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Peripheral T cell responses in treatment with immune checkpoint inhibitors

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Table of contents

Abstract

Relevance

Immune checkpoint inhibitors (ICI) have become one of the main pillars in cancer treatment but determining whether patients will profit from this treatment concerning response and immune-related adverse events remains difficult. Also, the current strategy of treating irAEs with immunosuppressants is detrimental to the anti-tumour response, so alternative treatment options are needed.

Aim

Elucidating the peripheral T cell responses induced by ICI could provide information on the underlying mechanism in response and toxicity, as well as help find potential biomarkers that predict toxicity and treatment outcomes. In addition, it could help identify new targets in the treatment of irAEs.

Study design

In this study, we investigated the peripheral T cell responses of cancer patients under monotherapy (anti-PD-1) or combination therapy (anti-PD-1 + anti-CTLA-4) of which half of the patients developed toxicity (irAEs ≥ 2). We performed FACS analysis on PBMCs from patient samples taken at baseline and after the first and second dose of treatment or when toxicity occurred.

Results

Increased CD4+/CD8+ T cell ratio at baseline is positively linked to response without toxicity. LAG-3+CD8+ T cells were found to be elevated in patients without clinical benefit. ICI treatment induced a stronger decrease in IFN-γ-expressing CD8+ T cells in toxicity-developing patients. More IFN-γ- CD8+ T cells expressing co-inhibitory receptors were also observed in this group. Granzyme-B-producing PD-1+, LAG-3+, and memory CD8+ T could be associated with toxicity and its severity. Combination therapy induced more (PD-1+)CD4+ memory T cells compared to monotherapy which could possibly contribute to toxicity development as well.

Conclusion

These findings will need to be confirmed and clarified in further research but could contribute to the knowledge of the underlying mechanisms in ICI treatment, as well as aid in the search for predictive biomarkers for toxicity and response.

Layman's summary

Relevance and explanation

Immune checkpoint inhibitors (ICI) are a potent treatment against cancer. The immune system has immune checkpoints in place to keep the balance between inducing an immune response and maintaining tolerance. Cancer can exploit these immune checkpoints by inducing tolerance, making it hard for the immune cells to effectively clear cancer cells. ICI were developed to block this cancer-induced tolerance and unleash a potent anti-tumour immune response. This treatment has proved to be very efficacious and has transformed the field of cancer treatment. However, not all patients are responsive, and the treatment is often accompanied by toxicities called immune-related adverse events (irAEs). ICI-induced toxicity is partly dependent on the type of ICI treatment. Still, most patients develop an irAE ranging from mild to lethal, independent of treatment type. The current treatment of these irAEs is with immunosuppressants that dampen the immune response alongside with the anti-tumour response. This calls for alternative treatment strategies for irAEs. In addition, we must find tools that can guide clinicians in assessing what (ICI) treatment is most beneficial for the patient, concerning response and the risk of developing toxicity.

Aim of study

Comparing the dynamics of T cells in patients' blood, before and during treatment, could clarify some of the underlying mechanisms in ICI treatment regarding response and toxicity. It might also aid in finding potential biomarkers that predict toxicity and treatment outcomes. Additionally, it could help identify new targets in the treatment of irAEs.

Study design

In this study, we investigated the peripheral T cell responses of cancer patients under treatment with ICI. We used the blood samples of cancer patients receiving two different types of ICI treatment: monotherapy or combination therapy. Within these two groups, half of the patients developed irAEs that required medical attention, while the other half did not. We assessed the immune cells retrieved from their blood samples, taken before they started treatment and after the first and second dose, or when they developed toxicity.

Results

We found that an increased CD4+/CD8 +T cell ratio is increased in patients that respond to treatment without developing toxicity. Secondly, we found that the levels of LAG-3+CD8+ T cells were elevated at baseline, in patients without clinical benefit. This subset could have potential predictive value in treatment outcomes. ICI treatment induced a stronger decrease in IFN-y-expressing CD8+ T cells in toxicity-developing patients. More CD8+ T cells expressing co-inhibitory receptors, unable to produce IFN-y were also observed in this group. Granzyme-B-producing PD-1+, LAG-3+, and memory CD8+ T could be associated with toxicity and its severity. Combination therapy induced more (PD-1+)CD4+ memory T cells compared to monotherapy which could possibly contribute to toxicity development as well.

Conclusion

We identified interesting trends in peripheral T cell responses related to toxicity and response. These findings will need to be confirmed and clarified in further research but could contribute to the knowledge of the underlying mechanisms in ICI treatment, as well as aid in the search for predictive biomarkers for toxicity and response.

Introduction

Significance of ICI

Ever since the FDA approved immune checkpoint inhibitors (ICI) in 2011, they have transformed the field of cancer treatment. Immunotherapy has proven to be a potent tool by increasing the long-term survival in patients with advanced malignancies and inducing durable complete responses in a portion of the patients[1]. ICI have become one of the main pillars on which cancer treatment relies next to surgery, radio- and chemotherapy. Currently, relevant ICI in the clinic are monoclonal antibodies (mAbs) blocking co-inhibitory receptors CTLA-4 (ipilimumab), PD-1 (nivolumab and pembrolizumab), and its ligand PD-L1 (avelumab, atezolizumab, durvalumab) [2]. Nivolumab and ipilimumab are often combined and referred to as combination therapy. ICI are used against many different types of cancers, e.g., melanoma and non-small cell lung cancer (NSCLC), as well as in non-solid cancers like lymphoma[3]. And fortunately, new promising ICI are underway. The RELATIVITY-047 trial, a phase 2-3 clinical trial, investigated relatlimab (anti-LAG-3 antibodies) in combination with nivolumab in advanced melanoma patients. This showed that relatlimab and nivolumab together improved the PFS in these patients, compared to nivolumab alone [4].

The working mechanism of ICI

The attenuation of immune responses is mediated through immune checkpoints. Post-infection immune responses against pathogens need to be tuned down and self-tolerance needs to be maintained. Therefore, CTLA-4, PD-1, and its ligands PD-L1/PD-L2 are crucial elements in self-tolerance and immune homeostasis[5,6]. Cancer has managed to exploit these immune checkpoints and cripple anti-tumour immunity. ICI reinstate potent anti-tumour responses by blocking these immune checkpoints.

PD-1

PD-1 is described as the gatekeeper of peripheral tolerance in immune cells. PD-L1 and PD-L2 are expressed on immune cells as well as on non-hematopoietic cells. In cases of tissue damage after infection, the PD-1-PD-L1/PD-L2 pathway prevents autoimmunity and prolonged effector T cell activity[7]. Tumour cells use this mechanism to their advantage by expressing PD-L1, so when PD-1 on T cells binds it, T cell activation is hampered. Upon PD-1 activation, its immune receptor tyrosine-based inhibitory motif (ITIM) and immune receptor tyrosine-based switch motif (ITSM) become phosphorylated at the cytoplasmic tail. These motifs recruit and activate SHP2, which dephosphatizes proteins associated with the cytoplasmic tails of the T-cell receptor (TCR) and CD28, inhibiting T-cell proliferation, differentiation, cytolytic activity, and cytokine production [8–10].

Tumour-specific CD8+ T cells are inhibited when infiltrating the tumour because cancer cells sense their IFN-γ release and upregulate PD-L1 in response. Other tumour-infiltrating immune cells, like antigen-presenting cells (APCs), regulatory T-cells (Tregs) and other lymphocytes might also express PD-1, PD-L1, and/or PD-L2, creating an immunosuppressive tumour microenvironment (TME) [11]. Other cells like cancer-associated fibroblasts (CAFs), tumour-associated macrophages (TAMs), and myeloid-derived-suppressor cells (MDSCs) are known to also add to this immunosuppressive environment [12].

Nivolumab and pembrolizumab bind PD-1, blocking PD-L1/PD-L2 ligation and reactivating T cells at the tumour site, resulting in increased expansion, migration, and (effector) functioning. Studies have shown that proliferation of effector CD8+ T cells in the TME in response to PD-1 blockade, can inhibit tumour growth and reduce its size.
Memory CD4+ and helper T cells are rescued from this PD-1-mediated inhibition as well, which is implied by the expansion and increased IFN-γ signatures of these cell populations in melanoma patients under anti-PD-1 therapy[9,13].

Exhaustion

Overexpression of PD-1 is correlated with impaired effector functioning in T cells. CD8+ effector T cells are critical in the clearance of cancer cells. Their functionality has been described as a continuum between overly functional at one end and dysfunctional at the other. On this spectrum, the dysfunctional CD8+ T-cells are viewed as exhausted, while overly functional CD8+ T-cells cause auto-immunity. The exhausted state of CD8+ effector T cells is characterized by upregulation of co-inhibitory receptors like PD-1, LAG-3, CTLA-4, and TIM-3; reduced cytokine production (IFN-γ, TNF, and IL-2) and decreased proliferative potential, all adding up to diminished effector functioning [14]. Exhaustion is also relevant in chronic infection, which implies that prolonged antigen exposure is the main driver of this phenomenon. Other factors could be suboptimal priming with antigen, the combination of inflammatory signals with anti-inflammatory (IL-10 and TGF-ß), and hypoxia in the TME [15]. Studies in mice with Lymphocytic choriomeningitis virus (LMCV) have shown that reducing antigen exposure, without changing the viral load, is sufficient to ameliorate exhaustion. Additionally, the persistence of antigen proved to be critical in maintaining exhausted T cells[16]. Exhaustion could have protective aspects, playing a role in limiting overt immune responses, as prolonged effector T cell functioning is harmful to the host.
At a certain point, the dysfunctional state becomes irreversible, making the CD8+ T cells terminally exhausted. However, there is way more heterogeneity within exhausted T cells. Many exhausted subtypes have been described, based on single-cell RNA and protein profiles, creating a path to exhaustion rather than a border for T cells to cross [14]. Furthermore, there is also evidence that tumour-associated CD8+ cells, expressing co-inhibitory markers, are still potent at IFN-γ production. Studies have shown that these 'pre-exhausted' or 'progenitor exhausted' T cells can show enhanced anti-tumour reactivity *ex vivo*, suggesting that maybe pre-exhausted cells manage tumour control, although maybe not as effective as CD8+ T cells would in acute infection [17]. Terminally exhausted T cells are believed to be insensitive to ICI treatment but targeting these pre-exhausted cells with ICI could still unleash potent anti-tumour immunity[5].

