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Original research

Atypical B cells (CD21-CD27-IgD-) correlate with lack of response to checkpoint inhibitor therapy in NSCLC

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ABSTRACT

Introduction: Checkpoint inhibitor (CI) therapy has revolutionized treatment for non-small cell lung cancer (NSCLC). However, a proportion of patients do not respond to CI therapy for unknown reasons. Although the current paradigm in anti-tumor immunity evolves around T cells, the presence of tertiary lymphoid structures and memory B cells has been positively correlated with response to CI therapy in NSCLC. In addition, double negative (DN) ($CD27^-$ IgD^-) B cells have been shown to be abundant in NSCLC compared to healthy lung tissue and inversely correlate with the intratumoral presence of memory B cells. Nonetheless, no study has correlated DN B cells to survival in NSCLC.

Methods: In this study, we evaluated the presence and phenotype of B cells in peripheral blood with flow cytometry of patients with NSCLC and mesothelioma before receiving CI therapy and correlated these with clinical outcome.

Results: Non-responding patients showed decreased frequencies of B cells, yet increased frequencies of antigen-experienced CD21- DN (Atypical) B cells compared to responding patients and HC, which was confirmed in patients with mesothelioma treated with CI therapy.

Conclusions: These data show that the frequency of CD21- DN B cells correlates with lack of response to CI therapy in thoracic malignancies. The mechanism by which CD21- DN B cells hamper CI therapy remains unknown. Our findings support the hypothesis that CD21- DN B cells resemble phenotypically identical exhausted B cells that are seen in chronic infection or function as antigen presenting cells that induce regulatory T cells.

1. Introduction

Checkpoint inhibitor (CI) therapy has revolutionized treatment of non-small cell lung cancer (NSCLC) and has led to improved overall survival (OS) [1]. However, there are patients that do not benefit from CI therapy. A better understanding of the mechanisms hampering response to CI is needed. The role of T cells in anti-tumor immunity has been well established and is documented as the paradigm of the cancer immunity cycle [2]. Extensive research over the last decade has elucidated that B cells can exert anti-tumor immunity through several mechanisms such as enhancing T cell function, complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity [3]. However, the prognostic role of intratumoral B cells remains variable and has been reported to be dependent on the organizational structure in which B cells reside [4,5]. The phenotype and thereby function of B cells is determined by the maturity – presence of germinal centers - of tertiary lymphoid structures (TLS). B cells within mature TLS results in plasma cell differentiation and antibody production [5], which is correlated with favorable prognosis in NSCLC [6] and correlates with response to CI therapy in melanoma, sarcoma and NSCLC [6–9]. Specifically, memory B cells and plasma cells were found to be correlated with favorable outcome to CI therapy in melanoma and NSCLC [7,8]. Immature TLS induce regulatory B cells with immunosuppressive capacities that have been correlated with worse clinical outcome in bladder cancer [5]. In NSCLC, double negative (DN) B cells, lacking expression of CD27 and IgD, are abundant in the tumor compared to healthy lung tissue and negatively correlate with the number of memory B cells [10]. Due to the unavailability of clinical response data, no correlation between the presence of intratumoral DN B cells and clinical outcome was made in that study.

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DN B cells are part of a heterogeneous group of B cells, also described as atypical B cells (ABC), tissue-like memory B cells, or age-associated B cells. These cells are diversely characterized by (a combination of) phenotypic markers such as CD11c and T-bet expression, and the lack of CD27, IgD, and CD21 [11,12]. ABCs differentate from naïve B cells under the influence of an inflammatory environment inducing exposure to specific cytokines, interleukins and toll-like receptors that subsequently determine the functionality the ABC [13,14]. These ABCs have been described in elderly, auto-immunity and chronic infection and the functionality varies from hyperresponsive and auto-reactive to exhausted [12–17].

Although literature on intratumoral B cells and cancer has become quite extensive, perturbations in B cell subtypes in peripheral blood of cancer patients and correlations with response to CI therapy have not been studied in detail. Only a low number of total B cells prior to therapy and a decrease in CD21- B cells after start of therapy has been correlated to worse clinical outcome [18,19].

Therefore, we studied B cells, with a focus on memory- and DN B cells, in the peripheral blood of responding (R) and non-responding (NR) NSCLC patients to anti-PD-1 CI therapy.

