased after doxorubicin treatment (Fig. 3B). Overall, localized doxorubicin treatment of established tumors enhances the accumulation and proliferation of CD8 T cells responding to cognate tumor antigen in secondary lymphoid organs.
Tumor-infiltrating CD8 T cells and IFN-γ production are enhanced by doxorubicin treatment.

We harvested AT3^{OVA} tumors from mice that had received intratumoral doxorubicin or PBS treatment and naive OT-I T cell adoptive transfer, to determine if localized doxorubicin administration increased the proportion and function of CD8 tumor infiltrating lymphocytes (TILs). A significant increase in the proportion of total CD8 T cells amongst AT3^{OVA} TILs was observed following doxorubicin treatment (Fig. 4A), and a similar increase was observed for infiltrating Ag-specific OT-I cells (Fig. 4B). Doxorubicin treatment also induced Ag-specific IFN-γ production from CD8 TILs, observed after in vitro re-stimulation with cognate antigen (Fig. 4C). No cytokine was detected from CD8 TILs in the absence of re-stimulation (data not shown). These experiments are amongst the first to directly isolate and demonstrate the increase in the proportion of tumor-localized and functional CD8^+ T cells following treatment with doxorubicin.

Doxorubicin treatment requires IL-1β and IL-17, but not IL-23p19

We have recently demonstrated that IL-1β production is pivotal for a robust anti-tumor response to chemotherapy, by facilitating the priming of tumor-specific CD8 T cells (10). We now have extended these findings in a range of mammary adenocarcinomas to show that IL-1β/IL-1R signaling is also critical to the therapeutic outcome of doxorubicin treatment (Fig. 5). Since IL-1β is known to regulate IL-17 responses (35-37), we utilized both IL-17A^-/- mice and an anti-IL-17Rα mAb, to also demonstrate a critical role for IL-17 in doxorubicin treatment of transplantable mammary tumors (Fig. 5). In concert with the mammary carcinoma data, a
critical role for IL-1β and IL-17A in doxorubicin anti-tumor activity was also illustrated using fibrosarcomas generated de novo by MCA (Fig. 6B; Fig. 6D; Supplementary Fig 4). Interestingly, the absence of IL-1 or IL-17 signaling had little or no direct effect on the growth of untreated established tumors. By contrast IL-23, another immunoregulatory cytokine known to cooperate with IL-1β in regulation of IL-17 responses (35-37), was dispensable in these models as no alteration in doxorubicin efficacy was observed in IL-23p19−/− mice or by neutralising IL-23 in wildtype mice using an anti-IL-23 mAb (Fig. 6C; Supplementary Figs. 2 and 4). In addition, by comparison the anti-tumor efficacy of doxorubicin in transplanted tumors was partially reduced in the absence of the IL-12p35 subunit (Supplementary Fig. 2).

**Doxorubicin therapy requires γδ T cells, but not type I NKT cells.**

Both NKT cells and γδ T cells have been implicated in innate immune surveillance of tumors (38, 39) and are also a potent early source of IL-17 in response to IL-1β cytokine stimulation (35, 36). We assessed the requirement for these innate lymphocytes in doxorubicin therapy of established AT3 mammary tumors and MCA205 fibrosarcomas (Supplementary Fig. 3). The absence of type I Jα18+ NKT cells in TCRJα18−/− mice did not perturb the anti-tumor effects of doxorubicin, however deficiency of γδ T cells in TCRδ−/− mice resulted in significant inhibition of doxorubicin activity in both AT3 and MCA205 tumors. The loss of NKT cells or γδ T cells did not alter the outgrowth of these tumors in the absence of therapy (Supplementary Fig. 3).
Clinical relevance using gene expression data from human breast cancer patients.

