Research paper

IgM-Rheumatoid factor confers primary resistance to anti-PD-1 immunotherapies in NSCLC patients by reducing CD137+T-cells

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A R T I C L E   I N F O

A B S T R A C T

Background: ICIs have strongly improved the outcome of NSCLC patients. However, primary and secondary resistance occur during treatment in most of the patients, with several of them developing fast progressions. Autoantibodies can be related with a dysfunctional immune system, although their association with immune-based anti-cancer therapies has never been investigated. Moreover, so far no reliable predictive factor is currently available to aid in treatment selection. CD137+T-cells are largely known to be the anti-tumor activated effector cells, but they have never been associated with the response to immunotherapies.
Methods: Forty-two patients with metastatic NSCLC receiving anti-PD-1 ICIs at Sant'Andrea Hospital and Policlinico Umberto I, from June 2016 to September 2018 were enrolled. Circulating levels of IgM-Rheumatoid Factor were evaluated at baseline and correlated with patients clinical response following the anti-PD-1 treatment. IgM-RF interaction and effect on T-cells in vivo and in vitro were investigated.
Findings: IgM-RF in NSCLC patient sera strongly predicted the development of early progression to ICIs. Also, a significant reduction of progression-free survival rate in anti-PD-1 treated patients could be identified when patients were stratified based on IgM-RF positivity and titers. IgM-RF bound preferentially circulating naïve and central memory T-cells and a significant reduction of CD137+ anti-tumor T effector cells was found in IgM-RF positive patients. In addition, a higher percentage of CD137+T-cells in peripheral blood of NSCLC patients at baseline resulted as a strong independent prognostic factor for a better outcome in terms of PFS and OS after the anti-PD-1 treatment. Furthermore, T-cells exposed to IgM-RF showed a robust defect in their migratory ability in response to CCL19 chemokine.
Interpretation: In this study we showed that serum IgM-RF can be regarded as predictive factor for the development of early progression and prognostic factor of a reduced progression-free survival and overall-survival in anti-PD-1 treated NSCLC patients. The ability of IgM-RF to bind naïve and central memory T-cells and impair their migration could make account for the reduction of the tumor-reactive CD137+ T-cells population that may cause a non-effectiveness of these T-cells targeting drugs.
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1. Introduction

Despite recent progresses in cancer treatment obtained with novel immunotherapies, several patients show a primary or secondary resistance, leading to fast progressions. The mechanisms of this resistance remain unknown, but different hypothesis can be outlined. Doubtless, host related factors and the fitness of patient immune system may contribute [1]. Indeed, the identification of immune biomarkers that can aid clinicians in the choice of the right therapy to reach the maximum benefit for the patient is of utmost importance.

The relation between autoimmune diseases and cancer is currently an emerging area of interest since it still remains a clinically challenging issue. In fact, some of the pathogenic mechanisms are shared between these two conditions, although direct proof of their role as causative factors is lacking, as in the case of serum autoantibodies [2].

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Research in context

Evidence before this study

We searched PubMed on Sept 4, 2018, using the following terms: "Immune Checkpoint Inhibitors AND NSCLC AND resistance OR early progression OR autoimmunity OR autoantibodies" AND "Rheumatoid Factor AND cancer" AND "Rheumatoid Factor AND isotypes OR role OR healthy subjects". The search was not limited by date, but was limited to articles and abstracts published in English only. From these studies we could point out that several NSCLC patients undergoing an ICI treatment developed fast resistance to the therapy leading to fast progressions; however it was not possible to identify a biomarker capable of predicting the response to the immunotherapy. A thorough review of the literature also showed as RF in patient sera could be associated with an higher risk of developing cancer, but the biological mechanism was unknown. Moreover, even if the relation between autoimmune diseases and cancer was already established, researches about the possible association between the presence of autoantibodies in sera and the response to the immunotherapies were lacking.

Added value of this study

Results of this retrospective study show that IgM-RF in patient sera is both a predictive factor for the development of early progression in response to an anti-PD-1 treatment and also an important prognostic factor for a progressive disease, probably due to its ability of impairing central memory and naïve T-cell migration and a subsequent reduction of CD137$^+$ anti-tumor T effector cells in RF-positive patients. The percentage of CD137$^+$ T-cells at baseline strongly influenced NSLC patients' outcome in response to the anti-PD-1 treatment. This study represents, to the best of our knowledge, the first evidence of IgM-RF as a possible biomarker that can predict the T-cell fitness of NSCLC patients and thus their capability to respond to an anti-PD-1 treatment. We also demonstrated for the first time that the CD137+ T-cell population is a key factor in driving the NSCLC patients response to the immunotherapies. Moreover, this study identifies IgM-RF as a novel prognostic factor for a progressive disease and a worse outcome in NSCLC patients.

Implications of all the available evidence

Our data suggest that the dosage of IgM-RF in NSCLC patients can be introduced in the routine clinical practice in order to select the correct therapeutic plan to get the best patient response. Moreover, our study indicates that the presence of IgM-RF and the percentage of CD137$^+$ T-cells at baseline can be considered together with the other well established prognostic factors in order to improve NSCLC patients stratification. Further studies including higher numbers of patients and different types of tumors will be needed to extend the knowledges about this new biomarker.

Rheumatoid Factors (RFs) are autoantibodies which bind the constant regions (Fc) of IgGs and mainly occur as IgM antibodies (IgM-RF). RF was initially discovered in serum of patients with Rheumatoid Arthritis (RA) of which IgM-RF is still considered a fundamental marker for the diagnosis and prognosis [3,4]. Later, it was highlighted that RFs could also be found in high titers in sera of patients with other diseases (both autoimmune and non-autoimmune) in the same way as healthy subjects [5,6].

About the possible role of RFs in cancer, little is known. Some studies suggested the possible correlation between RFs concentrations in patient sera and the increase of cancer risk [7−10], tumor recurrence and load [11,12], but no biological mechanism has been described. Moreover, the clinical association between RFs and the response to immunotherapies has never been investigated. This can be a crucial issue in ICI treatments, since RFs are autoantibodies directed against IgGs (the belonging class of ICIs monoclonal antibodies) and, differently from other autoantibodies, can be found in high titers in healthy subjects.

The CD137$^+$T-cell population has been largely referred as the real T effector cells population activated against cancer cells [13,14], but its role as a possible driver of the response to the immunotherapies in oncologic patients has never been probed.

In this study, we retrospectively assayed the IgM-RF levels in the sera of non-small cell lung cancer (NSCLC) patients in treatment with two anti-PD-1 immune-checkpoint inhibitors (ICIs), Nivolumab and Pembrolizumab, in order to identify a possible association between the failure of T-cells based therapies and an altered immune system.

2. Methods

2.1. Patients

Patients with metastatic NSCLC receiving Nivolumab or Pembrolizumab at "Sant'Andrea" Hospital and "Policlinico Umberto I", "Sapienza" University of Rome from June 2016 to September 2018 were enrolled in this study. Inclusion criteria were: age > 18 years; histologically-documented diagnosis of NSCLC; metastatic disease; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; measurable disease; adequate cardiac, pulmonary, renal, liver and bone marrow function; patients with stable and asymptomatic central nervous system metastases were eligible. Exclusion criteria were: autoimmune diseases; symptomatic interstitial lung disease and any other significant comorbidity; systemic immunosuppression; prior treatment with immune-stimulatory antitumor agents including checkpoint-targeted agents.