2. Methods

2.1. Study design

The MULTOMAB study (local ethics board study number MEC16–011) was originally designed to sample peripheral blood mononuclear cells (PBMCs) and serum to analyze pharmacokinetics and immune cells subsets in patients receiving CI therapy. Patients that were asked to participate in the reported analysis are suffering from NSCLC or malignant pleural mesothelioma (MPM) and received treatment in the form of nivolumab (Opdivo®, 3 mg/kg every 2 weeks) or pembrolizumab (Keytruda®, 2 mg/kg every 3 weeks). Written informed consent was obtained from all participants prior to inclusion into the study.

2.2. Patient response evaluation

Radiological tumor evaluation was performed 6 weeks after start of therapy. Dependent on CT scan evaluation, a follow-up scan was performed 4–12 weeks later. For NSCLC, best overall response (BOR) was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 and modified RECIST was used for MPM. Progression free survival (PFS) was defined as time from start of CI therapy until radiological progression or death and OS was defined as time from start of CI therapy until death.

2.3. Patient selection and data collection

For the initial cohort, NSCLC patients were selected to create a group of responding (R) and non-responding (NR) patients to CI therapy between the 5th of May 2016 and the 2nd of December 2022. These patients were matched on age, histology, gender, PD-L1 expression and treatment. R were patients with a partial response (PR) or complete response (CR) as BOR to CI therapy and NR were patients with progressive disease as BOR. In total, 11 R and 8 NR were selected for analysis based on the availability of samples. For the second cohort, MPM patients were selected similar to NSCLC patients, resulting in 6 R and 10 NR, whereby one of the R had stable disease as BOR for more than 1 year. For both cohorts, 5 gender-matched healthy controls (HCs) were selected for analysis. Clinical data was collected retrospectively.

2.4. Peripheral blood collection and flow cytometry

Blood was drawn at baseline (prior to therapy) and PBMCs were isolated using ficoll gradient centrifugation and cryopreserved before analysis, using standard procedures. Fluorochrome conjugated antibodies are listed in Table S1. Flow cytometry analyses were performed on cryopreserved PBMC samples and all stainings were performed at 4 °C following previously described procedures[20] and adherent to general flow cytometry guidelines.[21] Cells were stained for cell surface markers for 30 min, followed by incubation with Fixable Viability Dye (eBioscience, ThermoFisher, Waltham, MA, USA) for 15 min at 4 °C. Next, cells were fixed and permeabilized using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience) and stained intracellularly for 60 min at 4 °C (Table S1). Acquisition was performed on a FACSymphony A5 (BD Biosciences, Franklin Lakes, NJ, USA) using BD FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Statistics

Median OS and PFS were estimated using a Kaplan-Meier curve in combination with a log-rank (Mantel-cox) test. We presumed a not-normal distribution. In graphs comparing R, NR and HC, we used a Mann-Whitney U test as we primarily tested for a difference between R and NR and did additional analysis to see if R or NR more likely resemble the phenotype frequencies of HC. In graphs comparing different cell types we used a Kruskall-Wallis test with Dunn's posttest as we primarily were interested in a difference between any of the several cell subsets. A P-value < 0.05 was considered significant. All reported P-values were two-tailed. Statistical analyses were performed using R 3.6.0 (R Foundation for Statistical Computing) or Graphpad Prism 8.0.

3. Results

3.1. Clinical parameters

Baseline characteristics of NSCLC patients are summarized in Table 1. Median PFS for NR was 1.6 months and 56.9 months for R. PD-L1 status was available in all pembrolizumab treated NSCLC patients and there was no significant difference in PD-L1 expression between R and NR.

