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ABSTRACT

Background Multidrug resistance-1 (MDR1) transporter limits the intracellular accumulation of chemotherapies (paclitaxel, anthracyclines) used in breast cancer (BC) treatment. In addition to tumor cells, MDR1 is expressed on immune cell subsets in which it confers chemoresistance. Among human T cells, MDR1 is expressed by most CD8+ T cells, and a subset of CD4+ T helper (Th) cells. Here we explored the expression, function and regulation of MDR1 on CD4+ T cells and investigated the role of this population in response to neoadjuvant chemotherapy (NAC) in BC.

Methods Phenotypic and functional characteristics of MDR1+ CD4 Th cells were assessed on blood from healthy donors and patients with BC by flow cytometry. These features were extended to CD4+ Th cells from untreated breast tumor by flow cytometry and RNA-sequencing (RNA-seq). We performed in vitro polarization assays to decipher MDR1 regulation on CD4 Th cells. We evaluated in vitro the impact of chemotherapy agents on MDR1+ CD4+ Th cells. We analyzed the impact of NAC treatment on MDR1+ CD4+ Th cells from blood and tumors and their association with treatment efficacy in two independent BC cohorts and in a public RNA-seq data set of BC tumor biopsies before and after NAC. Finally, we performed single cell (sc) RNAseq of blood CD4+ memory T cells from NAC-treated patients and combined them with an scRNAseq public data set.

Results MDR1+ CD4 Th cells were strongly enriched in Th1.17 polyfunctional cells but also in Th17 cells, both in blood and untreated breast tumor tissues. Mechanistically, Tumor growth factor (TGF)-β1 was required for MDR1 induction during in vitro Th17 or Th1.17 polarization. MDR1 expression conferred a selective advantage to Th1.17 and Th17 cells following paclitaxel treatment in vitro and in vivo in NAC-treated patients. scRNAseq demonstrated MDR1 association with tumor Th1.17 and Th with features of cytotoxic cells. Enrichment in MDR1+ CD4+ Th1.17 and Th17 cells, in blood and tumors positively correlated with pathological response. Absence of early modulation of Th1.17 and Th17 in NAC-resistant patients, argue for its use as a biomarker for chemotherapy regimen adjustment.

Conclusion MDR1 favored the enrichment of Th1.17 and Th17 in blood and tumor after NAC that correlated to clinical response.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Multidrug resistance-1 (MDR1) expression limits the toxicity of chemotherapies (paclitaxel, anthracyclines) used in breast cancer (BC) treatment. In addition to tumor cells, MDR1 is expressed by a majority of CD8+ T cells and a subset of CD4+ T helper (Th) cells suggesting a particular resistance of these Th cells to chemotherapy.

WHAT THIS STUDY ADDS

⇒ MDR1+ CD4+ Th cells are strongly enriched in Th1.17 polyfunctional cells and Th17 cells, both in blood and breast tumor tissues. Mechanistically, Tumor growth factor (TGF)-β1 is required for MDR1 induction during Th17 or Th1.17 polarization. MDR1 confers resistance to Th1.17 and Th17 cells in vitro and in vivo in patients with neoadjuvant-chemotherapy-treated BC favoring their enrichment in blood and tumor that correlates with clinical response.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Absence of modulation of Th1.17 and Th17 early under neoadjuvant chemotherapy treatment could be used as a biomarker for chemotherapy regimen adjustment. Chemotherapy substrate of MDR1 should be favored for combination with immunotherapy.

INTRODUCTION

The multidrug resistance-1 (MDR1) efflux pump belongs to the ABC transporters family.[1 2] MDR1 is expressed in several tissues (intestine, liver, kidney, brain and testis barriers) where it plays a protective role against xenobiotics.[1] MDR1 actively excretes numerous toxic substances, including

anti-proliferative and immunosuppressant drugs[3] and confers resistance to chemotherapy in tumor cells, which has led to the development of MDR1 inhibitors such as zosuquidar[4] to restore drug sensitivity. In solid tumors, expression of MDR1 was described on infiltrated immune cells especially on T lymphocytes.[5 6] Among human T cells, MDR1 is preferentially expressed by $CD8^+$ T cells[7] but also by a subset of $CD4^+$ T helper (Th) cells, but not regulatory T (Treg) cells.[8] Specifically, MDR1 has been linked to a Th1.17 subset (known as pathogenic Th17) enriched in the blood of patients with Crohn's disease (CD)[9] or acute myeloid leukemia.[10] Unlike Th17 cells endowed with defense against pathogens and tissue-repair functions, Th1.17 cells display polyfunctional inflammatory properties. Th1.17 cells are characterized by the co-expression of the chemokine receptors CCR6 and CXCR3, transcription factors RORγ-T and T-bet but also the co-secretion of interferon (IFN)-γ and interleukin (IL)-17A as well as Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and IL-22.[5 11 12] Several studies reported a controversial impact of Th17 cells on the anti-tumor immune response in patients with cancer, mainly due to the approximate identification of Th17 cells through IL-17 positivity only.[13] In contrast, studies characterizing $CD4^+$ tumor infiltrated lymphocytes (TILs) revealed a positive correlation between the frequency of $CD4^+$ T cells expressing IL-17, encompassing Th17 and Th1.17 cells, and better prognosis in prostate,[14] cervical[15] and liver[16] carcinoma. Moreover, IFN-γ secreted by Th17 cells play an essential role for immune response against tumors in melanoma and ovarian cancer.[17 18]

Neoadjuvant chemotherapy (NAC) with cycles of combined doxorubicin (MDR1 substrate) and cyclophosphamide (CTX), followed by paclitaxel (MDR1 substrate) only, before surgical resection remains the most efficient therapy in aggressive breast cancer (BC).[19] Despite important lymphopenic and neutropenic side effects, NAC treatment may have beneficial immune activity through induction of tumor immunogenic cell death (ICD).[20] Park *et al* report that TILs density increased after one NAC cycle in patients with BC.[21] Moreover, in residual disease post-NAC, the presence of TILs is associated with better prognosis in patients with triple negative breast cancer (TNBC).[22] In patients with NAC-treated BC, the reduction in Treg cells correlates with pathological complete response (pCR),[23] however the composition of the non-regulatory $CD4^+$ T-cell subsets has not been extensively characterized.

In this context, we investigated the role of MDR1 in the enrichment of specific $CD4^+$ Th cell subpopulations, in the blood and the tumor microenvironment (TME) of patients with BC after NAC treatment, and their association with treatment efficacy.

We first confirmed that MDR1 expression on $CD4^+$ Th cells was associated with polyfunctional Th1.17 and Th17 in the blood and the TME. Its expression was induced by TGF-β1 on Th17 and Th1.17 polarizing conditions. $MDR1^+$ Th cells were resistant to the cytotoxic effect of chemotherapy in vitro leading to an enrichment in Th1.17 and Th17 cells. In addition, in patients with BC, NAC treatment induced a decrease in Treg cell frequency and an increase in $MDR1^+$ Th1.17 and Th17 cells, both in blood and in tumor samples, which positively correlated with pathological response. Finally, using own single cell (sc) RNAseq of blood $CD4^+$ memory T cells from NAC treated tumors combined with the reanalysis of an scRNAseq public data set of BC tumors, we demonstrated MDR1 association with tumor Th1.17 and Th with cytotoxic potential. Altogether these results suggest that, by resisting to NAC treatment, $CD4^+$ $MDR1^+$ Th cells might contribute to the therapeutic response observed in NAC-treated patients. Absence of early modulation of Th1.17 and Th17 in blood of NAC-resistant patients, argue for its implementation as a biomarker for chemotherapy regimen adjustment.

METHODS

Human samples

Healthy donors' (HD) blood was purchased from the Etablissement Français du Sang (EFS). Fresh blood and primary tumors from patients with BC were obtained through the biological resource center (BRC) of Léon Bérard Center, after written informed consent, in accordance with the Declaration of Helsinki. Liquid nitrogen stored peripheral blood cells (PBMC) from 25 patients with BC enrolled in the "Breast-Immun" clinical trial (NCT01440413) were obtained from BRC. All surgery tumor tissues from the Breast-Immun trial and the prospective cohort were analyzed by a pathologist to calculate the residual cancer burden (RCB) (http://www3.mdanderson.org). Major clinical patients' characteristics are presented in online supplemental table 1.

Purification of $CD4^+$ T-cell subpopulations

Memory and naïve $CD4^+$ T cells were purified from HD PBMC using respectively the Human $CD4^+$ Memory T Cell Isolation Kit (BioLegend) and the Human Naïve $CD4^+$ T Cell Isolation Kit II (STEMCELL). For functional studies, $MDR1^+$ and $MDR1^{neg}$ Th cells were purified from blood $CD4^+$ memory T cells, by cell sorting (FACSAria III, Becton Dickinson (BD)) using Rh123 efflux as a reporter of MDR1 expression. Antibodies against CD25, CD127 were used to exclude Treg cells. Primary cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 glutamax medium (Invitrogen) supplemented or not with Fetal calf serum (FCS, Eurobio) or human serum AB^+ (sAB, EFS).