We went on to explore if these genes were important for therapeutic efficacy of anthracycline treatment in breast cancer patients using publicly available gene expression datasets. The first cohort consisted of patients diagnosed at multiple different hospitals who had their tumor biopsies taken at surgery. The samples examined here are representative of the global breast cancer population as there was a mixture of different tumor sizes, nodal status and estrogen receptor (ER) expression (Supplementary Table 1). As these women received no systemic treatment, the true prognostic effects of these genes could be examined without treatment confounders. Therefore, we could examine the prognostic significance of CD8A, CD8B, IFNγ, IL1B, IL17A and IL23p19 genes independent of anthracycline therapy in patients who had received no systemic treatment (19-22) (Table 1). As observed, only high IFN-γ levels were associated with a good prognosis in these women – i.e. high levels were associated with fewer long-term breast cancer relapses.

We then went on to examine these genes in the context of treatment with anthracycline chemotherapy. The second cohort consisted of women enrolled into a breast cancer clinical trial specifically designed to investigate biomarkers to anthracycline chemotherapy. As a consequence, all of the women in this study were negative for expression of ER (as this is the more chemo-responsive breast cancer population) and all received single agent epirubicin, an anthracycline type chemotherapy commonly used in clinical practise. Tumor biopsies were taken with core biopsy needles and were subject to gene expression profiling prior to chemotherapy (27). In these breast cancer patients increasing levels of IFN-γ, and also CD8α and CD8β were
associated with a better response to therapy, as measured by the amount of tumor left at surgical resection after four cycles of therapy. Notably, the complete disappearance of invasive tumor after neoadjuvant chemotherapy was associated with an excellent survival from breast cancer and is an accepted surrogate for long-term survival from breast cancer (40). There was no significance for the other genes in these cohorts. Overall, this data supports further the role of IFN-γ, CD8α and CD8β in the clinical outcomes in breast cancer patients who have received anthracycline-type chemotherapy.
DISCUSSION

Anthracyclines are front-line chemotherapeutic agents in the treatment of breast cancers and sarcomas. In this study, we have provided an extensive amount of new data exploring the mechanism of action of doxorubicin in a series of transplantable mammary carcinomas and fibrosarcomas generated de novo by MCA. The new data in AT3, H2N100, and E0771 mammary tumor models demonstrated the critical role of host CD8+ T cells and IFN-γ, additionally supported by novel human clinical data in two different cohorts of women with breast cancer. Our study is the first to describe the specific importance of CD8+ T cells in vivo, as opposed to broadly assessing adaptive immunity by employing RAG-deficient or nude mice that are deficient in many immune components including all T cell subsets and B cells. Furthermore, analysis included CD8α- and CD8β-specific depletions to specifically define the importance of CD8+ T cells. Doxorubicin treatment enhanced the proliferation of CD8 T cells in the tumor DLN, in a cognate antigen-specific manner. Moreover, localized doxorubicin treatment increased the proportions of CD8 T cells infiltrating the tumor and enhanced tumor antigen-specific IFN-γ production from these CD8 TILs. Combined, this data is amongst the strongest to indicate that tumor cell death associated with doxorubicin treatment enhances the generation and functional activation of tumor-reactive CD8 T cells that are required for the anti-tumor activity of doxorubicin.

We also completed the most extensive characterization of mechanism of action of doxorubicin in tumors established de novo by carcinogen, including a comparative evaluation of the role of
CD8\(^+\) T cells, IFN-\(\gamma\), IL-1\(\beta\), IL-17A, and IL-23. These types of established tumors generated de novo are arguably more relevant than any short term transplanted tumors that have been used to previously establish the principles that chemotherapy may be immunogenic. Our study has clearly shown that CD8\(^+\) T cells, IFN-\(\gamma\), IL-1\(\beta\), and IL-17A, but not IL-23, were critical in the anti-tumor activity of doxorubicin. We further illustrated the key role of IL-1\(\beta\), and IL-17A, but not IL-23, in doxorubicin control of a series of mammary carcinomas. Our data extend our recently published data that illustrated the importance of IL-17A in the anti-tumor activity of doxorubicin against transplanted MCA205 tumors and tracked the generation of IL-17 producing \(\gamma\delta^+\)T cells following doxorubicin treatment (41). We confirmed the role of \(\gamma\delta^+\) T cells, but not type I NKT cells, in the mechanism of action of doxorubicin using transplantable mammary carcinomas.