3.2. NR show decreased B cell frequencies, but increased frequencies of CD27- IgD- DN B cells

We studied circulating B cell subtypes in R and NR patients with NSCLC and in HC. The gating strategy can be found in Fig. S1. Immunophenotypic analyses of PBMC samples revealed lower B cell frequencies in NR compared to R and HC (Fig. 1A). Next, the distribution of naïve B cells (CD27-IgD+), unswitched memory B cells (CD27+IgD+ or/and IgM+), switched memory (SM) B cells (CD27+IgD-IgM-), and DN B cells (CD27-IgD-) was analyzed between the different groups (Fig. 1B). NR had significantly higher frequencies of DN B cells and lower frequencies of naïve B cells compared to R, whilst R and HC showed similar frequencies in these B cell subsets. No correlation between age and the abundance of DN B cells (Fig. S2A) was found. As the frequency of total B cells was lower in NR compared to R and HC, the frequencies of B cell subsets were also plotted as fractions of live cells (Fig. S2B). This analysis showed that due to the lower abundance of B cells in NR patients, DN B cells as a fraction of total live cells was similar in NR, R and HC (Fig. S2B). This implies that, regarding DN B cells, specifically the distribution within the B cell compartment associates with response to CI therapy. In addition, particularly the frequency of naive B cells as a fraction of total live cells was reduced in NR, compared with R and HC (Fig. S1B). Finally, the proportion of proliferating Ki67+ cells within total B cells was significantly higher in NR than in R and a similar trend was seen in the other B cell subsets analysed (Fig. 1C).

Table 1
NSCLC Patient baseline characteristics.

	R (n = 11)	NR (n = 8)	Total (n = 19)	HC (n = 5)
Age (years)	68,7	68,4	68,6	29
Male	45% (n = 5)	38% (n = 3)	42% (n = 8)	60% (n = 3)
Histology	64% adeno (n = 7)27% squamous (n = 3) 9% unknown (n = 1)	50% adeno (n = 4)25% squamous (n = 2)12,5% mixed (n = 1)12,5% unknown (n = 1)	61% adeno (n = 11)28% squamous (n = 5)5% mixed (n = 1)11% unknown (n = 2)	NA
Therapy	45% nivolumab (n = 5)55% pembrolizumab (n = 6)	38% nivolumab (n = 3)62% pembrolizumab (n = 5)	42% nivolumab (n = 8)58% pembrolizumab (n = 11)	NA
PD-L1%	72,5 (n = 6)	78 (n = 5) (p = 0.91)	80,5 (n = 11)	NA
PFS (months)	56,9 months	1,6 months (p < 0.0001)	NA	NA
OS (months)	73,9 months	3,7 months (p < 0.0001)	NA	NA

Data are presented as percentage and absolute number, unless stated otherwise. The P values for PD-L1 is for the comparison of R vs. NR with a Mann-Withney U test. P values for survival are for the comparison of the survival curves of R vs. NR. Abbreviations: R: responders, NR: non-responders, HC: healthy control, PFS: progression free survival, OS: overall survival, NA: not applicable.

3.3. A high frequency of antigen-experienced CD21- DN B cells is associated with a lack of response to CI therapy

To phenotype the DN B cells in more detail, we analyzed CD21 expression in a subgroup of patients. For 5 individuals in R, NR and HC, PBMC samples were available for analysis of the CD21 marker. We found that the frequency of CD21$^-$ DN B cells was increased in NR, compared with R and HC, but the frequency of CD21+ DN B cells did not differ between the three groups (Fig. 1D). Finally, the frequency of CD21- DN B cells inversely correlated with the frequencies of total B cells (Fig. S2C), indicating that the two specific characteristics for NR, i.e. reduced frequencies of total B cells and increased frequencies of CD21- DN B cells, are linked.

As ABCs are described to be antigen-experienced, we analyzed isotype distribution and observed that CD21- DN B cells are poised towards an IgG isotype (Fig. 1E). Especially CD21- DN B cells in NR are IgG+ as compared to R and HC (Fig. 1E), resulting in a substantial and significantly increased frequency of IgG+ CD21- DN B cells in NR compared to R an HC, even as a fraction of total live cells (Fig. 1F). The latter is quite impressive as the number of total B cells in NR is very low compared to R and HC (Fig. 1A).

To further elucidate on the potential function of CD21- DN B cells, we looked at the expression of Ki67, HLA-DR and CD86 in comparison with their CD27 + counterpart; SM B cells. Expression of Ki67 and CD86 was higher on CD21- DN B cells compared to SM B cells and HLA-DR expression trended to be higher (Fig. 1G). The expression of these markers on CD21- DN B cells did not correlate with response to CI therapy (data not shown).

3.4. Increased frequencies of CD21- DN B cells in NR patients is not restricted to NSCLC

To explore whether high frequencies of CD21- DN B cells are also found in NR patients with other tumors, we analyzed PBMCs from MPM patients receiving CI therapy. Median PFS for NR was 1.7 months and 24.5 months for R (Table S2). Similar to NSCLC, NR trended towards lower B cell frequencies than R and significant lower frequencies than HC (Fig. 2A). The frequency of DN B cells in NR was significantly higher compared to R and HC (Fig. 2B), which was again not age-associated (data not shown). Finally, NR had a significantly higher proportion of CD21- DN B cells than R and HC (Fig. 2C), indicating that CD21- DN B cells are more frequent in blood of patients with thoracic malignancies that do not respond to CI therapy.