Tumor digestion

Tumor samples were mechanically dilacerated in RPMI, according to a standard calibration (2 mL/g of tumor) and soluble tumor milieu (STM) were stored at −80°C. Tumor dilacerates were then enzymatically digested for 45 min at 37°C using collagenase-IV (200 U/mL; Invitrogen) and DNase-I (25 µg/mL; Merck) in RPMI

containing 20% FCS. Dilacerates were filtered on 70 µm strainer to obtain TME single-cell suspension. For RNA sequencing, MDR1^{+} and MDR1^{neg} TA-Th cells were Fluorescence Activated Cell Sorter (FACS)-sorted using mAbs against MDR1, CD45, CD3, CD4, CD25, CD127, CD45RA, and zombie (BioLegend) as viability marker (online supplemental table 2).

Flow cytometry analyses

Cells were stained with specific mAbs and zombies (online supplemental table 2). For optimal MDR1 detection with UIC2 mAb, staining was done in the presence of ciclosporin A (25 µM; Tocris).[5] Stained cells were fixed with 2% formaldehyde (Merck). In case of intracellular stainings, FOXP3 Fixation and Permeabilization kit (Invitrogen) was used (online supplemental table 2). Cells were analyzed on LSR-Fortessa or LSR-Fortessa X20 (BD) with identical settings throughout the entire study. Raw data were analyzed using FlowJo V.10.4.2 software (Tree Star).

Intracellular cytokine detection assay

Intracellular cytokines detection was performed after short-term reactivation at 37°C in the presence of Phorbol-myristate-acetate (PMA, 50 ng/mL), ionomycin (1 µg/mL) (Merck) and a transport inhibitor (GolgiPlug 1 µg/mL, BD). After extracellular staining, cells were fixed and permeabilized with FOXP3 kit for intracellular cytokines detection. Th cell polyfunctionality (cytokines co-production) was evaluated using the Boolean method on the FlowJo software and then processed using Pestle and SPICE V.5.3 softwares (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA).

Rh123 efflux assay

MDR1 activity was analyzed on CD4^{+} memory T cells with Rh123 efflux assay as previously described.[5] In some conditions, inhibitors of MDR1 (zosuquidar, 1 µM), MRP-1 (MK571, 5 µM) and BCRP-1 (KO-143, 50 nM) (all from Tocris Bioscience) were added before the efflux phase to confirm the MDR1-specific efflux.

Drug resistance assay

FACS-sorted MDR1^{+} and MDR1^{neg} Th cells, were stained with Cell Tracers (CTY or CTV) (Invitrogen) and used either alone or mixed at physiological blood ratio (80% of MDR1^{neg} and 20% MDR1^{+} Th cells). Cells were cultured in 96-well U-bottomed plates (50,000 cells/well) in RPMI supplemented with 5% sAB in the presence of anti-CD3/anti-CD28 beads (ratio beads to cell 1:4, Invitrogen) and rhIL-2 (50 IU/mL; Proleukin, Clinigen), with increasing doses of chemotherapies (paclitaxel (1–20 nM) or cisplatin (1–5 µM), CLB) at 37°C with or without zosuquidar. After 4 days, cells were harvested and stained for flow cytometry (FC) analyses or reactivated for intracellular cytokines analysis. Similar experiments were also performed with FACS-sorted MDR1^{+} and MDR1^{neg} CD8^{+} T cells but also naïve and memory purified CD4^{+} T cells, respectively, stained with CTV and CTY and mixed at ratio 25/75.

Analysis of public BC RNA-sequencing and scRNAseq data sets

A recent public RNA-sequencing (RNA-seq) data set[21] of 46 SBR2/3 BC tumor biopsies was used to quantify tumor infiltration by MDR1^{+} and MDR1^{neg} TA-Th cells before (T1) and after one cycle (T2) of treatment with anthracyclines/CTX. Previously identified differentially expressed gene (DEG) were used to define MDR1^{+} and MDR1^{neg} gene signatures (*MDR1* gene was removed from MDR1^{+} TA-Th cell signature because of its high expression by tumor cells). Infiltration score was defined for each pair of samples (T1 vs T2) using single sample gene set enrichment analysis (ssGSEA).

Publicly available breast tumor single cell (sc)RNAseq data sets (GSE114727,[24] GSE110686,[25] Pekin university (PKU) study[26]) were reanalyzed using the PKU online bioinformatic tool (http://cancer-pku.cn:3838/PanC_T/)[26] to investigate Immune checkpoint (ICP) expression and proliferation status of the *ABCB1*-enriched the CD4^{+} T-cell meta-clusters.

Statistics

All values are reported as medians or as means±SEM. Statistical analyses were performed with Prism (GraphPad, USA) using appropriate tests specified in each figure legend. Significant changes were annotated with *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 or ns for non-significant.

RESULTS

MDR1 expression is associated to Th1.17 and Th17 cells and induced on polarization with TGF-β1

Using PBMC from HD, MDR1 was detected on 75% of CD8^{+} T cells while only 20% of CD4^{+} T cells expressed MDR1 (online supplemental figure 1, figure 1A). Among CD8^{+} T cells, MDR1 was expressed by most naive cells but with lower mean fluorescence intensity (MFI) compared with memory cells positive at 75% (online supplemental figure 1). In contrast, on CD4, MDR1 was mostly detected on Th cells but not on FOXP3^{high} Treg cells or naïve (CD45RA^{+} CCR7^{+}) T cells (figure 1B).

Based on CD45RA and CCR7 expression,[27] MDR1 was equally distributed among memory CD4^{+} (figure 1C) and CD8^{+} (online supplemental figure 1D) subsets with a slightly higher MFI on the effector memory T cell (T_{EM}, CD45RA^{neg} CCR7^{neg}) population (figure 1D, online supplemental figure 1D). In vitro, the efflux of Rh123, a fluorescent substrate of MDR1, was highly correlated to the expression of MDR1 on CD4^{+} Th cells (figure 1E) and CD8^{+} memory T cells (online supplemental figure 1E) and blocked by the selective MDR1 inhibitor zosuquidar, but not by other ABC transporter blockers (MK571 (MRP-1) and KO143 (BCRP-1))[8 28 29] (figure 1F), demonstrating

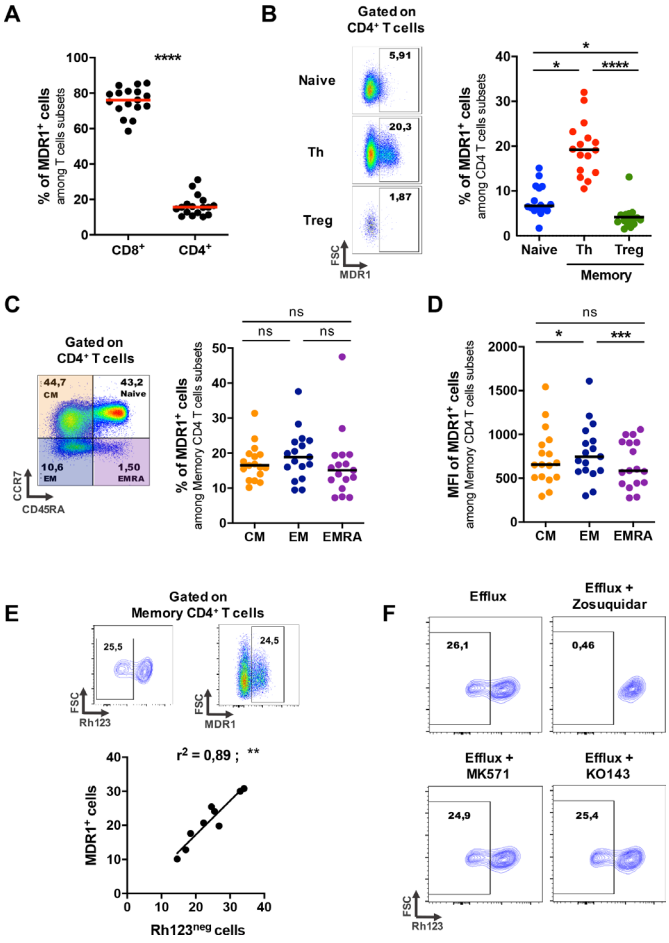

Figure 1 MDR1 is expressed by a subset of non-regulatory memory CD4$^+$ T cells and is functional. (A–D) Proportions of MDR1$^+$ cells in HD blood (n=17) among CD4$^+$ and CD8$^+$ T cells compartment: (A) CD4$^+$ T cells subsets of naive, Th and Treg cells (B) memory CD4$^+$ T cells subsets (T central memory ($_{CM}$), T effector memory ($_{EM}$), Teffector memory RA+ ($_{EMRA}$) based on CD45RA and CCR7 expression, in frequency (C) and expression intensity (MFI) (D) E) Correlation between proportions of MDR1$^+$ cells and rhodamine 123 effluxing cells (Rh123^{neg} cells) among all memory CD4$^+$ T cells of HD blood (n=10) (F) Representative dot plot of Rh123 efflux capacity of memory CD4$^+$ T cells, according to ABC transporter inhibitors (zosuquidar: MDR1; MK571: MRP-1; KO143: BCRP-1). Statistical analyses: Wilcoxon (A) Friedman (B, C and D) and Spearman (E) (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001). HD, healthy donors'; MDR1, multidrug resistance-1; Th, T helper cells; Treg, regulatory T cells. T_{CM}: T central memory; T_{EM}:T effector memory; T_{EMRA} : T effector memory RA$^+$; FSC : Forward side scatter

MDR1 functionality on both CD4$^+$ and CD8$^+$ T cells (online supplemental figure 1F).