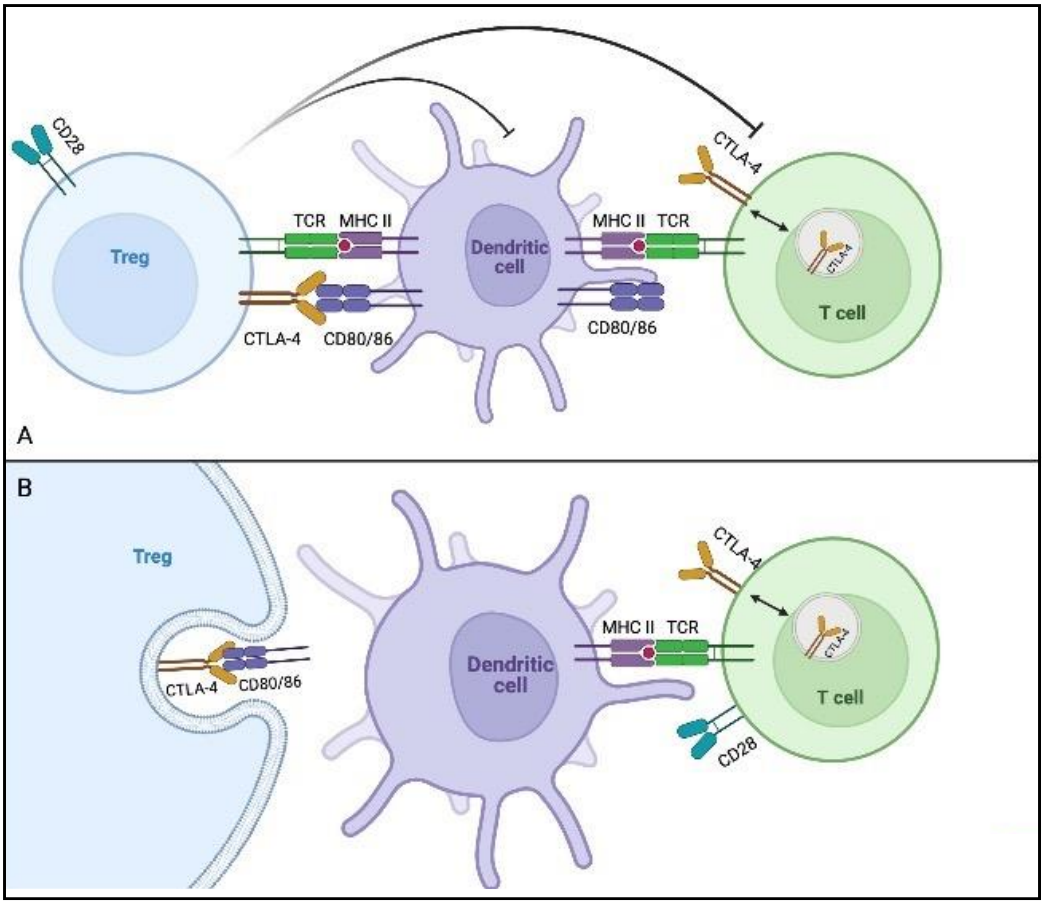
Recent view on PD-1 blockade

However, new views on PD-1 and PD-L1 blockade have risen based on recent data. PD-1 activation does not only inhibit the CD3-TCR complex, but it also inhibits CD28, just like CTLA-4. So anti-PD-1 treatment may not only relieve inhibition in the periphery but also at the priming phase [5].
Mouse studies have shown that response to anti-PD-1 treatment was lost when dendritic cells (DCs) were eliminated. This shows that anti-PD-1 treatment might be dependent on DC-T cell priming in the lymph nodes (LN) or in the TME. Adding to the importance of DCs, was the finding that tumour-resident DCs producing IL-12 and IFN-γ were shown to be crucial for tumour regression in this model. Single-cell transcriptomics in basal cell carcinoma has shown that the

CD8+ effector T cells responding to anti-PD-1 treatment were not the (pre-)exhausted in the TME, but instead newly primed CD8+ T cells that later became exhausted in the TME. This contributes to the implication that anti-PD1/PD-L1 treatment does not (only) reactivate exhausted CD8+ T cells in the TME, it contributes to the priming of new anti-tumour CD8+ T cells in tumour-draining lymph nodes. More evidence for this theory was provided in other mouse studies when these newly primed CD8+ T cells were trapped in the lymph nodes and the response of anti-PD-L1 treatment was lost. Another notion in line with this theory is that anti-PD-L1 treatments are also effective in patients that suffer from PD-L1-negative tumours, again pointing to the positive effects on priming activity by anti-PD1/PD-L1 blockade[5]. In conclusion, ICI might work on multiple levels: peripheral, central, in effector functioning, and at the priming phase. More research is needed to clarify the exact working mechanisms.

CTLA-4

Anti-CTLA4 treatment (ipilimumab) can relieve the immunosuppression on T-cell activation in the phase of T-cell priming. This effect is most profound in the LN where naive T-cells become activated by specific antigens presented by DCs together with co-stimulatory molecules and cytokines. CTLA-4 is widely expressed by Tregs and upregulated on activated T-cells. It competes with CD28 for binding to co-stimulatory CD80 and CD86 on APCs. When CD28 binds CD80 or CD86, this induces further T-cell activation, proliferation, and cytokine production[3,9]. CTLA-4 has a higher affinity for CD80 and CD86 but is continuously internalized and re-expressed by T-cells. When CTLA-4 is upregulated, the chances of it outcompeting CD28 increase, preventing the activation of T-cells. Besides this mechanism of immune inhibition, several research groups have shown that Tregs can 'steal' CD80 and CD86 molecules from APCs by CTLA-dependent trans-endocytosis, leaving the APCs with diminished abilities[18,19]. An immune synapse is created upon CTLA-4-CD80/CD86 binding which then facilitates the extraction of CD80/CD86 from the APC. This is another mechanism to prevent CD80/CD86-CD28 mediated activation of T-cells but it also increases unbound PD-L1 on the surface of APCs (figure X). It has been suggested that PD-L1 forms heterodimers with CD80 that bind CD28 but not CTLA-4 or PD-1. Furthermore, this complex can then induce T-cell activation while being unaffected by these immune checkpoints. Thus, CTLA-4-mediated inhibition of the formation of these heterodimers will add to a suppressive immune response [18,20]. Whether ipilimumab influences trans-endocytosis also remains unclear. The working mechanism of ipilimumab is not yet fully elucidated. The notion that its effect is a consequence of relieving T cell inhibition in the LN is overall established. But previous studies have suggested that CTLA-4 causes Treg depletion in the LN and TME, causing an inflammatory response which also provides anti-tumour immunity [21].



(made with BioRender)

Figure 1. **A**. Naive T cells can express and internalize CTLA-4 while Tregs constitutively express this on their surface. Upon APC-T cell contact, CTLA-4 can be upregulated and inhibit T-cell activation. **B.** Tregs use CTLA-4-mediated trans-endocytosis to abstract the co-stimulatory molecules from the APC, leaving it less able to activate T cells.

Toxicity

Administering ICI treatment is like cutting the brakes of the immune system, and naturally, this is not without consequence. ICI treatment is accompanied by off-tumour-directed inflammatory responses, which can cause severe toxicity. ICI-mediated toxicities strongly resemble auto-inflammatory and auto-immune disorders and are called immune-related adverse events (irAEs). Organs of the gastrointestinal tract, the skin, and endocrine systems are most affected by ICI-mediated toxicity. Less common irAEs are pneumonitis, myocarditis, myositis, nephritis, and acute rheumatological syndromes [22,23]. IrAEs can be unpredictable in when they occur and are very heterogenous in their severity and the underlying mechanism. The time of onset of IrAEs ranges from within the first 3 months after treatment until long after treatment termination [23]. Patients can develop multiple irAEs at the same time or subsequently. The frequency and severity of irAEs can differ per ICI treatment. While anti-PD1 treatment gives irAEs in 57-85% of the patients, combination therapy gives toxicity in 95% of the cases. IrAEs are graded by the Common Terminology for Adverse Events (CTCAE) from 1 (mild) to 5 (death) [23,24] IrAEs from grade 2 or higher are viewed as clinically relevant.

The underlying mechanism and kinetics of irAEs need to be further investigated, as it is complex and multifactorial. As mentioned above, ICI treatment type has a substantial impact on irAE development. Combination therapy induces more toxicity than either treatment alone. More irAE-dependence on ICI type is illustrated by the fact that anti-CLTA-4 treatment more often

induces hypophysitis, while anti-PD-1 and anti-PD-L1 treatment is correlated with thyroiditis and pneumonitis. CTLA-4 is expressed in on the pituitary gland cells, so direct binding of ipilimumab can lead to hypophysitis[23,25]. This is just one of the mechanisms by which ICI cause irAEs. IrAEs can also be more easily established when a patient has (undiscovered) autoimmune conditions or is more prone to develop autoimmune disease, e.g., by already having high amounts of auto-reactive lymphocytes[26]. Overt inflammation in response to pathogens or microbiome due loss of CTLA-4 or PD-1 induced tolerance may also add to the development of irAEs [25].

The tumour type can be of influence irAE development as well. Melanoma patients are more likely to develop skin-related irAEs, while there is an increased incidence of NSCLC patients developing respiratory irAEs, which might be linked to epitope-spreading in the context of ICI[22]. Expansion of tumour-directed effector T cells can cause tissue damage when attacking the site of the tumour. The inflammation releases tissue-specific self-antigens which can more easily give rise to auto-reactive T and B cell clones now self-tolerance is weakened by ICI. By blocking peripheral tolerance with anti-PD1 treatment, healthy tissue becomes more prone to the attack of auto-reactive lymphocytes. Overt inflammation because of the infiltration of more immune cells, cytokine release, and antibody-mediated toxicity cause irAEs to develop[12,23]. This could be how anti-tumour immunity, e.g., specific for neo-antigens in melanoma, expands to autoimmunity against melanocytes[26]. The findings of a small study by Läubli et al. are in line with this[27]. They found that TCRs overlap between TILs and T cells infiltrating the pulmonary tissues affected by ICI-induced toxicity[27]. These findings indicate that epitope-spreading could be correlated with some irAEs but should be interpreted with caution. It has been pointed out TCR overlap is not necessarily always biologically relevant, as TCR overlap can also be found in unaffected tissue, unrelated to the tumour[28]. On top of this notion, accurate diagnosis remains challenging, as irAEs are hard to distinguish from pre-existing comorbidities, infections, and other possible etiologies[24]. Furthermore, by far not all irAEs are related to tumour type. Tissue destruction causing epitope spreading can give rise to self-antigen that can cause cross-reactivity in different tissues. Furthermore, highly mutated cancers can give rise to neo-antigens causing cross-reactivity with healthy tissues as well. Additionally, because anti-CTLA-4 treatment non-specifically relieves inhibition at the initial stage of T cell activation, the threshold for any self-reactive T cell is lowered[29]. Now that recent observations indicate that anti-PD1 treatment also relieves the inhibition in T cell priming, this mechanism for irAE development has become more probable [5]. Besides, PD-1 is also expressed on Tregs, natural killer cells (NKs), B cells, innate lymphoid cells (ILCs), and T follicular regulatory cells. Anti-PD-1 treatment may interfere with the functioning of all these cells, adding to the break of self-tolerance and the induction of irAEs [25].

Treatment of irAEs

While some irAEs, like a rash starting within weeks after treatment, can be self-limiting, more severe irAEs need treatment with immunosuppressants. Management of severe ICI-mediated toxicity differs per irAE. Colitis, which is one of the most prominent irAEs, has served as an example to treat other irAEs. Persistent grade 1 or grade 2 and higher colitis are initially treated with 1 or 2 mg/kg/day of corticosteroids. If the symptoms are not relieved within 48-72 hours or keep returning, ICI treatment is discontinued, and infliximab (anti-TNF antibodies) may be given to decrease symptom duration and corticosteroid exposure. With no improvement of symptoms, tocilizumab (anti-IL6R antibodies) or vedolizumab (anti-$\alpha_4\beta_7$ integrin antibodies) can

be administered[23]. ICI treatment might be continued if symptoms are relieved. However, severe damage to endocrine systems (e.g., causing insulin deficiency) may result in life-long immunosuppressive and hormone-replacing therapy. Treating irAEs has proven to be a challenge in the clinic because of factors like late diagnosis, the toxic effects of immunosuppressants, and their dampening effect on anti-tumour immunity[12]. More research into the mechanism of irAEs is needed to find other treatment options and strategies.

Predicting response and toxicity

ICI is becoming increasingly relevant in the field of cancer treatment. Yet only a part of treatment-receiving patients develops good response rates while almost all patients suffer from any-grade irAEs. Therefore, predictors in the form of biomarkers could be extremely valuable. Predictors at baseline or during treatment, that anticipate severe irAE development, give clinicians the chance to alter treatment strategy. When baseline values indicate that patients have an increased risk of severe irAE development, this could be a reason to refrain from ICI treatment or use nivolumab alone instead of together with ipilimumab and closely monitor the patient. If patient values point towards severe toxicity during treatment, the treatment could be terminated before severe irAEs develop, or the patient could be closely monitored and perhaps treated prophylactically.

Some interesting biomarkers include increased neutrophil-to-lymphocyte ratio's, which are correlated with less treatment response. High T cell clonality at baseline or high expansion of T cell clones are correlated with increased irAEs risk, but interestingly also with increased treatment response. Overall, IrAE development is linked to better treatment responses and prolonged survival in multiple types of cancers [23].
More examples are cytokine levels, microbiome, and the detection of auto-antibodies. Unfortunately, most biomarkers lack the predictive power and mechanistic understanding to be translated to the clinic.