4. Discussion

Our study shows that a decreased frequency of B cells in peripheral blood is correlated with worse clinical outcome in NSCLC patients treated with CI therapy. Most importantly, a high frequency of CD21- DN B cells in peripheral blood of NSCLC and MPM patients is associated with a lack of response to CI therapy and is inversely correlated with the total number of B cells. CD21- DN B cells are mainly class switched towards IgG, indicating these B cells are antigen-experienced.

The correlation between the scarcity of peripheral B cells and lack of response to CI therapy confirms earlier work by Xia et al.[19]. The abundance of CD21- DN B cells inversely correlating with the number of B cells, complements a study in melanoma in which an increase in CD21- B cells correlated with a decrease in the number of B cells [22]. As ABCs in other diseases are prone to Fas ligand-induced cell death due to high expression of CD95 [23–28], we hypothesize that CD21- DN B cells in our study are short lived as well. NR have numerically higher frequencies of Ki67+ expression in all B cell subsets, especially in naive B cells, indicating their homeostatic proliferation [29].

The (IgG+) CD21- DN B cells we found in NSCLC and MPM phenotypically resemble the ABCs found in the elderly, auto-immunity and chronic infection [12–17]. As our patients were age-matched and there was no correlation between age and the presence of DN B cells, it seems unlikely that the DN B cells found in cancer patients resemble classical age-associated B cells, but are rather induced by the tumor. To distinguish if ABCs in our study resemble ABCs in chronic infection or autoimmunity, one would have to look at their functionality. ABCs in autoimmunity are hyperresponsive to toll-like receptor (TLR) signaling and differentiate easily, without BCR stimulation, into auto-reactive antibody-producing plasma cells [12,14]. In chronic infections such as HIV, malaria, long COVID and HCV, the ABCs have an exhausted phenotype as a consequence of chronic antigen exposure and have high affinity thresholds for activation [13,15,16,30]. As ABCs in our study are subject to chronic tumor-antigen exposure and correlate with lack of response to CI therapy, it is most likely that they have functional similarities with ABCs seen in chronic infection, but this remains to be further investigated.

Apart from potentially being exhausted and having a high affinity threshold for activation, CD21- DN B cells in our study express relatively high levels of CD86 and HLA-DR compared to their CD27 expressing counterparts (SM B cells). CD86 has also been described to be high on CD21- B cells in HIV, but after stimulation B cells are less capable of increasing CD86 expression and are unable to activate T cells, leading to poor CD4 T cell activation and proliferation [31]. However, research has shown that in NSCLC tumor tissue CD21- CD27- B cells are capable of presenting antigen to CD4 T cells, but induce regulatory CD4 T cells (FoxP3+ IFN-γ-) rather than active CD4 T cells (FoxP3- IFN-γ +)[32]. In a pan-cancer study, a decrease of CD21- B cells after start of CI therapy is correlated with longer survival [18]. In conclusion, lack of CD21 expression on B cells seems to be detrimental for the process of inducing