Blood samples and sera has been collected from the patients before the beginning of anti-PD-1 treatment (T0) and at different time-points during the treatment for IgM-RF dosage. IgM-RF and anti-cyclic citrullinated peptide (anti-CCP) autoantibodies were detected by using commercial ELISA kits (Diamedix, 720,710 for IgM-RF and DELTA BIOLOGICALS, CD-117 for anti-CCP): the results were evaluated according to the manufacturers' instructions. Peripheral Blood Mononuclear Cells (PBMCs) have been collected and frozen for 32 patients at T0 and then thawed for flow cytometry analysis.

2.2. Treatment, efficacy and safety assessments

Nivolumab was administered intravenously at a standard dose of 3 mg/kg every 2 weeks and Pembrolizumab was administered intravenously at a standard dose of 200 mg every 3 weeks until disease progression or development of unacceptable toxicity. Tumor response was assessed according to clinical practice thereafter until disease progression using immune-related Response Evaluation Criteria in Solid Tumors Criteria (i-RECIST) and classified according to disease control (complete response, partial response and stable disease) and progressive disease (PD). Safety assessments were performed at day 1 of every cycle until the end of treatment and toxicities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0).

2.3. Objectives and outcomes

Progression-free survival (PFS) was defined as the time from ICIs commencement until the first documented tumor progression or death from any cause, whichever occurred first. Early progressor patients were defined as those experiencing disease progression

within 3 months from the beginning of ICIs treatment. Overall survival (OS) was defined as the time from Nivolumab or Pembrolizumab commencement to death from any cause. Data for patients who were alive and did not have disease progression or who were lost to follow-up were censored for the analysis of progression-free survival and overall survival at the time of the last imaging assessment.

2.4. In vitro T-cells assays

Human T-lymphocytes were purified from PBMCs of healthy donors (Ethical Committee Protocol, RIF.CE: 4212) after Hypaque/Ficoll (Cedarlane, CL5020) gradient by EasySep^{TM} Human CD3 Positive Selection Kit II (StemCell Technologies, 17,851) and cultured (100.000 cells/100μl) in RPMI 1640 (Hyclone, R8758) supplemented with 2 mM L-glutamine (Sigma-Aldrich, G8540), penicillin 100 U/ml, streptomycin 100 μg/ml (Penicillin-Streptomycin solution, Sigma-Aldrich, P4333) with 5% heat-inactivated Fetal Bovine Serum (FBS) (Hyclone, SH30071.01). T-cell purity (>90%) was checked prior to each experiment by flow cytometer (FC).

IgM-RFs prepared from human plasma (Athens Research and Technology, 16−16−180,603) and control IgM prepared from plasma of multiple myeloma (MM) patients (Athens Research and Technology, 16−16−090,713-M) (the antibodies have been dialyzed prior to the usage to remove sodium azide) were added to T-cells cultures for 24 h at the concentration of 50 μg/ml (based on IgM-RF titers in positive patient sera). Nivolumab (Bristol-Myers Squibb, BMS) was added for 24 h at the concentration of 25 μg/ml.

For the proliferation assay, PBMCs were stimulated with PHA (5 μg/ml) (Sigma-Aldrich, 61,764) for 4 days at 37 °C in the presence or absence of IgM-RF and the control IgM; PBMCs were pre-treated with CarboxyFluorescein Succinimidyl Ester (1 μM, CFSE) (Life Technologies, C34554) and cell proliferation was monitored through progressive halving of fluorescence in CD3$^+$ labelled cells using FACSCantoII flow cytometer running FACS-Diva data acquisition and analysis software (Becton Dickinson, BD).

For the migration assay purified CD3$^+$ T-cells were activated with PHA (5 μg/ml) for 3 days at 37 °C and cultured for other 24 h with IgM-RF, control IgM or left untreated; cells were then collected, washed twice, re-suspended in migration media (RPMI, 0,1% FBS, 10 mM HEPES) and then were allowed to migrate for 2 h through a 5 μm pore size Transwell inserts (Corning, CLS3421−48). Cells from the bottom chamber were thus harvested and counted for 1 min using FACSCantoII flow cytometer; migration ability was assessed as the percentage of migrated T cells.

Cell viability was checked by 7-AAD (BD, 559,925) prior to each experiment.

2.5. T-cell phenotype

T-cell phenotype was performed by FC employing the following monoclonal antibodies (MoAbs): anti-CD3-BV510 (BioLegend, 317,332), anti-CD4-ApcH7 (BD, 560,158), anti-CD8-Percp5.5 (BioLegend, 344,710), anti-CCR7-FITC (BioLegend, 353,216), anti-CD45RA-Apc (BioLegend, 304,112), anti-PD-1-PE (BioLegend, 621,608), anti-IgM-BV421 (BioLegend, 314,516), anti-CD137-PeCy7 (BioLegend, 309,818) and 7-AAD (BD, 559,925). Cells were incubated with FC-block (BioLegend, 101,302) for 5 min and surface staining was performed at 4 °C for 15 min. At least 10^4 events were evaluated using a FACSCantoII flow cytometer.

2.6. Statistical analysis

To compare early progressions, Odd ratios with associated 95% confidence intervals and p values were calculated with the use of a Woolf logit model and Pearson's Chi-square test; when a group resulted in zero event, it was computed as 0,5 for the OR estimate and its 95% CI. We performed pre-specified clinical subgroups analyses to assess the power of IgM-RF as predictive factor, using ORs that were estimated from a Woolf logit model.

We evaluated the possible association between IgM-RF positivity and the other clinical characteristics using ORs with associated 95% confidence intervals and p-values that were estimated from a Woolf logit model.

The impact of clinic-pathological variables on progression-free survival (PFS) was analyzed by both the univariate and multivariate analyses (UVA and MVA, respectively). With regards to UVA, patients' PFS was analyzed using the Kaplan−Meier method and log-rank tests to obtain Hazard Ratios with associated 95% confidence intervals and p values. Prognostic clinic-pathological variables deemed of potential relevance in the univariate analysis (corresponding to a cut-off of $p < 0.10$) were included in the multivariate Cox proportional hazards regression analysis. The forward stepwise analysis was conducted with a P-IN = 0.05 and a P-OUT = 0.10.

Patients' Overall Survival (OS) was analyzed using the Kaplan−Meier method and log-rank tests to obtain Hazard Ratios with associated 95% confidence intervals and p values.

To evaluate the impact of the percentage of CD137$^+$ T-cells at T0 on PFS and OS, we first calculated the median value of the percentages of CD137$^+$ T-cells at T0 in our patients and then we divided the patients into two groups: > or < the median value. Patients' PFS and OS was thus analyzed using the Kaplan−Meier method and log-rank tests to obtain Hazard Ratios with associated 95% confidence intervals and p values.

For the other experiments, Student's t-test and one-way Anova test were used. A $p<0.05$ was considered statistically significant. When more than two groups have been compared, p values were corrected for multiple comparisons.

The calculated p-value have been corrected for multiple tests using the Bonferroni correction, so the calculated p-value needs to be multiplied for the number of tests and compared with the cutoff p-value of 0.05.