More mechanistic understanding can be gained by further investigation of peripheral immune responses induced by ICI. Other studies investigating the peripheral expansion of T cells and their expression patterns have already found links to response, OS, and toxicity. Decreased CD8+ T cell levels at baseline were associated with longer PFS as well as clinical benefit and response[30,31]. In studies investigating anti-PD-1 therapy, more contradicting findings were found on whether on-treatment increases of peripheral CD4+/CD8+ T cells with co-inhibitory receptors like PD-1 and TIM-3 are positively or negatively linked to response, clinical benefit, and OS [32–34]. The clonal expansion of CD8+ T cells foregoing irAE development in patients treated with ipilimumab was observed as well [35]. But not many studies have linked peripheral immune responses to toxicity. Hence, it becomes clear that more research is needed to elucidate the dynamics and functionality of blood-based immune cell populations in the context of ICI treatment, so we can clarify how they relate to toxicity and treatment outcome.

Aim

All in all, multiple challenges present themselves with the increasing importance of ICI treatment. The working mechanisms of ICI therapies and how they lead to irAE development are not fully clarified. This adds to the complexity of treating patients with ICI and their irAEs. The current treatment options for irAEs are far from ideal so new options and strategies are

needed. Biomarkers with adequate predictive power that are translational to the clinic are scarce but also very much necessary.

Further investigation of peripheral immune responses during ICI treatment could be valuable when comparing responders and non-responders and patients with clinically relevant toxicity and patients who stay toxicity-free. This information could indicate new predictive values and tell us more about the mechanism behind response and toxicity. An important topic in the context of peripheral immune responses induced by ICI is the functionality of CD4+ and CD8+ T cells in the context of coinhibitory receptor expression and potential exhaustion. Their role remains incompletely understood within ICI and clarification could improve treatment strategies and response rates.

In this study we aim to shine a light on the underlying mechanisms in ICI treatment by characterizing the peripheral immune responses in T cells *ex vivo,* using flow cytometry. We will do this by analyzing the PBMCs of patients treated with either monotherapy or combination therapy. We measured three samples with PBMCs per patient, taken at three different time points: at baseline, after the first and after the second dose of treatment, or when patients had developed clinically relevant toxicity. Healthy donors are taken along to serve as controls. Half of the patients in our cohort developed clinically relevant toxicity while the other half did not, allowing us to compare the peripheral immune responses between these two groups. In addition, the differences between responders and non-responders and those who experience clinical benefit and those who do not will be investigated as well. By doing this, we hope to provide a piece of the information that is needed to increase response rates, and improve treatment strategies but also find new targets to treat irAEs and predict their occurrence.

Methods section

Sample collection and study approval

This study uses the PBMCs of cancer patients, treated with either monotherapy with nivolumab or pembrolizumab or combination therapy with nivolumab and ipilimumab. Adult patients with solid malignancies in stage III or IV, undergoing immunotherapy for the first time, in the UMC Utrecht can be included in the UNICIT study. Patient blood is collected before the start of treatment (baseline), 3 weeks after the first dose, 3 weeks after the second dose, and/or when patients develop ICI-mediated toxicity. All patients signed informed consent compliant with the Declaration of Helsinki Principles. PBMCs are isolated from patient blood and cryopreserved in the biobank for the UNICIT study (UNICIT, UMC Utrecht). This study was approved by the Biobank Research Ethics Committee (TCbio). Approval for patient sample biobanking was granted with Biobankprotocol 18-123, and later requested and granted with protocol TCBio 19-704. Blood samples from healthy donors that served as controls were provided through the Mini Donor Service in the University Medical Center Utrecht.

Definition of toxicity, response, and clinical benefit

The development of toxicity was considered clinically relevant when a patient suffered from irAEs graded 2 or higher and had to interrupt ICI treatment and needed to be hospitalized and/or receive steroids of at least 0.5mg/kg. A grade 1 irAE was not considered clinically relevant and not classified as toxicity in this study.

Patient response was classified by RECIST 1.1. Patients with a complete response (CR) or partial response (PR) as best overall response were considered responders. Non-responders were classified as patients with a best overall response of either progressive disease (PD) of stable disease (SD). Patients were considered to experience clinical benefit when the outcome of their best overall response was CR, PR or SD.

Patient characteristics

43 patients with solid malignancies in stage III or IV were included in this study. 21 out of 43 (48,8%) patients had no or grade 1 irAEs and are referred to as the ‘non-toxicity group’. 11 (52,4%) of the patients in the non-toxicity group were treated with anti-PD1 treatment and 10 (47,6%) were treated with combination therapy. 22 out of 43 (51,2%) patients suffered from clinically relevant toxicity and are referred to as the ‘toxicity group’. 12 (54,5%) patients in the toxicity group were treated with monotherapy and 10 (45,5%) were treated with combination therapy. Other details regarding treatment setting, previous treatment, treatment outcome, toxicity, and deceased patients are given in the table below (table 1).

Patient characterictics	HD (n=10)	No toxicity (n=21)	Toxicity (n=22)
Age, median (range)	50 (39 -61)	64 (56-72)	65 (50-73)
Gender, n(%)			
Male	3 (30)	18 (85,7)	16 (72,7)
Female	7 (70)	3 (14,3)	6 (27,3)
Primary tumor, n(%)			
Melanoma		10 (47,6)	18 (81,8)
NSCLC		0	1 (4,5)
RCC		7 (33,3)	2 (9,1)
Urothelial carcinoma		3 (14,3)	0
Other		1 (4,8)	1 (4,5)
Stage, n(%)			
Stage III		3 (14,3)	5 (22,7)
Stage IV		18 (85,7)	17 (77,3)
Setting, n(%)			
Adjuvant		5 (23,8)	4 (18,2)
Palliative		16 (76,2)	18 (81,8)
Previous proinflammatory treatment		3 (14,3)	3 (13,6)
Previous treatment with chemotherapy		2 (9,5)	0
ICI treatment type, n(%)			
anti-PD1 (monotherapy)		11 (52,4)	12 (54,5)
anti-PD1 and anti-CTLA-4 (combination therapy)		10 (47,6)	10 (45,5)
Best overall response, n (%)			
CR (complete response)		1 (4,8)	2 (9)
PR (partial response)		6 (28,6)	4 (18,2)
SD (stable disease)		3 (14,3)	6 (27,3)
PD (progressive disease)		9 (42,9)	5 (22,7)
N/A		2 (9,5)	5 (22,7)
Days between treatment initiation and toxicity development, median (range)			42 (28,25 - 69,25)
Type of toxicity, n (%)			
Colitis or diarrhea			4 (11,8)
Dermatitis or other skin condition			2 (5,9)
Hepatitis			6 (17,7)
Inflammation of the GI tract			8 (23,5)
Other			14 (41,2)
Highest grade of toxicity, n (%)			
Grade 2			8 (36,4)
Grade 3			11 (50)
Grade 4			3 (13,6)
Number toxicities, n (%)			
1 toxicity			14 (63,6)
2 toxicities			5 (22,7)
3 or more toxicities			3 (13,6)
Died, n (%)		7 (33,3)	9 (40,9)
Months between treatment initiation and death or censoring, median (range)		20 (10-31)	15 (10-33)

Table 1. Table with patient characteristics

Sample processing and assessment

PBMCs isolation

To isolate the PBMCs, Ficoll-Paque density gradient centrifugation was used. After centrifugation, the ring of PBMCs was collected, Ficoll was washed away, and the PBMCs were counted and resuspended in freezing medium. Aliquots of approximately 10 million PBMCs per 1mL were frozen at −80°C before cryopreservation in liquid nitrogen in the biobank.

Thawing and stimulation of patient PBMCs

Vials with patient PBMCs were thawed at 37 °C. The samples were diluted in culture medium, counted, washed with wash medium, and brought to a concentration of 5 x 10^6 cells/mL in culture medium before plating them in round-bottom 96-wells plates. The cells were stimulated with PMA (20ng/mL) and Ionomycin (1 µg/mL) at 37°C for 3,5 hours. After the first

30 minutes of stimulation, GolgiStop (BD Biosciences, diluted 1500 times) was added to trap the produced cytokines in the cytoplasm.

Immunophenotyping with flow cytometry

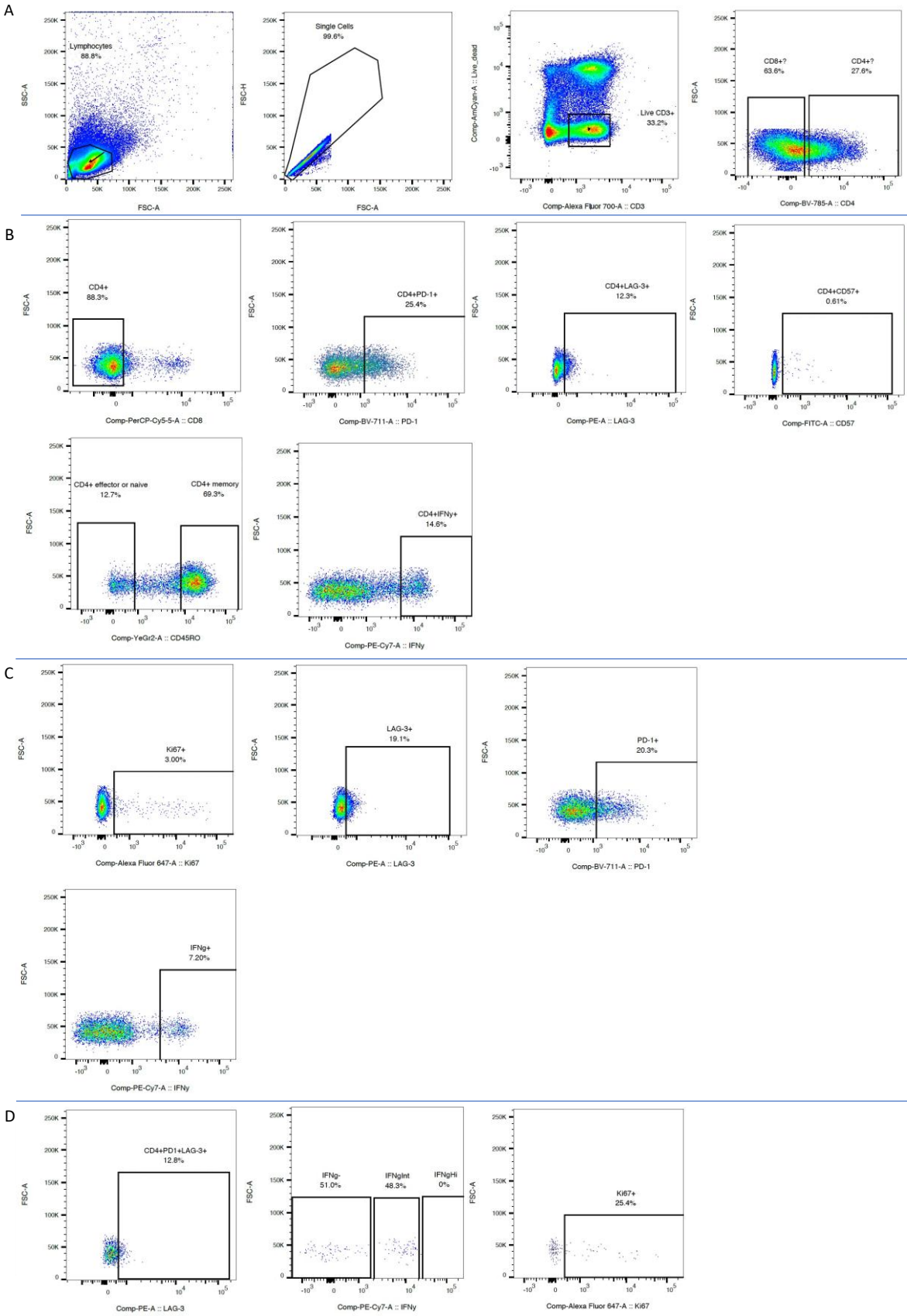
Stimulated and unstimulated conditions were stained with cell surface markers from nine different panels to assess several different cell populations. However, in this report, we will only focus on the T-cell exhaustion panel (table 2). First, the cells were stained with Fixable viability dye for 30 minutes at 4°C. Surface and intracellular staining mixes were supplemented with mouse or rat serum to prevent a-specific antibody binding. In addition, brilliant stain buffer was added to the surface staining mix to prevent a-specific polymer binding. Samples were incubated with surface staining mix for 25 minutes at 4°C. Panels that included PD-1 were stained with PD1-BV711 as well as IgG4-BV711 to visualize PD-1 expression as well as nivolumab-bound PD-1 expression. This method of staining PD-1 is further described by Osa et al. and was tested again within this research project[36]. Samples stained with this method required an extra staining step with anti-biotin-streptavidin-conjugated antibodies that binds as a secondary antibody to IgG4-BV711. Incubation with Fixation and Permeabilization reagent for 30 minutes at 4°C, allowed intracellular antibody staining. Intracellular staining was performed for 30 minutes at 4°C. After intracellular staining, the samples were resuspended in 200 µL FACS buffer and analyzed on an LSR Fortessa ™ cytometer (Becton Dickinson). Each time samples were measured; Rainbow calibration particles were also measured to check the stability of the cytometer's lasers and ensure comparability between measurements.