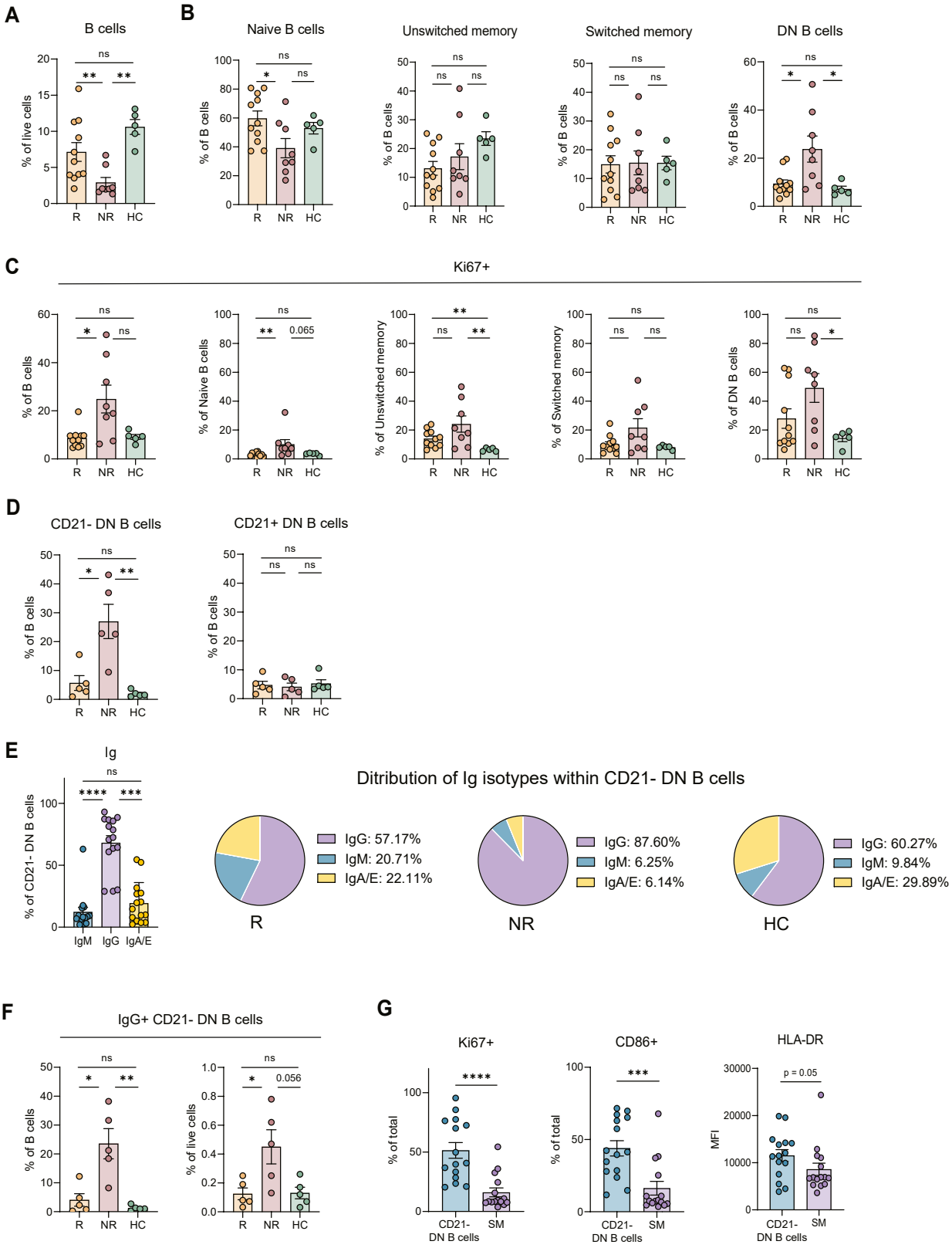

Fig. 1. Perturbations of B cell subtypes in NSCLC. (A-B) Frequencies of B cells (A) and double negative (DN) B cells, naïve B cells, unswitched memory B cells and switched memory B cells (B). (C) Percentage of Ki67 + cells of B cells and B cell subsets. (D) CD21- DN B cells as fraction of total B cells (left) and as a fraction of live cells (right). (E) Immunoglobulin isotype expression on CD21- DN B cells (left) and pie charts showing isotype distribution of CD21- DN B cells per patient group (right). (F) IgG+ CD21- DN B cells as fraction of total B cells (left) and as fraction of total live cells (right). (G) Percentage of Ki67 + (left), CD86 + cells (middle) and MFI of HLA-DR per cell subset. Means and SEMs are shown and Mann-Whitney U test or Kruskall-Wallis test withs Dunn's posttest were performed indicating statistical significance. * $P < .05$, * * $P < .01$, * ** $P < .001$, * ** * $P < .0001$. R; responders, NR; non-responders, HC; healthy controls, DN; double negative, SM; switched memory B cell, Ig; immunoglobulin.

