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Stereotactic Ablative Radiation Therapy in 3 Fractions Induces a Favorable Systemic Immune Cell Profiling in Prostate Cancer Patients

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ABSTRACT

The impact of radiotherapy (RT) on immune cell status in prostate cancer (PCa) is only partially determined. The aim of this study was to assess the effect of different RT strategies on peripheral B, T, and Natural killer (NK) lymphocytes at precise longitudinal time-points in PCa. 18 patients treated with stereotactic body radiation therapy (SBRT) (40 Gy/3FRX), definitive moderate-hypofractionation (62 Gy/20FRX), or post-operative conventional-fractionation RT (66–69 Gy/30FRX) were prospectively evaluated for the immune cell profile in terms of immune cell composition, differentiation stage, cytokine production and inhibitory receptor (IR) expression. The immune-monitoring of the 18 patients revealed that RT affects the balance of systemic immune cells, with the main differences observed between SBRT and conventionally fractionated RT. SBRT favorably impacts immune response in term of increased B cells, central-memory and effector-memory $CD8^+$ T cells, along with decreased Treg cells after treatment. On the contrary, conventional fractionated RT had a long-term negative effect on the systemic immune profile, including a decrease of total lymphocyte counts accompanied by an increase of neutrophils-to-lymphocytes ratio. Total B and T cells decreased and Treg-to-$CD8^+$ ratio increased. Functionality of T lymphocytes were not affected by any of the 3-fractionation schedules. Interestingly, SBRT significantly up-regulates the expression of V-domain immunoglobulin suppressor of T-cell activation (VISTA) in $CD8^+$ T cells in the absence of other IRs. Our results indicate the relevance of systematic immunomonitoring during RT to identify novel immune-related target to design trials of combined radio-immunotherapy as a promising strategy in the clinical management of PCa.

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Introduction

Prostate cancer (PCa) is the second most common cancer and the fifth cause of cancer related deaths overall.[1] Radiotherapy (RT) plays a pivotal role in the treatment of localized disease, either as adjuvant-salvage therapy after surgery or as curative treatment option alone or combined with androgen deprivation (AD).

Different fractionation schedules and volumes of treatment can be delivered depending on disease stage. Stereotactic body radiation therapy (SBRT), defined as a dose per fraction higher than 5 Gy,[2] is an accepted treatment modality for localized PCa.[3] Controlled trials have shown favorable clinical results in terms of local control and gastro-intestinal and genitourinary toxicity rates,[4–6] compared to conventionally fractionated RT.[7]

Moreover, several studies have found that peripheral immune cells might be significantly modified by conventionally fractionated RT[8] though the clinical role of this immune cell modification is yet to be determined. Moreover, the effect on peripheral immune cells of a higher dose per fraction in SBRT treated patients is still poorly investigated.

To enhance SBRT efficacy, studies are warranted to investigate the effect of this high dose per fraction on patients' immune cells, by collecting and analyzing longitudinal blood samples. An in-depth immunomonitoring may in fact highlight "the fine tuning" of immune response during RT and may allow to define complementary strategies of treatment.

Immunotherapy and in particular immune checkpoint inhibitors (ICI) have revolutionized the systemic treatment of cancer, though the survival benefit in PCa patients remains elusive. To date, ICI in PCa have been only tested in advanced castration resistant metastatic state and failed to provide clinical benefit when used as monotherapy.[9] The mechanism of resistance, mainly related to the tumor microenvironment (TME) characteristics[10,11] is still under investigation, even though the combination of Nivolumab and Ipilimumab has improved clinical outcomes at the cost of higher toxicity.[12]

The aim of this prospective study is to evaluate the immunological changes on peripheral T, B and Natural killer (NK) lymphocytes at precise time-points in PCa patients treated with

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3 different treatment strategies, in order to identify new therapeutic targets to be combined with radiotherapy.

Materials and methods

Patients and Treatments

This prospective study was conducted at a single Institution between February 2019 and May 2021 (Clinicaltrials.gov NCT04774133). The study was approved by the Institutional Review Board (RS1163/18). Eligibility criteria were: histologically confirmed PCa undergoing RT, age >18 years. Exclusion criteria were: previous malignancies, chemotherapy, autoimmune and hematological disease in leukemic phase. All accrued patients were treated according to our Institutional guidelines/ongoing research protocol: SBRT (40 Gy/3 fractions (FRX); Group-1); definitive moderately hypofractionationated RT (62 Gy/20FRX; Group-2); post-operative conventionally fractionated RT (66–69 Gy/30FRX; Group-3). Target volumes were: prostate only (Group-1), prostate + seminal vesicles (SV) (Group-2), prostate bed + pelvic lymph nodes (Group-3), including bilateral internal, external and common iliac lymph nodes as well as pre-sacral lymph nodes. Androgen deprivation (AD) was prescribed to selected patients. RT was delivered as follow: SBRT, every other day; definitive moderately hypofractionationated RT, four days per week; conventionally fractionated RT, five days per week.

Peripheral blood collection

30 ml of blood were collected at different timepoints (Figure 1a) from 18 treated patients and 12 sex/age-matched healthy donors (HD), after written informed consent, in EDTA vacutainer tubes (BD Bioscience, 367864). 10 ml of whole blood EDTA was analyzed for complete blood count (CBC) (DXH 900, Beckman Coulter) and flow cytometry staining. Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml of blood using Ficoll-paque separation (Euroclone, DVCL5020) and cryopreserved in 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650) until use.

Flow cytometry

200 µl of fresh blood was stained with a combination of antibodies (mAbs) (Supplementary Table S1) and acquired on Lyse-Wash-Assistant (LWA) of Becton Dickinson (BD Pharm Lyse, 555899). Surface-staining of isolated PBMC was performed for 30 min at 4°C using combinations of mAbs (Supplementary Table S1). 3×10^5 cells were activated with plate-bound anti-CD3 mAb (2 µg/ml) (CBT3 IgG2a), in the presence of Golgistop (BD Bioscience 554724) and Golgiplug (BD Bioscience 555029), for 5–6 h. Intracellular staining was performed by the use of Intrasure kit (BD Bioscience, 641778) according with the manufactory instruction, combining surface and intracellular mAbs (Supplementary Table S1). Dead cells were excluded by Fixable Viability Stain 700 (BD Bioscience 564997). Cells were acquired in the BD FACSCelesta and BD FACSCantoII flow cytometers and analyzed by BD FACSDiva and Flowjo software.

Statistical analysis

For the comparison of two or more groups non-parametric Mann-Whitney U-test and Kruskal-Wallis test were used. For intra-individual comparison among different times the Wilcoxon U-test and Friedman test were used. Box-graphs represent the 25th and 75th percentiles, median and outliers are shown. Statistical analysis was performed by comparing different timepoints versus baseline and follow-up (6 or 12 months) versus treatment-end. A P value ≤.05 was considered significant. Significance is denoted as *p ≤ .05, **p ≤ .01, ***p ≤ .001. p value supporting a trend toward statistical significance is shown as values. Statistical evaluation was performed with SPSS 21.0 (SPSS Inc., Chicago, IL, USA) for Windows. Barplot and boxplot were generated using R environment.