Based on chemokine receptors CXCR3 and CCR6,[30] we observed MDR1 expression on 58.5±14% of Th1.17 (CXCR3$^+$CCR6$^+$) and 33.8±10% of Th17 (CXCR3^{neg}CCR6$^+$) cells whereas lower proportions were found among Th1 (CXCR3$^+$CCR6^{neg}) (13.5±3%) and other Th (CXCR3^{neg}CCR6^{neg}) (5.8±2 %) subsets (figure 2A, online supplemental figure 2A). Based on RORγt and T-bet expression, we confirmed the enrichment of MDR1$^+$ cells within Th1.17 (RORγt$^+$T-bet$^+$) and Th17 (RORγt$^+$T-bet^{neg}) cells (figure 2B). Analyzing cytokine production pattern by FC (after PMA/ionomycin reactivation) we confirmed preferential MDR1 expression on IFN-γ$^+$IL-17A$^+$ (59.5±12 %) and IFN-γ^{neg} IL-17A$^+$ (45.7±9 %) cells. MDR1 was also detected on 26±7% of IFN-γ$^+$IL-17A^{neg} and 16±5% of IFN-γ^{neg}IL-17A^{neg} cells (figure 2C).

Boolean cytokine analysis highlighted the higher polyfunctionality of MDR1$^+$ CD4$^+$ Th cells (figure 2D). Indeed, 50% of MDR1$^+$ Th cells produced at least two cytokines among IFN-γ, IL-17A, IL-22 and Tumor necrosis factor (TNF)-α, compared with 32% for the MDR1^{neg} counterpart. Moreover, MDR1$^+$ Th cells produced higher levels of IL-22 (online supplemental figure 2D), very low levels of IL-4 and no IL-13 (online supplemental figure 2B). Consistent with these results, MDR1$^+$ Th cells secreted higher levels of Th1.17 cytokines and decreased levels of Th2 cytokines following in vitro T cell receptor (TCR) stimulation (online supplemental figure 2C).

Using in vitro Th polarization assays, we found that one round of polarization of naïve CD4$^+$ T cells under Th17 condition (online supplemental material) strongly induced MDR1 expression, whereas the Th1.17 cocktail induced only low MDR1 expression and Th1 and Th0 cocktails did not (figure 2E). Interestingly, Th17 polarized cells maintained MDR1 expression irrespective of the second polarization cocktail used (Th17, Th1 or Th0). This suggests that soluble factors common to Th17 and Th1.17 differentiation cocktails (IL-6, TGF-β1) might participate to MDR1 induction on CD4$^+$ T cells but are dispensable for its sustained expression (figure 2E). Blocking TGF-β1 signaling but not IL-6 signaling, completely abolished MDR1 expression (figure 2F), thereby demonstrating the essential role of TGF-β1 during naive CD4$^+$ T-cell polarization into MDR1$^+$ Th cells. However, exposure to TGF-β1 did not induce MDR1 expression on sorted MDR1^{neg} Th cells (online supplemental figure 2D), suggesting that the induction of MDR1 expression might happen early after naïve CD4$^+$ T-cell priming. It has to be noticed that induced Treg (iTreg) polarization cocktail (TGF-β1, IL-2) also induced the expression of MDR1 on these iTreg cells (online supplemental figure 2E).

MDR1$^+$ Th cells present in the breast TME are enriched in Th1.17 cells

Next, we examined the expression of MDR1 on CD4$^+$ Th cells in the blood and paired tumors of patients with untreated BC. While the proportions of MDR1$^+$ Th cells were similar in the blood and TME (online supplemental figure 3A), MDR1 was expressed at higher levels in the latter (online supplemental figure 3B). The frequency of Th1.17 cells was unchanged between both sites but the frequency of Th1 cells was significantly higher in the TME. In contrast, the proportions of Th17 and other Th subsets were reduced in the TME (online supplemental figure 3C).

To better define the differences between MDR1$^+$ and MDR1^{neg} Th cells in the TME, we performed a

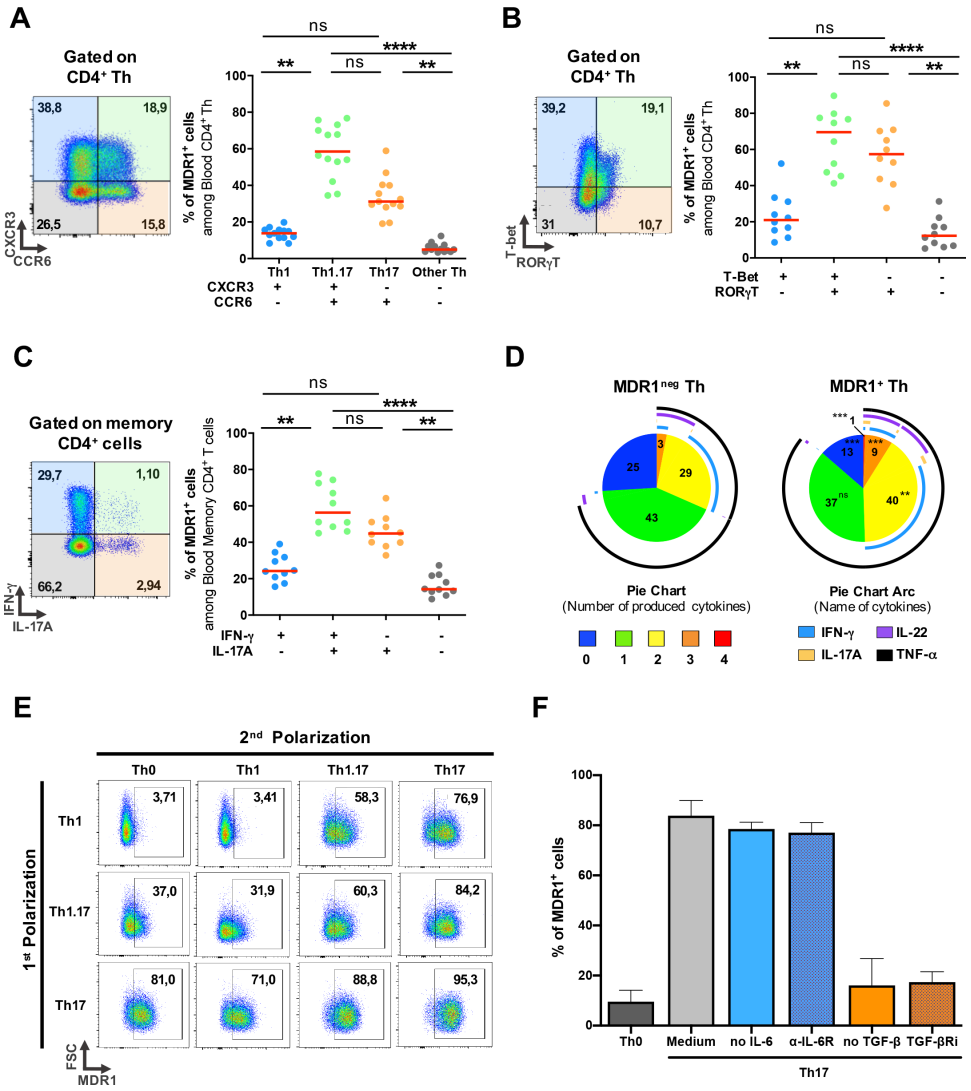

Figure 2 MDR1 expression delineates highly polyfunctional population enriched in Th1.17 and Th17 cells. (A–B) MDR1 expression among Th cell subsets from HD blood according to their expression of CCR6 and CXCR3 (n=11) (A) and RORγT and T-bet (n=12) (B). (C) MDR1 expression among IFN-γ and IL-17A expressing cells after PMA/ionomycin reactivation in HD blood (n=10) (D) SPICE representation of MDR1^{+} and MDR1^{neg} Th cell cytokines polyfunctionality (n=10) after PMA/ionomycin reactivation. (E) Representative dot plots of MDR1 expression on purified naive CD4^{+} T cells successively polarized with different Th polarizing cocktails (n=3). (F) MDR1 expression on Th0 and Th17 polarized naive CD4^{+} T cells with more or less TGF-β and IL-6 and in the presence of anti-IL-6R (RoActemra) or TGF-βR inhibitor (galunisertib). Statistical analyses: analysis of ANOVA-2 (A, B and C) and Wilcoxon (D). (**p<0.005, ***p<0.0005, ****p<0.0001). HD, healthy donors'; IFN, interferon; IL, interleukin; MDR1, multidrug resistance-1; Th, T helper cells.

transcriptome analysis of those two subsets isolated from untreated (UT) BC tumors (online supplemental figure 4A,B). Our analysis identified 157DEG segregating MDR1^{+} from MDR1^{neg} Th cells (online supplemental figure 4C). Among the 104 upregulated genes in MDR1^{+} subset were genes such as *CCR6*, *CTSH*, *CCL20*, *RORC* and *IL-17A* previously described in the core (Th17-Th1.17) common transcriptomic signature[9] and others such as *SLC4A10*, *ADAM23*, *LTK*, *IL4I1*, *KIT*, *IL23R*, *IL18RAP* and *DPP4* characteristics of a Th1.17 gene signature,[9 31] further supporting the Th1.17 polarization of MDR1^{+} Th cells in the TME. We confirmed by real-time (RT)-qPCR the specific enrichment of *RORC*, *IL4I1*, *CEBPD* and *LTK* in sorted MDR1^{+} Th1.17 (online supplemental figure 4D). Accordingly, an enrichment in Th1.17 specific as well as core (Th17-Th1.17) common transcriptomic signatures but not Th17-specific,[9 31] Th1 and Th2 signatures[32] was also identified by ssGSEA in MDR1^{+} TA-Th cells (online supplemental figure 4E).

