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RESEARCH ARTICLE

Cancer Therapy and Prevention

Immune landscape of vulvar cancer patients treated with surgery and adjuvant radiotherapy revealed restricted T cell functionality and increased IL-17 expression associated with cancer relapse

Selina Gies[1] | Patrick Melchior[2] | Russalina Stroeder[3] | Tanja Tänzer[1] | Laura Theobald[1] | Maike Pohlers[1] | Birgit Glombitza[1] | Martina Sester[4] | Erich-Franz Solomayer[3] | Barbara Walch-Rückheim[1]

[1]Center of Human and Molecular Biology (ZHMB), Institute of Virology, Saarland University, Homburg, Saar, Germany

[2]Department of Radiation Oncology, Saarland University Medical Center, Homburg, Saar, Germany

[3]Department of Obstetrics and Gynecology, Saarland University Medical Center, Homburg, Saar, Germany

[4]Department of Transplant and Infection Immunology, Saarland University, Homburg, Saar, Germany

Correspondence
Barbara Walch-Rückheim, Center of Human and Molecular Biology (ZHMB), Kirrbergerstraße, Saarland University, Institute of Virology, Building 47, D-66421 Homburg/Saar, Germany.
Email: barbara.walch@uks.eu

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Abstract

For vulvar cancers, radiotherapy is targeting cancer cells, but also affects the host immune system. As this may affect treatment outcome, in this prospective study, we characterized the individual T cell immune milieu induced by surgery and adjuvant radio +/− chemotherapy (aRT) systemically in the blood of vulvar cancer patients and found increased frequencies of Interleukin (IL)-17-producing $CD4^+$ and $CD8^+$ T cells after aRT while frequencies of Th1 and perforin-producing $CD8^+$ killer cells were strongly diminished. Phenotypic characterization revealed enhanced expression of the ectonucleotidase CD39 on Th17 and Tc17 cells as well as $CD8^+$ $perforin^+$ cells after aRT. Furthermore, the aRT cohort exhibited increased proportions of Programmed Cell Death Protein (PD-1) expressing cells among Th1 and $CD8^+$ $perforin^+$ cells, but not among Th17 and Tc17 cells. High post-therapeutic levels of Th17 and Tc17 cells and low proportions of Th1 and $CD8^+$ $perforin^+$ cells expressing PD-1 was associated with reduced recurrence free survival on follow-up. In conclusion, our study defines individual therapy-induced changes in the cellular immune milieu of patients and their association with cancer relapse. Our results may help to explain differences in the individual courses of disease of vulvar cancer patients and suggest PD-1 and IL-17 as targets for immunotherapy in vulvar cancer.

KEYWORDS

cancer relapse, radiation, T cells, Th17 cells, therapy, vulvar cancer

Abbreviations: aRT, adjuvant radiotherapy; FIGO, International Federation of Gynecology and Obstetrics; HC, healthy controls; HPV, human papillomavirus; IFN-γ, interferon-γ; IL, interleukin; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death protein.

What's new?

Vulvar cancer is a rare gynecologic tumor treated with radiotherapy that affects the host immune system. Here, we provide new insight into systemic therapy-induced individual immunity of patients, revealing increased frequencies of tumor-promoting interleukin(IL)-17-producing $CD4^+$ and $CD8^+$ T cell subsets after radiotherapy, while other T cell subsets important for cancer cell elimination, like T-helper (Th)1 and cytotoxic T cells, were decreased and showed high expression of the Programmed Cell Death Protein (PD) 1 indicating restricted functionality. Moreover, this imbalance within the post-therapeutic immune milieu was associated with shorter recurrence free survival suggesting IL-17 and PD-1 as target for immunotherapy in vulvar cancers.

1 | INTRODUCTION

Vulvar cancer is a relatively rare tumor representing 2% to 5% of all gynecological malignancies[1] with increasing worldwide incidence over recent decades resulting in 45.240 new cases and more than 17.000 vulvar cancer-related deaths in 2020.[2] Squamous cell carcinoma is the most common histological subtype accounting for 90% of all malignancies of the vulva (VSCC).[3] Non-keratinized squamous cell carcinomas are often associated with the human papilloma virus (HPV) and mainly affect younger woman[4] while keratinized squamous cell carcinomas affecting older woman are usually HPV-independent and due to a chronic genital inflammatory disease, such as lichen sclerosus.[5] Based on German cancer registries the annual incidence of invasive cancers nearly doubled between 1999 and 2011.[6] Although vulvar cancer is described to primarily affect postmenopausal women,[2] an increase in incidence rate of vulvar cancer in woman of all ages was found until 2007 in other high-income countries.[7] This indicates that women younger than 60 years were also diagnosed with vulvar cancer, what could be partially explained by an increased HPV prevalence. Approximately 21% to 40% of vulvar cancers worldwide are related to HPV infections.[2]

Most patients with vulvar cancer are primarily treated with a wide local excision of the primary tumor, often accompanied by adjuvant radiotherapy. As prognosis and therapeutic strategies depend on the pathological status of the inguinal lymph nodes and whether spread to adjacent structures has occurred, evaluation of the pathological status of lymph nodes performed by sentinel node biopsy or elective inguino-femoral lymphadenectomy is essential for adjuvant treatment decision and clinical outcome of vulvar cancer patients. While in early-stage VSCCs, therapy consists of radical excision of the primary tumor, adjuvant radiotherapy (aRT) to groins and pelvis is applied in case of advanced nodal involvement.[8] However, radical surgery can lead to reduced quality of life and morbidity.[9] Prognosis for patients with locally advanced tumors and positive lymph nodes remains poor as 46% of these patients developed VSCC recurrence after 10 years.[10] In patients with operable disease without lymph node involvement, the overall survival (OS) rate is 90%, however, in patients with nodal involvement, the 5-year OS rate is approximately 50% to 60%.[11] Thus, predictive biomarker and target-based immunotherapies are needed to improve the clinical outcomes, especially in advanced cancers.

In search for prognostic markers for vulvar cancers, high numbers of activated T helper cells and tumor-infiltrating $CD4^+$ and $CD8^+$ T cells[12] were associated with better clinical outcome, while tumor-infiltrating regulatory T cells (Tregs) with cyclooxygenase-2 (COX-2) expression[2] and leucine zipper downregulated in cancer 1 (LDOC1)[13] or combined COX-2/PPARγ expression of vulvar cancer cells[14] were linked with poor prognosis. Furthermore, the immune landscape of tumor-draining lymph nodes with immune suppressive features is associated with metastatic spread.[15]

Adjuvant radiotherapy reduced the risk of recurrence for patients with advanced nodal involvement,[16] nevertheless, not all patients achieve cure. RT is applied to kill cancer cells, however, different studies described that therapeutic approaches differentially affect subsets of human and murine immune cells.[17,18] Particularly, different T cell subsets react in a differential way to therapeutic approaches showing enhanced sensitivity or survival.[18-20] This could result in individual therapy-induced immune alterations in the patients' blood with increased frequencies of Th17 cells and Tregs associated with poor prognosis for the patients[20,21] potentially reducing antitumor effector functions favoring cancer progression. In this prospective study, we evaluated frequencies of $CD4^+$ and $CD8^+$ T cell subsets in the blood of vulvar cancer patients receiving surgery alone or surgery followed by adjuvant radiotherapy and analyzed their phenotypical and functional characteristics to clarify the systemic immunity during therapy and its association with cancer progression. We demonstrate that aRT increased frequencies of IL-17-expressing $CD4^+$ and $CD8^+$ T cells subpopulations while Th1 cells and perforin producing $CD8^+$ killer cells decreased. Phenotypic characterization revealed increased CD39, but no enhanced PD-1 expression on Th17 and Tc17 cells after aRT. In contrast, PD-1 expression increased on Th1 and perforin-producing $CD8^+$ killer cells after aRT. Finally, post-therapeutic levels of Th17 and Tc17 cells as well as high percentages of PD-1^+ Th1 and $CD8^+$perforin$^+$ cells were associated with early cancer relapse.