Results

Effect of different RT strategies on complete blood counts

Eighteen patients were included in this analysis (Group-1 3FRX, 5 pts; Group-2 20FRX, 6 pts; Group-3 30FRX, 7 pts). Patients, treatment characteristics and disease status after a median follow-up of 38.4 months (range 33.7–44.1 months) are reported in Table 1. Six patients were additionally treated with AD (Group-2, 4 pts; Group-3, 2 pts) for 6 to 24 months according to disease stage.

To identify which immune cell subsets might be modulated by the 3 RT strategies, including different fractionation and treatment volumes (Group-1, 40 Gy/3FRX, prostate; Group-2, 62 Gy/20FRX, prostate + SV; Group-3, 66–69 Gy/30FRX, prostate bed + pelvic lymph nodes), we performed an in-depth immune profiling at different time-points, before, during, at the end and post-RT in peripheral blood (Figure 1a).

Figure 1b shows the results of CBC analysis from baseline to 12 months after RT. At baseline, immune cell counts did not show significant differences among Groups except for a trend in highest monocytes count in Group-1 (Figure 1b-f, middle panels). White blood cell count (WBC) increased in Group-1 during RT, and returned to baseline at 6 and 12 months (Figure 1b, left panel). This was also evident after the normalization of counts calculated as intra-individual ratio between 12 months and baseline (Figure 1b, right panel). Differently, in Group 2 and 3 WBC significantly decreased at treatment-end, but did not return at baseline after 1 year (Figure 1b). Absolute lymphocyte counts underwent a slight reduction during RT in Group-1, recovering to baseline 6 months after RT (Figure 1c). In both Group-2 and Group-3 a significant drop was observed at treatment-end. After 1 year of follow-up (POST 12 months) we evidenced a recovery in Group-2 and only a partial recovery not reaching baseline value in Group-3 (Figure 1c).

Monocyte's analysis did not show significant changes during RT (Figure 1d). Neutrophil counts increased in Group-1 from 3 h and returned to baseline after 6 months (Figure 1e), as evidenced in neutrophil-to-lymphocyte ratio (Figure 1f).

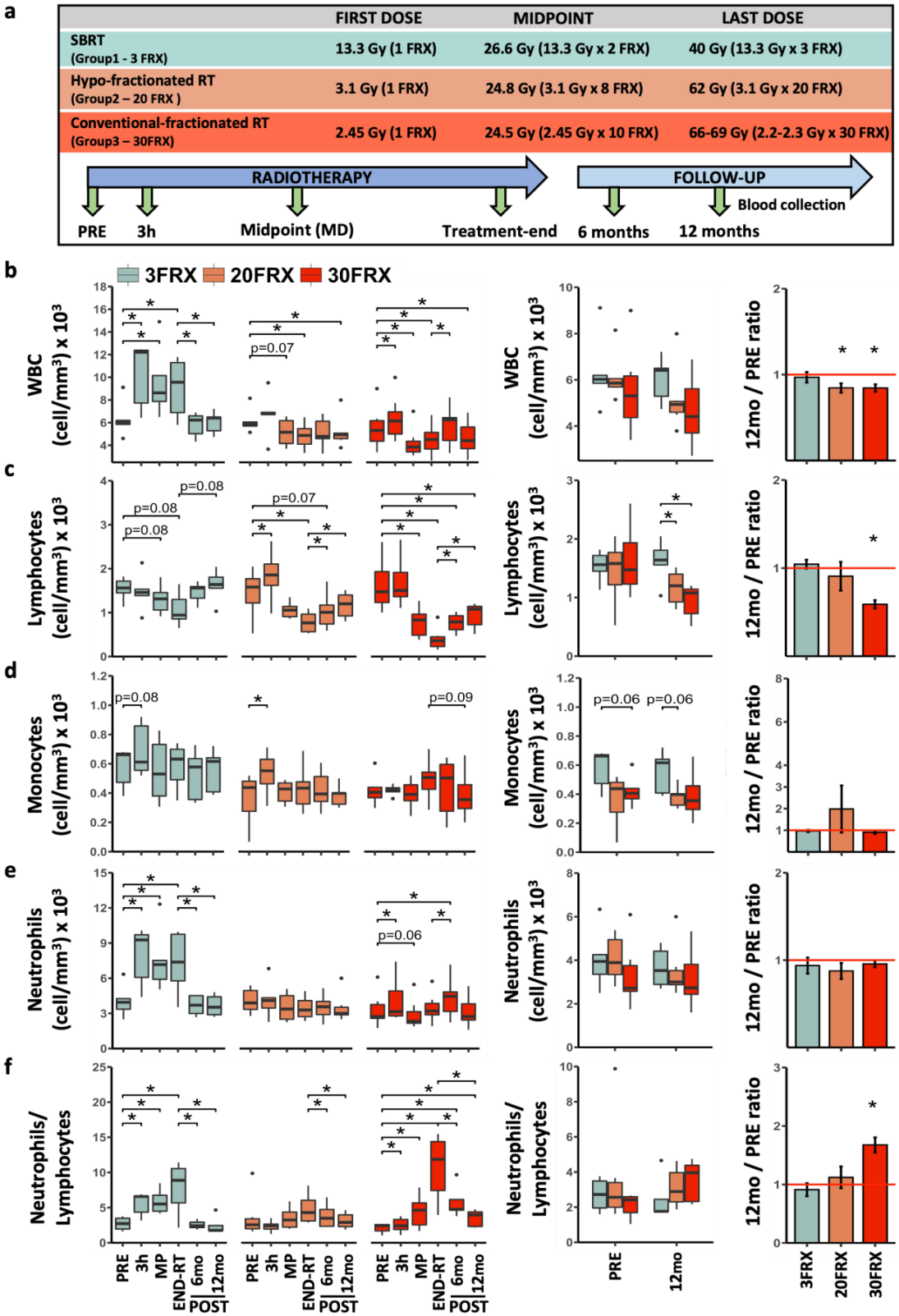

Figure 1. Flow chart of the study and CBC analysis. (a) Treatment schedule and blood sample collection. (b-f) Longitudinal analysis of immune cells in 18 patients (Group1-3FRX, n = 5; Group2-20FRX, n = 6; Group3-30FRX, n = 7), determined by full blood counts (Wilcoxon U-test). Middle panels, comparison among Groups (Mann-Whitney U-test). Right panels, normalization calculated as intra-individual patient ratio between 12 months (POST) and baseline level (POST/PRE ratio). The red line indicates a ratio of 1, representing no change between PRE and POST treatment. The P value is calculated by Wilcoxon U-test between POST versus baseline and follow-up versus treatment-end. *p ≤ .05. *Abbreviation*: FRX = fraction; WBC = white blood cells; MP = Middle point; mo = months.

Table 1. Clinical characteristics of patients.