We also investigated the expression of *ABCB1* gene transcripts within CD4^{+} T-cell meta-clusters, using the recently published "pan-cancer single-cell landscape of tumor-infiltrating T cells" data set by Zheng *et al* in breast tumor samples from UT patients.[24–26] *ABCB1* was detected on meta-clusters annotated as c14-Th17-SLC4A10" and "c15-Th17-IL23R", sharing Th17 and Th1.17 specific genes confirming their Th17 and Th1.17 identity (online supplemental figure 4F) but also on

"c13-TEMRA CX3CR1" meta-cluster enriched in characteristic cytotoxic genes previously reported on human $CD4^+$ cytotoxic cells, including hallmark features such as granzymes and granulysin (for review[33]) (online supplemental figure 4G).

MDR1$^+$ Th cells resist to paclitaxel treatment in vitro leading to Th17 and Th1.17 cell enrichment

To investigate whether MDR1 expression conferred $CD4^+$ Th cells with resistance to chemotherapy, HD blood $CD4^+$ Th cells were FACS-sorted based on their Rh123 efflux capacity. Purified $Rh123^{neg}$ ($MDR1^+$) and $Rh123^+$ ($MDR1^{neg}$) Th cells were stimulated with TCR/CD28 signal and cultured 4 days with increasing doses of chemotherapy drugs. Paclitaxel (MDR1 substrate) treatment, did not alter the viability of $MDR1^+$ Th cells whereas it strongly reduced, in a dose-dependent manner, that of $MDR1^{neg}$ Th cells (figure 3A). As a result, when $MDR1^{neg}$ and $MDR1^+$ Th cells were mixed together at a physiological ratio (mix 80/20), paclitaxel treatment led to a dose-dependent enrichment of $MDR1^+$ cells with higher levels of MDR1 at the end of the culture (figure 3B,C). In contrast, cisplatin (not MDR1 substrate) treatment similarly reduced the viability of both populations (figure 3D) and did not modulate their respective ratio in the mix 80/20 (figure 3E,F). $MDR1^+$ Th cells maintained their proliferative capacity in the presence of paclitaxel, in contrast to the $MDR1^{neg}$ fraction (figure 3G), whereas the proliferation of both subsets was inhibited by cisplatin (figure 3H). Moreover, the addition of zosuquidar restored sensitivity of $MDR1^+$ Th cells to paclitaxel, impeding their proliferation as well as their enrichment in the mix 80/20 (figure 3B,C and G). Interestingly, the addition of paclitaxel in a mix (25/75) of purified naïve and memory $CD4^+$ T cells blocked the proliferation of the naive compartment devoid in MDR1 whereas it allows that of purified memory T cells containing $MDR1^+$ cells (online supplemental figure 5A). Importantly, paclitaxel treatment favored a dose-dependent selection of $CXCR3^+CCR6^+$ Th1.17 population (figure 3I) associated with an increase of cells producing IFN-γ, IL-17A and IL-22 (10-fold increase in IFN-γ^+IL-$17A^+$ cells) (figure 3J). Together, this demonstrates that MDR1 expression provides a survival and proliferation advantage to Th1.17 cells and Th17 cells following exposure to chemotherapy substrate of MDR1.

NAC treatment selects functional MDR1$^+$ Th cells enriched in Th1.17 and Th17 cells in blood and tumor of patients with BC

To evaluate the translation of our observations in vivo, we analyzed blood samples from patients with NAC-treated BC (NAC) at time of surgery (online supplemental table 1). As a control, we analyzed samples from patients with UT BC (online supplemental table 1) with SBR 2/3 grade, as well as from age-matched and sex-matched HD. While the proportion of $CD3^+$ T cells was stable (online supplemental figure 6A), NAC treatment reduced the proportion of $CD4^+$ T cells (figure 4A), accompanied by an increased frequency of memory $CD4^+$ T cells compared with UT patients and HD (figure 4B) and a higher proportion of $MDR1^+$ cells (figure 4C). Proportions of $CD8^+$ T cells among total T cells were increased in NAC-treated patients (online supplemental figure 6B), in accordance with the higher frequency of $MDR1^+$ cells in $CD8^+$ T cells. We also observed a slight decrease in Treg cells frequency (figure 4D) and an increase in $MDR1^+$ Th cells proportion (figure 4D), with a particular increase of central memory T cells (T_{CM}) (online supplemental figure 6C). Within the memory $CD4^+$ Th cells, the proportions of Th1.17 and Th17 cells were significantly increased in NAC-treated patients whereas Th1 and other Th cells decreased (figure 4E,F, online supplemental figure 6D,E).

To ascertain the direct impact of NAC treatment on these observations, we performed similar analyses on paired frozen PBMC samples obtained before and after the second cycle of chemotherapy with anthracyclines in patients enrolled in the Breast-Immun cohort (online supplemental table 1). We confirmed the selective enrichment of $MDR1^+$ Th cells post-chemotherapy (figure 4G,H), associated with a decrease in Treg cell frequency (figure 4I). Moreover, we confirmed the increase in Th1.17 and Th17 cells proportions among $MDR1^+CD4^+$ Th cells using both chemokine receptors expression (figure 4I) and cytokine production (figure 4J) whereas the proportion of Th1 cells was reduced. All other major modulations observed in the prospective cohort were confirmed (online supplemental figure 6F–H). Altogether, these results demonstrate that NAC treatment of patients with BC with an MDR1 substrate reshapes the blood $CD4^+$ T-cell compartment with an enrichment in Th1.17 and Th17 cells.

To determine if the alterations of the $CD4^+$ Th cell compartment in the periphery could also be detected at the tumor site, we analyzed the tumor infiltrate of UT-treated and NAC-treated patients. Similar to the blood, there were no changes in the proportions of total T cell between UT-treated and NAC-treated patients (figure 5A). However, while there was a 25% reduction in the proportion of $CD4^+$ among tumorassociated (TA)-T cells in NAC-treated tumors (figure 5B), the frequency of $MDR1^+$ TA-Th cells as well as its MFI were increased by 1.8-fold in NAC-treated tumors (figure 5C). We also observed a reduction in the frequency of TA-Treg cells (figure 5D). This correlated with a strong enrichment in Th1.17 cells (75% increase) and in cells co-producing IFN-γ and IL-17A or producing IL-17A only (respectively, 5.24-fold and 6.62-fold) and a decrease in Th1 cells (25% reduction) (figure 5E,F).

Using a recent public RNA-seq data set[21] of BC tumor paired biopsies collected before (T1) and after one cycle (T2) of treatment with anthracycline/CTX, we further investigated the direct impact of NAC treatment on the enrichment in the different Th signatures in the TME. NAC treatment induced an enrichment in Th1.17 and Th17 selective and core (Th17-Th1.17) signatures but not