2.7. Ethics

The study was conducted in accordance with good clinical practice guidelines and the declaration of Helsinki. The final version of the protocol was approved by the Institutional Ethics Committee of the two "Sapienza" Hospital Centers involved (Ethical Committee approval, protocol n° 805/16, RIF.CE: 4181).

All the patients gave written informed consent.

2.8. Role of the funding source

3. Results

3.1. Patients characteristics

From June 2016 to September 2018 a total of 42 metastatic NSCLC patients who were about to begin an anti-PD-1 therapy with Nivolumab or Pembrolizumab as first, second, third or fourth line treatment were enrolled. Five patients were administered with Pembrolizumab, while 37 were administered with Nivolumab. Response to the immunotherapy has been monitored, evaluating in particular early progressions (within 3 months). Table 1 reports patient clinical characteristics.

Among these patients, 57.1% had Squamous-cell carcinoma, while 38.1% had Adenocarcinoma. The average age was 67.8 years, 92.9% of

Table 1
Characteristics of the patients at enrollment, according to IgM-RF positivity.

Characteristic	All patients $N = 42$	Patients RF_{neg} $N = 31$	Patients RF_{pos} $N = 11$
Age - yr			
Mean ± SD	67.8 ± 9.8	66.8 ± 10.7	70.9 ± 5.9
Sex - no (%)			
male	28 (66.6)	19 (61.3)	9 (81.8)
female	14 (33.3)	12 (38.7)	2 (18.2)
Histologic diagnosis - no (%)			
Adenocarcinoma	16 (38.1)	10 (32.2)	6 (54.5)
Squamous-cell carcinoma	24 (57.1)	20 (64.6)	4 (36.4)
Other	2 (4.8)	1 (3.2)	1 (9.1)
Smoking status - no (%)			
Never	3 (7.1)	2 (6.4)	1 (9.1)
Former or current	39 (92.9)	29 (93,6)	10 (90,9)
Previous surgery - no (%)			
yes	10 (23.8)	7 (22.6)	3 (27.3)
No	32 (76.2)	24 (77.4)	8 (72.7)
Adjuvant chemotherapy ± radiotherapy - no (%)			
Yes	4 (9.6)	2 (6.4)	2 (18.2)
No	38 (90.4)	29 (93.6)	9 (81.8)
ECOG Performance status score - no (%)			
0	17 (40.5)	14 (45.2)	3 (27.3)
1	20 (47.6)	14 (45.2)	6 (54.5)
2	5 (11.9)	3 (9.6)	2 (18.2)
Sites of metastasis at beginning of ICI treatment - no (%)			
1 site	7 (16.7)	5 (16.1)	2 (18.2)
2 sites	19 (45.3)	12 (38.7)	7 (63.6)
3 sites	8 (19)	8 (26)	0 (0)
> 3 sites	8 (19)	6 (19.2)	2 (18.2)
ICIs line of treatment - no (%)			
First line	4 (9.6)	2 (6.4)	2 (18.2)
Second Line	34 (80.9)	26 (84)	8 (72.7)
Third line	1 (2.4)	1 (3.2)	0 (0)
Fourth line	3 (7.1)	2 (6.4)	1 (9.1)

them were former or current smokers, 23.8% had a previous surgery, while 9.6% had an adjuvant chemotherapy ± radiotherapy.

At the UVA, the ECOG PS and the sites of metastasis at the beginning of ICIs treatment resulted significant prognostic factors for a Progressive Disease (PD), as already shown [15] (**Fig. S1**).

3.2. IgM-RF predicts early progression and is a prognostic factor for a progressive disease in NSCLC patients

IgM-RF titers were evaluated in the enrolled NSLC patients at T0: 11 out of 42 (26.2%) were positive for IgM-RF (RF_{pos}, values ≥ 16 IU/ml). Our patients were also tested for anti-CCP autoantibodies: none of them resulted positive. None of the RF_{pos} patients could be classified as affected by RA or other autoimmune diseases. We further stratified RF_{pos} patients in highly positive (RF_{high} subgroup, values ≥ 50 IU/ml) and slightly positive (RF_{low} subgroup, values ≥16 and < 50 IU/ml) (Table 2).

When looking at the possible associations between IgM-RF positivity and the other patients clinical characteristic (Table S1), not surprisingly it resulted significantly associated (OR, 4.83; 95% CI, 0.89 to 25.92; p = 0.05; Woolf logit model and Pearson's Chi-square test) only with an older age (≥ 68 years) of the patients, as previously reported [16−19].

IgM-RF levels did not change through different time-points of ICI treatment (both Nivolumab and Pembrolizumab) for each patient (Fig. S2), thus suggesting that the production and the levels of the autoantibody were not influenced neither by the ICI treatment itself nor by the progression and tumor load. Surprisingly, the presence of IgM-RF in patient sera strongly correlated with the development of a fast resistance to the anti-PD-1 treatment and thus with early progressions; in addition, all the patients with high IgM-RF levels were early progressors (Table 2 and Fig. 1a). Indeed, early progression was observed in 73% of RF_{pos} patients, while it occurred in only 26% of RF-negative (RF_{neg}) patients (p = 0.005, Chi-square test). The power of IgM-RF as a predictive factor for the risk of developing early

progression was calculated with the estimate of the odd ratio, comparing RF_{pos} vs RF_{neg} patients. Considering that 1 represents the risk in RF_{neg} patients, the power of IgM-RF positivity as predictive factor resulted high in both the overall population (OR, 7,66; 95% CI, 1.62 to 36.18; p = 0.005; Woolf logit model and Pearson's Chi-square test) and in most of clinical subgroups (Fig. 1b). IgM-RF resulted not relevant in patient ≥ 68 years and with ECOG PS =2, two subgroups in which clinical and immunological parameters are most probably already compromised. On the other side, it gained validity (OR, 51.67; 95% CI, 1.61 to 1657; p = 0.0008; Woolf logit model and Pearson's Chi-square test) in the group characterized by a young age (< 68 years), thus strengthening its role as independent predictive factor even when its presence is not accompanied by other compromising factors.

In addition, at the UVA, the IgM-RF positivity was a significant prognostic factor for a PD; indeed, the PFS was significantly shorter (p = 0.035, Kaplan−Meier method and log-rank tests) with a median PFS of 75 days in RF_{pos} patients compared to RF_{neg} patients, for which the median PFS was 240 days (Fig. 2a, b). A significant increase of the probability to develop a PD following ICI therapy and a lower PFS rate (p = 0.0024, Kaplan−Meier method and log-rank tests) was also associated to IgM-RF titers when patients were divided in RF subgroups (Fig. 2a, b). Moreover, at both the MVA methods we used, the RF positivity or RF values and the ECOG PS retained their statistical significance as prognostic factors; both MVA tested methods resulted equally valid and significant (p = 0.001, Cox proportional hazards regression analysis) (Fig. 2b). Sites of metastases has also been included as input variable in the multivariate analyses, being a significant factor in the univariate analysis. For the forward stepwise analysis, a P-IN = 0.05 and a P-OUT = 0.10 were used. Of note, the forward stepwise selection method did not retain this variable in the final models because they did not significantly improve the prediction of the two calculated models. Therefore, only the variables with statistically significant results were added in the table, reporting their HR and 95% CI.

Table 2
IgM-RF values and early progression.