Group	Age* (IQR)	Treatment	Volumes	RT** doses	FRX	iPSA (IQR)	GGG (%)			Clinical T stage (%)			Pathological T stage (%)			Margins at RP (%)		bNED (%)
							1	2 or 3	4 or 5	cT1	cT2	cT3	pT2	T3a	pT3b	R0	R1	
Group 1 (n = 5)	72 (68–73)	SBRT	Prostate	40 Gy	3	6.9 (5.3–7)	2 (40)	3 (60)	0 (0)	4 (80)	1 (20)	0 (0)	NA	NA	NA	NA	NA	5 (100)
Group 2 (n = 6)	75.5 (75–80.5)	Definitive moderately hypofractionated RT	Prostate + seminal vesicle	62 Gy	20	5.8 (5.4–8.3)	1 (16.7)	5 (83.3)	0 (0)	1 (16.7)	4 (66.6)	1 (16.7)	NA	NA	NA	NA	NA	6 (100)
Group 3 (n = 7)	70 (68.5–71.5)	Coventional fractionated RT	Prostate bed + pelvic lymph nodes	66–69 Gy	30	7 (5.5–12.6)	1 (14.3)	4 (57.1)	2 (28.6)	NA	NA	NA	2 (28.6)	2 (28.6)	3 (42.8)	4 (57.1)	3 (42.9)	5 (71.4)

*, years; ** RT was delivered as follow: SBRT, every other day; definitive moderately hypofractionationated RT, four days per week; conventionally fractionated RT, five days per week. IQR, interquartile range; RT, radiotherapy; FRX, fractions; PSA, prostatic specific antigen; GGG, Gleason Grade Group; RP, radical prostatectomy; bNED, biochemical non-evidence of disease; NA, not applicable.

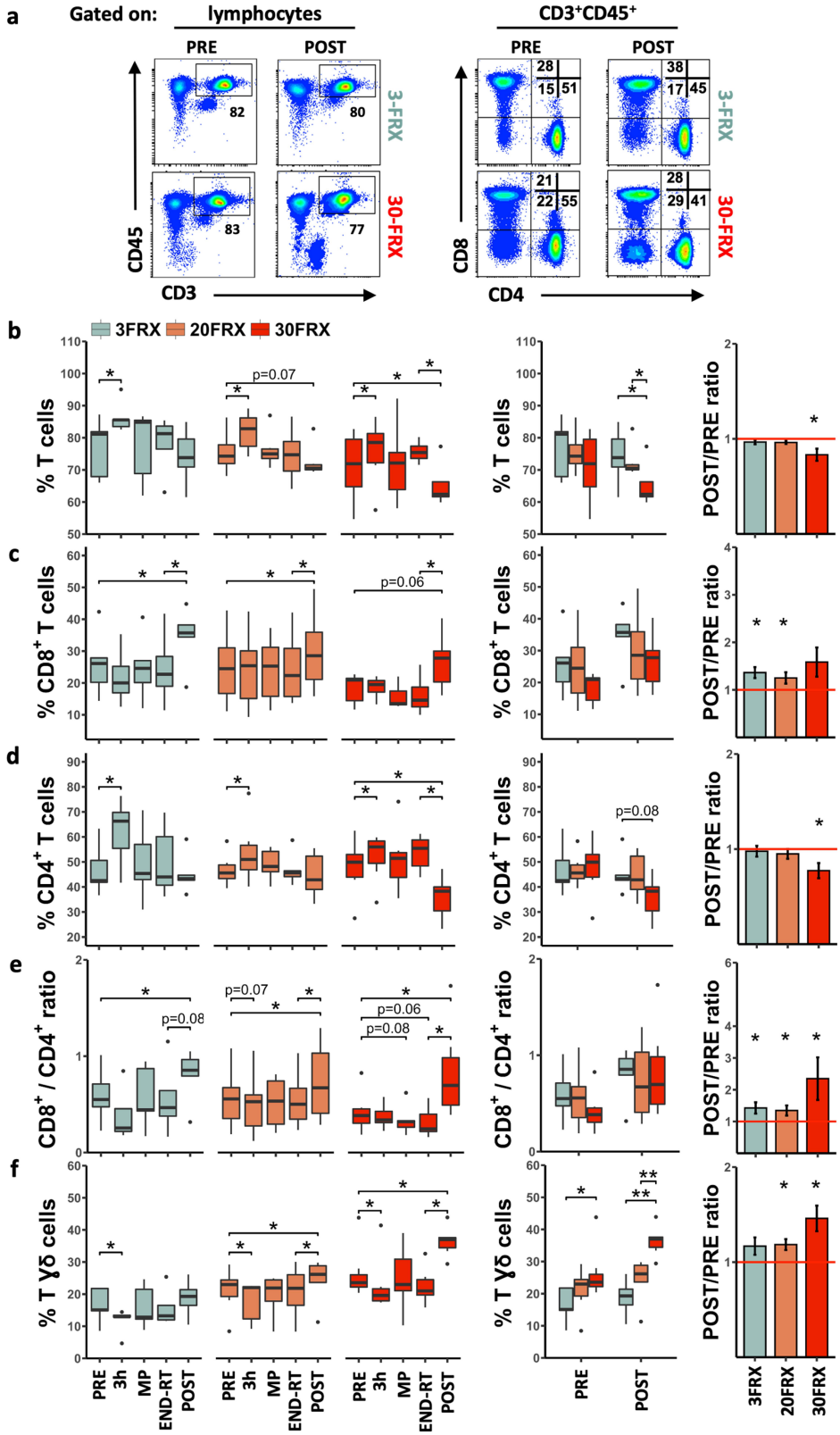

Figure 2. Impact of RT-strategies on immune cell frequency. (a, g) Dot-plots representing immune cell population, evaluated by multicolor flow cytometry, in two patients, at PRE and POST (6mo). (b-f, h-m) Longitudinal analysis of 18 patients (Group1-3FRX, n = 5; Group2-20FRX, n = 6; Group3-30FRX, n = 7), showing the frequency of total T cells ($CD3^+CD45^+$ on total lymphocytes) (b), $CD8^+$ T cells (on $CD3^+CD45^+$ T cells) (c), $CD4^+$ T cells (on $CD3^+CD45^+$ T cells) (d), CD8-to-CD4 ratio (e), γδ T cells ($CD3^+CD4^-CD8^-$ on $CD3^+CD45^+$ T cells) (f), Treg cells ($CD4^+CD25^{high}CD127^-$ on $CD3^+CD45^+CD4^+$ T cells) (h), Treg-to-$CD8^+$ ratio (i), NK cells ($CD3^-CD56^+$ on $CD45^+$ lymphocytes) (l) and B cells ($CD3^-CD19^+$ on $CD45^+$ lymphocytes) (m) (Wilcoxon U-test). Middle panels, comparison among Groups (Mann-Whitney U-test). Right panels, normalization calculated as intra-individual patient ratio between 12 months (POST) and baseline level (POST/PRE ratio). The red line indicates a ratio of 1, representing no change between PRE and POST treatment. The P value is calculated by Wilcoxon U-test between POST versus baseline and follow-up versus treatment-end. *p ≤ .05. *Abbreviation*: FRX = fraction; MP = Middle point; POST = 6 months after RT.