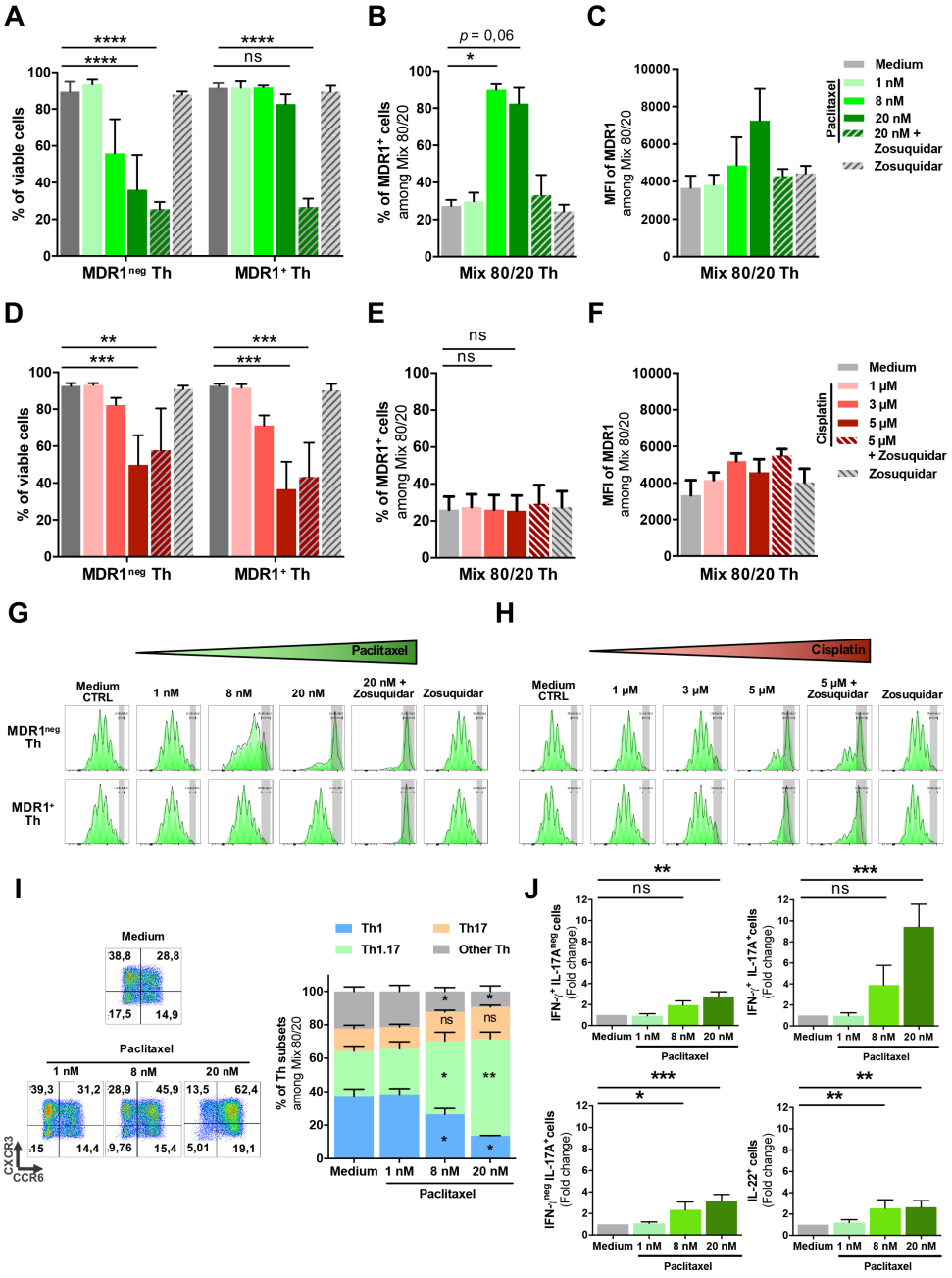

Figure 3 MDR1⁺ Th cells resist to paclitaxel treatment and proliferate favoring an enrichment in Th1.17 and Th17 cells. (A and D) Percentage of viable cells among purified MDR1⁺ and MDR1^{neg} Th cells according to paclitaxel (A) or cisplatin (D) concentrations and zosuquidar. (B, C, E and F) Percentage (B and E) and MFI (C and F) of MDR1⁺ cells after treatment of a physiological mixed Th population (80% MDR1^{neg} 20% MDR1⁺) with different concentrations of paclitaxel (B and C) or cisplatin (E and F) and zosuquidar. (G–H) Proliferation (assessed by CTY dilution) of purified MDR1⁺ and MDR1^{neg} Th cells according to paclitaxel (G) or cisplatin (H) concentrations and zosuquidar. (I–J) Phenotypic (I) and functional (J) Th cell subset enrichment in a mixed Th population (80% MDR1^{neg} 20% MDR1⁺) after paclitaxel treatment (n=3 for each experiment). Statistical analyses: analysis of ANOVA-2 (A and D) and Friedman (B, E and J) (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001). IFN, interferon; IL, interleukin; MDR1, multidrug resistance-1; Th, T helper cells.

Th1 and Th2 ones (figure 5G) in adequacy with results in the Breast-Immun cohort (figure 3I,J).

Using STM and a sensitive multiplex electrochemiluminescence method (online supplemental material), we detected both IFN-γ and IL-17A in a higher proportion (26.3%, 5/19) of NAC-treated tumors compared with UT ones (16.6%, 8/48), IFN-γ alone was also increased with, respectively, 47.36% and 37.5% whereas IL-17A alone was never detected (online supplemental figure 7A).

In NAC non-responding patients, tumor infiltrating MDR1⁺ Th cells do not present signs of high activation

Using frozen tumor cell suspensions of five NAC-treated patients with available fresh material at surgery but who

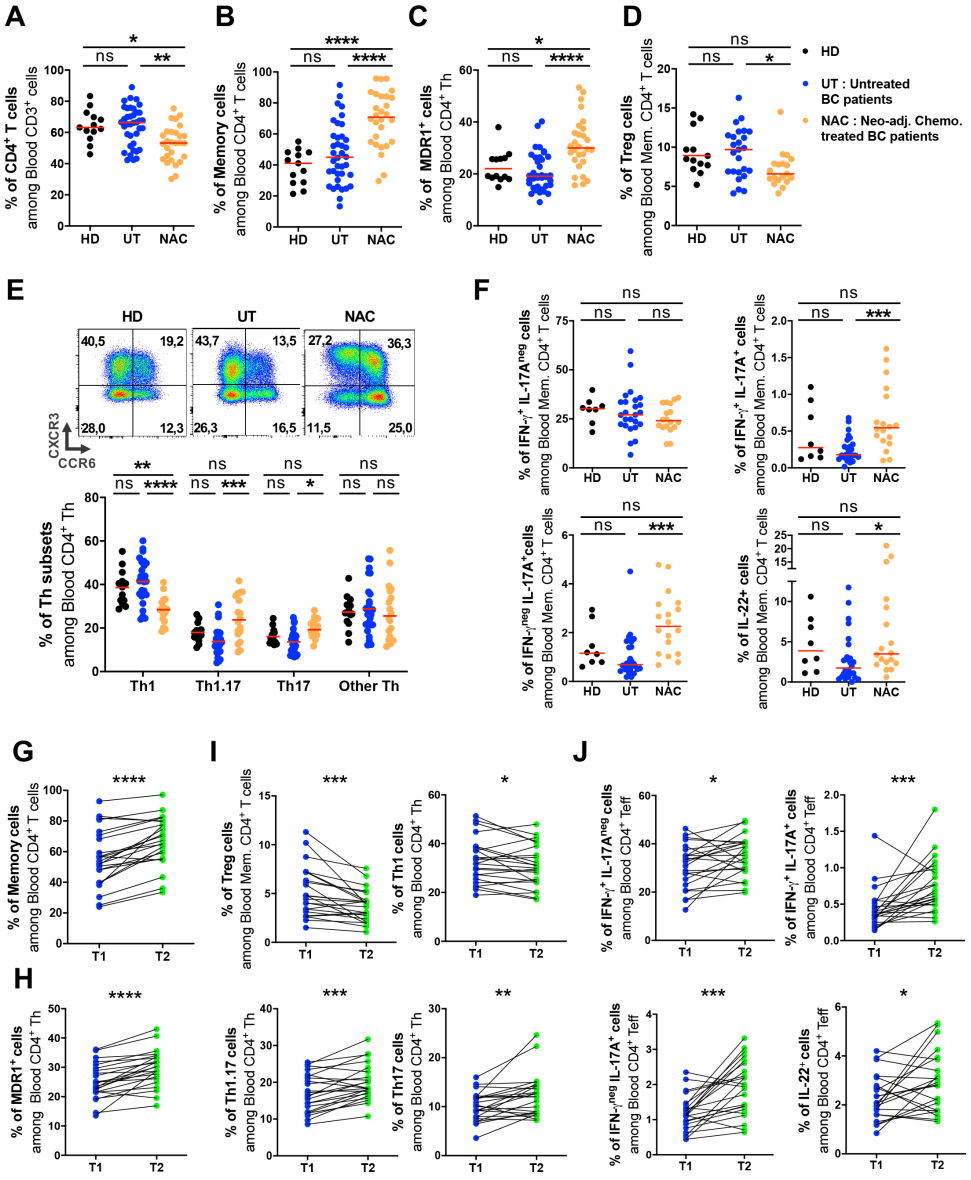

Figure 4 NAC selects functional MDR1$^+$ Th enriched in Th1.17 cells and increases IFN-γ$^+$+IL-17A$^+$ and IL-17A$^+$ cells in patients with BC's blood. (A–F) Comparison of blood samples from HD (n=13), untreated (UT; n=38) or patients with NAC-treated BC (NAC) (n=28): (A–E) Percentages of CD4$^+$ T cells (A) memory CD4$^+$ T cells (B) MDR1$^+$ Th cells (C) Treg (D) and Th cell subsets (E). Proportion of IFN-γ$^+$IL-17A^{neg}, IFN-γ$^+$IL-17A$^+$, IFN-γ^{neg}IL-17A$^+$ and IL-22$^+$ cells after PMA/ionomycin reactivation (F). (G–J) Analysis of patients' blood from Breast-Immun cohort before (T1) and after (T2) NAC. Proportions of memory CD4$^+$ T cells (G) MDR1$^+$ Th cells (H) Treg and Th cell subsets based on phenotype (I) and IFN-γ$^+$IL-17A^{neg}, IFN-γ$^+$IL-17A$^+$, IFN-γ^{neg}IL-17A$^+$ and IL-22$^+$ cells after PMA/ionomycin reactivation (J). Statistical analyses: Kruskal-Wallis (A to D and F) analysis of ANOVA-2 (E) Wilcoxon (G to J). (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001). BC, breast cancer; HD, healthy donors'; IFN, interferon; IL, interleukin; MDR1, multidrug resistance-1; NAC, neoadjuvant chemotherapy; Th, T helper cells; Treg, regulatory T cells; UT, untreated.