Patient RF subgroup	Patient	IgM-RF value (IU/mL)	Early Progression
RF$_{neg}$ (<16 IU/ml)	PT 3	2.08	No
	PT 4	6.07	No
	PT 6	4.21	Yes
	PT 7	4.05	No
	PT 8	11.93	No
	PT 9	11.84	No
	PT 11	15.72	No
	PT 12	1.75	Yes
	PT 14	5.28	No
	PT 15	3.57	No
	PT 16	3.97	No
	PT 17	4.75	No
	PT 19	11.63	No
	PT 20	4.54	No
	PT 22	5.87	Yes
	PT 24	11.57	No
	PT 25	9.23	Yes
	PT 26	11.93	Yes
	PT 28	1.13	No
	PT 29	13.54	No
	PT 30	3.78	No
	PT 31	9.47	No
	PT 32	1.46	No
	PT 33	5.41	No
	PT 35	1.04	Yes
	PT 36	0.43	Yes
	PT 38	13.87	No
	PT 39	3.20	No
	PT 40	9.89	No
	PT 41	1.49	No
	PT 42	2.93	Yes
RF$_{low}$ (≥16 IU/ml and < 50 IU/ml)	PT 5	17.81	Yes
	PT 10	30.88	Yes
	PT 13	34.51	No
	PT 18	17.61	No
	PT 21	20.59	Yes
	PT 27	33.92	Yes
	PT 34	18.24	Yes
	PT 37	35.51	No
RF$_{high}$ (≥ 50 IU/ml)	PT 1	122.25	Yes
	PT 2	95.18	Yes
	PT 23	161.81	Yes

We finally evaluated also the impact of IgM-RF positivity on patients' OS (Fig. S3). RF$_{pos}$ patients resulted in a statistically significant reduction of the OS (p = 0.034, Kaplan−Meier method and log-rank tests) showing a robust decrease of the median survival (90 days versus 300 days) when compared to RF$_{neg}$ patients. This data confirmed that the presence of IgM-RF is a prognostic factor for a poor outcome of NSCLC patients.

To conclude, based on the rate of early progression calculated using the data shown in table 2 (i.e. 73% vs. 26% in the RF positive and negative group in 11 and 31 patients, respectively), assuming an alpha value of 0.05, the post-hoc power of our study is 80.9%.

3.3. IgM-RF affects T-cells function and reduce CD137$^+$ T-cells in NSCLC patients

3.3.1. IgM-RF does not interfere with the anti-PD-1 binding to T-cells

Since RFs are autoantibodies with an affinity for the Fc fragment of the IgG class, we first hypothesized that IgM-RF could trap the circulating therapeutic antibodies (both belonging to the IgG$_4$ subclass), avoiding the engagement between the drug and its target receptor PD-1 on T-cells and therefore predisposed RF$_{pos}$ patients to become resistant to anti-PD-1 immunotherapy. To test this hypothesis, PD-1 expression was induced by PHA stimulation in CD3$^+$ cells isolated from PBMCs of healthy donors; then T-cells were exposed to Nivolumab and IgM-RF alone or in combination and finally stained with a fluorescent anti-PD-1 antibody. The presence of Nivolumab alone, but not of IgM-RF, completely abrogated the anti-PD-1 staining, due to its ability to cover the receptor. When IgM-RF and Nivolumab were used in combination, again the anti-PD-1 staining was not detected, thus indicating that IgM-RF did not interfere in the engagement of Nivolumab with its target on T-cells (**Fig. 3a**), coherently with other observations already published [20].

3.3.2. IgM-RF binds to naïve and central memory T-cells in RF$_{pos}$ NSCLC patients

We thus hypothesized that IgM-RF could exert a direct effect on T-cells. Indeed, the Fcμ receptor is expressed by T-cells in which it mediates the IgM binding with different effects on their functions [21−26]. To test whether IgM-RF could bind to T-cells, we first stained with a fluorescent anti-IgM antibody T-cells isolated from PBMC of healthy donor and cultured in vitro in the presence of both IgM-RF and a control IgM isolated from MM patients. IgM-RF bound more efficiently T-cells than the control IgM (p = 0.004, Student's t-test) (Fig. 3b). This is probably due to the fact that IgM from MM patients are highly sialylated and quickly internalized [21,22], while IgM-RF are not. To confirm this hypothesis, T-cells were incubated with IgM-RF and stained with a fluorescent anti-IgM antibody at different time-points, demonstrating an increase of IgM-positive cells throughout the experiment (Fig. 3c). This prompt us to verify whether IgM-RF binding occurred also in vivo in NSCLC patients. As a confirmation, RF$_{pos}$ patients showed higher percentage of IgM-positive T-cells than RF$_{neg}$ patients (p<0.0001, Student's t-test) (Fig. 3d).

a. Percentage of early progressors.

b. Odds Ratios for development of Early Progressions in clinical subgroups.

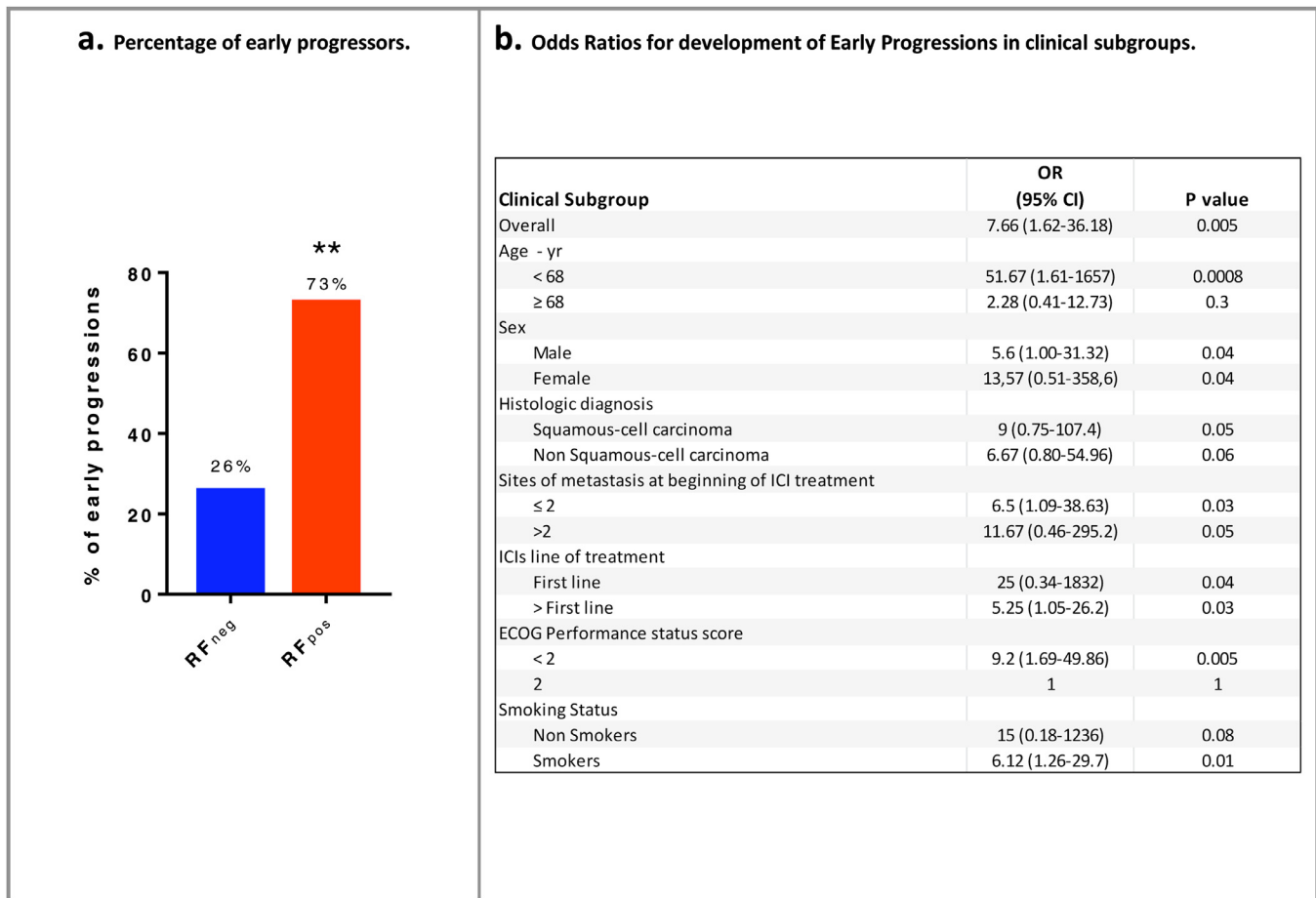| Clinical Subgroup	OR (95% CI)	P value
Overall	7.66 (1.62-36.18)	0.005
Age - yr		
< 68	51.67 (1.61-1657)	0.0008
≥ 68	2.28 (0.41-12.73)	0.3
Sex		
Male	5.6 (1.00-31.32)	0.04
Female	13,57 (0.51-358,6)	0.04
Histologic diagnosis		
Squamous-cell carcinoma	9 (0.75-107.4)	0.05
Non Squamous-cell carcinoma	6.67 (0.80-54.96)	0.06
Sites of metastasis at beginning of ICI treatment		
≤ 2	6.5 (1.09-38.63)	0.03
>2	11.67 (0.46-295.2)	0.05
ICIs line of treatment		
First line	25 (0.34-1832)	0.04
> First line	5.25 (1.05-26.2)	0.03
ECOG Performance status score		
< 2	9.2 (1.69-49.86)	0.005
2	1	1
Smoking Status		
Non Smokers	15 (0.18-1236)	0.08
Smokers	6.12 (1.26-29.7)	0.01