In human breast cancer patients, whilst IFN-\(\gamma\) levels seemed relevant for patient clinical outcome independent of anthracycline, the importance of both CD8 T cells and IFN-\(\gamma\) in therapeutic efficacy was supported by the observation that increasing levels of CD8\(\alpha\), CD8\(\beta\) and IFN-\(\gamma\) gene expression correlated with a better response to anthracycline chemotherapy. Notably, here the tumor biopsies were taken prior to chemotherapy, supporting the concept that an intact immune system aids therapeutic response to anthracyclines. However, it would be most interesting to also see the relative changes in gene expression before and shortly after administration of therapy. Whilst the analyses in human breast cancer patients presented here are limited by the use of retrospective datasets, non-randomized treatment cohorts and patients treated with epirubicin rather than doxorubicin, which is a different type of anthracycline chemotherapy, it supports our
laboratory observations and a recent study (42) of the clinical relevance of anti-tumor immunity for better clinical outcomes from anthracycline therapy in breast cancer.

It is likely that the context in which IL-17A is produced, particularly the constituents of the tumor microenvironment, will determine the outcome of IL-17 secretion. IL-17 production, particularly from innate T lymphocytes such as NKT cells and γδ T cells, is dependent on IL-1R signaling (36, 43) and has been shown to boost CD8 T cell IFN-γ responses (44). Given the requirement for IL-1β and CD8 T cells in the anti-tumor immune response following doxorubicin treatment, we assessed the role for IL-17 as an intermediary molecule in this pathway. IL-17A or IL-17Rα blockade resulted in loss of doxorubicin efficacy, very similar to that of IL-1β/IL-1R blockade, indicating that IL-17 is probably a major downstream response cytokine to IL-1β secretion. Interestingly, IL-23, itself a modulator of anti-tumor immunity (45-48), but also an important co-factor along with IL-1β in the stimulation of IL-17 production from memory T cells (37), NKT cells (35) and γδ T cells (36), was dispensable in doxorubicin treatment of breast cancers and fibrosarcomas, with no alteration in therapeutic effect following IL-23 blockade. Notably, neither IL1B, IL17A, nor IL23 gene expression was associated with clinical outcomes to doxorubicin treatment in human breast cancer patients, but perhaps this was not surprising given that the tumor biopsies were taken prior to doxorubicin treatment, and these cytokines were likely to be induced following treatment. In a parallel study, we have very recently shown that IL-17 from γδ T cells is critical for the efficacy of chemotherapy and preceded the activation and polarization of CD8 T cells for IFN-γ production (41). Also, γδ T cells that lacked IL-1R lost the capacity to amplify the action of chemotherapy, confirming the
causal relationship of IL-1β production from APC causing IL-17 production from γδ T cells, and resulting in IFN-γ production from tumor-specific CD8 T cells. The current study provides important additional data in mammary tumor transplant and de novo fibrosarcoma models to support a role for IL-17A in doxorubicin mechanism of action. In doing so, we also demonstrate the relevance of γδ+ T cells, but not type I NKT cells.

Overall, our in vivo mouse data and human genetic analysis demonstrates the role of host CD8 T cells and IFN-γ in doxorubicin mechanism of action. The study also strongly supports our most recent work on the role of IL-17A in the mechanism of action of chemotherapy (41), particularly with respect to novel data concerning doxorubicin and mammary carcinoma. In addition, it extensively characterizes mechanism of action of doxorubicin in tumors established de novo by carcinogen, including comparative evaluation of the role of CD8+ T cells, IFN-γ, IL-1β, IL-17 and IL-23. These types of established tumors are arguably more relevant than any short-term transplanted tumors that have been used to previously establish the principle that chemotherapy may be immunogenic. The work corroborates recent studies showing that numbers of TILs in breast cancer are a predictor of response to neoadjuvant chemotherapy (49, 50). Similar outcomes in mouse sarcomas suggest more widespread applicability of these findings to other tumor cell and tissue types. Future work should address how to facilitate the potential development of sustained tumor-specific, adaptive immunity by anthracycline chemotherapy. This includes the generation of long-lived memory T cells and identification of the patients who are best candidates for such immunotherapeutic approaches since enhancing tumor immune
surveillance by chemotherapy may allow extended immune-mediated control of the malignancy beyond the treatment period of the drug.
Acknowledgements