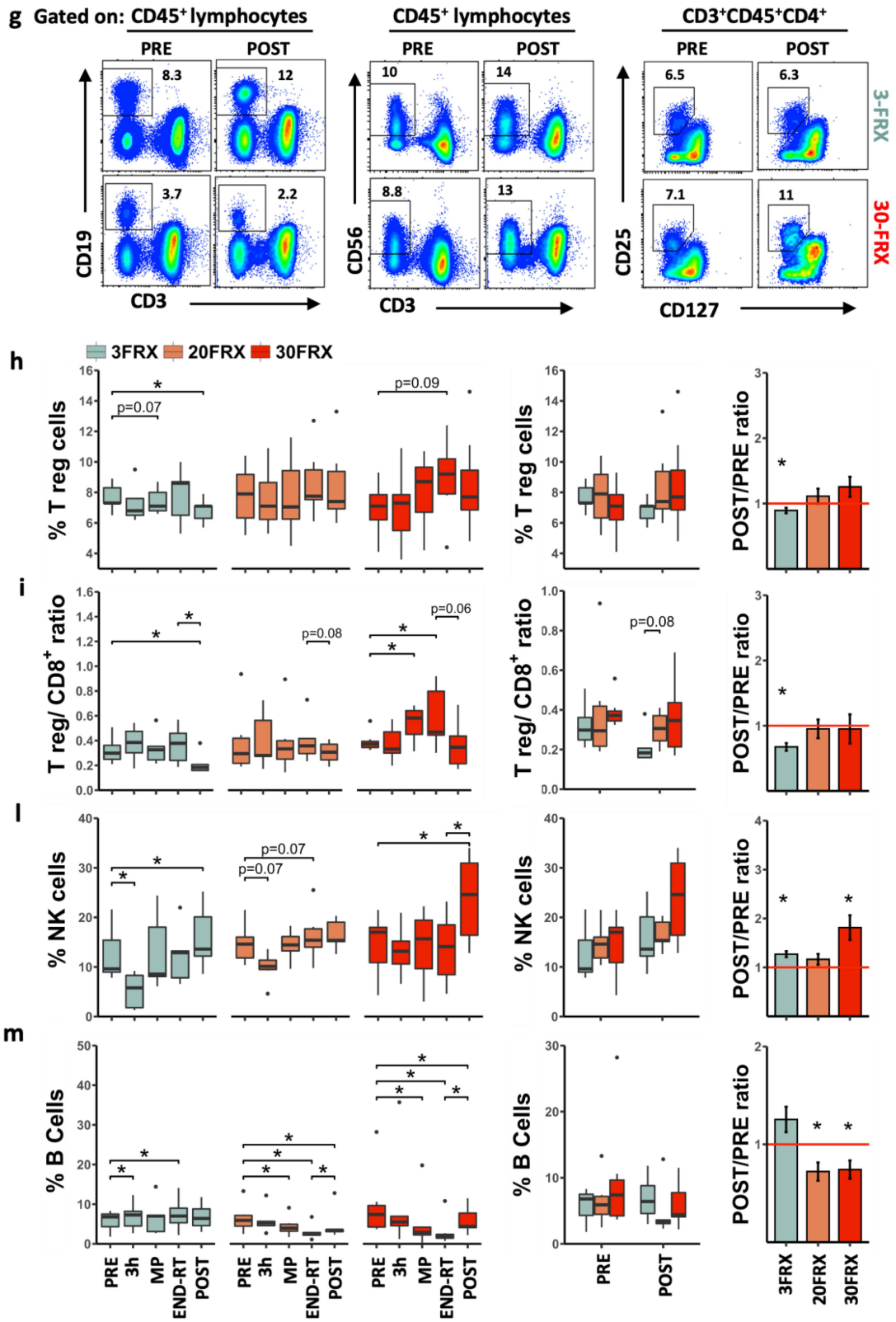

Figure 2. (Continued)

A significant increase was observed as well at 3 h and 6 months in Group-3 (Figure 1e), with a higher neutrophil-to-lymphocyte ratio at treatment-end, which was not fully restored after 1 year (Figure 1f).

Comparison of CBC after 1 year among the 3 groups clearly showed that lymphocyte count was higher in Group-1 than in Groups-2 and 3 (Figure 1c, middle panel). The comparison of CBC between the 18 patients (at baseline) and 12 sex/age-matched HD did not evidence statistically significant differences (Supplementary Figure S1).

Effect of RT strategies on immune cell frequency and functionality

Immunophenotyping of peripheral immune cells was performed by multicolor flow cytometry (gating strategy Supplementary Figure S2) and results are shown in Figure 2. The frequency of T and B lymphocytes (CD3, CD4, CD19) were detected in whole fresh blood and isolated PBMCs, with comparable results (Supplementary Figure S3).

Immune cells frequency did not show significant differences among Groups at baseline (Figure 2, middle panels), similarly to CBC, with the exception of γδ-T cells higher in Group-3.

A significant increase of total T cells ($CD3^+CD45^+$) and conventional $CD4^+$ T cells was observed in all Groups (Figure 2b and d) at 3 h. At treatment-end, both subsets returned to baseline but Group-3 showed a significant decrease after 6 months compared to baseline and treatment-end values (Figure 2b and d). Conversely, within $CD8^+$ T cells a slight decrease was observed in Group-1 at 3 h and treatment-end, showing a significant recovery after 6 months compared to baseline. Groups-2 and 3 showed a significant increase of $CD8^+$ cells after 6 months, accompanied by an increment of CD8-to-CD4 cell ratio in all groups at 6 months (Figure 2c and e).

Looking at Treg subset, defined as $CD3^+CD4^+CD25^{high}CD127^-$, a progressive increase during RT was noticed with highest value at treatment-end, although not reaching statistical significance in any groups. Notably, only in Group-1 a significant decrease of Treg was observed at 6 months compared to baseline (Figure 2h). Furthermore, while in Group-1 the Treg-to-CD8 ratio remained stable during treatment and then significantly decreased at 6 months, in Group-3 an increase was observed during and at treatment-end, recovering after 6 months (Figure 2i).

The percentage of γδ-T cells, evaluated in terms of $CD3^+CD4^-CD8^-$, showed a reduction at 3 h in all groups, then increased at 6 months in Group-2 and Group-3 with respect to baseline and treatment-end (Figure 2f). The evaluation of total NK cells ($CD3^-CD56^+$) showed a significant increase in Group-1 and 3 at 6 months (Figure 2l).

As regard to B-cell frequency ($CD3^-CD19^+$), we observed a progressive increase in Group-1 and a progressive decrease in Groups-2 and 3 during RT. Notably, while in Group-1 the proportion of B cells at 6 months resolve to baseline, in Groups-2 and 3 there is only a partial recovery that not reached baseline value (Figure 2m).

Comparison of immune cell frequency among different groups after 6 months of follow-up showed significant differences in $CD3^+$ T-cell subset, higher in Groups-1 and 2 then in Group-3, and in γδ-T cells, higher in Group-3 (Figure 2, middle panels).

No significant differences were observed in the proportion of T and NK cells between patients before treatments and sex/age-matched HD. Otherwise, B-cells were higher in HD (Supplementary Figure S5), in line with literature.[8,13,14]

To determine whether different RT treatment strategies may influence functionality of T and B cells, we performed an intracellular staining for cytokines production (IFN-γ, TNF-α and IL-10) after anti-CD3 activation (Figure 3 and Supplementary Figure S4 a-c). In Group-1 and 3 functionality of $CD8^+$ T cells was not affected by RT treatments, with only a slight reduction of IFN-γ and TNF-α 3 h after RT in Group-1 (Figure 3a and b). Differently, $CD8^+$ T cells from Group-2 showed a decrease in IFN-γ and TNF-α production along the treatment, with a significant drop at 6 months, compared with treatment-end and baseline. Although at baseline the frequency of T cells was comparable in all groups (Figure 2, middle panels), we noticed that $CD8^+$ T cells of Group-3 showed a baseline higher level of IL-10, stable at treatment-end and significantly higher with respect to Group-1 (Figure 3a and b).