Fig. 1. IgM-RF positivity and early progressions. Panel a shows the percentage of early progressions in 42 NSCLC patients divided in RF_{pos} ($n = 32$) vs RF_{neg} ($n = 11$); p value was calculated with Chi-square test. Panel b shows the odd ratios (with 95% confidence intervals) with p values for development of early progression in pre-assigned clinical subgroups; OR and associated p value was calculated with Woolf logit model and Pearson's Chi-square test. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$.

When analyzing the IgM positivity on the different subsets of both CD4$^+$ and CD8$^+$ T-cells from RF_{pos} patients, we noticed that IgM-RF preferentially bound to naïve (CCR7$^+$CD45RA$^+$) and central memory (CCR7$^+$CD45RA$^-$) T-cells when compared to effector (CCR7$^-$CD45RA$^+$) and effector memory (CCR7$^-$CD45RA$^-$) T-cells (Fig. 3e), coherently with the higher expression of the Fcμ receptor on these two T-cell subsets [23,26]. IgM-RF could also bind with similar affinity the same T-cells subsets *in vitro* (Fig. S4).

3.3.3. IgM-RF bound to T-cells impedes T-cell migration but not proliferation

We then sought to assess if this binding of IgM-RF to T-cells could affect their functions. Therefore, we tested *in vitro* the proliferative and migratory ability of T-cells after exposure to IgM-RF. Since IgM-RF preferentially binds naïve and central memory T-cells, both positive for the CCR7 receptor, we used the CCL19 chemokine as chemoattractant in a migration assay. IgM-RF significantly decreased T-cells migration ($p = 0.0001$, Anova test) without affecting both their proliferation and CCR7 expression. IgM from MM patients used as control were able to reduce T-cell proliferation, as previously shown [22], without affecting their migration (Fig. 3f). This seems to be also coherent with the physiological role of RFs in limiting an overstated immune response [27,28].

3.3.4. CD137$^+$T-cells are reduced in IGM-RF positive patients and affect NSCLC patients' outcome

We then reasoned that IgM-RF could impact the activation status of T-cells also *in vivo*, by modulating naïve and central memory

T-cell migration. We therefore performed the immunophenotype of T-cell subsets of our NSCLC patients at T0, finding that RF_{pos} patients had the same amount of PD-1$^+$T-cells, targeted by anti-PD-1 ICIs (data not shown), but significantly lower percentages of CD137$^+$ T-cells ($p = 0.02$, Student's *t*-test) (Fig. 3g). This T-cell subset has been largely identified as the real tumor-reactive T-cell population, even when present in peripheral blood [13,14,29]. In line with that, when we divided our patients based on the percentage of CD137$^+$ T-cells at T0, we found that patients with the higher percentage of this population had a marked benefit in terms of PFS ($p = 0.0064$, Kaplan−Meier method and log-rank tests) with a median survival of 240 days for patients that had more than 0.65% of CD137$^+$T-cells versus 82.5 days for patients with less than 0.65% of CD137$^+$ T-cells (Fig. 4a). An higher percentage of CD137$^+$T-cells in peripheral blood of patients at baseline resulted also as an important independent prognostic factor for a better OS ($p = 0.0027$, Kaplan−Meier method and log-rank tests) after the anti-PD-1 treatment, with a sharply better median OS for patients that had more than 0.65% of CD137$^+$T-cells (460 days vs 130 days) (Fig. 4b).