2 | MATERIALS AND METHODS

2.1 | Study participants and study design

Forty patients diagnosed with vulvar cancer and treated from 2017 to 2020 in the Saarland University Hospital as well as

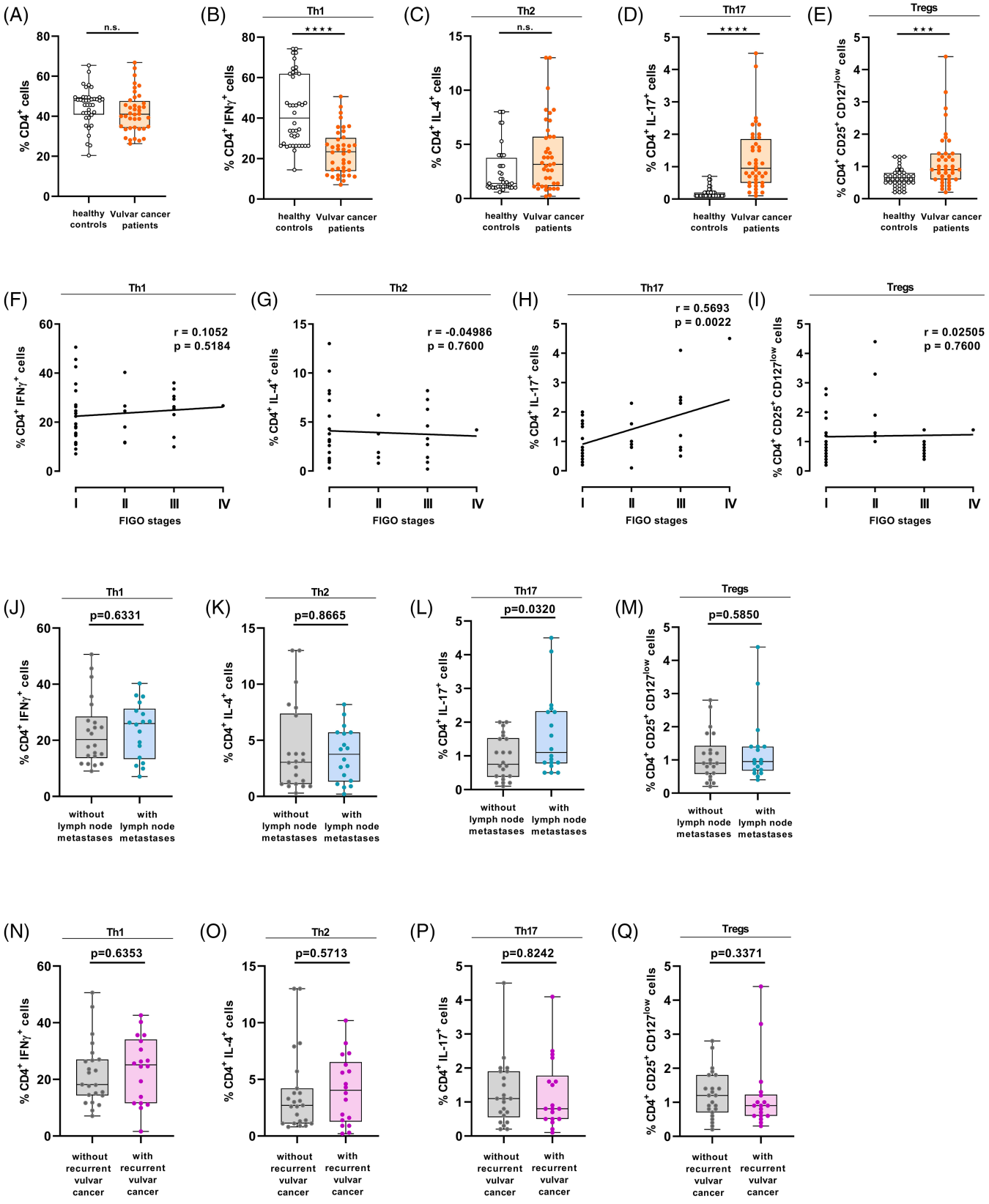
TABLE 1 Clinicopathologic characteristics of patients.

Participants (n)	Healthy controls (n = 40)	Treated with surgery (n = 22)	Treated with aRT (n = 18)
Age of diagnosis/participation (mean ± SD)	61.0 ± 5.7	63.9 ± 15.2	62.8 ± 11.7
Diagnosis			
Squamous cell carcinomas (n = 40)	/	n = 22	n = 18
Tumor stage			
T1	/	n = 19	n = 14
T2	/	n = 2	n = 3
T3	/	/	n = 1
Nodal stage			
N0	/	n = 22	n = 5
N1	/	n=/	n = 7
N2	/	n=/	n = 6
Stage (FIGO)			
I	/	n = 18	n = 5
II	/	n = 4	n = 3
III	/	n=/	n = 9
IV	/	n=/	n = 1
Grading			
G1	/	n = 3	n=/
G2	/	n = 13	n = 7
G3	/	n = 5	n = 10
Data unavailable	/	n = 1	n = 1
Radiotherapy			
Mean dose in Gray	/	/	51.1
Range	/	/	45-66 Gray
Concurrent chemotherapy	/	/	n = 3 (cisplatin 40 mg/m^2 body surface area)

40 aged-matched female healthy controls (HC) were included in this study. Peripheral blood samples were obtained from 40 vulvar cancer patients during different therapeutic approaches as well as samples from untreated female immunocompetent controls and used for monitoring of T cell subsets constitution. Within the 40 vulvar cancer patients, 22 patients were treated with surgery alone, and 18 patients received surgery followed by adjuvant radiotherapy, of which three received concurrent chemotherapy (cisplatin 40 mg/m^2 body surface area). Results of these 3 patients are illustrated by black edging within the aRT cohort. Blood samples were obtained at primary diagnosis, 1 day after primary surgery, before the onset of aRT, and directly after completion of aRT. The applied irradiation in RT ranged from 45 bis 66 Gray (Gy) (single dose 1.7-2.25 Gy). Clinicopathologic and demographic data are listed in Table 1. Absolute numbers of T cell subtypes/µl were determined using blood counts. Absolute numbers of T cell subtypes/µl were calculated by incorporating the respective frequency values per absolute number of CD4$^+$ or CD8$^+$ T cells.

2.2 | Isolation and staining of peripheral blood mononuclear cells and flow cytometry

PBMCs were isolated from heparinized blood samples by Pancoll (Pan Biotec) density gradient centrifugation. For long-term storage, cells were cryoconserved in 90% fetal calf serum (FCS) and 10% DMSO in liquid nitrogen. PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA; 5 ng/ml)/ionomycin (500 ng/ml) (both from Sigma-Aldrich, Taufkirchen, Germany) for 6 h. After 2 h, brefeldin A (10 mg/ml; Sigma) was added. Cells were fixed using BD Bioscience Cytofix/Cytoperm Kit and stained using anti-CD4-PE (AB_395752), anti-CD8-FITC (AB_395769), anti-IL-17-APC (AB_1603584), anti-IFN-γ-BV421 (AB_2738952), anti-IL-4-APC (AB_2726799), anti-perforin-AlexaFluor647 (AB_2738287), anti-CD25-BV510 (AB_2744336), anti-CD127-BB700 (AB_2744279), anti-TCRγδ-FITC (AB_2733699), anti-CD39-BV421 (AB_2738368) and anti-PD-1-BB700 (AB_2744348) or respective isotype control antibodies (BD Biosciences, Heidelberg and Miltenyi Biotec,



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Bergisch-Gladbach, Germany) and analyzed by flow cytometry (FACSCantoII; BD Biosciences).

2.3 | Statistics

All statistical analyses were performed using the GRAPHPAD Prism 8 (GRAPHPAD Software) program. To evaluate the statistical differences between the analyzed groups, a Mann-Whitney *U*-test was applied for the comparison of nonparametric data between two groups and the Kruskal-Wallis test for comparison of nonparametric data of >2 groups. Paired analyses of T cell levels were performed using the Wilcoxon matched pairs test in the surgery only cohort. The paired Friedman test was used to compare differences in T cell levels in the aRT cohort before surgery, after surgery and after aRT. Correlation between tumor International Federation of Gynecology and Obstetrics (FIGO) stages and the number of T cell subtypes was done using Spearman rank correlation. Best cutoffs to discriminate patients regarding T cell frequencies and with or without recurrent vulvar cancers were identified by receiver operator characteristics (ROC) analysis and Youden's index calculation. Comparison of survival curves between different groups were evaluated by a Kaplan-Meier survival curve with log-rank (Mantel-Cox) test. A P value <.05 was considered statistically significant. Significances are indicated by asterisks (*P < .05; **P < .01; ***P < .001; ****P < .0001).