did not respond to treatment, we highlighted, by FC, lower levels of Programmed cell Death protein1 (PD-1), CD39 and CD38 on MDR1$^+$ Th cells compared with MDR1^{neg} Th cells whereas no significant expression of OX40 and ICOS was found independently of their MDR1 phenotype (figure 6A). Low proliferation was observed on both Th cell populations (figure 6A) that strongly contrasted with high proliferation observed on Treg cells (figure 6B). This is in adequacy with scRNAseq public data set analysis (online supplemental figure 4F) demonstrating that *ABCB1*-enriched meta-clusters did not express genes coding for proliferation or markers of activation or exhaustion except *PDCD1* in contrast to Treg meta-clusters (c20 and c21) (online supplemental figure 4F). In contrast, the c13 meta-cluster, also associated to *ABCB1* expression, contained a high number of features associated with cytotoxic CD4$^+$ T cells[33] (online supplemental figure 4G).

In addition, an scRNAseq analysis of patients with BC's blood CD4$^+$ memory T cells identified two clusters (c8, c9) enriched with cytotoxic features. Both clusters expressed *PRF1*, *GNLY* and *NKG7*. In addition, the cluster

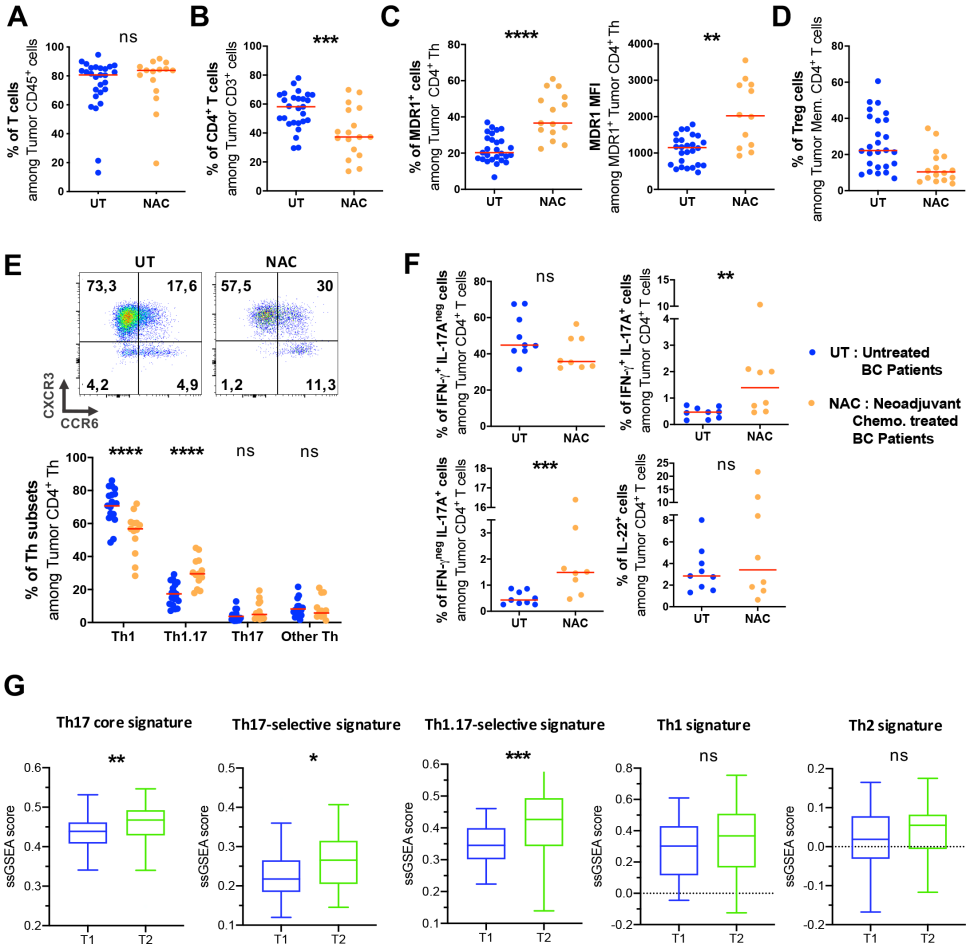

Figure 5 MDR1$^+$ Th are present in BC tumors and selected by NAC. (A–F) Comparison of tumor associated (TA) T-cell subsets on UT or patients with NAC-treated (NAC) BC: Percentage of TA-T cells (A) CD4$^+$ TA-T cells (B) MDR1 expression TA-Th cells in frequency and MFI (C) TA-Treg (D) and TA-Th cell subsets (E). Proportion of IFN-γ$^+$IL-17A^{neg}, IFN-γ$^+$IL-17A$^+$, IFN-γ^{neg}IL-17A$^+$ and IL-22$^+$ TA-CD4$^+$ T cells after PMA/ionomycin reactivation (F). (G) ssGSEA analyses of Th17-selective, Th1.17-selective, (Th17-Th1.17) core, Th1 and Th2 signatures on RNA-sequencing data set on 46 paired tumor samples from Park *et al* data set (GSE123845).[21] Statistical analyses: Mann-Whitney (A to D and F), ANOVA-2 (E), Kruskal-Wallis (G) (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001). BC, breast cancer; HD, healthy donors'; IFN, interferon; IL, interleukin; MDR1, multidrug resistance-1; NAC, neoadjuvant chemotherapy; ssGSEA, single sample gene set enrichment analysis; Th, T helper cells; Treg, regulatory T cells; UT, untreated.

9 expressed *GZMK* and *KLRK1* but not *GZMB*, whereas the cluster 8 expressed *GZMB* and *GZMH* but low levels of *GZMK* and not *KLRK1*. *ABCB1* was found selectively enriched in the cluster 9 (online supplemental figure 8A–D). Moreover, the comparison at the messenger RNA (mRNA) level of blood (scRNAseq) and tumor-associated (RNA-seq) MDR1$^+$ Th cells (online supplemental figure 8E), highlighted the expression of *KLRB1* gene (coding for CD161) specifically on blood MDR1$^+$ Th cells. This was confirmed at the protein level, MDR1$^+$ Th cells from either HD or patients with NAC-treated BC's blood expressing high proportion of CD161 (online supplemental figure 2F, figure 7C), previously described on Th1.17 cells[9] but this expression was sharply reduced in the TME from NAC-treated patients suggesting they received specific signal in the TME (figure 6C).

Only two genes were found shared by blood and tumor MDR1$^+$ T cells, including *PDE4D* coding for the phosphodiesterase that degrades cyclic AMP.[34] This could be connected to their higher expression levels of CD73 producing Ado that signals through AdoR and adenylate cyclase (figure 6D).

Clinical response to NAC treatment is associated with an increase in MDR1$^+$ Th cells, Th1.17 and Th17 cells in both TME and blood

In light of these results, we investigated the possible association between TA-Th subsets composition and clinical response to NAC therapy. Using RCB, as a measurement of response to NAC treatment,[35] patients were segregated into responders (RCB-I/RCB-II), and non-responders (RCB III). Of note, patients with pCR could not be included in this analysis due to the absence of remaining tumor tissue. The proportion of CD4$^+$ TA-T cells among total TA-T cells was strongly reduced (by two-fold) in responding patients (figure 7A,B) and a reduction even though not significant was also observed for TA-Treg cells (figure 7C). Interestingly, MDR1$^+$ TA-memory CD4$^+$ T