The authors thank Qerime Mundrea and Ben Venville for maintenance of the mice, Nicole Haynes for generation of the AT3OVA cell line, Jennifer Towne (AMGEN, Inc) for providing the anti-agp3, anti-IL-23 and anti-IL-17Rα mAbs and Benjamin Haibe-Kains for bioinformatics assistance.

Grant Support

This work was supported by The Victorian Cancer Agency, The Victorian Breast Cancer Consortium, and the Susan G. Komen Breast Cancer Foundation. S.R.M. was supported by a Balzan Foundation Fellowship. S.L. was supported by a National Health & Medical Research Council (NH&MRC) clinical fellowship. Y.M. was supported by China Scholarship Council. L.Z. was supported by LIGUE labellisee, INFLACARE FP7 EU grant, INCa, Fondation pour la Recherche Medicale and Fondation de France. M.J.S. received support from a NH&MRC Australia Fellowship.
REFERENCES

FIGURE LEGENDS

Figure 1. Doxorubicin therapy requires CD8\(^+\) T cells and IFN-\(\gamma\). Groups of 5 syngeneic WT or gene-targeted mice as indicated were injected subcutaneously with (A) 5 x 10\(^5\) AT3 mammary adenocarcinoma cells, (B) 5 x 10\(^5\) H2N100 mammary adenocarcinoma cells, (C) 5 x 10\(^5\) EO771 mammary adenocarcinoma cells, (D) 8 x 10\(^5\) MCA205 fibrosarcoma cells, or (E) 1 x 10\(^5\) MCA2 fibrosarcoma cells. Mice then received either intratumor PBS or DOX (50 µl, 2 mM) on (A) day 7, (B) day 8, (C) day 14, and (D) day 7 after tumor inoculation or PBS or DOX (2 mg/kg) i.v. on (E) day 7 and 14 after tumor inoculation. Some mice received control Ig (cIg), anti-CD8\(\alpha\), anti-CD8\(\beta\) or anti-IFN-\(\gamma\) (100 µg i.p.) on day -1, 0 and weekly thereafter relative to initial PBS/DOX treatment. Tumor size was measured as indicated. Data shows means of 5 mice per group ± standard errors. Data for AT3, H2N100, EO771 (parts A-C), and MCA2 (part E) are representative of two independent experiments. Statistical analyses were performed at the time point indicated on the figure using Mann-Whitney test (*p<0.05; **p<0.01).

Figure 2. Tumors induced de novo by carcinogen respond to doxorubicin in a CD8\(^+\) T cells and IFN-\(\gamma\)-dependent manner. Groups of 15-30 male BALB/c WT mice were injected s.c. on the flank with 400 µg MCA on day 0. When sarcomas had established (the second week of palpable tumor – 0.20-0.45 cm\(^2\)) BALB/c mice received either intratumor PBS (left column) or DOX (50 µl, 2 mM- right column) once a week for 2 weeks. Some mice received (A) control Ig (anti-agp3), (B) anti-CD8\(\alpha\), or (C) anti-IFN-\(\gamma\) (100-500 µg i.p.) on day -1, 0 and weekly thereafter for 6 weeks relative to initial PBS/DOX treatment. Mice were then monitored for...
tumor development over 250 days and recorded as the growth curves (tumor size in cm²) of individual mice with tumor in each group.