3 | RESULTS

3.1 | Pre-therapeutic immune landscape of vulvar cancer patients and its association with tumor FIGO stages, lymph node metastases and cancer progression

We analyzed peripheral blood specimens obtained from 40 vulvar cancer patients before therapy and 40 age-matched female HC (Table 1; gating strategy and representative dot plots are shown in Figure S1). We found that frequencies of $CD4^+$ T cells did not significantly differ between vulvar cancer patients and healthy controls (Figure 1A), however, frequencies of Th1 cells were significantly lower in vulvar cancer patients (Figure 1B). Analysis of further $CD4^+$ T cell subpopulations revealed slightly, but not significantly, higher Th2 frequencies (Figure 1C) as well as significantly higher frequencies of Th17 cells (Figure 1D) and $CD25^+$ $CD127^{low}$ regulatory $CD4^+$ T cells (Tregs) in vulvar cancer patients (Figure 1E). Same tendencies we obtained regarding absolute numbers/µl showing decreased numbers of Th1, comparable numbers of Th2 and increased numbers of Th17 cells and Tregs in the patients' blood before therapy in comparison to HC (Figure S2A-E).

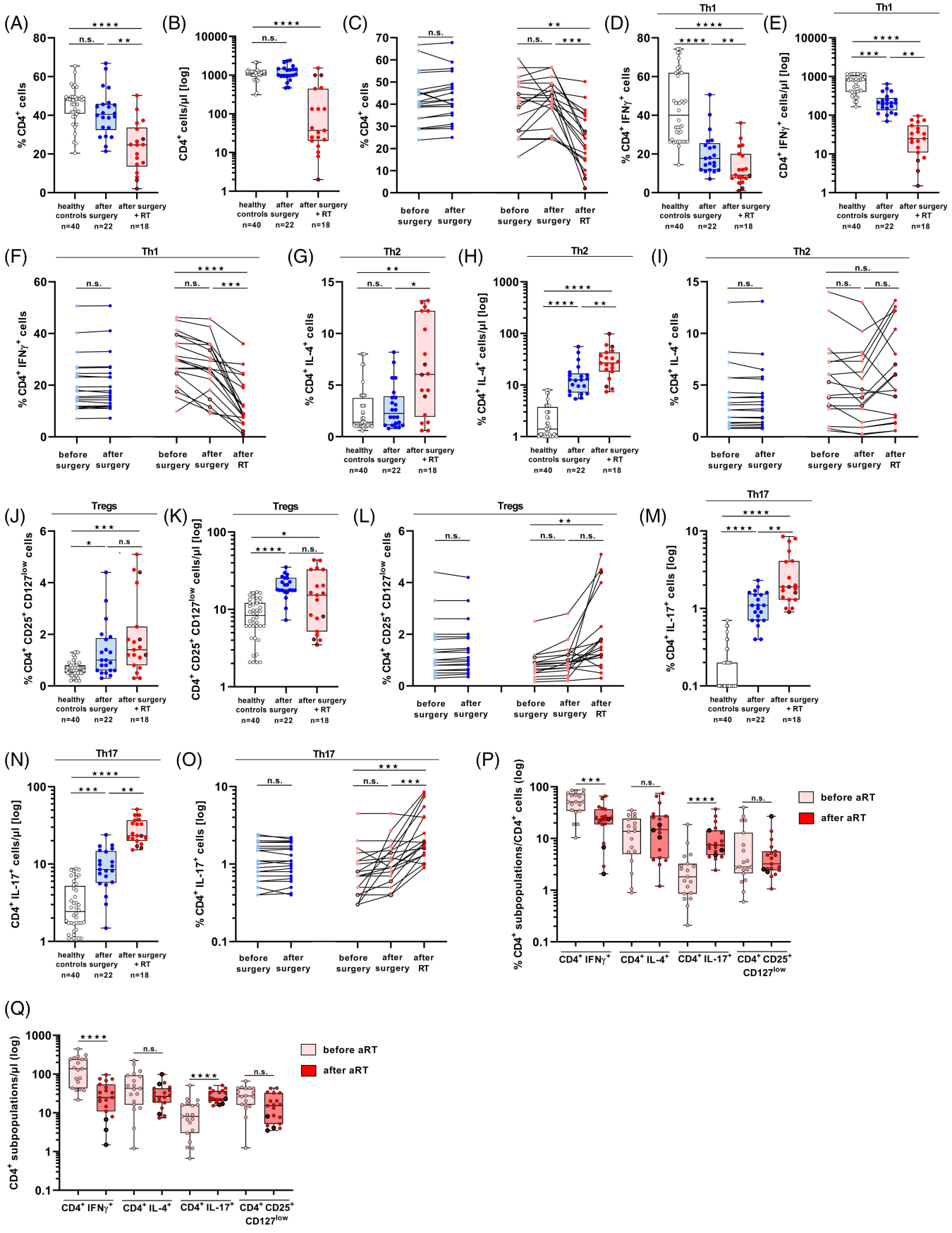
We were interested whether the immune cell constitution in the patients' blood before therapy is associated with course of disease. While frequencies of Th1, Th2 and Tregs in the patients' blood did not significantly differ in patients with different tumor FIGO stages (FIGO stages I-IV) there was no significant correlation between T cell frequencies and advanced FIGO stages in correlation analysis (Figure 1F,G,I). However, patients with more advanced vulvar cancers (FIGO stages III-IV) exhibited higher Th17 frequencies resulting in a significant correlation between Th17 frequencies and advancing tumor FIGO stages (Figure 1H; $r = 0.5693$, $P = .0022$). Higher Th17 frequencies we also found in the blood of vulvar cancer patients with lymph node metastases in comparison to patients without metastases (Figure 1L), whereas frequencies of Th1, Th2 and Tregs cells did not significantly differ between these two groups (Figure 1J,K,M). However, frequencies of Th1, Th2, Th17 cells or Tregs did not significantly differ before therapy in the blood of patients who retrospectively developed vulvar cancer relapse in comparison to patients without recurrence (Figure 1N-Q).

3.2 | Radiotherapy decreased Th1 but increased Th2 and Th17 frequencies

Next, we were interested whether therapeutic approaches affect the T cell immune landscape in the blood of vulvar cancer patients. We analyzed peripheral blood specimens obtained from 40 vulvar cancer patients (Table 1) treated with surgery alone (n = 22) or adjuvant RT (aRT) in addition to surgery (n = 18) and age-matched 40 female HC. We found that frequencies and absolute numbers of $CD4^+$ T cells were not affected by surgery alone (Figure 2A, B; blue dots) in comparison to HC. However, in the cohort of aRT (red dots) percentages and absolute numbers of $CD4^+$ T cells/µl were significantly lower (Figure 2A, B). Monitoring of patients during therapy revealed no differences in $CD4^+$ T cell frequencies after surgery in the surgery only and aRT cohort, but a significant decrease in $CD4^+$ T cell frequencies (by 43%) took place in all 18 patients after completion of aRT (Figure 2C).

Regarding the CD4 T cell subpopulations we found significantly lower frequencies and absolute numbers of Th1 cells in patients after surgery (Figure 2D,E; blue dots) in comparison to HC, and aRT further

FIGURE 1 Pre-therapeutic T cell immune landscape of vulvar cancer patients and its association with course of disease. PBMCs of 40 vulvar cancer patients (orange dots) and 40 female age-matched healthy controls (white dots) were analyzed by flow cytometry. Frequencies of (A) $CD4^+$ T cells, (B) Th1 ($CD4^+$ $INF\gamma^+$) cells, (C) Th2 ($CD4^+$ $IL\text{-}4^+$) cells, (D) Th17 ($CD4^+$ $IL\text{-}17^+$) cells, and (E) Treg ($CD4^+CD25^+CD127^{low}$). (F-I) Correlation of Th1, Th2, Th17, and Treg with tumor FIGO stages. (J-M) Frequencies of Th1, Th2, Th17, and Treg in patients with (blue dots) and without (gray dots) lymph node metastases. (N-Q) Pre-therapeutic frequencies of Th1, Th2, Th17, and Treg in patients who retrospectively developed vulvar cancer recurrence (purple dots) or not (gray dots). P value according to the nonparametric Mann-Whitney *U*-test (A-E, J-Q) or Spearman rank correlation with linear regression (F-I). Asterisks represent statistical significances: ns: not significant; *P < .05; **P < .01; ***P < .001; ****P < .0001.