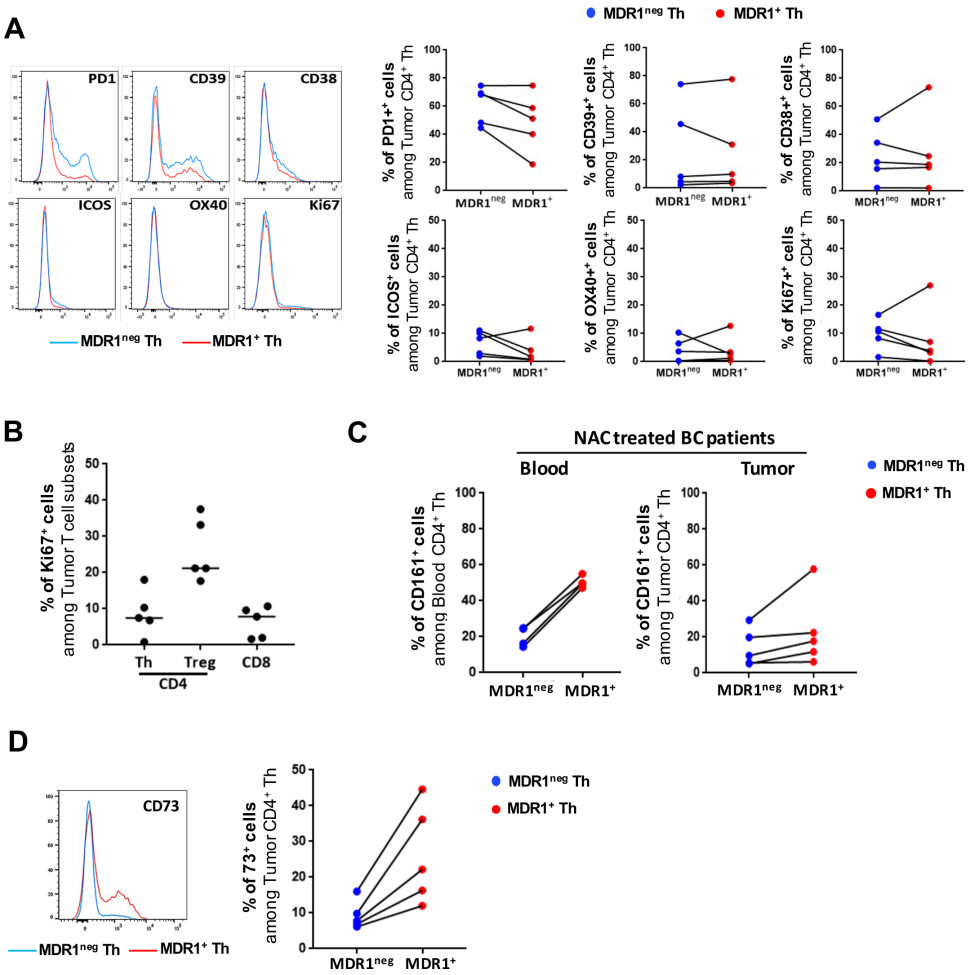

Figure 6 Analysis of ICP and proliferation capacity of MDR1$^+$ CD4$^+$ Th cells in the NAC-treated breast tumor environment (A) Expression of ICP on MDR1$^+$ and MDR1^{neg} Th cells from NAC-treated breast tumors (n=5). (B) Compared Ki67 expression of Th cells, Tregregulatory T cells and CD8$^+$ T cells in the tumor environment of NAC-treated tumors (n=4). (C) CD73 expression on MDR1$^+$ and MDR1^{neg} Th cells from NAC-treated tumors (n=4). (D) Differential expression of CD161 on MDR1$^+$ and MDR1^{neg} Th cells in blood (n=4) and tumor environment (n=5) of patients with NAC-treated BC. BC, breast cancer; MDR1, multidrug resistance-1; NAC, neoadjuvant chemotherapy; Th, T helper cells.

cells, Th1.17 and Th17 cells, but not Th1 cells, were significantly enriched in responding patients (figure 7D,E).

Finally, we assessed whether the treatment-induced changes in blood CD4$^+$ Th cell composition were associated with response to chemotherapy. Within the Breast-Immun cohort (online supplemental table 1, figure 7F) we highlighted, after two cycles of chemotherapy, a significant T2/T1 fold-change increase in MDR1$^+$ Th cells (figure 7G) but also in Th1.17 and Th17 cells (figure 7H) according to RCB. In contrast, no differences were observed for Th1 cells. Moreover T2/T1 fold-change of Th cells producing IFN-γ only, IFN-γ/IL-17A and IL-17A only (figure 7I) were also increased in patients with RCB-0/pCR compared with those with RCB-III, values for RCB-I/II being intermediate.

DISCUSSION

Transcriptome analysis of MDR1$^+$ TA-Th cells isolated from untreated tumors confirms their Th1.17 and Th17 hallmarks. Moreover, analysis by scRNAseq of blood memory CD4$^+$ T cells confirmed that *ABCB1*$^+$ cells are enriched in Th1.17 and Th17 clusters. In line with our results, a small subset of Th17 cells also expressing MDR1 (Rh123^{neg}) was also detected apart from the major Th1.17 population in CD.[9]

Interestingly, while CD161 is expressed by MDR1$^+$ Th cells, its expression is strongly reduced in the TME which might be the result of TCR engagement as described recently.[36] However, the reanalysis of scRNA-seq BC public data sets as well as the FC analysis of NAC-treated BC tumor samples failed to identify activated or proliferating cells within MDR1$^+$ TA-Th cells. Alternatively, CD161 downregulation could also result from the presence of TGF-β1 in the TME (online supplemental figure 7B).[36] Finally, the elevated IL-18Rβ expression on MDR1$^+$ Th cells detected in our RNA-seq data set could suggest their capacity to respond to IL-18 contributing to the downregulation of CD161 in the TME.[37] In this context,

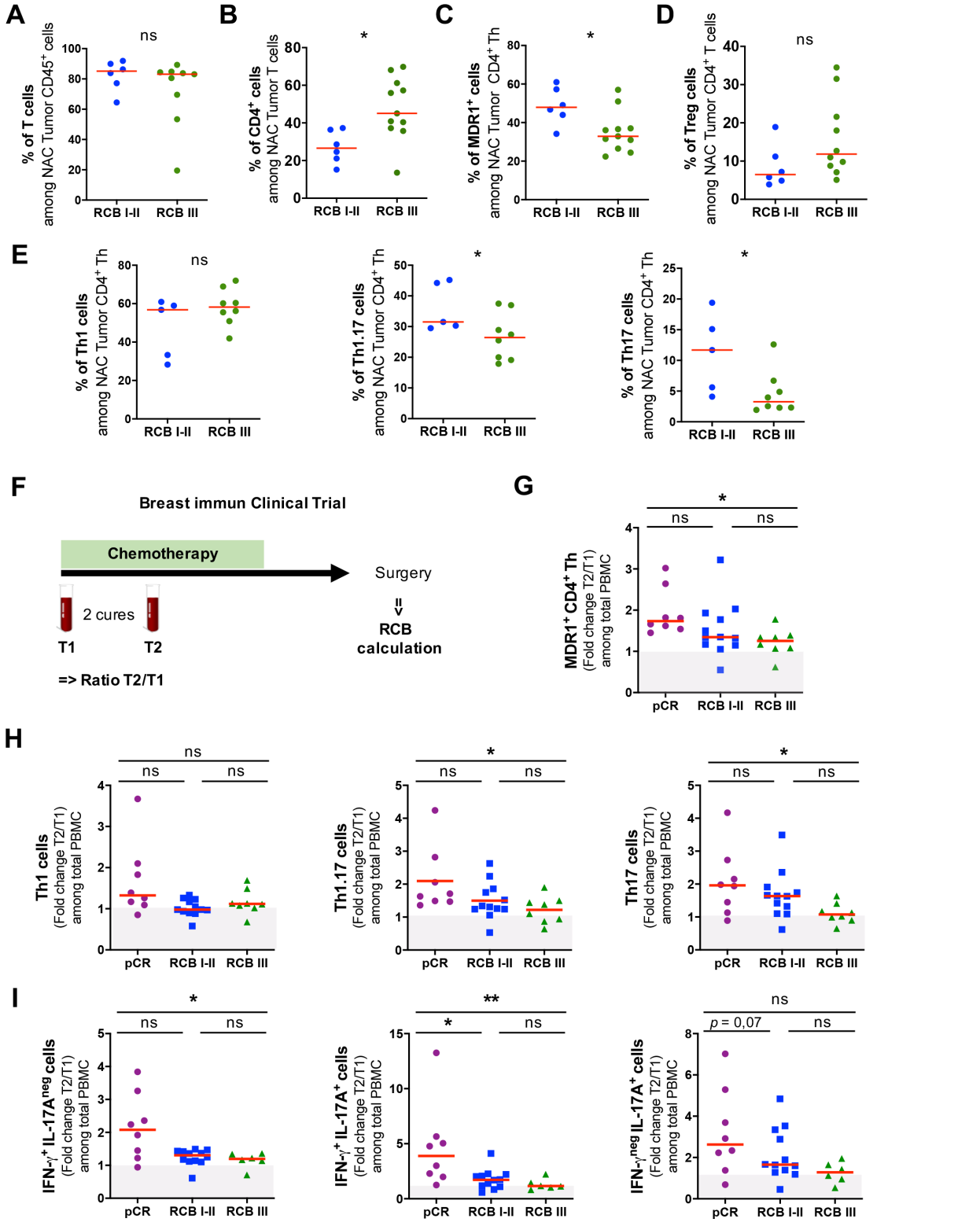

Figure 7 Response to chemotherapy is associated with an increase in MDR1$^+$ Th, Th1.17 and Th17 cells in the TMEtumor microenvironment and an increase in IFN-γ$^+$+IL-17A$^+$ and IL-17A$^+$ producing cells in the blood. (A–E) Comparison of TA-T cell subsets from patients with NAC-treated BC according to their response to chemotherapy (response: RCB I-II; no response: RCB III): proportions of TA-T cells (A) CD4$^+$ TA-T cells (B) MDR1$^+$ cells (C) TA-Treg (D) and TA-Th subsets (phenotypically) (E). F–I) Evolution of blood cell proportions under treatment (ratio (T2/T1) in patients with BC from Breast-Immun cohort, according to their response to NAC: blood sample collection scheme in the Breast-Immun cohort (F). Evolution of the ratio (T2/T1) of MDR1$^+$ cells (G) Th1, Th1.17 and Th17 cells (H) (G) and IFN-γ$^+$IL-17A^{neg}, IFN-γ$^+$IL-17A$^+$, IFN-γ^{neg}IL-17A$^+$ after PMA/ionomycin reactivation (I). Statistical analyses: Mann-Whitney (A to E); Kruskal-Wallis (G to I) (*p<0.05, **p<0.005). BC, breast cancer; IFN, interferon; IL, interleukin; MDR1, multidrug resistance-1; NAC, neoadjuvant chemotherapy; PBMC, peripheral blood cells; pCR, pathological complete response; RCB, residual cancer burden; TA, tumor associated; Th, T helper cells.