**Figure 3. Doxorubicin treatment enhances CD8 T cell proliferation to cognate tumor antigen.** C57BL/6 mice were inoculated s.c. with either AT3EV (1 x 10⁶) or AT3OVA (1 x 10⁶) tumors. After 30 days, groups of mice (n=5) were i.t. treated with doxorubicin (1 mM) or PBS, and the following day all mice received i.v. transfer of purified, CFSE-labeled CD8+ OT-I T cells (2 x 10⁶). Five days after OT-I cell transfer tumor draining lymph nodes (TDLN) were excised and FACS analyses on Kb-OVA tetramer-reactive CD8 T cells were performed. (A) Representative FACS plots of CD8+KbOVA-tetramer+ cells in the DLN of mice harbouring AT3EV or AT3OVA tumors. Data shown is the percentage of OT-I cells within the lymphocyte population in individual mice with the indicated tumors and treatment. *p<0.02, t-test. (B) FACS histograms showing representative CFSE dilution within the gated CD8/KbOVA-tetramer+ transferred OT-I cell population in DLN of mice harbouring AT3EV or AT3OVA tumors after prior treatment with PBS (*top panels*) or DOX (*bottom panels*). OT-I cell proliferation indices with means are shown in the underlying graph. ***p<0.0001, t test. All graphs are representative of three independent experiments.

**Figure 4. Local doxorubicin treatment enhances numbers of CD8 TILs and IFN-γ production.** C57BL/6 mice were inoculated s.c. with AT3OVA (1 x 10⁶) tumors. After 42 days, groups of mice (n=5) were treated i.t. with doxorubicin (1 mM) or PBS, and the following day received i.v. transfer of purified, CD8+ OT-I T cells (2 x 10⁶). Seven days after OT-I cell transfer...
tumors were excised and FACS analyses on CD8+ TILs were performed. CD8 T cells (A) and KbOVA tetramer reactive CD8 T cells (B) as a percentage of total TILs for individual mice treated with PBS or DOX, is shown. (C) Antigen-specific IFN-γ cytokine production from CD8 TILs measured by intracellular staining after overnight in vitro stimulation of AT3OVA tumor cell suspensions with OVA peptide + IL-2. **p<0.01; ***p<0.0001, \(t\) test. Data is representative of three independent experiments.

**Figure 5. Doxorubicin therapy requires IL-1R and IL-17A.** Groups of 5 syngeneic WT or gene-targeted mice as indicated were injected subcutaneously with (A) 5 x 10^5 AT3 mammary adenocarcinoma cells, (B) 5 x 10^5 H2N100 mammary adenocarcinoma cells, or (C) 5 x 10^5 EO771 mammary adenocarcinoma cells. Mice then received either intratumor PBS or DOX (50 µl, 2 mM) on (A) day 7, (B) day 8, and (C) day 14 after tumor inoculation. Some mice received control Ig (cIg), anti-IL-1β, or anti-IL-17RA (500 µg i.p.) on day 7, 8, 15 and 22 relative to tumor inoculation. Tumor size was measured as indicated. Data shows means of 5 mice per group ± standard errors, representative of two independent experiments. Statistical analyses were performed at the time point indicated on the figure using Mann-Whitney test (*p<0.05; **p<0.01).

**Figure 6. Tumors induced de novo by carcinogen respond to doxorubicin in an IL-1β and IL-17-dependent manner.** Groups of 15-30 male BALB/c WT mice were injected s.c. on the flank with 400 µg MCA on day 0. When sarcomas had established (the second week of palpable tumor – 0.20-0.45 cm^2) BALB/c mice received either intratumor PBS (left column) or DOX (50 µg i.p.) on day 7, 8, 15 and 22 relative to tumor inoculation. Tumor size was measured as indicated. Data shows means of 5 mice per group ± standard errors, representative of two independent experiments. Statistical analyses were performed at the time point indicated on the figure using Mann-Whitney test (*p<0.05; **p<0.01).
µl, 2 mM- right column) once a week for 2 weeks. Some mice received (A) control Ig (anti-agp3), (B) anti-IL-1β, (C) anti-IL-23p19, or (D) anti-IL-17Rα (100-500 µg i.p.) on day -1, 0 and weekly thereafter for 6 weeks relative to initial PBS/DOX treatment. Mice were then monitored for tumor development over 250 days and recorded as the growth curves (tumor size in cm²) of individual mice with tumor in each group. Control Ig graphs in (A) are reproduced from Figure 2A.
Table 1
Association of genes with clinical outcome in breast cancer patients treated with and without anthracycline chemotherapy.