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decreased frequencies and numbers of Th1 cells (red dots). Monitoring of patients before and after surgery of the surgery only as well as aRT cohorts showed that Th1 frequencies were not significantly affected by surgery. However, Th1 cells decreased in all 18 patients after completion of aRT (Figure 2F). In contrast to the decrease of Th1 cells, we found an increase in Th2 and Th17 frequencies after aRT (Figure 2I, M-O). While surgery had no effect on Th2 frequencies in comparison to HC, aRT resulted in heterogeneous, but in general significantly increased Th2 frequencies (Figure 2G, I, red dots; 9/18 patients) and increased absolute numbers of Th2 cells/µl (Figure 2H, red dots). Tregs frequencies were higher in the surgery only cohort in comparison to HC with no significant differences in frequencies and numbers after aRT (Figure 2J, K, L). The strongest increase was found for Th17 cells. While in patients with surgery only frequencies and absolute numbers of Th17 cells were 3.6-5.5-fold higher than in HC, aRT led to a further increase in Th17 frequencies (Figure 2M; 1.7-fold) and absolute numbers/µl (Figure 2N; 2.7-fold). An aRT-induced enhancement in Th17 frequencies were observed in 17/18 patients (Figure 2O).

In summary, we found a decrease in frequencies and absolute numbers of $CD4^+$ T cells after aRT. Moreover, surgery only and aRT in addition to surgery affected $CD4^+$ T cell subpopulations in a differential way. Generally, surgery only did not affect frequencies and absolute numbers of the analyzed $CD4^+$ T cell subpopulations. However, evaluation of the proportions of the respective T cell subpopulations after aRT revealed that proportions of Th1/$CD4^+$ T cells (Figure 2P) and absolute numbers of Th1 cells (Figure 2Q) significantly decreased in addition to surgery (2.2- and 8.4-fold reduction, respectively). Proportions per $CD4^+$ T cells and absolute numbers of Th2 or Treg cells were not affected by aRT. The strongest increase was found in percentages of Th17 cell/ $CD4^+$ T cells and Th17 numbers/µl (4.1- and 2.7-fold increase, respectively) after aRT. Thus, our results clearly showed a shift in frequencies and absolute numbers/µl in the blood of vulvar cancer patients after aRT resulting in individual therapy-induced immune milieus.

3.3 | Frequencies of γδT17 and Tc17 cells after radiotherapy in vulvar cancer patients

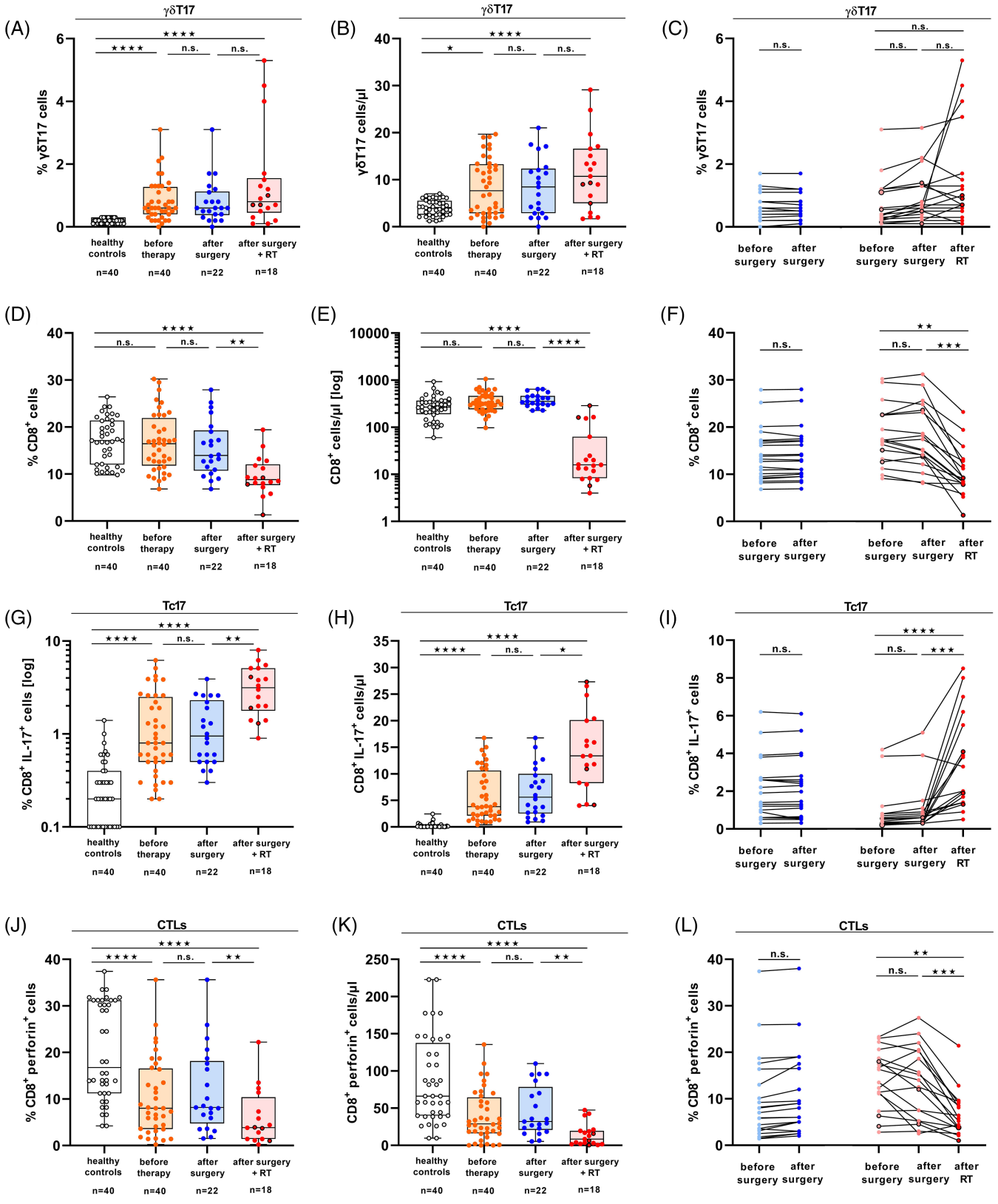
As we found a strong increase in Th17 frequencies after aRT, we analyzed whether further IL-17-expressing cells were enriched in the blood of vulvar cancer patients after aRT. We first focused on the presence of γδT17 cells that represent known producers of IL-17 and are linked to inflammatory conditions and tumor-promoting activity in different cancers.[22] We found higher frequencies and numbers of γδT17 cells before therapy in comparison to HC (Figure 3A, B, orange dots). However, neither surgery only nor surgery with subsequent aRT significantly affected circulating γδT17 frequencies and numbers (Figure 3A-C).

Besides Th17 and γδT17 cells, IL-17 expressing $CD8^+$ T cells (Tc17 cells) represent a further source of IL-17 and their presence is associated with tumor-promoting functions in cancers, including cervical cancer.[23,24] Considering frequencies of $CD8^+$ T cells, there was no significant difference between samples from patients before therapy and HC (Figure 3D; orange and white dots). Likewise, surgery had no effect on $CD8^+$ T cells (Figure 3D, E, F, blue dots) while aRT led to a decrease in $CD8^+$ T cells (red dots). Regarding Tc17 cells, patients exhibited significantly higher Tc17 frequencies than HC (Figure 3G). Surgery only did not affect Tc17 cells, however, and in contrast to $CD8^+$ T cells, frequencies and absolute numbers of Tc17 cells significantly increased after aRT (Figure 3G, H; red dots). 17/18 patients showed an increase in Tc17 frequencies after aRT (Figure 3I). In summary, our results identified an increase of a further IL-17-expressing T cell population besides Th17 cells in the blood of vulvar cancer patients after aRT.

3.4 | Decreased frequencies of perforin producing $CD8^+$ T cells in vulvar cancer patients

Since frequencies of $CD8^+$ T cells decreased during aRT we analyzed perforin expression of $CD8^+$ T cells during therapy involved in killing capacity. Before therapy, patients showed diminished frequencies of perforin expressing $CD8^+$ T cells in comparison to HC (Figure 3J, orange dots). After therapy, patients of the surgery only group showed comparable frequencies and numbers of $CD8^+$ $perforin^+$ cells to untreated stages (blue dots). However, aRT further reduced frequencies and numbers of perforin producing $CD8^+$ T cells (Figure 3J, K). Monitoring of patients during therapy revealed that surgery did not significantly affect frequencies of $CD8^+$ $perforin^+$ cells but aRT led to a general 2.6-fold reduction in all 18 patients (Figure 3L). Thus, we found a radiation-induced decrease in $CD8^+$ T cell frequencies with reduced perforin but increased IL-17 expression.