Within total $CD4^+$ T cells, we observed a weak functional decrease of TNF-α production in Group-1 at 6 months from RT (Figure 3c and d), likely to ascribe at the reduction of Treg cells (Figure 2h, i). In agreement, Group-3 showed higher production of all three cytokines in $CD4^+$ T-cell population at 6 months compared to Group-1, suggesting that 30FRX among the CD4 pool advantages Treg frequency (Figure 2h and i) and functionality (Figure 3d).

Looking at B-cells we found an increase of IL-10 production, at 3 h and treatment-end only in Group-3 (Figure 3e and f). No significant differences were observed in the cytokine production between PCa patients before treatments and HD, with the exception of IL-10 that was lower in $CD4^+$ T cells of PCa patients (Supplementary Figure S5).

Effect of RT strategies on maturative differentiation

Radiotherapy has been reported to preferentially eliminate $CD45RA^+$ T-cells,[15] thus we assessed the longitudinal differentiation status of $CD8^+$ and $CD4^+$ T lymphocytes, based on CD45RA and CCR7 expression (Figure 4 and Supplementary Figure S4 d,e). Baseline values showed no significant differences among groups (Figure 4b,d right panels).

As regard to total $CD8^+$ T cells, we observed a progressive increase of central-memory (CM, $CD45RA^-CCR7^+$) $CD8^+$ T cells until the end of RT in all groups, returning to baseline after 6 months for Groups-2 and 3, with a trend of higher percentage in Group 1. Notably, only in Group-1 we observed an increase of the effector-memory subset (EM, $CD45RA^-CCR7^-$), accompanied by a decrease of naïve (-$CD45RA^+CCR7^+$) and terminally-effector cells (EMRA, $CD45RA^+CCR7^-$) at 6 months after RT (Figure 4a and b).

As regard to total $CD4^+$ T cells, similarly to $CD8^+$ lymphocytes, an increase of CM subsets occurred at treatment-end in

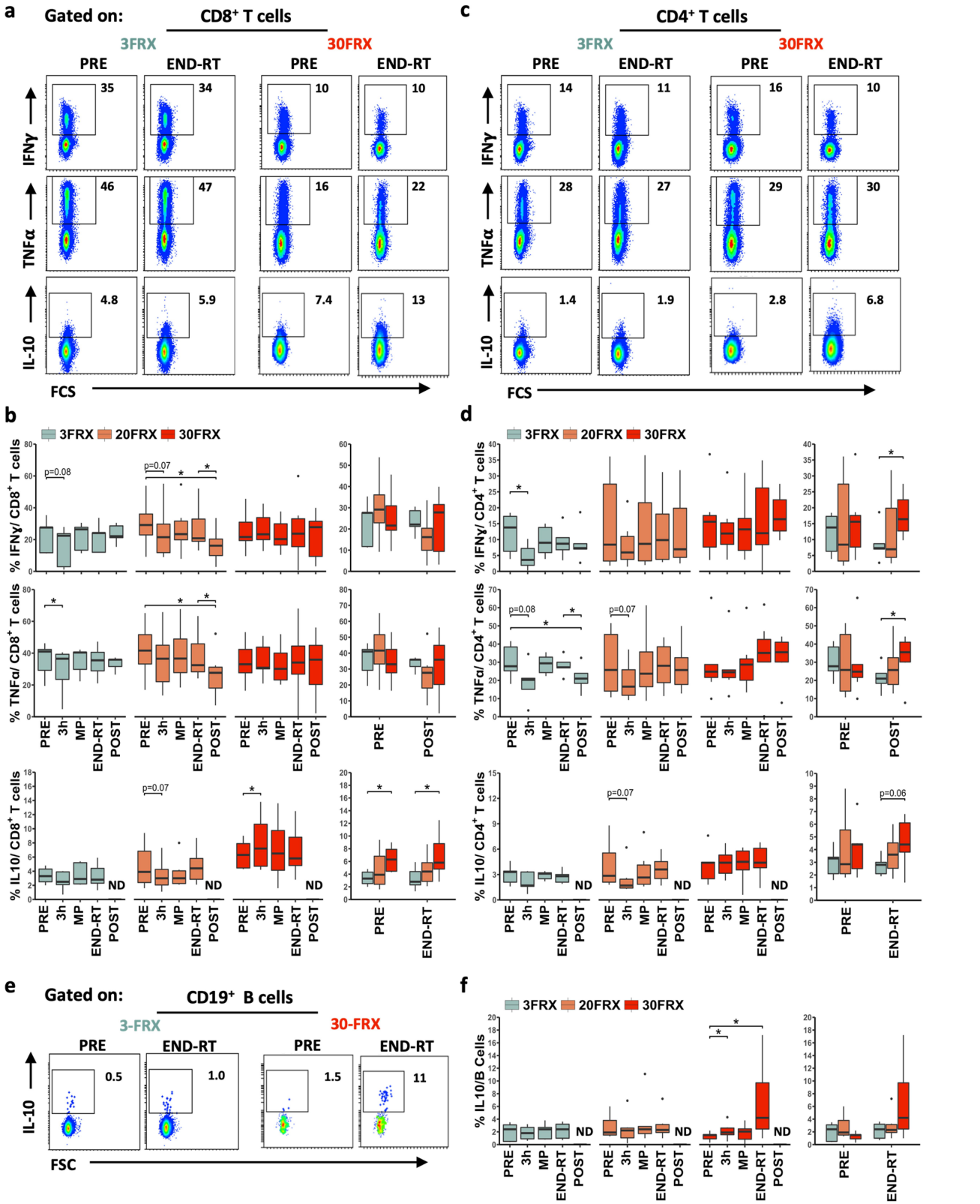

Figure 3. Intracellular multicolor staining for cytokine- production (IFN-γ, TNF-α and IL-10), after 5h of anti-CD3 activation, in $CD8^+$ (a-b), $CD4^+$ (c-d) and B (e-f) cells, on 18 patients (Group1-3FRX, n = 5; Group2-20FRX, n = 6; Group3-30FRX, n = 7). (a, c, e) Dot-plots representing cytokine production in gated $CD8^+$ (a), $CD4^+$ (c) and B (e) cells, from two representative patients, at baseline (PRE) and treatment-end (END-RT). (b-d) Proportion of $CD8^+$ (b) and $CD4^+$ (d) producing IFN-γ (top panels) TNF-α (middle panels) and IL10 (bottom panels), during treatment (left panels) (Wilcoxon U-test). (f) Proportion of B cells producing IL10 during the treatment (Wilcoxon U-test). Right panels, comparison among Groups, (Mann-Whitney U-test). *p ≤ .05. *Abbreviation*: FRX = fraction; MP = Middle point; POST = 6 months after RT; ND = not determined.