Altogether, these data suggest that the presence of IgM-RF in patient sera can interfere with migration of both naïve and central memory T-cells, thus impacting T-cells recirculation and leading to a decrease of tumor-specific T-cells. In this scenario, the therapy with ICIs directed against PD-1 results less efficient, being anti-tumor T effector cells the target of these immunotherapy drugs and in particular the CD137$^+$population a crucial factor in determining the patients' response.
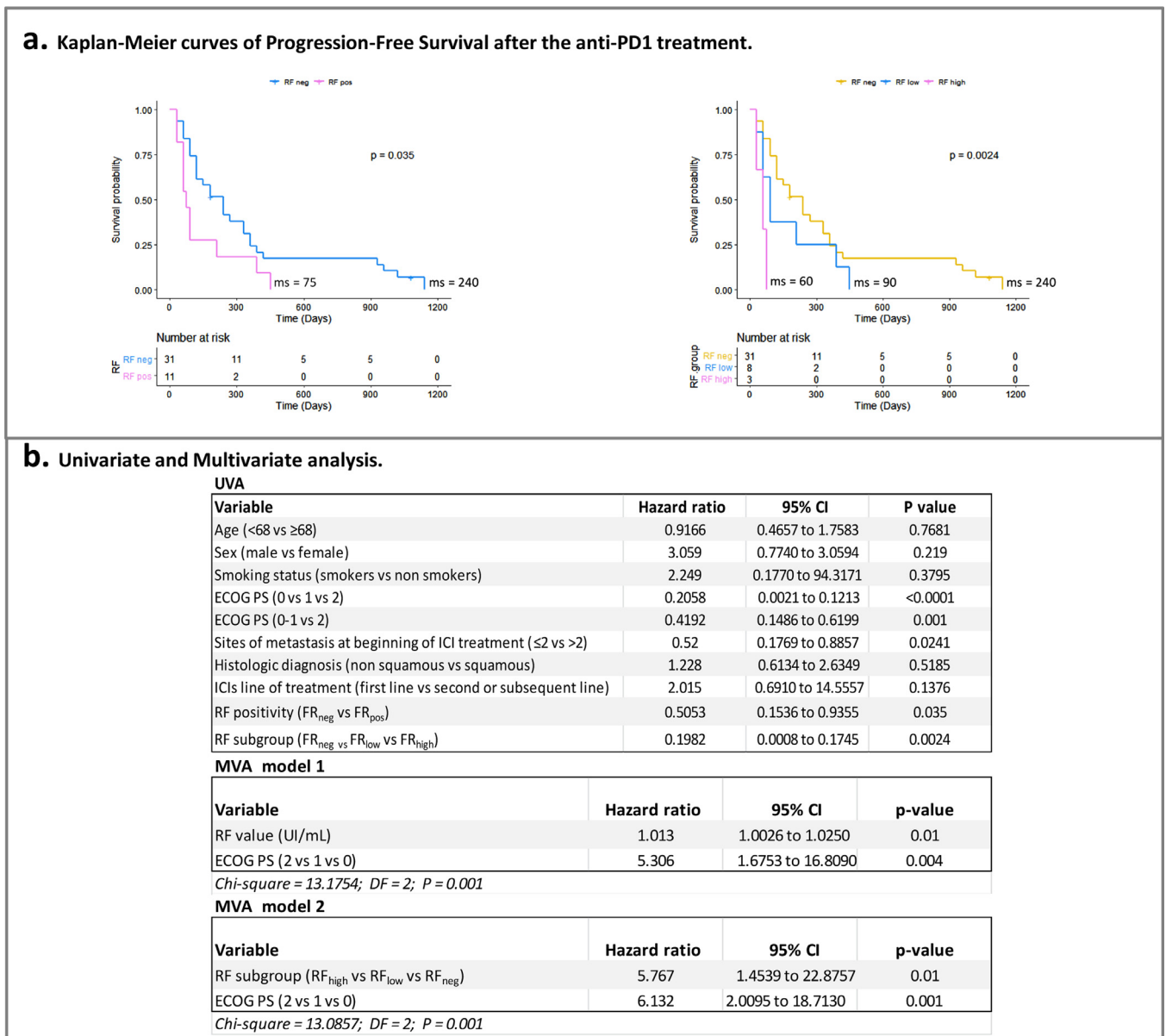
a. Kaplan-Meier curves of Progression-Free Survival after the anti-PD1 treatment.

b. Univariate and Multivariate analysis.

UVA

Variable	Hazard ratio	95% CI	P value
Age (<68 vs ≥68)	0.9166	0.4657 to 1.7583	0.7681
Sex (male vs female)	3.059	0.7740 to 3.0594	0.219
Smoking status (smokers vs non smokers)	2.249	0.1770 to 94.3171	0.3795
ECOG PS (0 vs 1 vs 2)	0.2058	0.0021 to 0.1213	<0.0001
ECOG PS (0-1 vs 2)	0.4192	0.1486 to 0.6199	0.001
Sites of metastasis at beginning of ICI treatment (≤2 vs >2)	0.52	0.1769 to 0.8857	0.0241
Histologic diagnosis (non squamous vs squamous)	1.228	0.6134 to 2.6349	0.5185
ICIs line of treatment (first line vs second or subsequent line)	2.015	0.6910 to 14.5557	0.1376
RF positivity (FR$_{neg}$ vs FR$_{pos}$)	0.5053	0.1536 to 0.9355	0.035
RF subgroup (FR$_{neg}$ vs FR$_{low}$ vs FR$_{high}$)	0.1982	0.0008 to 0.1745	0.0024

MVA model 1

Variable	Hazard ratio	95% CI	p-value
RF value (UI/mL)	1.013	1.0026 to 1.0250	0.01
ECOG PS (2 vs 1 vs 0)	5.306	1.6753 to 16.8090	0.004

Chi-square = 13.1754; DF = 2; P = 0.001

MVA model 2

Variable	Hazard ratio	95% CI	p-value
RF subgroup (RF$_{high}$ vs RF$_{low}$ vs RF$_{neg}$)	5.767	1.4539 to 22.8757	0.01
ECOG PS (2 vs 1 vs 0)	6.132	2.0095 to 18.7130	0.001

Chi-square = 13.0857; DF = 2; P = 0.001

Fig. 2. IgM-RF and Progression Free Survival. Panel a shows the Kaplan-Meier representation of Progression-free survival after the anti-PD-1 treatment among the patients divided into RF$_{pos}$ vs RF$_{neg}$ (left) and RF$_{neg}$ vs RF$_{low}$ vs RF$_{high}$ subgroups (right); ms = median survival; p values have been calculated using Kaplan−Meier method and log-rank tests. Panel b shows the univariate (UVA) and two different models of multivariate (MVA) analysis used to study the effect of the different clinic-pathological variables on patients' PFS; hazard ratios (with 95% confidence intervals) and p values are shown; p values have been calculated using Kaplan−Meier method and log-rank tests for UVA and Cox proportional hazards regression analysis for MVA.

4. Discussion

It is already well established that the success of the immunotherapies largely depends on the fitness of patients' immune system. However, no evidence has been provided so far about the possible presence of markers that can be the spy of a dysfunction of those immune cells that are directed against the tumor and are the target of ICI-based immunotherapies. The "quality" of T-cells, including optimal activation and trafficking to the target site, is crucial for all the immune-based drugs to be effective. Several of the immunotherapies that are available nowadays for clinical practice have indeed T-cells as target. The anti-PD-1 monoclonal antibodies Nivolumab and Pembrolizumab are doubtless two of the most exploited drugs [30]. Although the great benefits that the anti-PD-1 ICIs have brought in the treatment of advanced and metastatic NSCLC patients, several of them do not respond to the treatment. Since both Nivolumab and Pembrolizumab are directed against tumor-reactive T effector cells, an impairment of these cells can easily lead to the failure of the therapy. Various mechanisms of T-cells dysfunction in cancer have already been outlined and could serve in the future to develop novel strategies in order to improve the survival of patients undergoing T-cells-based immunotherapies [31]. Unfortunately, so far it is not possible to predict if a patient will respond or not to a certain drug.