FIGURE 2 $CD4^+$ T cell subpopulations in the blood of vulvar cancer patients during therapy. PBMCs of 40 vulvar cancer patients and 40 female age-matched healthy controls were analyzed for (A) CD4, (D) Th1, (G) Th2, (J) Treg, and (M) Th17 frequencies by flow cytometry. Absolute numbers/µl of (B) CD4, (E) Th1, (H) Th2, (K) Treg, and (N) Th17 cells. PBMCs of vulvar cancer patients which received surgery only (n = 22; blue dots) or aRT (n = 18, red dots; n = 3 patients with concurrent chemotherapy; black edged dots) were analyzed for (C) CD4, (F) Th1, (I) Th2, (L) Treg, and (O) Th17 frequencies. *P* value according to the nonparametric Mann-Whitney *U*-test or paired Wilcoxon matched pairs test or paired Friedman test. (P) Proportions of Th1, Th2, Th17, and Treg per total $CD4^+$ T cells before (light red dots) and after aRT (red dots; n = 3 patients with concurrent chemotherapy; thick black edged dots) were determined. (Q) Absolute numbers/µl of Th1, Th2, Th17, and Treg per total $CD4^+$ T cells before (light red dots) and after aRT (red dots) were evaluated. *P* value according to the nonparametric paired Wilcoxon test. Asterisks represent statistical significances: ns, not significant; ***$P < .001$; ****$P < .0001$.



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3.5 | CD39 expression increased on IL-17-producing T cell subpopulations and perforin expressing CD8$^+$ T cells after aRT

We were interested in phenotypic and functional characteristics of IL-17-expressing CD4$^+$ and CD8$^+$ T cell subpopulations after aRT. The ectonucleotidase CD39 was found on tumor-reactive CD4$^+$ and CD8$^+$ TILs in vulvar cancer patients.[25] CD39 mediates the conversion of ATP to immunomodulatory adenosine[26] and is regarded as a target for immunotherapy.[26-28] Vulvar cancer patients showed significantly higher CD39 expression on CD4$^+$ and CD8$^+$ T cells in the blood before therapy than HC (Figure 4A, B). While surgery only did not affect frequencies of CD39 expressing CD4$^+$ and CD8$^+$ T cells, aRT significantly increased CD39 expression (Figure 4A, B; red dots) and enhanced absolute numbers/µl (Figure S3A, B). When we analyzed CD39 expression on IL-17 expressing CD4$^+$ and CD8$^+$ T cell subtypes we found that aRT increased frequencies of CD39 expressing Th17 and Tc17 cells in comparison to the surgery only cohort or untreated patients (Figure 4C, D). In contrast, vulvar cancer patients exhibited increased frequencies of CD39$^+$ Tregs in their blood in comparison to HC, but their levels remained stable after surgery and aRT (Figure 4E). Interestingly, aRT increased percentages of CD39$^+$ cells of perforin expressing CD8$^+$ T cells indicating an exhausted, antigen-experienced phenotype of remaining CD8$^+$ killer cells after irradiation (Figure 4F).

3.6 | PD-1 expression of T cell subtypes from vulvar cancer patients

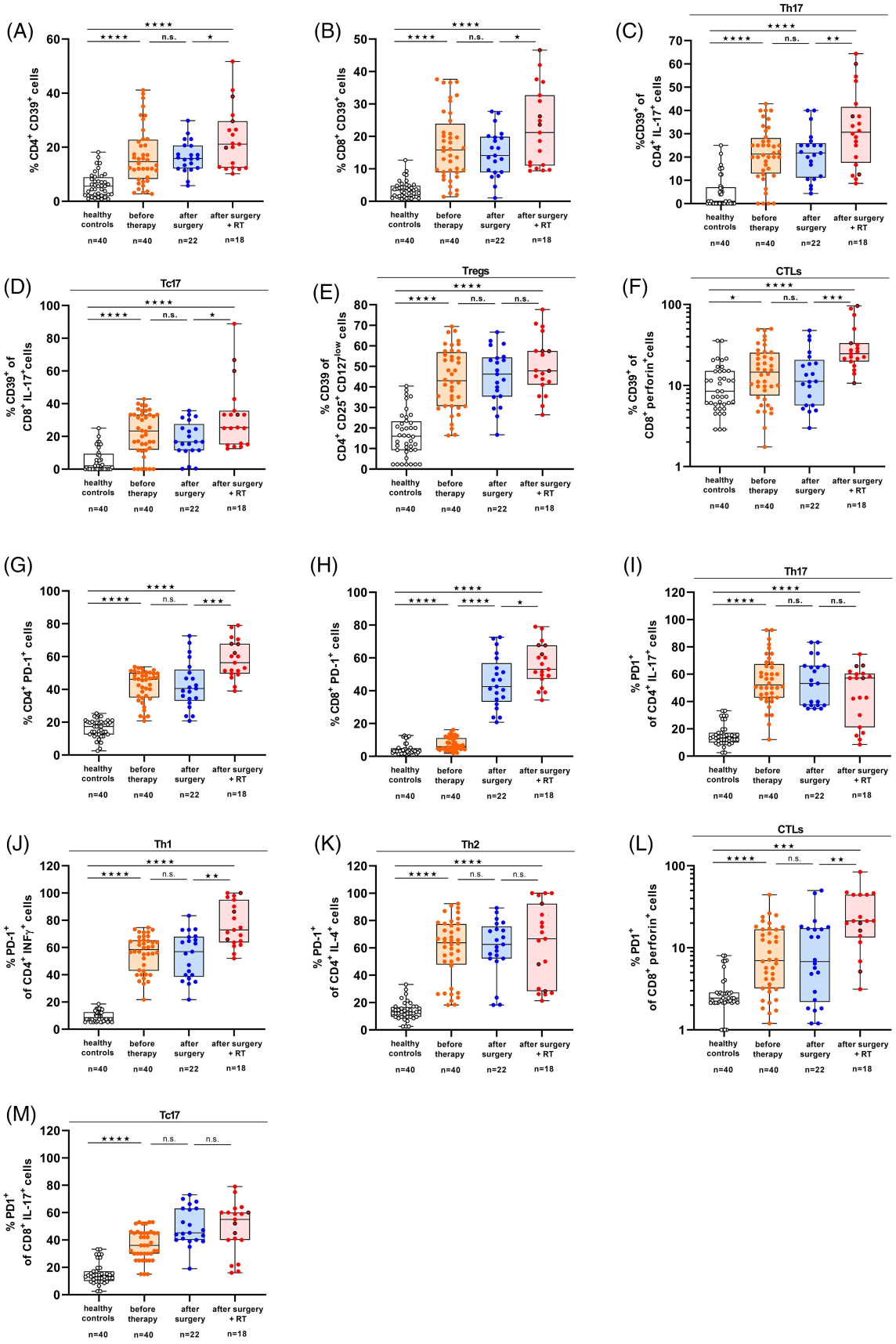
Next, we evaluated PD-1 expression on T cell subtypes during therapy, a known exhaustion marker and target for immunotherapy[29] and considered for treatment of vulvar cancers.[7] We found significantly higher pre-therapeutic frequencies (Figure 4G, H; orange dots) and numbers (Figure S3C, D) of PD-1 positive CD4$^+$ and CD8$^+$ T cells in comparison to HC (white dots). After therapy, patients with surgery only exhibited comparable levels of PD-1$^+$ CD4$^+$ T cells to pre-therapeutic values (Figure 4G; blue dots) but higher PD-1$^+$ CD8$^+$ frequencies (Figure 4H) and aRT in addition to surgery further increased PD-1 expressing CD4$^+$ and CD8$^+$ T cells (red dots; 1.4- and 1.3-fold, respectively). Interestingly, analysis of CD4$^+$ T cell subpopulations revealed no significant differences in PD-1 expression of Th17 cells between untreated patients and patients after surgery or aRT (Figure 4I). In contrast, we found increased PD-1 expression on Th1 cells after aRT compared to probes after surgery (Figure 4J; 1.3-fold increase), while Th2 cells exhibited heterogeneous PD-1 expression after aRT, comparable to untreated patients and the surgery only cohort (Figure 4K). Regarding CD8$^+$ T cell subpopulations, perforin expressing killer cells showed increased PD-1 expression after aRT in comparison to untreated patients or the surgery cohort (Figure 4L), while Tc17 cells exhibited slightly higher, but not significantly increased PD-1 expression (Figure 4M). Thus, we found a general increase in PD-1 expression on CD4$^+$ and CD8$^+$ T cells with variable PD-1 expression of T cell subtypes induced by aRT resulting in differentially exhausted T cell phenotypes.