Laser	channel	Filter	Fluorochrome	Target	Target location	Clone	Dilution	Company	Catalog Number
Panel 2 *T cell exhaustion*									
488	Blue 1	530/30	FITC	CD57	Extracellular	HNK-1	100	Biolegend	359603
	Blue 3	710/50	PerCP-Cy5.5	CD8a	Extracellular	SK1	100	BD	341050
640	Red 1	670/14	AF647	Ki67 / CD158b	Intracellular	B56	50	BD	558615
	Red 1/1	730/45	AF700	CD3	Extracellular	UCHT1	50	Biolegend	300424
	Red 2	780/60	APC-Fire750	GzmB	Intracellular	QA16A02	50	Biolegend	372210
405	Violet 1	450/40	eFluor450	CD95 (FAS)	Extracellular	DX2	50	Thermo Fisher Scientific	48-0959-42
	Violet 2	525/50	eFluor506	Fixable viability dye	__	__	1000	Thermo Fisher Scientific	65-0866-14
	__	__	Biotin	IgG4	Extracellular	HP6025	200	Invitrogen	A10663
	Violet 4	670/30	BV711	Streptavidin	Extracellular	__	100	Biolegend	405241
	Violet 5	710/50	BV711	PD-1	Extracellular	EH12.1	100	BD	564017
	Violet 6	785/60	BV785	CD4	Extracellular	RPA-T4	50	Biolegend	300554
561	Y/Gr 1	586/15	PE	LAG-3	Extracellular	__	25	R&D	FAB2319P
	Y/Gr 2	610/20	PE-Dazzle	CD45RO	Extracellular	UCHL1	200	Biolegend	304247
	Y/Gr 5	780/60	PE-Cy7	IFNg	Intracellular	4S.B3	200	BD	557844

Table 2. Panel for T cells and exhaustion

FACS analysis and gating strategy

Basic analysis to check whether surface and intracellular staining were successful and scan the data for any abnormalities, was done in BD FACS diva during measurements on the LSR Fortessa ™ cytometer. The gating strategy was done in Flow Jo software version 8 (figure 2). Alterations were made to the panels during data collection and analysis. We decided to use a Ki-67-AF647 antibody instead of a Ki-67-BV650 antibody because of improved fluorescent intensity. This means that Ki-67 expression for some samples was measured on a different antibody. Ki-67 expression is therefore not comparable between all samples. CD158b-AF647 was removed from the panel to make room for Ki-67-AF647. The gating strategy for T cells and exhaustion is given below (figure 2).


A
Lymphocytes
88.8%
SSC-A
FSC-A
Single Cells
99.6%
FSC-H
FSC-A
Comp-AmCyan-A :: Live_dead
Live CD3+
33.2%
Comp-Alexa Fluor 700-A :: CD3
CD8+?
63.6%
CD4+?
27.6%
FSC-A
Comp-BV-785-A :: CD4
B
CD4+
88.3%
FSC-A
Comp-PerCP-Cy5-5-A :: CD8
CD4+PD-1+
25.4%
FSC-A
Comp-BV-711-A :: PD-1
CD4+LAG-3+
12.3%
FSC-A
Comp-PE-A :: LAG-3
CD4+CD57+
0.61%
FSC-A
Comp-FITC-A :: CD57
CD4+ effector or naive
12.7%
CD4+ memory
69.3%
FSC-A
Comp-YeGr2-A :: CD45RO
CD4+IFNγ+
14.6%
FSC-A
Comp-PE-Cy7-A :: IFNγ
C
Ki67+
3.00%
FSC-A
Comp-Alexa Fluor 647-A :: Ki67
LAG-3+
19.1%
FSC-A
Comp-PE-A :: LAG-3
PD-1+
20.3%
FSC-A
Comp-BV-711-A :: PD-1
IFNg+
7.20%
FSC-A
Comp-PE-Cy7-A :: IFNγ
D
CD4+PD1+LAG-3+
12.8%
FSC-A
Comp-PE-A :: LAG-3
IFNg-
51.0%
IFNgInt
48.3%
IFNgHi
0%
FSC-A
Comp-PE-Cy7-A :: IFNγ
Ki67+
25.4%
FSC-A
Comp-Alexa Fluor 647-A :: Ki67

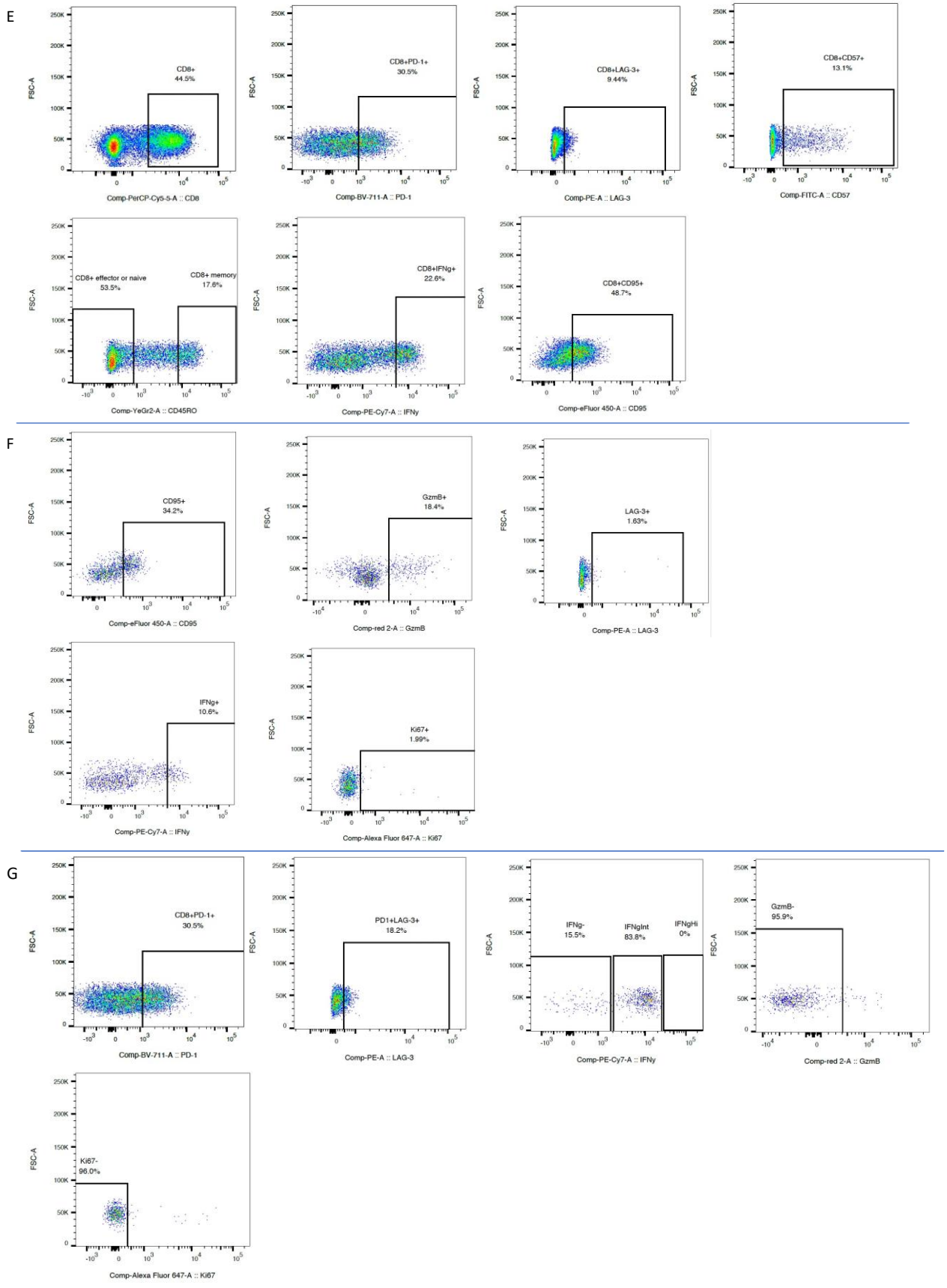


Figure 2. Gating strategy for T cells and exhaustion. **A.** Basic gating of lymphocytes. **B.** CD4+ T cells gated from live CD3+CD8- T cells and all markers gated within CD4+ T cells. **C.** Gating within effector or naïve (CD45RO-) and memory CD4+ T cells (CD45RO+). **D.** Gating for exhausted CD4+ T cells. **E.** CD8+ T cells gated from live CD3+CD4-

T cells and all markers gated within CD8+ T cells. **F.** Gating within effector or naïve and memory CD8+ T cells. **G.** Gating for exhausted CD8+ T cells.

Statistical analysis

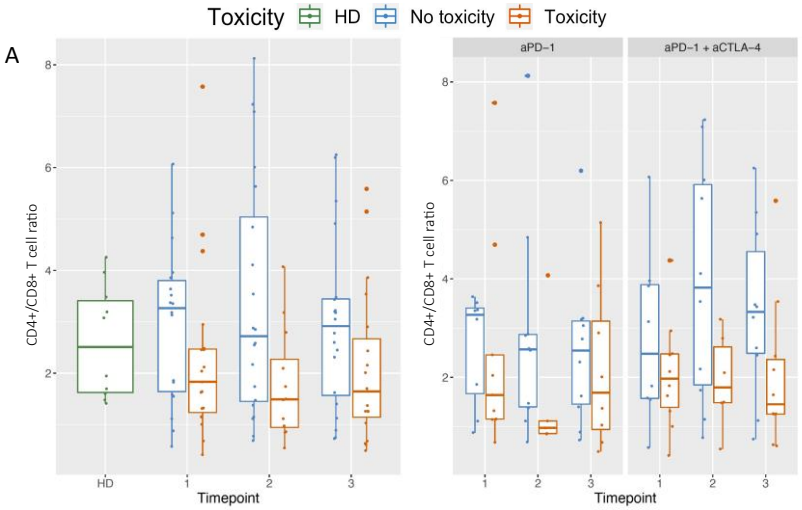
Mixed ANOVA and repeated-measures ANOVA were performed to find potentially significant differences. Differences between two groups of patients (e.g., responders versus non-responders, toxicity versus non-toxicity) within time points were assessed by doing a Wilcoxon-rank test (2-tailed, nonparametric test for non-paired samples) on the change scores between timepoints. For differences in baseline variables, the Wilcoxon-rank test was used to calculate *p*-values. $p < 0.05$ (*) and $p < 0.01$ (**) were considered statistically significant. For examples of how the statistical analysis was performed in R studio, see supplementary.