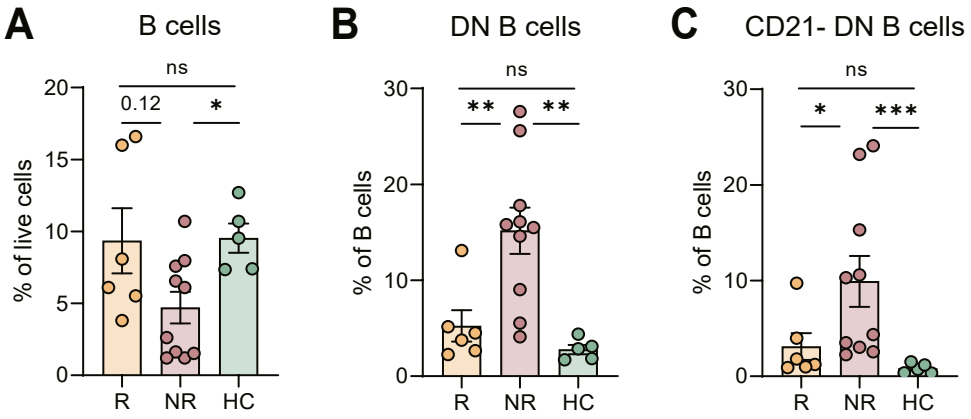

Fig. 2. Perturbations of B cell subtypes in MPM. (A-C) Frequencies of B cells (A), double negative (DN) B cells (B) and CD21- DN B cells as fraction of total B cells (C). Means and SEMs are shown and Mann-Whitney U test was performed indicating statistical significance. * $P < .05$, * * $P < .01$, * ** $P < .001$, * ** * $P < .0001$. R; responders, NR; non-responders, HC; healthy controls, DN; double negative.

an effective anti-tumor immune response.

We acknowledge that this study has some limitations. Firstly, the lack of tumor material precluded to study intra-tumoral immune cells. Establishing a correlation between our peripheral blood findings and intra-tumoral B cells is crucial to reveal if CD21- DN B cells locally hamper a functional anti-tumor response. Secondly, due to limited sample availability, we were only able to analyze small number of patients at one time point. Nonetheless, even with small numbers of patients we were able to identify and validate our results in NSCLC and MPM, implying that the differences in CD21- DN B cells are robust and not specific for one tumor type. In addition, the limited sample availability prevented us from conducting functional studies on CD21- DN B cells, e.g. immunoglobulin secretion capacity upon stimulation, or additional phenotyping Full validation with elaborate analyses of both the functional status and phenotype is recommended to determine if these cells can serve as a predictive biomarker. Fourth, recent literature has reported on the correlation between CD21- B cells and immune-related adverse events (irAEs) in melanoma patients treated with combination checkpoint inhibitors [5,22,33]. As patients in our study had no irAEs, we were not able to check for a correlation between the presence of CD21- DN B cells and irAEs.

In conclusion, the abundance of (IgG+) CD21- DN B cells in peripheral blood is correlated with lack of response and unfavorable clinical outcome in NSCLC and MPM patients treated with CI therapy. We speculate that naïve B cells are skewed by the tumor microenvironment – together with systemic changes that might be induced by the anti-tumor response – into CD21- DN B cells. This would parallel the proposed mechanisms in chronic infection and auto-immunity, whereby an inflammatory environment is also responsible for the induction of ABCs. The pathways by which these CD21- DN (atypical) B cells subsequently hamper CI therapy remains unknown. However, we hypothesize that CD21- DN B cells either resemble phenotypically identical exhausted B cells that are seen in chronic infection or function as antigen presenting cells that induce regulatory T cells. Future research on how CD21- DN B cells might hamper CI therapy response is needed.

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CRediT authorship contribution statement

Robert Belderbos: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Odilia Corneth**: Writing – review & editing. **Daphne Dumoulin**: Investigation, Resources. **Rudi Hendriks**: Supervision, Writing – review & editing. **Joachim Aerts**: Supervision, Resources, Writing – review & editing. **Marcella Willemsen**: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing.

Declaration of Competing Interest

Daphne Dumoulin reports personal fees from Roche, BMS, MSD, Pfizer, Astra Zeneca and Novartis outside the submitted work. Joachim Aerts reports personal fees and nonfinancial support from MSD, personal fees from BMS, Boehringer Ingelheim, Amphera, Eli-Lilly, Takeda, Bayer, Roche, Astra Zeneca outside the submitted work; in addition, Joachim Aerts has a patent allogenic tumor cell lysate licensed to Amphera, a patent combination immunotherapy in cancer pending, and a patent biomarker for immunotherapy pending; Joachim Aerts served as an unpaid editorial board member of Translational Lung Cancer Research from September 2019 to September 2021. The other authors do not have a conflict of interest to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.113428.

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