In this retrospective study, we describe for the first time the autoantibody IgM-FR as possible biomarker of T-cells fitness and capability to respond to ICIs in NSCLC. In fact, we showed that NSCLC patients positive for IgM-RF are more prone to develop an early progression after the anti-PD1 treatment. It is quite interesting to notice that in the past there have been few reports describing the possible association between RF and an increased risk of developing cancer [7−10], tumor recurrence and load [11,12]. Today we can probably speculate that the mechanisms underlying those observations can be
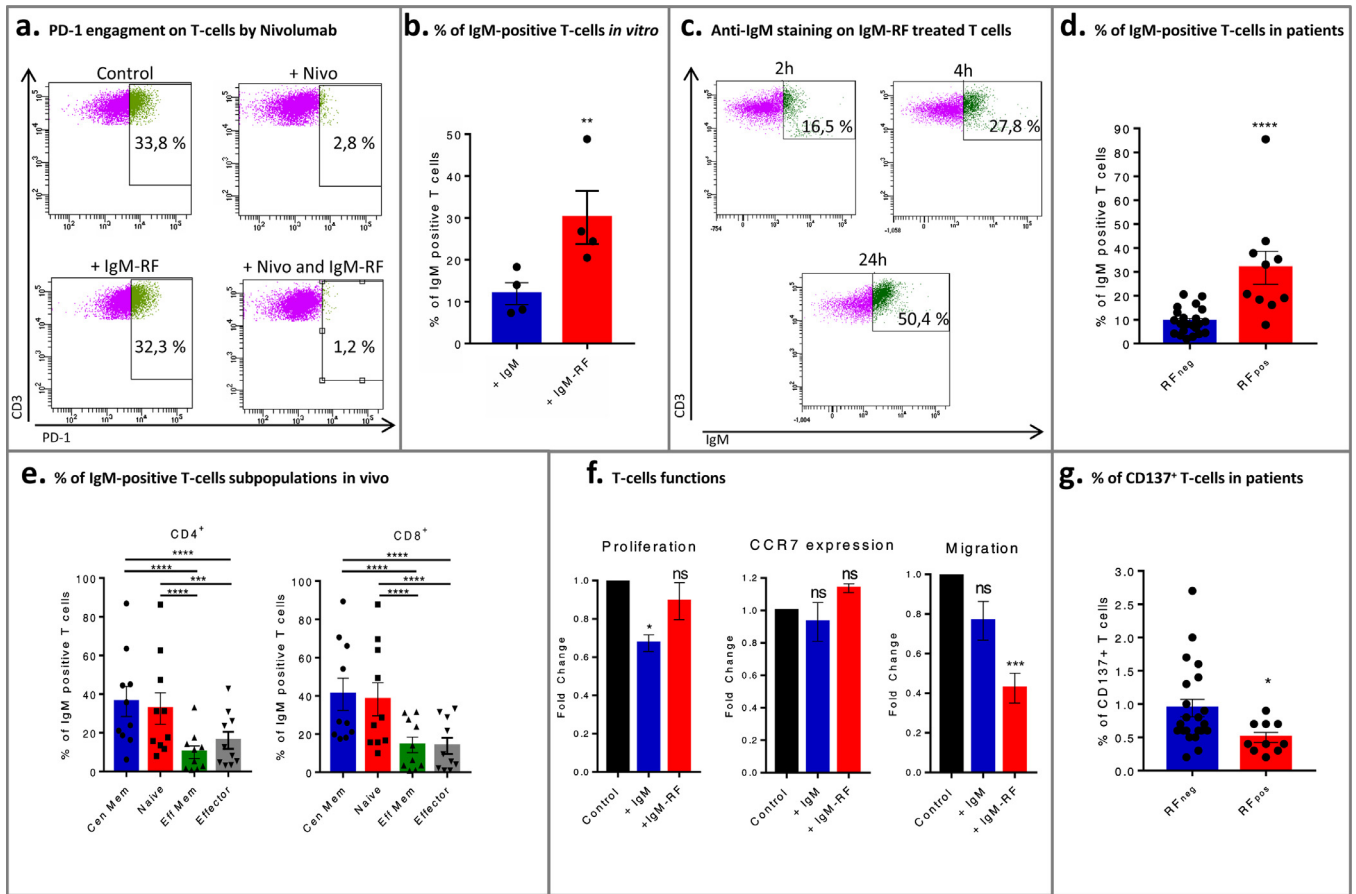
Fig. 3. IgM-RF effects on T-cells. Panel a shows the percentage of PD-1 positive PHA activated T-cells after flow cytometer analysis. Panel b shows the percentage of IgM-positive T-cells *in vitro*; CD3$^+$ cells were isolated from PBMCs of healthy donors, activated with PHA, treated with IgM-RF or control IgM and stained with an anti-IgM for a flow cytometer analysis; n = 4; mean with SEM is shown; p value was calculated with Student's *t*-test. Panel c shows the percentage of IgM positive T-cells 2, 4 and 24 h after the treatment with IgM-RF. Panel d shows the percentage of IgM-positive T-cells after the T-cells and anti-IgM staining on PBMCs isolated from 32 NSCLC patients at T0, divided into RF$_{pos}$ (n = 10) and RF$_{neg}$ (n = 22) groups; mean with SEM is shown; p value was calculated with Student's *t*-test. Panel e shows the percentage of IgM positive CD4$^+$ and CD8$^+$ T-cell subsets in vivo in RF$_{pos}$ (n = 10) NSCLC patients; PBMCs from 10 RF$_{pos}$ patients at T0 were isolated and stained for CD3, CD4, CD8, CCR7 and CD45RA to identify the different subsets and with an anti-IgM for a flow cytometer analysis; means with SEM are shown; p value was calculated with Anova test for multiple comparisons. Panel f shows the fold change compared to the experiment control (untreated sample) of the percentages of proliferated (left), CCR7 positive (middle) and migrated (right) T-cells of untreated, control IgM or IgM-RF treated T-cells; n = 5; means with SEM are shown; p value was calculated with Anova test for multiple comparisons. Panel g shows the percentage of CD137$^+$ T-cells after the T-cells and anti-CD137 staining on PBMCs isolated from 32 NSCLC patients at T0, divided into RF$_{pos}$ (n = 10) and RF$_{neg}$ (n = 22) groups; mean with SEM is shown; p value has been calculated with Student's *t*-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

attributed to the hampered immunesurveillance exerted by T-cells in individuals with high values of circulating RFs in an inflammatory milieu.

From our study, IgM-RF appears to be a predictive factor of early progression as shown by Odds ratios analysis both considering the overall patient cohort and when analyzing clinical subgroups. IgM-RF lose its validity as predictor biomarker in the subgroups identified by old age (≥68) and ECOG PS =2. These findings appear in agreement with those previously described in the literature concerning the independent negative impact of patients' general health conditions on ICIs efficacy [15,32,33]. On the contrary, it increases its power in the young population (<68 years) suggesting its independent role in predicting a progressive disease.

Also, the IgM-RF results as a prognostic marker for a reduced PFS in NSCLC patients treated with anti-PD-1 ICIs. Indeed, at the UVA its significance results similar to other well established clinical prognostic markers for NSCLC as the ECOG PS and Sites of metastasis at beginning of therapy [15,33]. In addition, when patients have been divided based on RF subgroups, high IgM-RF titers correlated with a worse outcome. Furthermore, the IgM statistical power as prognostic factor in NSCLC is confirmed at two different MVA methods that have been tested, including as a variable both the IgM-RF values and the

patients RF subclass. Finally, IgM-RF positivity resulted also as a significant prognostic factor for a reduced OS of NSCLC patients.