<table>
<thead>
<tr>
<th>Association with clinical outcome</th>
<th>Genes</th>
<th>Cohort 1:</th>
<th>Cohort 2:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endpoint: distant metastases</td>
<td>Endpoint: pCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. patients = 1062</td>
<td>No of patients= 114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment= none</td>
<td>Treatment= epirubicin monotherapy</td>
</tr>
<tr>
<td>Hazard Ratio (95%CI)</td>
<td></td>
<td>AUC (95%CI)</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.8 (0.65-0.95), p=0.031</td>
<td>0.69(0.56-0.81), p=0.016</td>
<td></td>
</tr>
<tr>
<td>CD8A</td>
<td>0.82(0.5-1.3), p=0.4</td>
<td>0.72 (0.59-0.84), p=0.005</td>
<td></td>
</tr>
<tr>
<td>CD8B</td>
<td>0.87(0.59-1.3), p=0.21</td>
<td>0.65 (0.52-0.78), p=0.049</td>
<td></td>
</tr>
<tr>
<td>IL17A</td>
<td>0.82 (0.67-1.1), p=0.051</td>
<td>0.5, p=0.44</td>
<td></td>
</tr>
<tr>
<td>IL1B</td>
<td>0.87 (0.7-1.06), p=0.18</td>
<td>0.39, p=0.19</td>
<td></td>
</tr>
<tr>
<td>IL23</td>
<td>0.88(0.75-1.03), p=0.13</td>
<td>0.56, p=0.42</td>
<td></td>
</tr>
</tbody>
</table>

Association of genes with clinical outcome in breast cancer patients treated with and without anthracycline chemotherapy. Cohort 1 consisted of 1062 patients from 4 gene expression datasets who received no systemic treatment after their surgery in order to determine the clinical outcome association independent of anthracycline. 70% of tumors were ER-positive, 85% were node negative at diagnosis. Univariate hazard ratios presented are stratified by dataset. Genes are correlated as continuous variables. Here, increasing expression of IFN-γ is correlated with a good prognosis or a longer time free from
distant metastases. Cohort 2 consisted of 114 patients treated in a neoadjuvant trial of the anthracycline epirubicin given as a sole therapy prior to surgery. All patients were ER-negative with 86% of tumors >2 cm and 54% with positive lymph nodes at study entry. Biopsies were taken prior to therapy. Here, increasing expression of IFN-γ, CD8A and CD8B are associated with a higher chance of a complete response from chemotherapy at surgery. In this second cohort, a pCR was strongly correlated with a better survival from breast cancer. (Full clinical information for cohorts 1 and 2 can be found in the Supplementary Table 1).

CI: confidence interval

pCR: pathological complete response: complete disappearance of invasive tumor at surgery after 4 cycles of epirubicin chemotherapy given at 100 mg/m².
Matarollo et al. Figure 4

A

% CD8 T cells of total TL

***

PBS treated DOX treated

B

% NK/NK-1 T cells of total TL

**

PBS treated DOX treated

C

Gated on CD8 TIL

PBS

0.28%

DOX

5.43%
Matterollo et al. Figure 6

A

B

C

D

PBS + anti-agp3

DOX + anti-agp3

PBS + anti-IL-1β

DOX + anti-IL-1β

PBS + anti-IL-23p19

DOX + anti-IL-23p19

PBS + anti-IL-17Ra

DOX + anti-IL-17Ra

Days after MCA inoculation

Tumor size (cm²)

Days after MCA inoculation

Tumor size (cm²)

Days after MCA inoculation

Tumor size (cm²)

Days after MCA inoculation

Tumor size (cm²)
Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors

Stephen R Mattarollo, Sherene Loi, Helene Duret, et al.

Cancer Res  Published OnlineFirst June 6, 2011.