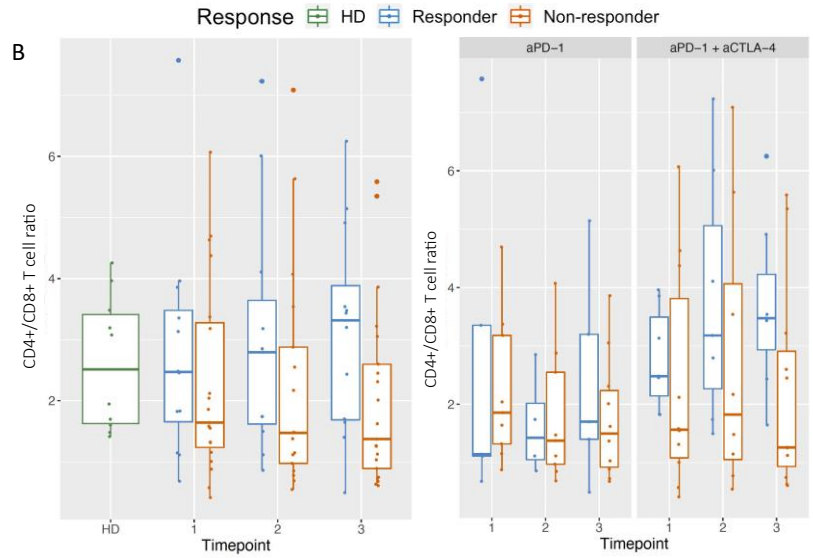
Results

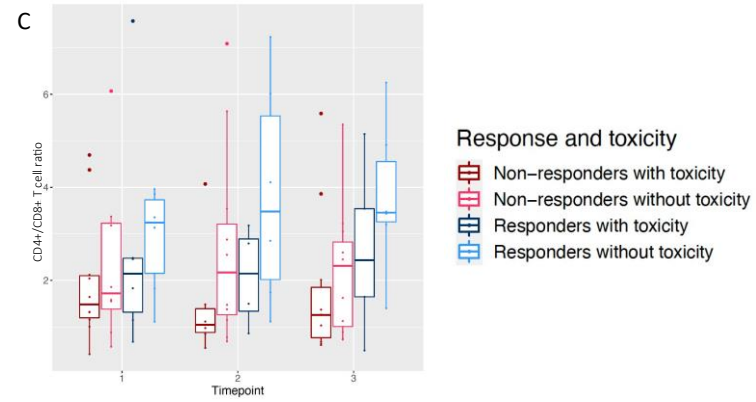
CD4+/ CD8+ T cells ratio at baseline is linked to treatment, response, and toxicity

We first investigated if there were any variables in peripheral blood that correlate with toxicity or response, that were already visible at baseline. Patients that don't develop toxicity have an overall higher CD4+/CD8+ T cell ratio compared to patients in the toxicity group. Combination therapy (anti-PD-1 + anti-CTLA4 treatment) induces an increased CD4+/CD8+ T cell ratio in patients that did not suffer from toxicity. Yet, in monotherapy (anti-PD-1 treatment), the CD4+/CD8+ T cell ratio stayed relatively stable for all patients (figure 3A). The increase in ratio induced by combination therapy is mainly due to an enlargement of the CD4+ T cell compartment(not shown, supplementary figure 1). The type of treatment does not result in a major difference in CD8+ T cells (not shown, supplementary figure 1). Within patients under combination treatment, a higher CD4+/CD8+ T cell ratio at baseline is seen in responders, meaning they had complete response (CR) or partial response (PR) compared to the non-responders that had progressive disease (PD) or stable disease (SD) (figure 3B). Overall, responders without toxicity had the highest CD4+/CD8+ T cell ratio at all time points (figure 3C). Four out of six patients in this group had combination therapy. No striking differences were found in the ratio between effector or naïve (CD45RO-) and memory (CD45RO+) cells, within CD4+ or CD8+ T cells between the toxicity group and non-toxicity group (not shown, supplementary figure 2).

CD4/CD8 T cell ratio in response and toxicity







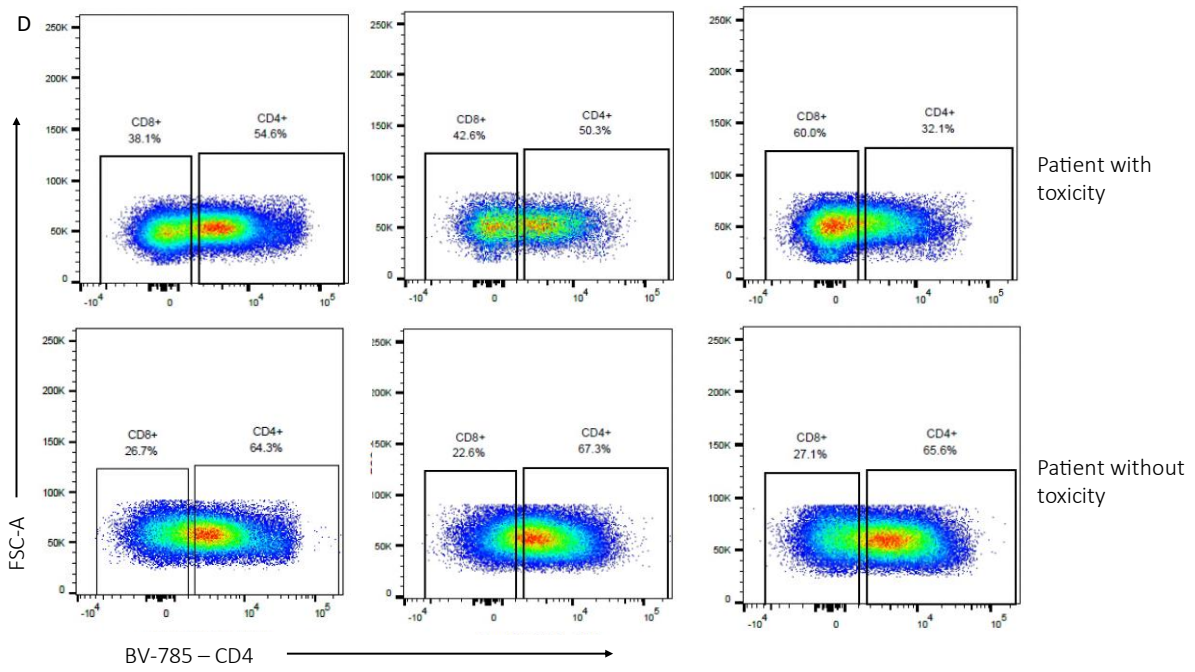


Figure 3. In all boxplots, the medians with interquartile range is shown. **A.** CD4+ and CD8+ T cells as a part of all lymphocytes (live CD3+) were measured before treatment (baseline)(Timepoint = 1), after the first (Timepoint = 2) and second cycle of treatment (Timepoint = 3) or when toxicity occurred (Timepoint = 3) and are given as a ratio. This ratio is also shown per treatment (anti-PD1 or anti-PD1 + anti-CTLA-4 treatment.) **B.** CD4+/CD8+ T cell ratio for responders and non-responders, also stratified per treatment type. **C.** CD4+/CD8+ T cell ratio for responders, non-responders in both the toxicity and non-toxicity groups. **D.** An representative image of the flow cytometry gating of the ratio between CD4+ and CD8+ T cell, comparing patients with and without toxicity.

Does (PD-1+)LAG-3+ CD8+ T cells predict at clinical benefit at baseline?

We further explored if there were any other variables that could have any predictive value. Figure 4 shows that having elevated LAG-3+ and PD-1+LAG-3+ fractions of CD8+ T cells at baseline, seem to be associated with not having clinical benefit (PR, CR or SD) from monotherapy. If treated with combination therapy, these fractions are not clearly associated with clinical benefit. As monotherapy is initiated, we see that the fractions of patients that had clinical benefit decrease more steeply in size compared to the few patients without clinical benefit.

(PD-1+)LAG3+ CD8+ T cells in clinical benefit

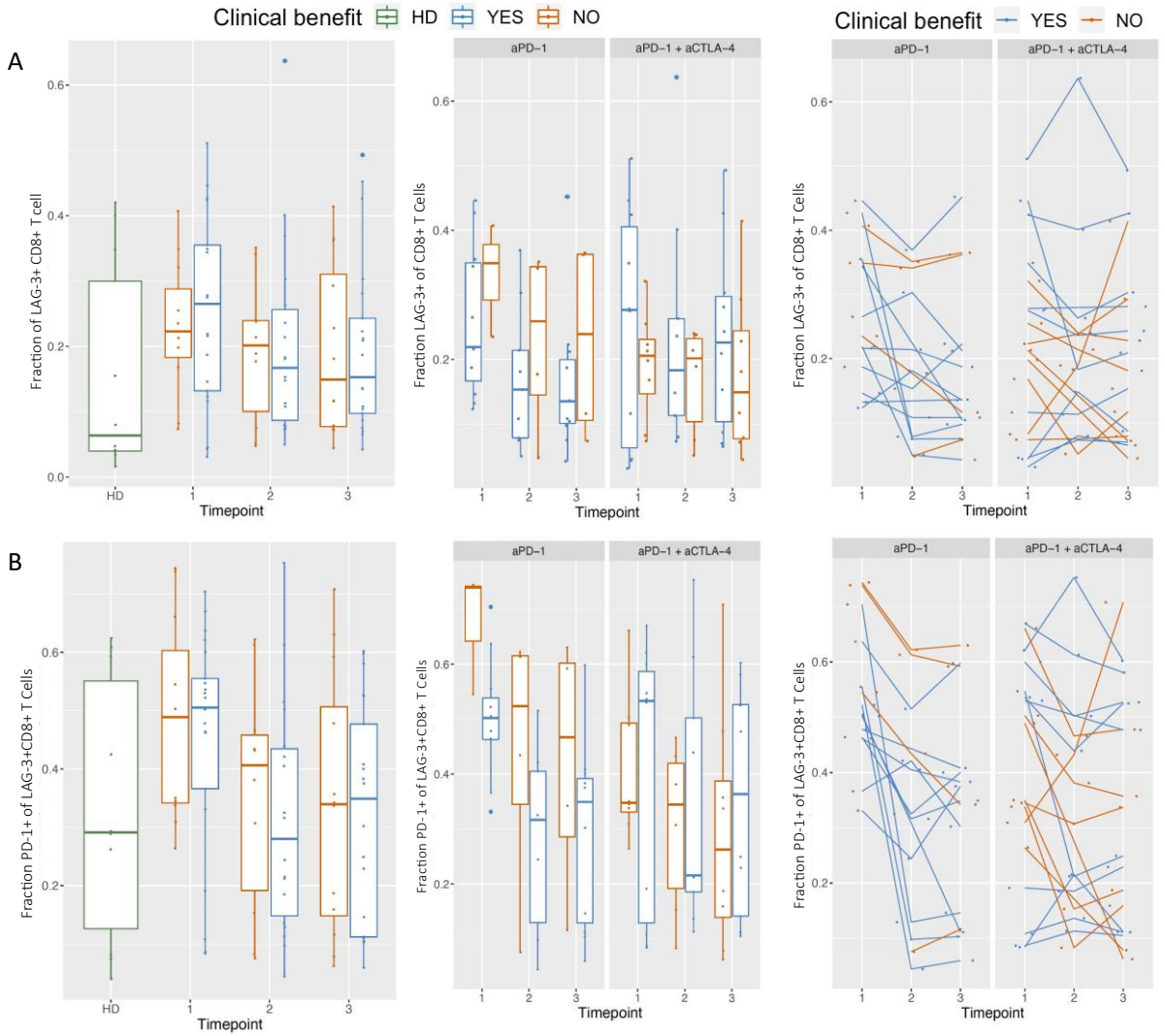
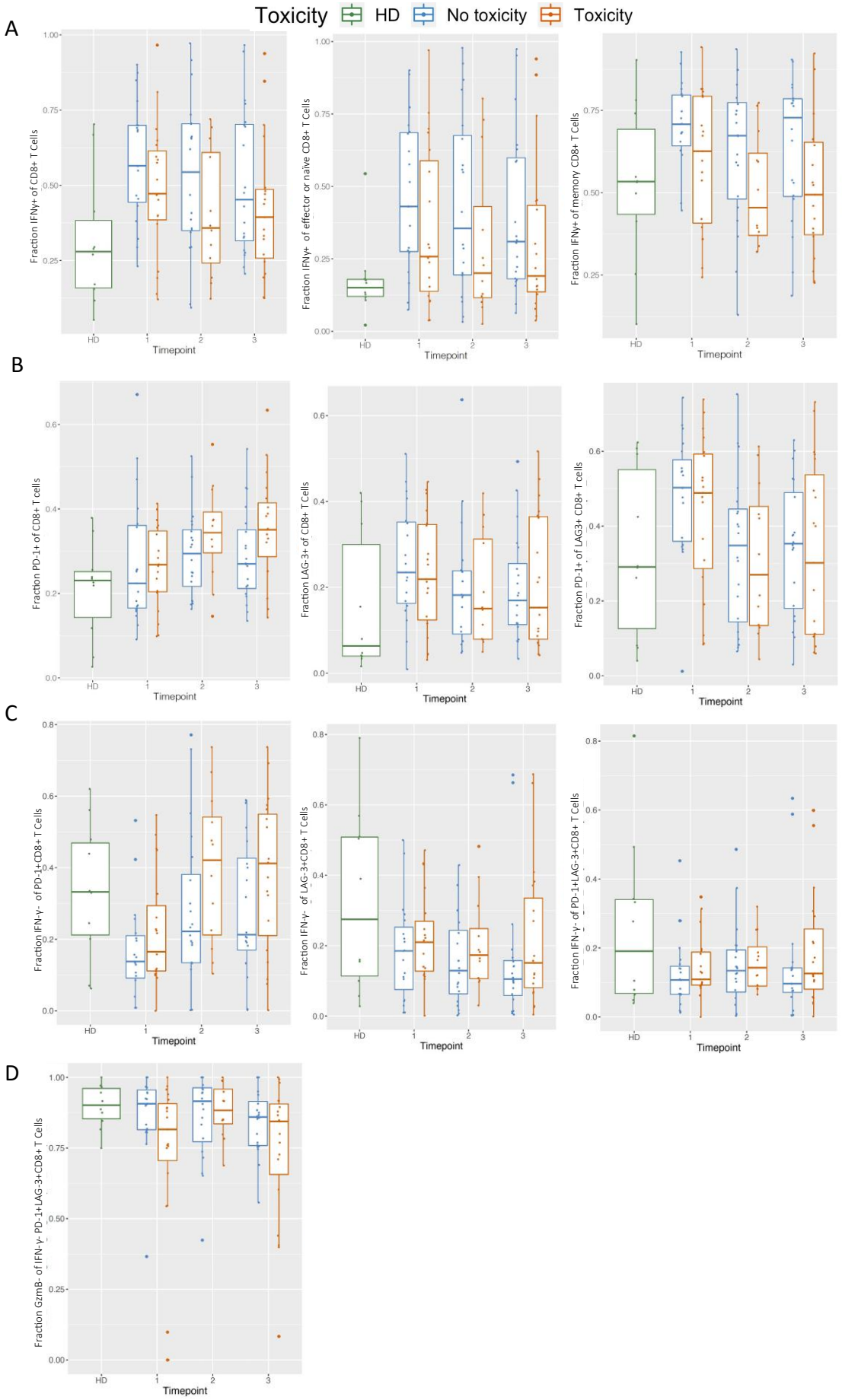