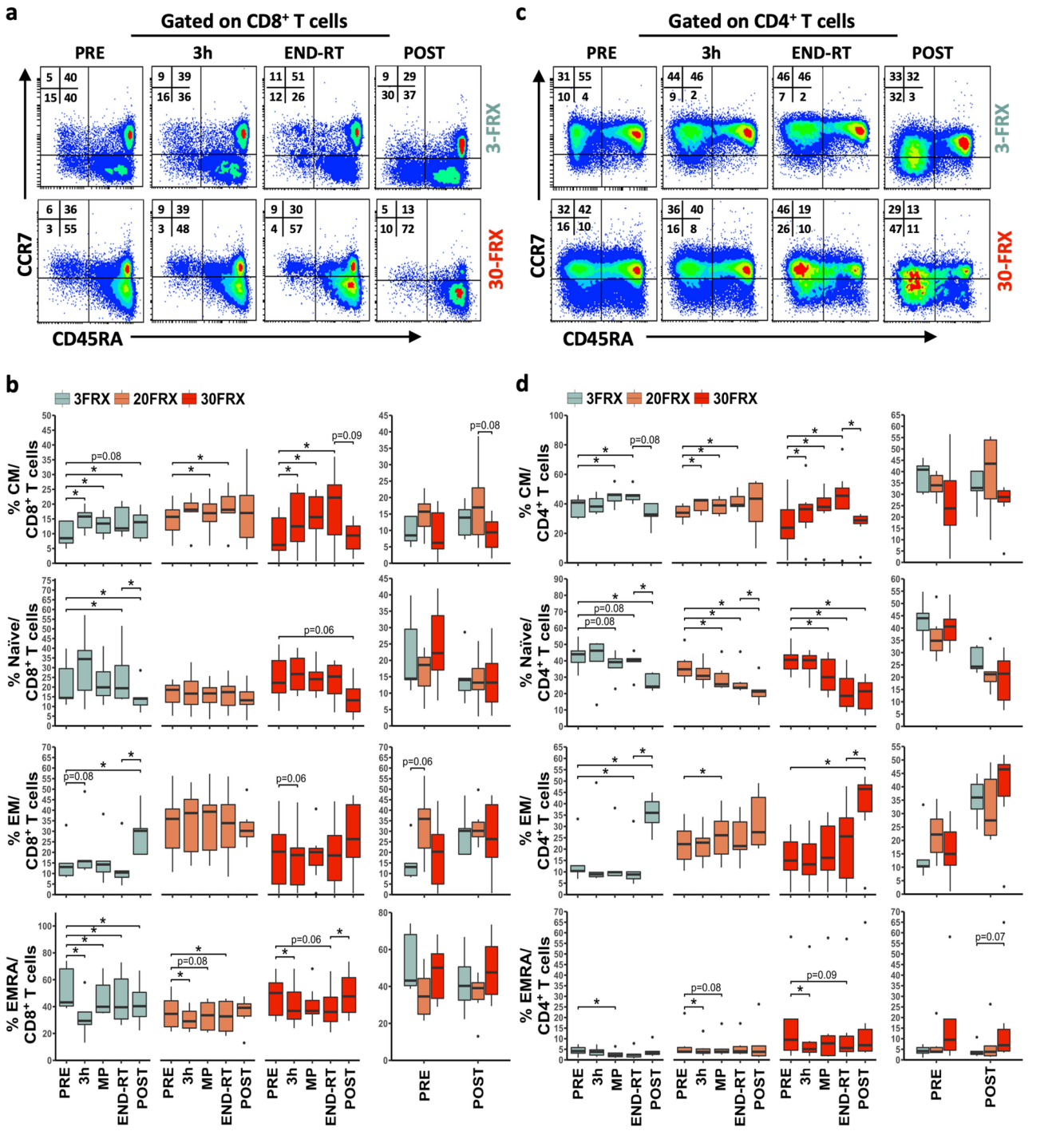

Figure 4. SBRT determines an increase of central-memory and effector-memory T cells. (a-c) Dot-plots showing CCR7 versus CD45RA staining, evaluated by multicolor flow cytometry, in gated $CD8^+$ (a) and $CD4^+$ (c) T cells from two representative patients, during and after RT. (b-d) Pooled results from 18 patients (Group1-3FRX, n = 5; Group2-20FRX, n = 6; Group3-30FRX, n = 7), showing the proportion of central-memory (CM, $CCR7^+CD45RA^-$), naïve ($CCR7^+CD45RA^+$), effector-memory (EM, $CCR7^-CD45RA^-$) and terminally-effector (EMRA, $CCR7^-CD45RA^+$) T cells, in gated $CD8^+$ (b) and $CD4^+$ (d) T cells (left panels) (Wilcoxon U-test). Right panels, comparison among Groups (Mann-Whitney U-test). *p ≤ .05. *Abbreviation*: FRX = fraction; MP = Middle point; POST = 6 months after RT.

all groups, returning to normal baseline after 6 months, along with a progressive decrease of naïve cells in all groups. Looking at EM subsets, we observed an increase, mostly evident in Group-1 and Group-3 at 6 months of follow-up, compared with baseline and treatment-end (Figure 4c and d).

From comparing patients versus HD emerged that patients have higher level of both $CD4^+$ CM and $CD8^+$ CM T-cells, and lower levels of $CD4^+$ EM T-cells (Supplementary Figure S5).

Effect of RT strategies on inhibitory receptor (IR) expression

The analysis of total single inhibitory receptor expression in $CD8^+$ T cells revealed that lymphocyte-activation gene-3 (LAG3) was the lowest receptor expressed at baseline, and was almost not affected by treatment, with only a trend of up-regulation in Group-3, similarly to mucin domain containing 3