3.7 | Association of therapy-induced alterations in T cell subtypes and their CD39 and PD-1 expression with vulvar cancer recurrence

To clarify the impact of therapy-induced immune milieu in the blood of patients for clinical outcome, 40 patients (with follow-up times of at least 36 months; n = 22 after surgery (dots) and n = 18 (triangles) after surgery and aRT) could be evaluated for vulvar cancer recurrence. The median follow-up over all was 67 months (range 36-72 months). Retrospectively, 18/40 patients developed vulvar cancer recurrence, including seven after surgery only and 11 after surgery and aRT. Patients with cancer relapse exhibited slightly, but not significantly advanced tumor FIGO stages (Figure S4A). When analyzing the association of the therapy-induced T cell immune milieu with vulvar cancer recurrence, we found significantly higher proportions of Th17 cells within the remaining CD4$^+$ T cell population after therapy in the blood of patients who retrospectively developed cancer relapse compared to patients without recurrence (Figure 5A; 5.8-fold increase). In contrast, patients with relapse showed no significant differences concerning Th2 and Treg frequencies, but percentages of Th1 cells were strongly reduced by 50%. The same tendencies we found regarding absolute numbers/µl (Figure S4B). Proportions of γδT17 cells after therapy did not significantly differ between patients with and without relapse (Figure 5B). Regarding CD8$^+$ T cell subpopulations after therapy, patients with vulvar cancer recurrence displayed significantly higher percentages of Tc17 cells but less perforin producing CD8$^+$ T cells after therapy than patients without relapse (Figure 5C).

As we found enhanced CD39 expression on distinct T cell subtypes after aRT (Figure 4), we included CD39 expression and found that Th17 cells (Figure 5D), Tc17 cells (Figure 5E) and CD8$^+$ perforin$^+$

FIGURE 3 Frequencies and absolute numbers of γδT17 cells and CD8$^+$ T cell subpopulations during therapy of vulvar cancer patients. PBMCs of 40 vulvar cancer patients and 40 female age-matched healthy controls were analyzed for (A) γδT17 frequencies by flow cytometry and (B) absolute numbers/µl of γδT17 cells were evaluated. (C) γδT17 frequencies were analyzed before and after surgery (n = 22; blue dots) and during aRT (n = 18; red dots; n = 3 patients with concurrent chemotherapy; black edged dots). PBMCs were analyzed for (D) CD8, (G) Tc17, (J) CD8$^+$perforin$^+$ frequencies by flow cytometry and absolute numbers/µl of (E) CD8$^+$ T cells, (H) Tc17 cells and (K) CD8$^+$perforin$^+$ cells were evaluated. PBMCs of vulvar cancer patients which received surgery only (n = 22; blue dots) or aRT (n = 18, red dots; n = 3 patients with concurrent chemotherapy; black edged dots) were analyzed for (C) γδT17, (F) CD8, (I) Tc17, and (L) CD8$^+$perforin$^+$ frequencies. *P* value according to the nonparametric Kruskal-Wallis test or paired Wilcoxon matched pairs test or paired Friedman test. Asterisks represent statistical significances: ns, not significant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.



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cells (Figure 5F) of patients who retrospectively developed cancer relapse exhibited significantly higher proportions of CD39 after therapy than patients without relapse.

A better discrimination between patients with and without relapse was obtained when stratified for PD-1 expression of distinct T cell subtypes (Figure 5G). While percentages of PD-1$^+$ cells of Th2 cells did not significantly differ between the two cohorts, patients with cancer recurrence exhibited significantly higher proportions of PD-1$^+$ Th1 cells, but lower percentages of PD-1$^+$ Th17 cells. The same results were found for Tc17 cells with reduced percentages of PD-1$^+$ Tc17 cells in patients with cancer relapse, however these patients exhibited high proportions of PD-1$^+$ perforin expressing CD8$^+$ killer cells.

To evaluate whether the presence of distinct T cell subtypes is linked to the course of disease, ROC analyses were performed to identify a cutoff value for Th1, Th17, Tc17 and CD8$^+$perforin$^+$ frequencies which discriminates between patients with and without recurrent cancers. The best discrimination was obtained for a cut-off value of percentages of Th1 per CD4$^+$ T cells <33.9% (77.78% sensitivity and 72.73% specificity; Figure S4C), Th17 per CD4$^+$ T cells >4.3% (66.67% sensitivity and 95.45% specificity; Figure S4D), Tc17 per CD8$^+$ T cells >6.2% (94.44% sensitivity and 81.82% specificity; Figure S4E) and CD8$^+$ perforin$^+$ per CD8$^+$ T cells <18.2% (77.78% sensitivity and 100% specificity; Figure S4F). The area under the ROC curve (AUC) value was 0.8119, 0.8838, 0.9192 and 0.9722, respectively. Applying the defined cut-off values for the 40 patients (Figure 5H-K), in the group of patients with high proportions of Th1 (>33.9%) (Figure 5H, light pink line) but low percentages of Th17 after therapy (<4.3%) (Figure 5I, light blue line) recurrence-free survival (RFS) was 77.9% and 75.1% after 5 years, respectively. In contrast, for patients with a low percentage of Th1 (<33.9%) (Figure 5H, pink line) and high percentages of Th17 cells (>4.3%) (Figure 5I, blue line) 5-years RFS was 27.5% and 7.7%, respectively. Regarding CD8$^+$ T cell subtypes, 5-years RFS of patients with low percentages of Tc17 cells (<6.2%) (Figure 5J, light gray line) and high occurrence of perforin expressing CD8$^+$ cells (>18.2%) (Figure 5K, light green line) was 94.7% and 100%, respectively. However, high levels of Tc17 cells (>6.2%) (Figure 5J, gray line) and reduced frequencies of perforin expressing CD8$^+$ cells (<18.2%) (Figure 5K, green line) resulted in 5-years RFS of 15.9% and 23.6%, respectively. Notably, high percentages of PD-1 on Th1 (>73%) and CD8$^+$ perforin$^+$ cells (>6.1%) further reduced 5-years RFS of patients (Figure 5H, K, purple lines; 16.7% and 6.6%, respectively), while high PD-1 expression on Th17 and Tc17 cells was associated with 5-years RFS of 29.9% and 32.9% (Figure 5I, J, purple lines). In conclusion, our data demonstrated a clear association between increased post-therapeutic Th17 and Tc17 cell levels, but reduced Th1 and perforin expressing CD8$^+$ T cell levels with high PD-1 expression and vulvar cancer recurrence.