Figure 4. A. LAG-3+ fraction of CD8+ T cells for patients with and without clinical benefit (PR, CR, SD), at all measured time points, stratified per treatment type. Including the individual patient trajectories. **B.** PD-1+LAG-3+ fractions of CD8+ T cells for patients with and without clinical benefit (PR, CR, SD), at all measured time points, stratified per treatment type. Including the individual patient trajectories.

Upregulation of co-inhibitory receptors in CD8+ T cells in toxicity

Secondly, in unraveling what mechanisms drive toxicity, we examined the functionality of CD4+ and CD8+ T cells. We observed a stronger treatment-induced decrease in IFN-γ-expressing CD8+ T cells in peripheral blood among patients with toxicity. When looking into different subsets of CD8+ T cells, it is shown that this is the case, especially in memory (CD45RO+) but also in effector or naïve (CD45RO-) CD8+ T cells (figure 5A). We also observed increased PD-1 expression in CD8+ T cells in patients with toxicity (figure 5B). The increase in PD-1 expression is visualized by an example of flow cytometry gating (figure 5E). LAG-3+ and PD-1+LAG-3+ fractions of CD8+ T cells are decreased in patients with toxicity compared to patients without toxicity (figure 5B). The IFN-γ- fractions of CD8+ T cells are higher in patients with toxicity. Almost all cells within PD-1+LAG-3+ CD8+ T cells were unable to produce granzyme B (figure 5D).

Upregulation of co-inhibitory receptors in CD8+ T cells



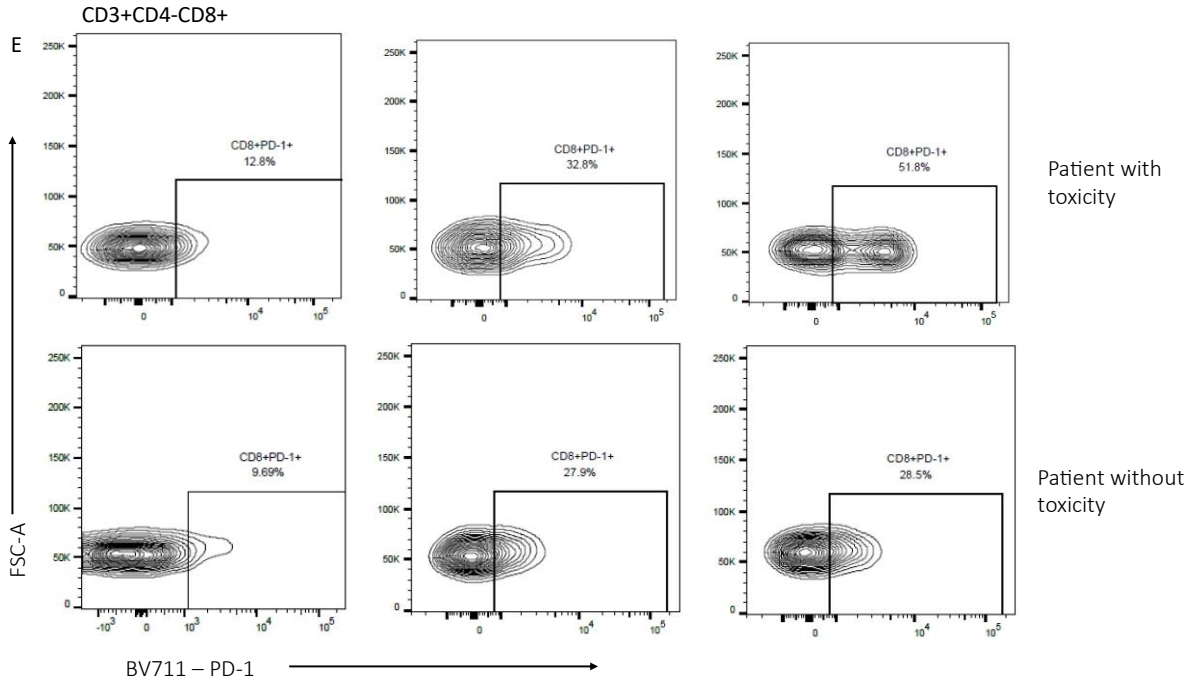
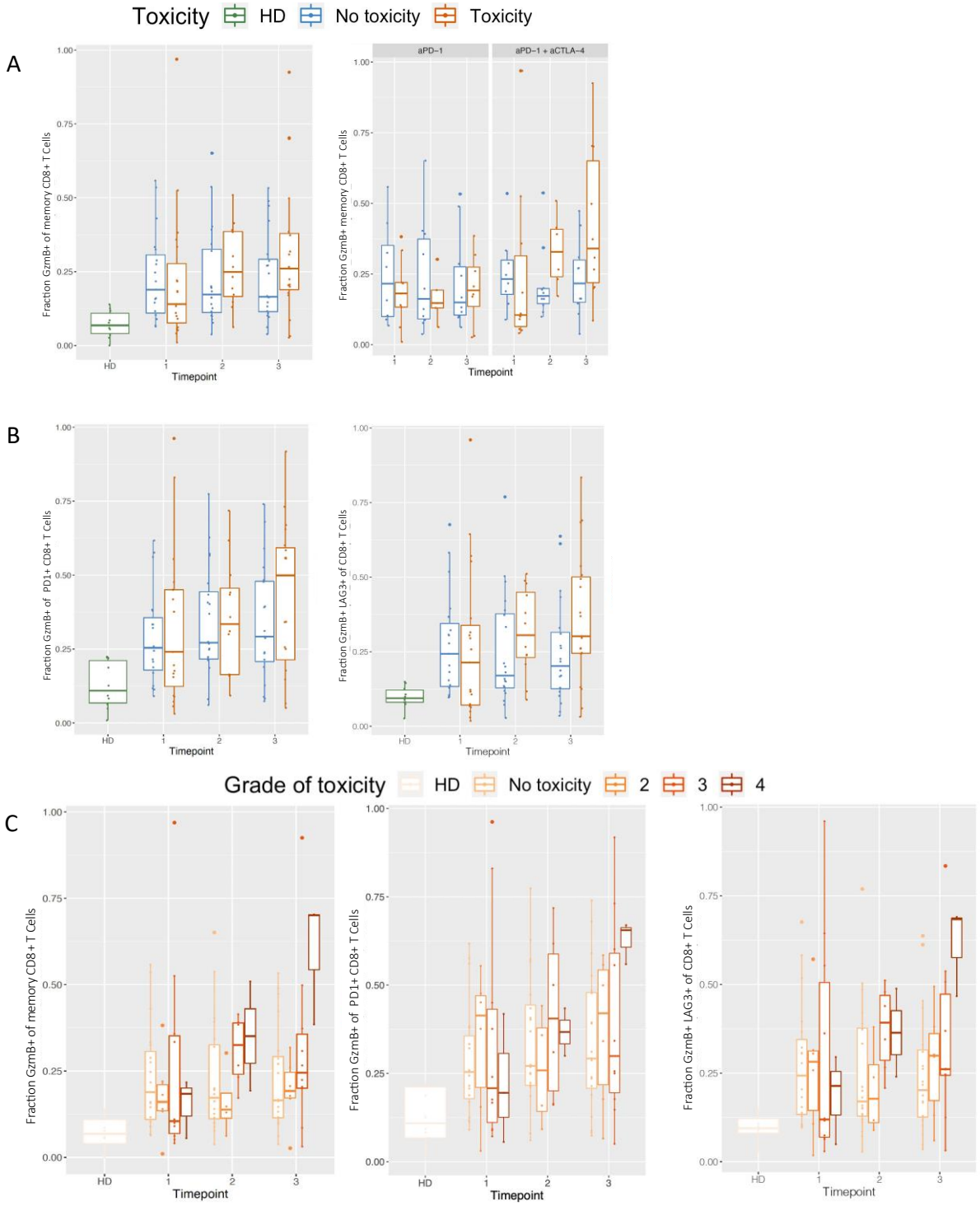


Figure 5. A. The IFN-γ+ populations of CD8+ T cells and its effector or naïve (CD45RO-CD8+) and memory (CD45RO+CD8+) subsets at all measured time points for the toxicity and non-toxicity groups. **B.** The PD1+, LAG-3+, and PD-1+LAG-3+ populations within CD8+ T cells at all measured time points for the toxicity and non-toxicity groups. **C.** The IFN-γ- populations within PD1+, LAG-3+, and PD-1+LAG-3+ fractions of CD8+ T cells at all measured time points for the toxicity and non-toxicity group. **D.** The granzyme B- population for the PD-1+LAG3+ IFN-γ- of all CD8+ T cells is shown at all measured time points for the toxicity and non-toxicity groups. **E.** A representative image of the flow cytometry gating of the PD-1 expression by CD8+ T cells in FlowJo, comparing patients with and without toxicity. Gating for PD-1 expression is set differently for baseline samples and on-treatments samples due to different use of antibody staining at these time points.