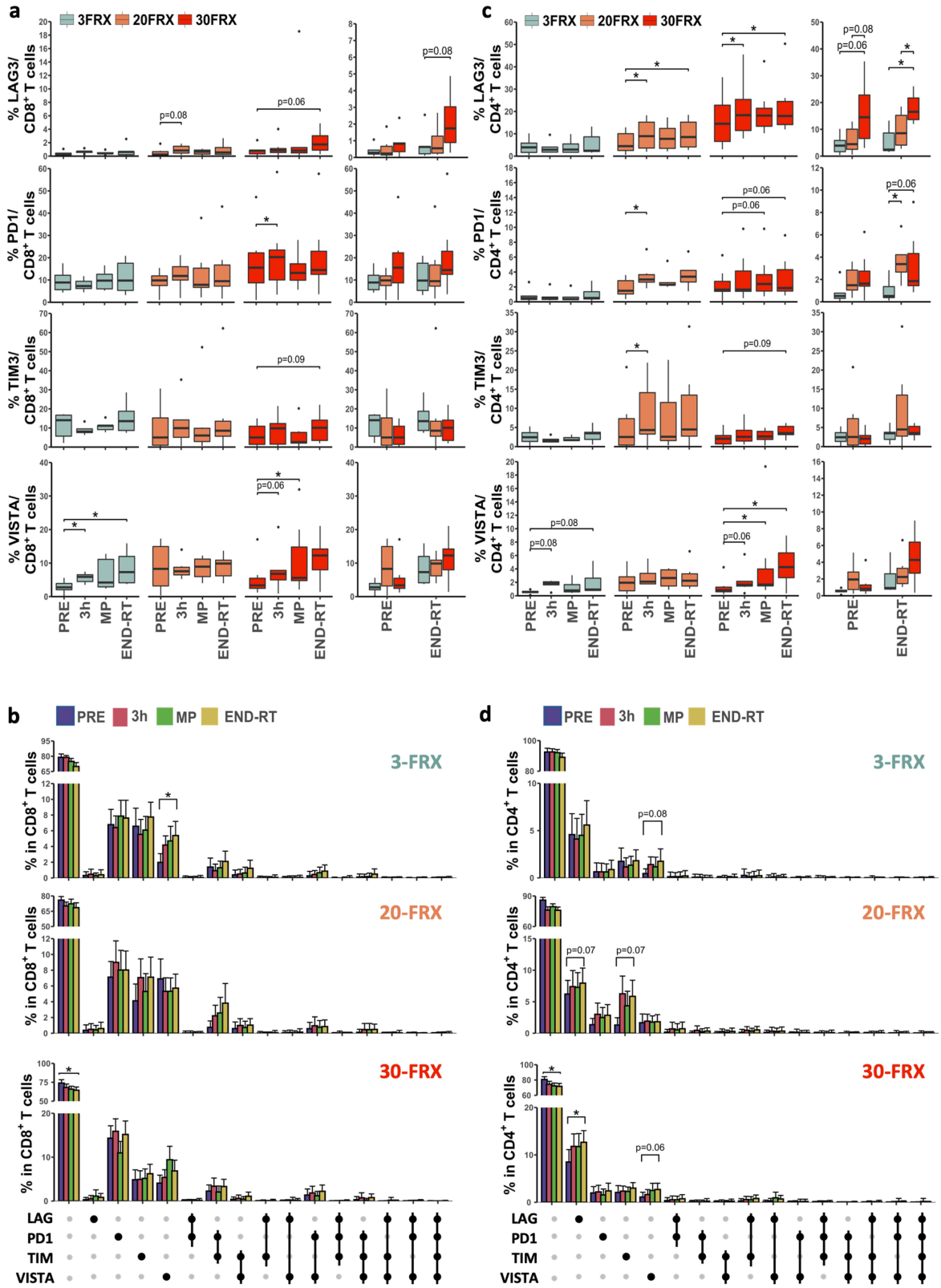

Figure 5. Impact of RT-strategies on inhibitory receptor (IR) expression. (a-c) Analysis of single IR expression (LAG3, PD1, TIM3 and Vista) performed by multicolor staining, in gated $CD8^+$ (a) and $CD4^+$ (c) T cells, in 18 patients during treatment (Group1-3FRX, n = 5; Group2-20FRX, n = 6; Group3-30FRX, n = 7) (Wilcoxon U-test). Right panels, comparison among Groups (Mann-Whitney U-test). (b-d) Quantification of 16 IR simultaneous co-expression in gated $CD8^+$ (b) and $CD4^+$ (d) T cells. Each bar represents the mean (± SEM) percentage (Wilcoxon U test). *p ≤ .05. *Abbreviation*: FRX = fraction; MP = Middle point.

(TIM3) (Figure 5a). Interestingly, the expression of V-domain immunoglobulin suppressor of T cell activation (VISTA) showed a significant up-regulation at 3 h and treatment-end in Group-1 and at midpoint in Group-3 (Figure 5a, lower panel). Differently, the expression of programmed cell death 1 (PD1) was not affected by RT, with the only exception of transient up-regulation at 3 h in Group-3.

When evaluating the concomitant expression of IRs we found that PD1, TIM3 and VISTA were merely expressed singularly, with a significant increase of $VISTA^{+}PD1^{-}LAG^{-}TIM^{-}$ $CD8^{+}$ T cells in Group-1 (Figure 5b). We did not observe simultaneous expression of the 4 IRs in any group.

Regarding total $CD4^{+}$ T cells, we evidenced that at baseline the IRs expression was almost absent in Group-1, whereas TIM3 and LAG3 were the most expressed in Groups-2 and 3 respectively (Figure 5c). Considering the effect of the different fractionation doses, Group-3 showed the most robust changes, with a significant up-regulation of LAG3 and VISTA, and a trend of increase of PD1 and TIM3. Group-1 displayed only a trend of rise for VISTA, while Group-2 showed a significant increase of LAG3 (Figure 5c). The analysis of simultaneous expression reflected the results from singular study, including a significant up-regulation of $LAG3^{+}PD1^{-}TIM3^{-}VISTA^{-}$ in Group-3 (Figure 5d).

No significant differences were observed in the IRs expression between PCa patients before treatments and HD (Supplementary Figure S5).

Overall, these results indicate that the different strategies did not affect the simultaneous expression of IRs in both $CD8^{+}$ and $CD4^{+}$ T cells, but selectively influenced the expression of single IRs, and in particular VISTA in $CD8^{+}$ T cells in patients treated with SBRT (Group-1).

Discussion

To define optimal combination strategies of radiotherapy and immunotherapy we need to gather information on the effect of different RT fractionation schedules on the systemic immune cell functions. To date, only few data are available in PCa, a tumor often treated with RT, and known to be resistant to immunotherapy approaches such as ICI.[9,16] Herein, we performed an in-depth immunomonitoring of peripheral immune cell composition, differentiation stage, cytokine production and IRs expression, from baseline to one year after three different RT treatment strategies.

We have previously shown that prostate SBRT in 3 fractions is feasible and tolerated at one year.[6] Here we report, for the first time, that this schedule (Group-1) exerts a favorable effect on immune cell functionality with respect to moderately-hypofractionated RT (Group2-20FRX) and conventionally-fractionated RT (Group3-30FRX). Although our SBRT setting was in 3 fractions, we argue that similar effect on immune cells may be obtained with SBRT in 5 fractions, as reported for other tumors.[17,18]

In the SBRT cohort we found a transient lymphocyte reduction and a neutrophil increase, returning to baseline within 6 months after RT. Conversely, patients treated with 30 fractions showed a more persistent lymphocyte toxicity with a partial recovery, and a higher neutrophils-to-lymphocytes ratio after 1 year of follow-up (Figure 1). These results are in agreement with those observed after 20–30 fractions.[8,13,15] Furthermore, these data suggest that larger irradiated immune-related volumes (bone marrow and vessels) may impair anti-tumor immunity.[19] Indeed, in the present analysis we cannot tease out the effects of dose/fractionation from those of treated volumes. Therefore, the results are to be interpreted as the consequence of a given treatment strategy rather than a single characteristic.

T cell multiparametric flow cytometry analysis highlights that total $CD3^{+}$ and $CD4^{+}$ T-cell frequency and functionality are not affected, differently from Groups-2 and 3 where a decrease of both cells was observed 6 months post-treatment (Figure 2). Notably, Treg frequency and Treg-to-$CD8^{+}$ ratio decreased only in Group-1, in agreement with data showing that SBRT treatment in lung cancer determines a Treg reduction,[18,20] thus supporting the hypothesis that the larger the fraction size the more is the effect on Tregs. On the other hand, the findings that, in patients treated with 30 fractions T-reg/$CD8^{+}$ ratio increases toward an immune-suppressive environment is consistent with the observation from PCa patients treated with multiple fractions over large pelvic treatment volumes.[8,19] This is in agreement with data showing that Tregs are more radio-resistant than effector T cells,[21] due to a reduced apoptosis and an increased proliferation ability, thus underscoring the relevance of monitoring Treg population during radiotherapy.[22]

Although we did not detect any difference in $CD8^{+}$ effector cell frequency modulation with respect to RT strategies, with a $CD8^{+}$ increase in all Groups, we found relevant differences evaluating the $CD8^{+}$ T-cell differentiation and maturation stage. Indeed, only after SBRT we observed a significant increase of both central-memory and effector-memory $CD8^{+}$ T cells (Figure 4a), a subset with a crucial role in long-lasting immunity and protection against tumor recurrence. We could hence speculate that this schedule may favor the induction of molecular pathways able to increase an efficient antigen-presentation and in turn amplify the anti-tumor specific immune response. This is in line with data from McGee and colleagues that observed an increase of activated memory $CD4^{+}$ and $CD8^{+}$ after SBRT[23] and from Evans et al. who described an increase of tumor-reactive $CD8^{+}$ T cells as protective against the risk of PCa progression when treated with SBRT.[24] The increase of effector-memory cells 14 days after SBRT was associated with superior overall survival in oligometastatic PCa.[25]