This is probably due to the effect of IgM-RF on naïve and central memory T-cells, of which it impairs their migratory but not their proliferative ability with no effect on PD-1 availability. Some authors highlighted how IgM can bind to T-cells both *in vitro* and *in vivo*, through the Fcμ (Toso) receptor [21−26]. The effect of this binding is however controversial. This is probably due to the diversity in posttranslational modification occurring between different types of IgM. Indeed, the sialylated IgM can be internalized by T-cells, impairing their ability to proliferate, while IgM with low sialylation remain on T-cells surface and do not impact cell proliferation [22]. Interestingly, it has already been demonstrated that autoantibodies in RA and in particular IgG-RF, have a lower sialylation level if compared to their normal counterpart [34,35], although direct proof of low sialylation of IgM RF is lacking.

IgM-RF preferentially binds naïve and central memory T cells, most probably affecting a correct homing and a subsequent expansion of a CD137$^+$ T-cells population directed against cancer cells. In this way the treatment with Nivolumab or Pembrolizumab loose its effectiveness, being their target population not properly activated. The hypothesized scenario appears somehow paradoxical, with T-
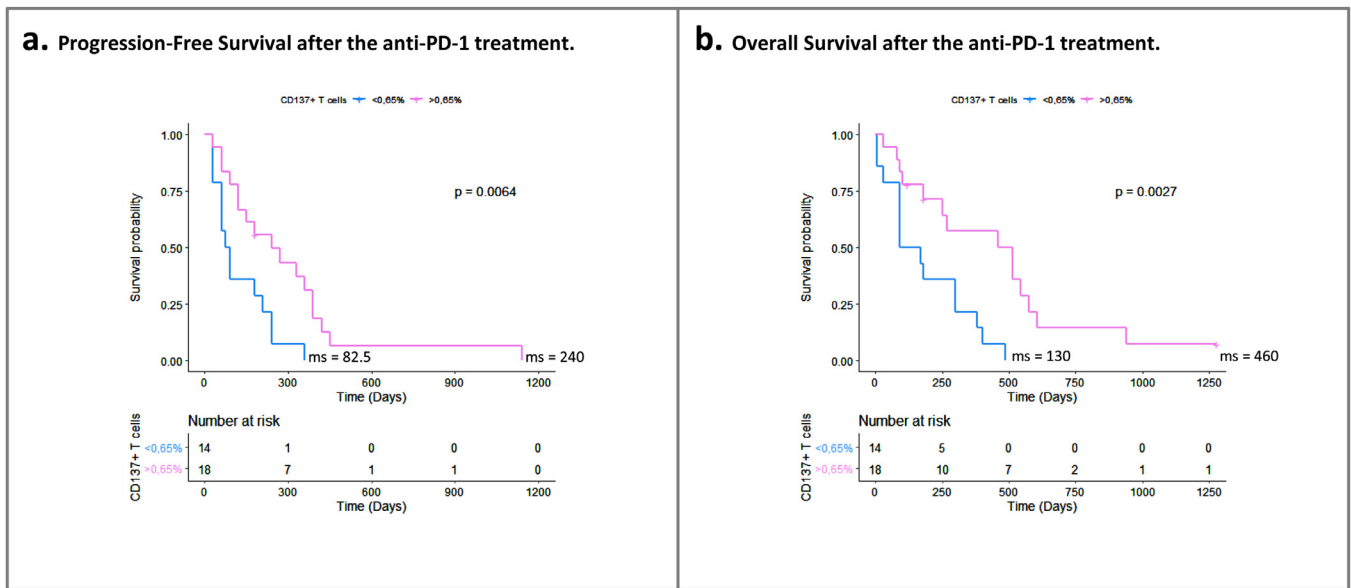
Fig. 4. Impact of the percentage of CD137⁺ T-cells at baseline on PFS and OS of NSCLC patients. Panel a shows Kaplan-Meier representation of Progression-free survival after the anti-PD-1 treatment based on the percentage of CD137⁺ T-cells at baseline; p values have been calculated using Kaplan−Meier method and log-rank tests. Panel b Kaplan-Meier representation of Overall survival after the anti-PD-1 treatment based on the percentage of CD137⁺ T-cells at baseline; p values have been calculated using Kaplan−Meier method and log-rank tests. ms = median survival.

cells theoretically capable to be unleashed by ICI treatment from a negative regulatory constrains but unable to exert a correct homing and thus being activated against target cells in a previous step. This hypothesis could most probably apply also for other drugs that relay on T-cells as final effectors and for other tumor settings, being T-cells recirculation and CD137⁺ T-cells expansion critic in most of tumor models [14,29]. As a confirmation, when we stratified our patients based on the percentage of CD137⁺ T-cells at a baseline, it clearly resulted that those patients that undergo the ICIs treatment with a larger expansion of this population are the patients that will benefit the most from the immunotherapy, in terms of both PFS and OS. These data strongly indicate that the expansion of the CD137⁺T-cell population is a critical element in driving the patients towards a positive outcome in response to the anti-PD-1 treatment.

We consider these findings fundamental from two different points of view. Firstly, it confirms that T-cells need to be present and in a perfect shape, with all their functions conserved, so that the immunotherapies can be truly effective. This fact should encourage oncologists to investigate the immune system fitness of their patients before starting an immune-based treatment, moving towards what should become a personalized immunotherapy, considering the patient and her/his own immune system as the keystone to orient the therapeutic plan. On the other side, we are providing a specific biomarker, already standardized for clinical use, that can be easily tested before the patient undergoes an immunotherapy and predict whether the patient is prone to respond to the immune treatment or not. In this way, it would be possible to modulate the therapeutic strategy in order to offer the patient more chances of becoming a responder.

The main limitation of this study is the reduced size of our sample of patients, which classify our results as preliminary. Given the number of statistical tests and the minimal sample size, the calculated p-values have been corrected for multiple tests using the Bonferroni adjustment. Unfortunately, the Bonferroni adjustment of p-value is recognized to be too conservative, so real differences might be missed. Nevertheless, our study is the first to provide some comparative insights on the impact of new variables such as IgM-RF and the percentage of CD137⁺ T-cells on PFS and OS of NSCLC patients in treatment with the anti-PD-1 and can be considered an exploratory

study. In fact, a better characterization of the mechanism and larger studies including higher numbers of patients and different types of tumors will be needed to definitely bring the dosage of IgM-RF in cancer patients in the routine clinical practice.

5. Contributors

Conceptualization, M.N., Fu.C., A.B. and A.U.; methodology, A.U., L.S., Fu.C., T.C. and H.R.; validation, A.U, I.G.Z. and L.S.; resources, P.M., G.V. and Fa.C.; data curation, A.U. and L.S.; writing - original draft preparation, A.U.; writing - review and editing, A.R., I.G.Z., M.N., Fu.C, A.B. and L.S.; visualization, A.R., M.N. and I.G.Z.; project administration, M.N.; funding acquisition, M.N.

All authors had access to the raw data and had responsibility for the decision to submit the article for publication. All authors approved the final version of the manuscript.

A.U., M.N., Fu.C. and A.B. can verify the accuracy of the raw data.

Declaration of competing interest

All authors declare no competing interests.

Acknowledgments

Data sharing

Individual participant data that underlie the results reported in this article, after de-identification, study protocol, informed consent form and statistical analysis plan will be available immediately following publication, with no end date, with researchers who provide a methodologically sound proposal, to achieve aims in the approved proposal. Proposals should be directed to marianna.nuti@uniroma1.it to gain access; data requestors will need to sign a data access agreement.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103098.

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