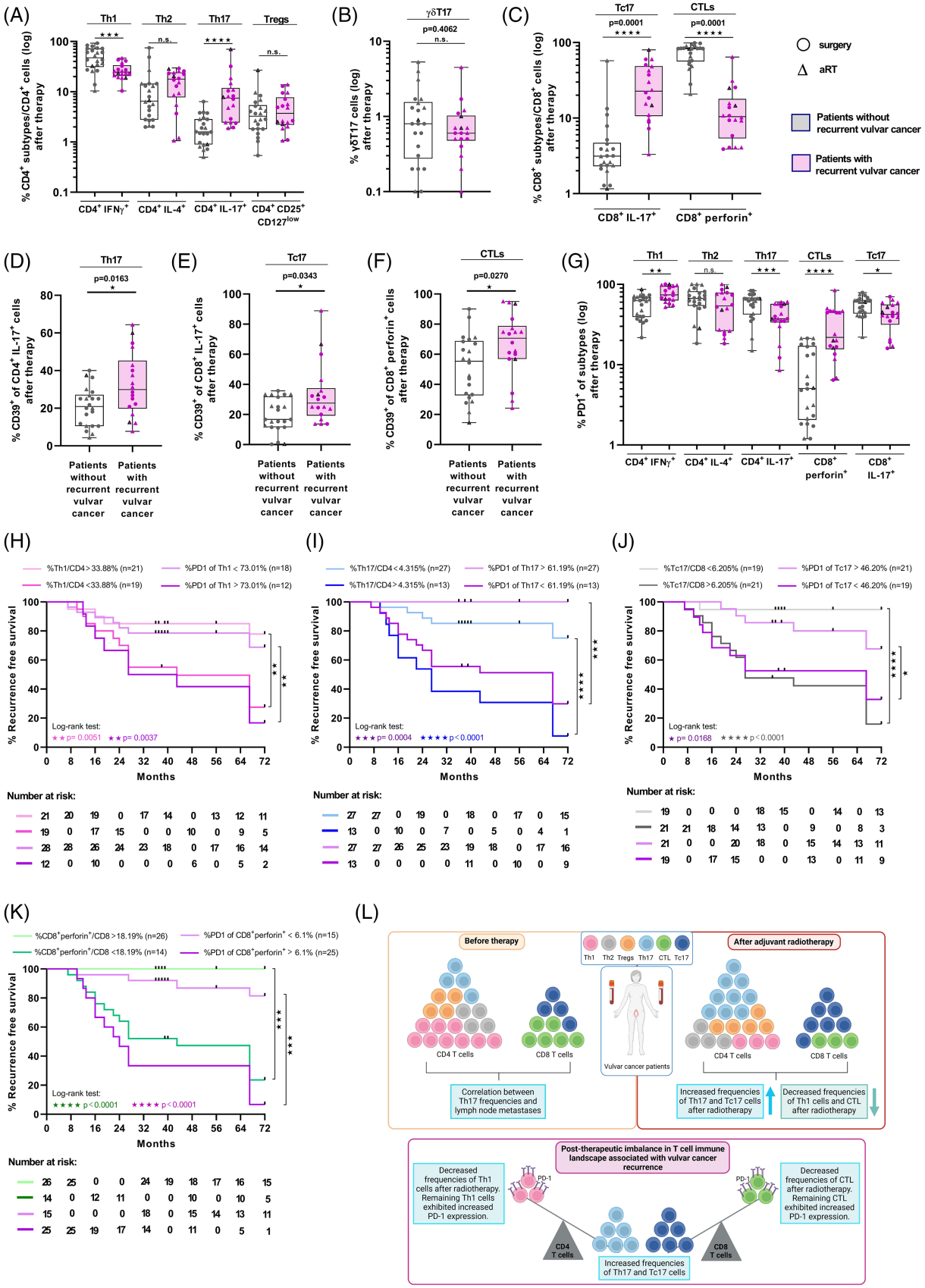
4 | DISCUSSION

After surgical removal of the initial tumor, the systemic immune milieu individually shaped by different oncological therapies provides a distinct environment potentially favoring cancer metastases and relapse. In this study, we investigated CD4$^+$ and CD8$^+$ T cell subpopulations in vulvar cancer stage-dependent therapy and showed that frequencies of IL-17-expressing T cells increased while Th1 and perforin producing CD8 killer cells decreased during aRT in the blood of patients. Notably, enhanced proportions of CD39$^+$ Th17 and Tc17 cells after therapy and lower proportions of Th1 and perforin-producing CD8$^+$ T cells with increased expression of PD-1 were associated with recurrent vulvar cancers. Figure 5L summarizes our findings concerning the T cell immune landscape during therapy of vulvar cancer patients.

Second line treatment options are limited in vulvar cancer patients and show often limited results with poor therapy responses, progression or distant metastases[30,31] underlining the need for new treatment approaches. Effects of oncological therapies on lymphocyte subsets have been of great interest, because of the possibility that therapy-induced changes in immune-cell balance might interfere with antitumor activity supporting therapy resistance and cancer progression. Regarding the systemic immune landscape as potential driver of carcinogenesis or target for immunotherapy, our study shows that vulvar cancer patients exhibit reduced Th1, but increased Th17 and Tregs frequencies in their blood before therapy in comparison to healthy controls. Interestingly, Th17 frequencies correlated with advancing tumor FIGO stages and patients with lymph node metastases, a crucial step in carcinogenesis critically affecting the course of disease,[32,33] exhibited increased Th17 frequencies. However, pretherapeutic frequencies of Th1, Th2, Th17 or Tregs cells were not associated with vulvar cancer relapse.

In line with previous studies describing Th17 cells as long living effector cells with high proliferative capacity and apoptosis-resistant phenotype toward different cell death inducers, such as the chemotherapeutic drug cisplatin,[19] Fas-mediated cell death in murine Th17 cells[34] or chemoradiotherapy in cervical cancer patients,[21] monitoring of vulvar cancer patients during therapy from this study revealed a decrease in CD4$^+$ T cells in the patients' blood, but a significant increase in Th17 frequencies and absolute numbers after aRT in comparison to patients treated with surgery alone. Besides Th17 cells,

FIGURE 4 Phenotypic characterization revealed CD39 expressing Th17 and Tc17 cells as well as PD1$^+$ Th1 and CD8$^+$perforin$^+$ cells after aRT in the blood of vulvar cancer patients. PBMCs of 40 vulvar cancer patients and 40 female age-matched healthy controls were analyzed for frequencies of CD39$^+$ (A) CD4$^+$ cells, (B) CD8$^+$ cells, (C) Th17 cells, (D) Tc17 cells, (E) CD4$^+$ Tregs and (F) CD8$^+$perforin$^+$ cells, and for frequencies of PD1$^+$ (G) CD4$^+$ cells, (H) CD8$^+$ cells, (I) Th17 cells, (J) Th1 cells, (K) Th2 cells, (L) CD8$^+$perforin$^+$ cells and (M) Tc17 cells. N = 22 (blue dots) received surgery and n = 18 aRT (red dots; n = 3 patients with concurrent chemotherapy; black edged dots). *P* value according to the nonparametric Kruskal-Wallis test or paired Wilcoxon matched pairs test or paired Friedman test. Asterisks represent statistical significances: ns, not significant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.



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frequencies of Tc17 cells but not γδT17 cells were also increased after aRT while frequencies of Th2 and Tregs were comparable to the surgery cohort. In contrast, we found a strong decline in frequencies of Th1 and perforin expressing $CD8^+$ T cells in the patients' blood after aRT. Analysis of Th17 and Tc17 cells within the remaining $CD4^+$ and $CD8^+$ T cells revealed enhanced proportions of Th17 per $CD4^+$ and Tc17 per $CD8^+$ cells. This accumulation was particularly pronounced in the aRT patient cohorts, implying that these IL-17-expressing subpopulations are resistant to RT. This is in line with the findings that human Th17 cells showed upregulation of anti-apoptotic genes after irradiation ex vivo[35] favoring a therapy-induced immune milieu potentially dominated by IL-17. Strikingly, our data show an association between enhanced Th17 and Tc17 frequencies but reduced proportions of Th1 and $CD8^+$ $perforin^+$ cells after therapy and vulvar cancer recurrence. Imbalances in Th1/Th2 and Th17/Treg ratios are associated with the development and progression of untreated cancers[36] including gynecological malignancies, like cervical cancers.[37] In other tumor entities, Th17 cells mediated anti-tumor properties by recruiting immune cells into tumors or stimulating effector $CD8^+$ T cells[38,39] as well as tumor-promoting responses driving proliferation, invasion, metastasis and angiogenesis.[40,41] Tc17 cells were found in patients with inflammatory diseases and different cancers.[23,24] Thus, our results refer to a link between systemic therapy-induced immunity and course of disease.

Phenotypic characteristics of the remaining T cell subtypes after therapy revealed enhanced occurrence of CD39 expressing $CD4^+$ and $CD8^+$ T cells in the blood of vulvar cancer patients in comparison to healthy controls and frequencies were further elevated by aRT. The ectonucleotidase CD39 was found on tumor-reactive $CD4^+$ and $CD8^+$ TILs in vulvar cancer patients.[25] Converting ATP to immunomodulatory adenosine[26] CD39 exhibited tumor-promoting capabilities and is regarded as marker of exhausted $CD8^+$ T cells[42] as well as a target for immunotherapy.[26-28] Higher CD39 expression we found on Th17 and Tc17 cells after aRT potentially providing these subpopulations with suppressive capacities[43] as found in the blood of patients with Crohn's disease whose $CD39^+$ Th17 cell levels directly correlate with clinical disease activity.[44] Furthermore, aRT increased frequencies of CD39 expressing $CD8^+$ $perforin^+$ killer cells indicating an exhausted, antigen-experienced phenotype possibly mediated by RT that is supposed to lead to more antigen presentation.[45] In contrast to other studies showing enhanced CD39 expression on Tregs after chemoradiotherapy of patients with head and neck squamous cell carcinomas[20] aRT did not increase CD39 expression on $CD4^+$ Tregs of vulvar cancer patients in our study.