Another hypothesis that could be generated is that the increase of memory T-cell subsets is due to a preferential elimination of naïve T cells by RT and the resistance of CD45RA T cells to RT-induced apoptosis.[15] These data support the rationale for combination of RT with antigen-based vaccination, also considering the several immunogenic antigens specific for PCa such as the prostatic acid phosphatase and the prostate-specific antigen.[26]

Considering the crucial role of B cells in anti-tumor immune response, B-cell frequency in SBRT-treated patients increases at the end of treatment while this effect was not detectable when RT was performed in 20 or 30FRX. Noteworthy, we found that only in Group-3, B cells produce IL-10 while Group-1 and 2 did not. Further studies are needed to evaluate the composition of B cell subsets and their maturation state, considering the recent role of plasma cells in prolonged recurrence-free survival after surgery for PCa.[27]

Interestingly, the frequency of NK cells, a key population of innate immune response, increased 6 months after RT (Figure 2l), independently from the schedule, indicating no toxic effect on this population. Our results are in line with several studies in PCa,[8,13] lung cancer,[20,28] and breast cancer.[29] A possible explanation for NK increase could be ascribed to the high intracellular glutathione levels of these cells, which allow to maintain homeostasis after radiation stress, as demonstrated in NK-resistance to antineoplastic agents.[30]

To date, there is still lack of consensus on the dose, fractionation and timing of RT in combination with immunotherapy. So far ICI therapy, alone or combined with RT, has been tested mostly in metastatic castration-resistant PCa,[9,16,31,32] and the translation to earlier stages of disease may raise further challenges. Gao et al. reported that patients treated with Ipilimumab had an increased expression of VISTA,[33] indicating that is crucial to in-depth analyze the impact of RT fractionation on the modulation of IRs expression in T cells and in turn in anti-tumor immune response. In the present study, the analysis of IRs showed a significant increase of VISTA, in absence of other IRs ($VISTA^{+}PD1^{-}LAG^{-}TIM\text{-}\ CD8^{+}$ subset, Figure 4b) at the end of SBRT. Therefore, these results confirm the potential role of VISTA, which is expressed as immune suppressive receptor on the myeloid and lymphoid lineages with an impact on both innate and adaptive immunities[34] and might represent an important new potential immunotherapy target in PCa. Moreover, our results suggest the potential role of VISTA as a new target after SBRT completion, also in consideration of the ability of this dose-fractionation to preserve central-memory and effector $CD8^{+}$ T-cell functions (Figure 2–4). Noteworthy, SBRT has been reported to promote immunogenic cell death, tumor antigens release and in turn immunotherapy efficacy.[35] However, differently from PD-1,[36] the precise role of VISTA as inhibitor[16,37] or activator receptor is still unclear in PCa. Indeed, Loeser et al.[38] showed that VISTA expression on tumor-infiltrating lymphocytes was correlated with improved overall survival within esophageal adenocarcinoma, suggesting that VISTA could represents a positive prognostic marker other that a therapeutic target in this cohort of patients.

Differently from what observed with SBRT, patients of Group-3 presented an up-regulation of $LAG3^{+}PD1^{-}TIM3^{-}VISTA^{-}$ (Figure 5d), likely to ascribe to the concomitant increase of immune suppressive Treg. Noteworthy, this phenotype is associated with other immune suppressive factors one year after treatment (Figure 1b,c,f and Figure 2m), suggesting that conventional-fractionated RT in PCa could negatively impact the immune response.

Our results argue on a favorable role of SBRT on the immune response, and support SBRT as the treatment choice for both localized[7,39] and oligometastatic PCa.[40] In the framework of this treatment with logistic convenience, shorter treatment time and equivalent efficacy compared to conventional RT,[7] the identification of immunological advantages may be of great impact for both patients and physicians.

It is important to note that this was an exploratory study without a clinical endpoint. The limits of our study were the small number of patients, and the heterogeneity between Groups, especially in terms of AD and treatment volumes. According to Hoffman and colleagues[19] the early suppression of anti-cancer immunity is directly related to large volumes of treatment, and in particular with immune related structures such as blood vessels and bone marrow. Recently two different head and neck studies highlight that lymphatic preserving treatments favor systemic antitumor immunity, whereas elective nodal irradiation may decrease immune cell functionality.[41,42] One may argue that this was the case of conventionally fractionated RT (Group-3) where both prostatic bed and pelvic lymph nodes were irradiated. Sini et al.[43] has shown how whole pelvis RT in PCa can induce hematological toxicity, and the use of specific constraints on bone marrow may help limiting this effect. In order to try to tease out the effect of treated volume from dose/fractionation, we have decided to contour these additional immune related structures for a further analysis on a larger cohort and to investigate dosimetric parameters which may correlate with a higher immune toxicity in all 3 Groups.

Finally, regarding AD, since in a previous analysis[43] no detrimental effect of AD was found in terms of both acute and late hematological toxicities, we do not believe this to be a major confounding factor.

We think that other than the comparison between Groups and the different RT strategies, one of the strengths of our results was the preservation of immune cells functionality in SBRT patients, where the effect measured was entirely pure without any potential influence of AD and/or larger volume with immune related structures irradiated. Further studies with more balanced Groups are needed to strongly confirm our results, and a new study directly comparing CFRT and SBRT in the salvage setting is recruiting patients.

Furthermore, it would be of major clinical interest to determine if all the changes in periphery could reflect microenvironment alteration, as reported by Kane et al.[44]

Conclusion

Our study provides a detailed analysis on the role of different RT strategies on systemic immune cell functions. The in-depth monitoring at precise timing evaluating frequency, differentiation stage and functionality of several immune cells, the analysis of four IRs, singularly or concomitantly expressed, were, to our knowledge, analyzed for the first time in PCa patients. Importantly, we found that SBRT, other than advantageous for shorter treatment schedule and the health care cost benefit, preserve central-memory and effector-memory T-cell functions and decrease the immunosuppressive Treg population. Further studies are needed to correlate the systemic immune changes with patient's clinical outcome and to define potential biomarkers of recurrence. Finally, the increase of VISTA observed after SBRT may provide a rationale for combining VISTA blockade after SBRT in PCa patients.

Trial registration

Clinicaltrials.gov NCT04774133, registered February 23 2021, retrospectively registered; https://clinicaltrials.gov/ct2/show/NCT04774133

Data availability statement

Research data are stored in an institutional repository and will be shared upon reasonable request to the corresponding author.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the 'Regina Elena' National Cancer Institute (protocol code RS1163/18).

Author contribution

B.P., A.F., M.B. P.N. and G.S. contributed conception and design of the study. P.N. and G.S. supervised the data. M.B., A.F. and G.S. enrolled and treated patients and collected the clinical information. B.P., I.C and P. N. designed the experiments. B.P., M.P. and S.M. performed the experiments and analyzed the data. B.P., M.B., A.F., I.C. and P.N interpreted data. I.S. and G.F performed statistical analysis. M.L.F. supplied blood samples from healthy donors. B.P., M.B. P.N. and G.S. wrote the manuscript. All authors read and agreed on the final version of the submitted manuscript.

Disclosure statement

The authors report there are no competing interests to declare

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