CD8+ T cells producing granzyme B might play a role in toxicity

Next, we analyzed what other cell populations changed after the initiation of ICI treatment. Within the toxicity group, we saw an increase in granzyme B producing PD-1+, LAG3+, and memory CD8+ T cells. In the non-toxicity group, these populations stay more or less stable (Figure 7A). This trend is treatment-independent in PD-1+ and LAG3+ CD8+ T cells (figure 7B). But the increase in granzyme B producing memory CD8+ T cells is specific for patients treated with combination therapy. There is another indication that this particular subset contributes to toxicity in CTLA-4 blockade. Within memory CD8+ T cells, it shows a trend in which more severe toxicity correlates to an overall bigger granzyme B + fraction that increases as treatment continues (Figure 7C). However, the groups being compared here are small and the spread among patients is substantial. An exemplary visualization in flow cytometry is shown, of a patient with grade 4 toxicity that shows how a substantial part of the memory CD8+ T cell population, eventually becomes granzyme B+ (Figure 7D).

Granzyme B producing CD8+ T cells subsets in toxicity



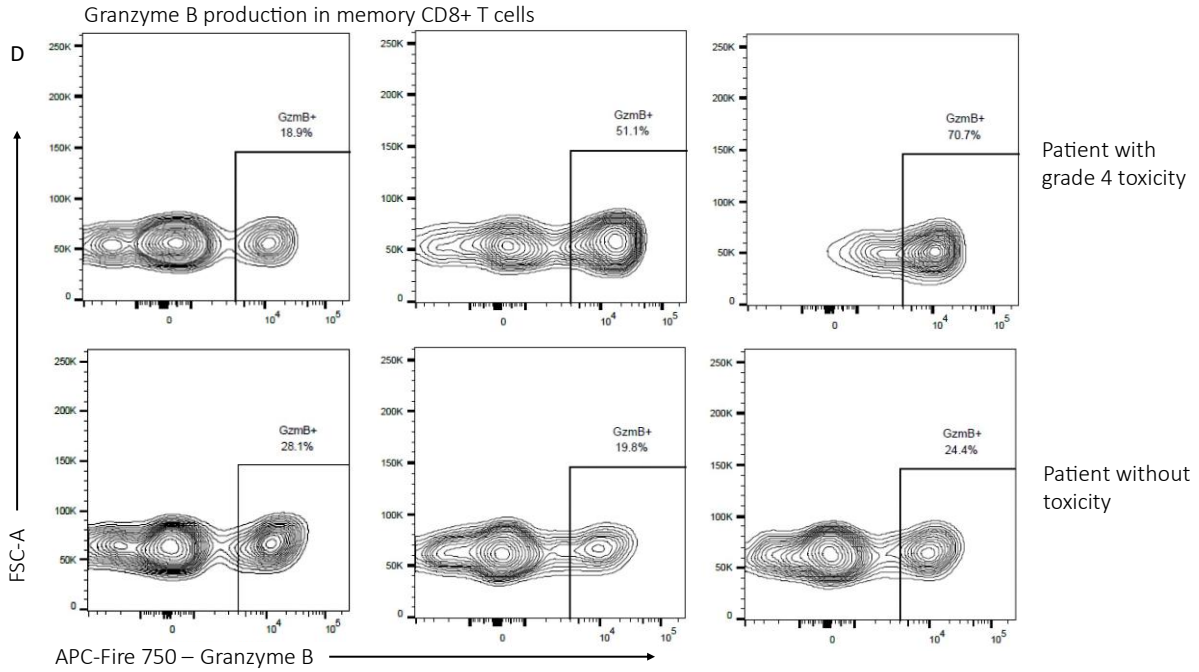


Figure 7. **A.** Granzyme B+ fractions of memory CD8+T cells for both cohorts at all measured time points for both treatment types. **B.** Granzyme B+ fractions of LAG3+ and PD-1+ CD8+T cells are shown for both cohorts at all measured time points. **C.** Graphs showing the granzyme B+ cells within populations of memory CD8+ T cells in patients with different toxicity severity (highest grade of toxicity by CTCAEv5 grading) at all measured time points. **D.** A representative visualization in flow cytometry of the granzyme B+ fraction of memory CD8+ T cells in a patient with grade 4 toxicity versus a patient without toxicity is shown.

Combination therapy induces more memory (PD-1+) T cells

Combination therapy induces larger fractions of PD-1 expressing CD4+ T cells in all patients (not shown, supplementary data, figure 4). In patients with toxicity, an elevated level of memory CD4+ T cells is already present at baseline. Within this group, we also observed a striking increase in PD-1 expression in memory CD4+ T cells (figure 8B). We observed a similar trend for PD-1 expressing memory CD8+ T cells (figure 8C).

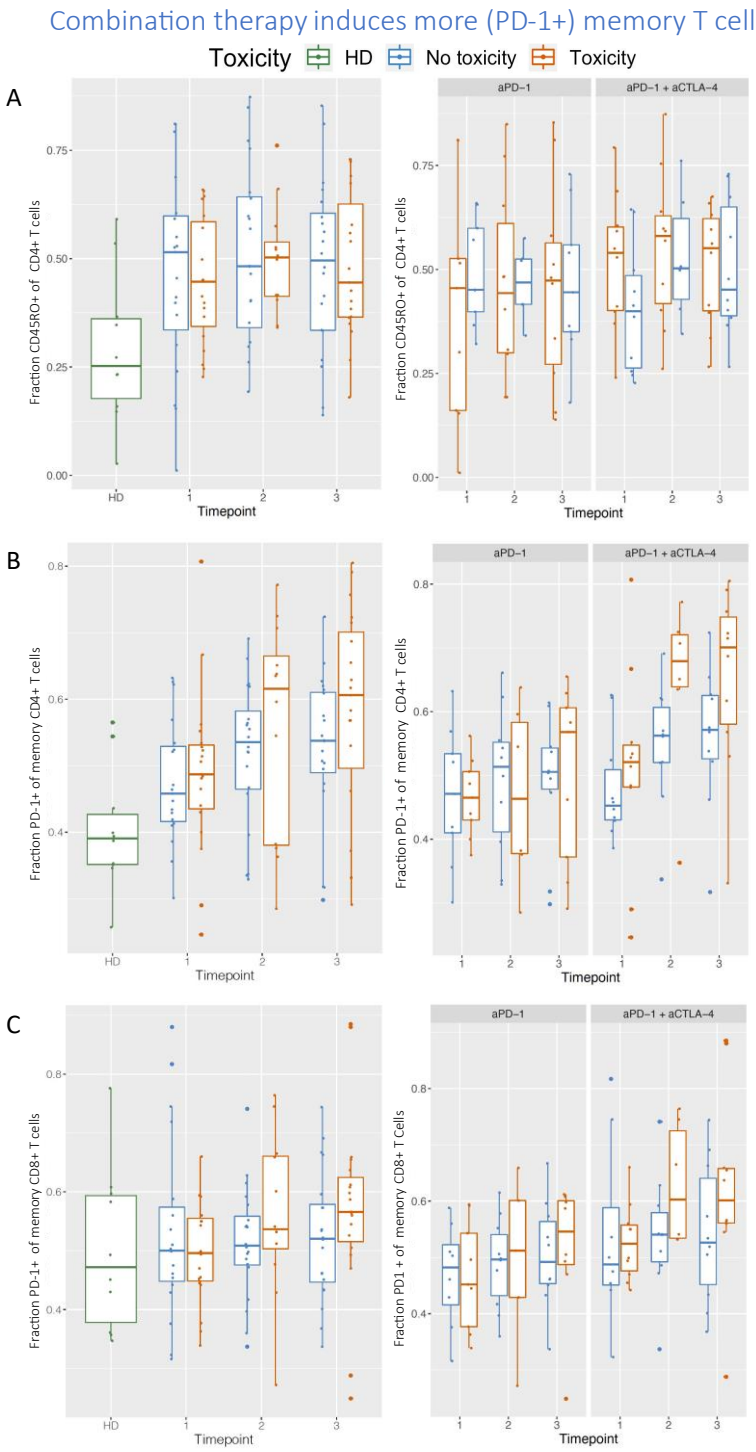


Figure 8. A. Fraction of memory CD4+ T cells of all CD4+ T cells, at all measured time points for the toxicity and non-toxicity group, stratified per treatment type. **B.** PD-1+ fraction of memory CD4+ T cells, at all measured time points for the toxicity and non-toxicity group, stratified per treatment type. **C.** PD-1+ fraction of memory CD8+ T cells, at all measured time points for the toxicity and non-toxicity group, stratified per treatment type.

Discussion

In this study, we aimed to characterize some of the peripheral T cell responses induced by ICI treatment and their link to toxicity and treatment outcomes. We found multiple indications that some baseline values could be associated with toxicity and treatment outcomes. Also, we observed treatment-induced changes in T cell populations that could be linked to toxicity and treatment outcome. Furthermore, we provide links between specific T cell subsets and specific toxicities. We also added suggestions for further research in the context of our findings.

CD4+/ CD8+ T cells ratio at baseline is linked to treatment, response, and toxicity

The ratio between peripheral CD4+ and CD8+ cells has often been described as a potential predictive biomarker in the context of cancer and ICI. A study by Liu et al. showed that a higher ratio of CD4+/CD8+ T cells was associated with early anti-tumour responses (within 6 months) after mono- and combination therapy. In addition, they showed a higher CD4+/CD8+ T cell ratio at baseline was linked to non-progressive disease, as well as a better OS and prognosis in gastrointestinal cancer [37]. Accordingly, within our study, we found that the CD4+/CD8+ T cell ratio at baseline in patients that received combination therapy was overall higher in responders compared to non-responders. Interestingly, this ratio was highest in the small group of responders without toxicity, independent of treatment type. CD4+/CD8+ T cell ratio as predictive value for toxicity is less well-described. The CD4+/CD8+ T cell ratio is lower in patients that develop toxicity. This suggests the clinic, that patients with a low CD4+/CD8+ T cell ratio might need to be monitored more closely for irAEs.

There are multiple theories on why a relative increase in the CD8+ T cell compartment contributes to toxicity. Auto-reactive cytotoxic T cells are often described to be at the base of toxicity. When immune checkpoints are inhibited, effector CD8+ T cells, potentially auto-reactive, could potentially be unleashed, causing tissue damage and inflammation which may lead to bystander activation of other cytotoxic T cells. CD8+ T cell-mediated anti-tumour responses can cause epitope spreading, leading to cross-reactivity with healthy tissue, and giving rise to toxicity. Also, clonal expansion in CD8+ T cells right before toxicity development is linked to toxicity. This was reported in a study with patients treated with ipilimumab[35]. This study showed that clonal expansion of CD8+ T cells after treatment was more evident in patients that later developed grade 2-3 irAEs compared to patients with grade 1 irAEs [35]. However, the reason why a low CD4+/CD8+ T cell ratio at baseline, could be predictive of toxicity remains incompletely understood. A theory could be that a relatively large CD8+ T cell population can simply enable more powerful cytotoxic effector functioning and damage in healthy tissues after ICI-mediated activation. In addition, the imbalance in CD4+/CD8+ T cell ratio might cause auto-reactive CD8+ T cells to be less well regulated by CD4+ T helper and regulatory cells, causing more effector functioning in an uncontrolled manner.

LAG-3+ and LAG-3+PD-1+ CD8+ T cell populations as a predictive value in clinical benefit

LAG-3+ and LAG-3+PD-1+ fractions of CD8+ T cells could potentially serve as a predictive value in patients under monotherapy. We observed increased levels of these subsets at baseline in patients without clinical benefit. Within the patients that do experience clinical benefit, these subsets decrease more in size upon treatment initiation compared to patients without clinical benefit. These findings indicate that high levels of T cells (co-)expressing these co-inhibitory receptors at baseline could point towards monotherapy resistance. Our results are based on small numbers of patients, but a study by Shen et al. found similar observations in a cohort with

188 melanoma patients under monotherapy and combination therapy [38]. After using multiparametric flow cytometry to stratify their cohort for their dominant immunotype; LAG+ (CD8+LAG3+), LAG- (CD8+LAG3-), and PRO(CD8+LAG3+Ki-67+), they found significant differences in treatment outcome. Patients with a LAG+ immunotype showed worse response rates, OS, and PFS compared to patients with other immunotypes. This was observed independent of treatment type as well as in monotherapy alone, but not in patients under combination therapy. In addition, they showed similar results in a smaller cohort of patients with urothelial cancer [38]. These findings also indicate that high LAG-3 expression on T cells might provide resistance against anti-PD-1 treatment. Still, the functionality of circulating LAG3+ and LAG-3+PD-1+ fractions of CD8+ T cells in the context of immunotherapy is incompletely understood.