significant IL-18 levels were detected in STM (online supplemental figure 7B).

MDR1 expression confers a survival advantage for CD8+ and polyfunctional CD4+ Th cells in response to NAC treatment at the expense of Treg cells

As recently reported in BC treated by adjuvant chemotherapy,[38] NAC treatment induces a significant enrichment in blood and TA-CD8+ T cells and TA-CD4+ T_{CM} in both prospective and Breast-Immun cohorts at the expense of Treg and naïve CD4+ T cells. The expression pattern of MDR1 on CD4+ and CD8+ T-cell subsets provides a mechanistic explanation to these observations, with a particular decrease in naïve T cells described in patients with metastatic BC treated by docetaxel, an MDR1 substrate.[39] These different data sets are consistent with the in vitro impact of paclitaxel on total CD4+ T cells, which favors a strong enrichment in memory Th cells at the expense of naïve cells, due to their lack of MDR1 expression. We also confirm in vivo, the higher sensitivity of Treg cells to MDR1 substrate after two cycles of NAC.

We also highlight, using a public RNA-seq data set,[21] the Th1.17 enrichment in tumors after one cycle of NAC. This correlates with our data showing a higher proportion of tumor supernatants from NAC-treated tumors that contain both IFN-γ and IL-17A as compared with UT.

The enrichment in MDR1+CD4+ Th cells observed in the TME further implements the scarce existing data reporting the modulation of T-cell subsets in the TME of patients with NAC-treated BC as most of them focused, mainly by immunohistochemistry or transcriptomic analyses, on total TILs.[40–42] MDR1 is likely responsible for the observed enrichment in "Th17 like" cells including both Th1.17 and Th17 cells recently reported in BC after adjuvant chemotherapy.[38]

We also highlight a significant increase in CCL20 and CXCL9 levels in STM from NAC-treated patients (online supplemental figure 7B), possibly associated with the increase in MDR1+CD4+ TA-Th cells. Indeed, CCL20 is a potent chemoattractant of Th17 and Th1.17 cells, both expressing high levels of CCR6 and CXCL9 is one of the three CXCR3 ligands favoring the recruitment of Th1 and Th1.17 cell subsets. Of interest, a positive correlation between high *CXCL9* mRNA levels and significant increased pCR rate[41] or relapse-free survival[40] was reported in tumors from patients with NAC-treated BC.

Enrichment in MDR1+ Th cells and Th17/Th1.17 cells in the TME and blood correlates with the therapeutic efficacy of NAC treatment

Using the Breast-Immun cohort, we report an increase in the proportion of circulating IL-17A+IFNγ+ Th cells between T2 and T1 in responding patients but not in non-responding ones. In our prospective NAC-treated cohort, we also detect significantly more MDR1+ TA-Th cells but also Th1.17 and Th17 cells in the TME from responding patients than from non-responding ones. It will be interesting to determine the contribution of Th1.17 and Th17 cells in the efficacy of NAC treatment as previously shown in gastric cancer.[43]

Whereas gene-expression module reflecting Th1 cells and IFN-γ detection in BC tumors has been largely associated with good prognosis,[44] the role of Th17 and Th1.17 cells remains controversial. Here, we propose a critical function of these subsets in antitumoral immunity in the context of NAC treatment in BC. Our analyses offer a mechanistic understanding of the impact of NAC treatment on T-cell subsets, and open new avenues for the immunotherapeutic potential of T helper subsets. However, it remains important to determine the role of MDR1+ TA-Th cells in the response to NAC. Based on our work and the literature, we hypothesize that these CD4+ Th cells may have antitumor functions linked to their cytokine pattern and cytotoxic potential. Indeed, the scRNAseq data (our data in blood and PKU reanalyzes in tumor) show that MDR1 is associated with Th1.17 and Th cells with features of cytotoxic cells expressing *GZMK* that could refer either to less differentiated cells as defined for CD8+ T cells[45] or to a population with a cytotoxic potential in a caspase-independent manner through either single-stranded DNA damage, mitochondrial dysfunction, reactive oxygen species or cell membrane damage (for review[46]). However, we did not detect signs of activation/proliferation on the tumor-associated MDR1+ CD4+ Th cells. One caveat of this analysis is that it was performed only on non-responding patients due to the lack of accessible fresh tumor material in NAC-responding patients. This is in line with data from Oh *et al*[47] in bladder tumors who demonstrated that CD4 cytotoxic clusters did not express ICPs such as TIGIT or TNFRSF4/9/18 but were endowed with polyfunctional features (co-expression of IFN-γ and TNF-α). However, the expression of IL-17A was not investigated in this study.

The absence of activation of MDR1+ CD4+ Th cells in non-responding patients might be linked to their upregulated expression of CD73 that could participate in their own regulation through adenosine production in a TME enriched in CD39+ Treg cells.[5] Indeed, Treg cells proportion is decreased in responding patients, and *PDE4D* gene selectively enriched in MDR1+ Th cells may also contribute, through cyclic AMP degradation, to resist to A2AR signaling, both events favoring MDR1+/Th1.17+ cell activation in responding patients. To address properly this question, a dedicated clinical trial with a mandatory biopsy before and after two cycles of NAC would have to be set-up.

Our observation could provide a strategy to identify, early in the treatment through liquid biopsies, patients who will not respond to anthracyclines/CTX chemotherapy and for whom a change in the chemotherapy regimen may be beneficial to increase antitumor response.

In metastatic BC, current therapeutic strategies aim to combine ICP blockers with chemotherapy to increase their efficacy.[48] Of interest, the TONIC trial[49] evaluating, on metastatic TNBC, the impact of an induction treatment with different chemotherapy regimen before anti-PD-1

reported a better objective response rate in doxorubicin-treated (35%) patients than in CTX-treated (8%) ones. This could result from ICD induced by anthracyclines[20] but also, as they express PD-1, from MDR1$^+$ T cells (CD4$^+$ and CD8$^+$ T cells) enrichment and Treg cell depletion by doxorubicin but not CTX. Moreover, results from the KEYNOTE-522 phase 3 trial highlighted that addition of anti-PD-1 to NAC with paclitaxel/carboplatin regimen in patients with early TNBC significantly increased pCR rate[50] suggesting that preservation, by chemotherapy, of MDR1-expressing cells (Th1.17 and Th17 but also CD8$^+$ Teff) could participate to the efficacy of anti-PD-1. Based on these results, chemotherapies substrate of MDR1 should be preferred to develop combination with immunotherapies in other tumor indications. In this context, etoposide, another MDR1 substrate, combined with anti-PD-1 ligand (PD-L1, atezolizumab) obtained Food and Drug Administration approval in 2019 for the treatment of advanced lung carcinoma based on IMpower133 trial results.[51] It could be of interest to investigate in such patients a link between blood Th1.17 cells enrichment and response to treatment to extend our observations.

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Contributors ADR performed and analyzed the majority of the experiments and contributed to the draft of the manuscript. MB initiated the study. MH performed all the bioinformatic analyses. CR generated the cytokine data. LB managed RNA-seq and scRNAseq experiments and part of flow cytometry analyses. YG-B and GL designed and supervised the polarization experiments. TD and DJC were involved in the confirmation of genes of interest by qPCR on purified Th1.17 cells. CD generated scRNAseq data sets and RS performed the associated bioinformatic analyses. IT established the tumor RCB after NAC treatment for both Breast Immun and prospective cohorts. SM and OT were highly involved in the generation of the BreastImmun cohort. YG-B, TD, MB and MH contributed to the critical reading and improvement of the manuscript. CM-C and CC conceived the study, supervised the project designed and analyzed the experiments and wrote the manuscript. CC and CM-C are joint last authors.CMC was the guarantor of this study.

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Patient consent for publication Consent obtained directly from patient(s).

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