FIGURE 5 Increased frequencies of CD39 expressing Th17 and Tc17 cells as well as high proportions of $PD1^+$ Th1 and $CD8^+perforin^+$ cells in the blood of patients with vulvar cancer relapse. (A) The post-therapeutic percentage of $CD4^+INFγ^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ and $CD4^+CD25^+CD127^{low}$ cells per $CD4^+$ T cells, (B) frequencies of γδT17 cells, (C) percentage of $CD8^+IL\text{-}17^+$ and $CD8^+$ $perforin^+$ cells per $CD8^+$ T cells of 40 patients who received surgery (circles; n = 22) or aRT (triangles, n = 18; n = 3 patients with concurrent chemotherapy; black edged dots) was evaluated. The percentage of the respective subpopulations was depicted for patients with recurrent vulvar cancers (n = 18; purple circles and triangles) in comparison with patients without relapse (n = 22; gray circles and triangles). (D) Percentages of $CD39^+$ Th17 cells, (E) $CD39^+$ Tc17 cells and (F) $CD39^+$ perforin expressing $CD8^+$ cells were evaluated and depicted for patients with recurrent vulvar cancers in comparison with patients without relapse. (G) Percentages of $PD1^+$ $CD4^+$ and $CD8^+$ T subpopulations were evaluated and depicted for patients with recurrent vulvar cancers in comparison with patients without relapse. *P* value according to the nonparametric Mann-Whitney *U*-test. Asterisks represent statistical significances: ns, not significant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. (H) Recurrence-free survival (RFS) of 40 patients who received surgery (n = 22) or aRT (n = 18) was determined for a cohort with high percentages of Th1 cells (light pink line) and for a cohort with low percentages of Th1 cells (pink line). Median RFS was 43 months for the cohort with low percentages of Th1 cells. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 7.827, *P* = .0051. RFS was evaluated for a cohort with percentages of $PD1^+$ cells per Th1 cells <73.01% (light purple line) and for a cohort of $PD1^+$ cells per Th1 cells >73.01% (purple line). Median RFS was 35 months for the cohort with $\%PD1^+$ of Th1 > 73.01%. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 8.431, *P* = .0037. (I) RFS of 40 patients which received surgery (n = 22) or aRT (n = 18) was determined for a cohort with %Th17/CD4 < 4.315% (light blue line) and for a cohort with %Th17/CD4 > 4.315% (blue line). Median RFS was 27 months for the cohort with high percentages of Th17 cells. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 18.98, *P* < .0001. RFS was evaluated for a cohort with percentages of $PD1^+$ cells per Th17 cells >61.19% (light purple line) and for a cohort <61.19% (purple line). Median RFS was 67 months for the cohort with $\%PD1^+$ of Th17 < 61.19%. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 12.68, *P* = .0004. (J) RFS of 40 patients who received surgery (n = 22) or aRT (n = 18) was determined for a cohort with %Tc17/CD8 < 6.205% (light gray line) and for a cohort >6.205% (gray line). Median RFS was 27 months for the cohort with high percentages of Tc17 cells. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 19.94, *P* < .0001. RFS was evaluated for a cohort with percentages of $PD1^+$ cells per Tc17 cells >46.2% (light purple line) and for a cohort <46.2% (purple line). Median RFS was 67 months for the cohort with $\%PD1^+$ of Tc17 < 46.2%. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 5.715, *P* = .0168. (K) RFS of 40 patients which received surgery (n = 22) or aRT (n = 18) was determined for a cohort with $\%CD8^+perforin^+$/CD8 > 18.19% (light green line) and for a cohort <18.19% (green line). Median RFS was 43 months for the cohort with low $\%CD8^+perforin^+$/CD8 cells. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 23.98, *P* < .0001. RFS was evaluated for a cohort with percentages of $PD1^+$ cells per $CD8^+perforin^+$ cells <6.1% (light purple line) and for a cohort >6.1% (purple line). Median RFS was 24 months for the cohort with $\%PD1^+$ of $CD8^+perforin^+$ cells >6.1%. Comparison of survival analysis was performed using log-rank (Mantel-Cox) test; chi-square: 42.37, *P* < .0001. (L) Schematic presentation of the T cell immune landscape during therapy and vulvar cancer relapse. Adjuvant radiotherapy reduced frequencies of Th1 and CTLs but increased occurrence of Th17 and Tc17 cells. Enhanced frequencies of Th17 and Tc17 cells and PD-1 expressing Th1 and CTLs were associated with vulvar cancer recurrence (scheme was generated with the BioRender software).

Currently, first studies indicated the relevance for immune checkpoint inhibitors,[46] like the PD-1 inhibitor pembrolizumab, in vulvar cancer treatment, especially in combination with radiotherapy.[7] First clinical studies with small patient numbers evaluated PD-1 inhibitors in combination with chemotherapy in vulvar cancer patients with varying, but promising results describing cancer remission and improved survival rates.[47,48] In line with[17] demonstrating high PD-1 levels on $CD4^+$ T cells after irradiation, our study showed increased PD-1 expression of $CD4^+$ and $CD8^+$ T cells after aRT. Additionally, we identified enhanced proportions of PD-1 expressing Th1 and perforin-producing $CD8^+$ T cells, but not of Th17 and Tc17 cells, which are associated with reduced recurrence free survival. Following the idea to use PD-1 inhibitors for immunotherapy of vulvar cancer patients, enhanced PD-1 expression on Th1 and $CD8^+$ killer cells in aRT-treated patients might be targeted by PD-1 inhibitors preventing interactions with PD-L1, which is found on vulvar cancers with poor prognosis,[49] and potentially restore their functionality. Interestingly, based on our findings showing no enhanced PD-1 expression on Th17 and Tc17 cells after irradiation, these IL-17 expressing subpopulations apparently exhibited no exhausted phenotype potentially avoiding PD-1-PD-L1-driven T cell dysfunctions. This might favor Th17-mediated activation of tumor-promoting pathways, like PI3K/AKT pathway,[21] currently discussed as potential treatment target for vulvar cancers.[46] Our study is limited by a low sample size and longitudinal analysis concerning stability of therapy-induced immunity is lacking. Furthermore, unspecific stimulation of PBMCs with PMA/ionomycin might represent a potential weakness. Nevertheless, differences in therapy-induced immunity between patients with and without cancer relapse are very pronounced and correlate well with the severity of the disease.

In conclusion, our findings identified individual therapy-induced immune milieus of patients and linked high proportions of Th17 and Tc17 cells in the patients' blood after irradiation with reduced recurrence free survival indicating the need for treatment approaches against IL-17 expressing T cells in vulvar cancers. Furthermore, similar studies with larger sample sizes are needed to evaluate the stability of therapy-induced immunity and its role for cancer recurrence potentially providing a systemic milieu favoring circulating cancer cells and metastases. Consistently, IL-17 is described to promote spheroid formation and self-renewal of $CD133^+$ cancer stem-like cells in ovarian cancer.[50] As antibodies against IL-17A or IL-17 receptor are approved for the treatment of psoriasis[51] and are currently being evaluated for treatment of inflammatory diseases[52] and different cancers,[53,54] these antibodies should also be considered for vulvar cancer immunotherapy.

AUTHOR CONTRIBUTIONS

Conceptualization, Russalina Stroeder, Patrick Melchior and Barbara Walch Rückheim; methodology, Selina Gies, Tanja Tänzer, Laura Theobald, Maike Pohlers, Birgit Glombitza and Barbara Walch Rückheim; validation, Selina Gies, Patrick Melchior, Russalina Stroeder and Barbara Walch Rückheim.; formal analysis, Selina Gies, Patrick Melchior, Russalina Stroeder and Barbara Walch Rückheim; investigation, Selina Gies, Tanja Tänzer, Laura Theobald, Maike Pohlers, Birgit Glombitza and Barbara Walch Rückheim.; resources, Patrick Melchior, Martina Sester, Erich-Franz Solomayer and Barbara Walch Rückheim.; data curation, Patrick Melchior, Barbara Walch Rückheim.; writing-original draft preparation, Barbara Walch Rückheim.; writing-review and editing, Selina Gies, Patrick Melchior, Russalina Stroeder, Laura Theobald, Martina Sester and Erich-Franz Solomayer; project administration, Barbara Walch Rückheim.; funding acquisition, Barbara Walch Rückheim. All authors have read and agreed to the published version of the manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study has been conducted according to Declaration of Helsinki principles. Usage of peripheral blood mononuclear cells (PBMC) of vulvar cancer patients and healthy controls were approved by the Ethics Committees of the Saarland Ärztekammer (Saarbrücken, Germany; 98/17). Written informed consent was provided by all study participants.

ORCID

Barbara Walch-Rückheim https://orcid.org/0000-0003-3355-8978

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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