A recent study by Alsalman et al. did investigate this but found no correlation between peripheral LAG3+CD8+ T cells and patient response or survival[39]. Another recent study did a transcriptomic analysis of the exhaustion signature in the TME of hepatocarcinoma[40]. According to their transcriptomic analysis, LAG3+CD8+ T cells were most associated with having an exhausted gene signature. Counterintuitively, they found that a higher density of this subset in the TME was associated with better progression-free survival (PFS) and overall survival (OS) in patients treated with pembrolizumab or nivolumab[40]. The notion that LAG-3 expressing T cells in the TME are exhausted was supported by multiple other studies for other tumour types [39,41]. Exhausted CD8+ T cells in the TME, co-expressing PD-1 and LAG-3, are one of the reasons anti-LAG-3 treatment (relatlimab) was introduced. One could assume, based on the described findings, that combining anti-LAG-3 and anti-PD-1 treatment could be more efficacious, at least in patients showing resistance to anti-PD-1 treatment alone. Indeed, in the RELATIVITY-047 trial, they showed improved treatment outcomes for melanoma patients when treated with this new combination[4]. However, it did also induce more toxicity. 18,9% of the patients treated with relatlimab–nivolumab had grade 3 or 4 irAEs versus in 9.7% of patients treated with nivolumab [4]. This suggests that future studies should investigate whether baseline values of LAG3+ and/or LAG-3+PD-1+ CD8+ T cells could predict treatment outcomes in patients under relatlimab-nivolumab therapy.

Upregulation of co-inhibitory receptors in CD8+ T cells might be related to toxicity

Within the toxicity group, we found slightly larger fractions of CD8+ T cells expressing co-inhibitory receptors that were unable to produce IFN-γ. We also observed a treatment-induced decrease in IFN-γ-expressing memory CD8+ T cells in this group. These findings might point to exhaustion, or it may reflect a portion of IFN-γ-expressing (memory) CD8+ T cells leaving the circulation and entering tissues to possibly give rise to toxicity or an anti-tumour response.

Subsets of LAG-3+ and PD-1+ CD8+ T cells show increased granzyme B production within patients with toxicity after the first dose of treatment. Perhaps the upregulation of co-inhibitory receptors does not always indicate exhaustion but instead reflects recent activation [5]. These LAG-3+ and PD-1+ subsets might represent (pre-exhausted) CD8+ T cells, that we (re)activated by ICI treatment. Within the subset of CD8+ T cells expressing both PD-1 and LAG-3, most cells had lost the ability to produce granzyme B. Subsets high in multiple co-inhibitory receptors might be further down the path of exhaustion and harder to reactivate, which could explain the lack of granzyme B production. Modern technologies like single-cell transcriptomics could provide more insight into the functionality of T cells expressing co-inhibitory markers in the

context of cancer, and to what extent this implies activation instead of exhaustion. Furthermore, memory CD8+ T cells show increased granzyme B production in response to combination therapy, perhaps again pointing towards reactivation by ICI treatment [42].

Increased granzyme B production in CD8+ T cells is linked to toxicity

We suspect that granzyme-B-producing subsets of CD8+ T cells might inflict toxicity by causing inflammation and perhaps being self-reactive. A study by Luoma et al. did a single-cell analysis on the colon biopsies from patients with melanoma and showed that certain subsets of CD8+ T cells with high expression levels of IFN-γ and granzyme B, were almost restricted to patients with ICI-induced colitis[43]. IFN-γ and granzyme B were elevated in all patients receiving ICI, but not as drastically as in those who developed colitis[43]. Similar observations were made in another recent study on ICI-induced colitis[44]. They suggested that in ICI-induced colitis, tissue-resident memory (TRM) CD8+ T cells in the gut, producing IFN-γ and granzyme B were the main drivers of tissue damage. Within patients under combination therapy, these TRM CD8+ T cells had increased expression of co-inhibitory receptors like PD-1 and LAG-3[44]. Luoma et al. et al provide more data in line with this[43]. They showed that the same colitis-associated CD8+ T cell clusters, high in granzyme B and IFN-γ, overlapped in TCR clonotypes with clusters of tissue-resident memory CD8+ T cells [43]. This indicates that TRM CD8+ T cells might not only activate other cytotoxic T cells but even differentiate towards this phenotype.

Interestingly, we observed that ICI treatment induced increased granzyme B+ fractions in LAG-3+, PD-1+, and memory CD8+ T cells. The increase of granzyme-B-producing fractions was also associated with the severity of toxicity. Overall, treatment induced the largest fractions of granzyme B producing memory CD8+ T cells for the highest grade of toxicity (grade 4 by CTCAEv5 grading). However interesting, these groups of patients are very small, so we can only speculate about the contribution of granzyme B to high-grade toxicity. Some of the patients with the most evident granzyme-B-producing subsets in our cohort suffered from colitis. Together with the findings described above, we could sketch an interesting model in which TRM CD8+ T cells from the colon, are somehow re-activated by ICI treatment. They might already express co-inhibitory receptors to maintain a tolerant environment, or upregulate these in response to activation. They regain effector functioning, expand and cause the influx and activation of other immune cells[42]. This could explain the increase of granzyme-B-producing CD8+ T cell subsets in peripheral blood of patients with colitis. Further research combining peripheral analysis with colitis biopsies could provide more insight into this hypothesis.

Memory (PD-1+) CD4+ and CD8+ T cells in toxicity

We found that combination therapy induces a stronger increase in PD-1 expression in CD4+ and CD8+ T cells compared to monotherapy. Within CD4+ and CD8+ memory T cells, these increases were most evident. Combination therapy overall induces a slightly stronger expansion in memory subsets compared to monotherapy. In our cohort, patients with toxicity had elevated levels of memory CD4+ T cells. PD-1 expression on these CD4+ and CD8+ T cells could again indicate recent activation instead of exhaustion [5]. This finding was in line with another study that found a stronger increase in the PD-1+ memory effector subset of CD4+ T cells after combination therapy[45]. This does not imply that memory CD4+ T cells are less important in the mechanism of action within anti-PD-1 treatment. Rather it emphasizes that the expansion of memory CD4+ T cells is boosted more by anti-CTLA4 treatment. Enhanced memory CD4+ T cell

functioning can enhance B-cell mediated antibody production and provide help to effector CD8+ T cells infiltrating the TME and healthy tissue, potentially adding to anti-tumour response and toxicity.

This theory becomes more probable when looking at the following recent findings[43,44,46]. First, the study of Lozano et al. stratified a cohort of melanoma patients treated with monotherapy and combination therapy, based on the development of severe irAEs (grade 3 or higher)[46]. They found that in the group that developed severe irAEs, the levels of effector memory CD4+ T cells were already elevated at baseline, implying that this subset could serve as a predictor of severe irAEs. Secondly, Luomo et al. found that alongside the expansion of cytotoxic CD8+ T cells, effector CD4+ T cells also expanded in ICI-induced colitis in response to combination therapy [43]. Similar observations were made by Sasson et al., that showed CD4+ T cells in ICI-induced colitis to have increased IFN-γ and TNF-α production [44]. These effector CD4+ T cells in colitis did not show a direct link to circulating effector memory CD4+ T cells. However, it was shown that gene expression linked to α4β7-integrin, which allows gut homing, was elevated in CD4+ T cell clusters associated with colitis[43]. Bello & Dougan proposed a model that connects the dots in these findings [47]. Effector memory CD4+ T cells that become activated by ICI treatment can navigate to the gut where they provide T cell help to TRM CD8+ T cells. These cause inflammation and tissue damage by the model described above. More inflammation might be facilitated by the lack of functional Tregs in the gut, which were weakened by ICI treatment. This model of toxicity development might be applicable in other organ systems that have a lot of TRM T cells like in the skin and lungs.

Limitations

There are several weaknesses in this study. Firstly, the obtained amount of PBMCs from patient samples after thawing was very inconsistent. While most patient samples could be plated out at 500.000 cells per well for FACS analysis, some samples were plated out with fewer cells which might have influenced the readout of certain variables.

Secondly, FACS analysis of PD-1 expression in patient PBMCs could be blurred by nivolumab-bound PD-1. The IgG4 antibody of nivolumab blocks the staining antibody PD-1-BV711 (clone EH12.1) from binding PD-1. Therefore, measuring PD-1 expression in on-treatment samples required a different staining protocol with anti-biotin-streptavidin-conjugated antibodies that binds as a secondary antibody to IgG4-BV711. This also resulted in different expression patterns for PD-1 between baseline and on-treatment samples. Consequently, we applied different gating in Flow Jo for baseline and on-treatment samples to make PD-1 expression between timepoints as comparable as possible. Still, it should be noted that there could be possible discrepancies within patients over time.

Another limitation is found in the analysis of peripheral blood. By analyzing PBMCs, immune responses from the entire body are merged with ICI-induced immune responses. Although healthy controls, as well as baseline samples, are taken along, this cannot fully uncouple general immune responses from tumour- or toxicity-specific ones. Adding data from the TME and/or from irAE-affected tissue could help to capture a more complete view of ICI-induced responses and toxicity.

Sex is not evenly distributed in our patient groups nor in the healthy controls. The donors that provided our controls were mostly women (70%), while most of our patients were male (79,1%). Lastly, there was a lot of heterogeneity in our group in terms of cancer types and toxicity. This is a strength and a weakness at the same time. Different tumour types might be affected by ICI treatment in different ways. In this analysis, they are taken all together, which could fade out specific tumour-treatment interactions. As for toxicity, the mechanisms in which skin-related toxicities develop could be distinct from colitis. The specific underlying mechanisms might not be picked up because we have generalized toxicity by defining it only by clinical relevance in most of our analyses. Additionally, the groups of patients with the same toxicity were too small to draw any strong conclusions. On the other hand, the diversity in our cohort allowed this study to find trends, applicable to many types of tumours and toxicities. This could have added value to more general treatment strategies in the clinic. Still, in future research, focusing on one type of toxicity or tumour could help unravel the assumptions already made in this study.

Conclusion

All in all, we have provided pieces of information that could potentially add to improving treatment outcomes and toxicity prediction in the future. Firstly, we showed that increased CD4+/CD8+ T cell ratios at baseline are positively linked to response without toxicity. Secondly, we observed that (PD-1+)LAG-3+ CD8+ T cells are elevated at baseline in patients without clinical benefit. This could potentially serve as a predictive value in patients under monotherapy and combination therapy with relatimab and nivolumab in the future. We observed higher fractions of CD8+ T cells expressing co-inhibitory receptors unable to produce IFN-γ in patients with toxicity, pointing towards exhaustion. However, we have also found increased granzyme-B-producing PD-1+, LAG-3+, and memory CD8+ T cells in this group, which could potentially be associated with toxicity severity. We observed that combination therapy induces more (PD-1+) CD4+ memory T cells compared to monotherapy and that having elevated levels of memory CD4+ T cells at baseline might add to combination therapy-induced toxicity. These findings were in line with a model that connects elevated memory CD4+ T cells in peripheral blood to TRM CD8+ T cells causing tissue damage and toxicity [47].

However, further investigation is needed to predict the occurrence of irAEs and find potential targets to treat them. In addition, more research on the predictive variables discussed here is needed to accurately evaluate their predictive power in toxicity and treatment outcomes. Hopefully, in the future, this will lead to more potent treatment strategies and improved treatment outcomes for cancer patients, treated with ICI.

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