An immunogenic first-in-human immune modulatory vaccine with PD-L1 and PD-L2 peptides is feasible and shows early signs of efficacy in follicular lymphoma

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ABSTRACT
Cells in the tumor microenvironment of Follicular lymphoma (FL) express checkpoint molecules such as programmed death ligands 1 and 2 (PD-L1 and PD-L2) and are suppressing anti-tumor immune activity. Stimulation of peripheral blood mononuclear cells (PBMC) with PD-L1 (IO103) or PD-L2 (IO120) peptides can activate specific T cells inducing anti-regulatory functions including cytotoxicity against PD-L1/PD-L2-expressing cells. In this study, we vaccinated eight FL patients with PD-L1 and PD-L2 peptides following treatment with standard chemotherapy. Patients experienced grade 1–2 injection site reaction (5/8) and mild flu-like symptoms (6/8). One patient experienced neutropenia and thrombocytopenia during pseudo-progression. Enzyme-linked immunospot detected vaccine-specific immune responses in PBMC from all patients, predominately toward PD-L1. The circulating immune composition was stable during treatment; however, we observed a reduction regulatory T cells, however, not significant. One patient achieved a complete remission during vaccination and two patients had pseudo-progression followed by long-term disease regression. Further examination of these early signs of clinical efficacy of the dual-epitope vaccine in a larger study is warranted.

ARTICLE HISTORY
Received 5 August 2021
Revised 30 August 2021
Accepted 30 August 2021

KEYWORDS
PD-L1; PD-L2; immune modulatory vaccine; anti-regulatory T cells; anti-Tregs; follicular lymphoma

Introduction
Despite important immune therapeutic advances in several types of lymphoma,1 follicular lymphoma (FL) is still considered incurable aside from limited-stage disease.2 Early progression of disease has been associated with particularly poor survival and the disease becomes more resistant and severe over time with a risk of transformation to a more aggressive lymphoma.2–5 FL patients with symptomatic disease and high tumor burden are traditionally treated with chemotherapy and rituximab which are associated with considerable side effects. With recurrent relapses, patients often require numerous treatments during their lifetime, highlighting the need for effective and less toxic treatment options. Therapies within the field of immunotherapy are promising in this matter and is already advancing treatment in many cancers including lymphoma.1 Immunotherapy is harnessing the ability of the immune system to recognize and kill cancer cells and by-pass the immune evasive mechanisms induced by the cancer cells. One of the important mechanisms of cancer immune escape is through the programmed death receptor 1 (PD-1) and its ligands (PD-L1 and PD-L2), and targeting this pathway has greatly improved the field of cancer immunotherapy.6 As in other tumors, PD-L1 and PD-L2 are often overexpressed in the microenvironment of FL compared to healthy tissues, and PD-1 expressing T cells in affected lymph nodes have decreased cytokine responses.7,8 FL patients with a more diverse immune infiltration in their tumors, including T cells, are less likely to progress within 24 months9 and occasionally the disease spontaneously regress, which is believed to be a regain of immunological control.10 This indicates that FL is vulnerable to immunological changes and might also be susceptible to immunotherapy. In the last decade, studies have described self-reactive, pro-inflammatory T-cells, known as anti-regulatory T-cells (anti-Tregs), that specifically target immune-suppressive cells.11,12 These natural occurring anti-Tregs recognize HLA-restricted epitopes derived from proteins expressed by regulatory immune cells at inflamed sites, such as PD-L113–16 and PD-L2.17 We have described that such specific T cells could recognize not only malignant cells but also both nonmalignant and malignant cells that expressed PD-L1 or PD-L2, and they reacted in a target concentration-dependent manner.14,16,18 It is therefore important to consider...
that both PD-L1 and PD-L2 are not only expressed by the malignant cells but also by other cell types in the tumor microenvironment that are taking part in the creation of an immunosuppressive milieu. The first attempt at activating such anti-Tregs was done with a peptide derived indoleamine-2,3-dioxygenase (IDO) and showed promising and long lasting in non-small cell lung cancer. Later a PD-L1 peptide has been proven safe in 10 patients with multiple myeloma and peptides derived from PD-L1 and IDO in combination with anti-PD1 antibodies as first-line treatment seems to markedly improve response rates compared to anti-PD1 antibodies alone in patients with metastatic melanoma (NCT03047928). In this study, we tested a long peptide derived from PD-L2 for the first time in humans in combination with a long PD-L1 peptide in patients with follicular lymphoma. With the promising activity of anti-regulatory peptide vaccines and the immune sensitive microenvironment of FL we hoped to tip the balance from immune escape to tumor elimination.

**Material and methods**

**Study design and patients**

This trial was an investigator initiated, single-center, single-arm, first-in-human, phase I trial, investigating the safety of the combination of IO103, a PD-L1 peptide, and IO120, a PD-L2 peptide (NCT03381768). The study was approved by the Ethics Committee of the Capital Region, Denmark, (H-17019141); the Danish Data Protection Agency (HGH-2017-082) and the Danish Medicines Agency (EudraCT: 2017–002000-28). The follow-up study from end of treatment (EOT) was an independent retrospective registry study approved by the Danish Data Protection Agency (P-2021-104). All patients signed an informed consent form before entering the trial and an external unit monitored the trial according to the principles of good clinical practice.

Patients with relapsed FL grade 1–3a were enrolled in the study after standard treatment to receive the vaccine in order to sustain or improve remissions. The study was subsequently amended to also include patients after first-line therapy and without a time limit between the standard treatment and inclusion. The final inclusion and exclusion criteria are listed in Supplemental Table 1. General requirements were a minimum of partial remission (PR) according to the Lugano criteria after latest treatment and without the need of other treatments. Patients with poor performance status, autoimmune diseases, or serious medical conditions were not included in the study. The primary end point was safety and the secondary endpoint was immunological response to the vaccine assessed by Enzyme-linked immunospot (ELISPOT). Clinical efficacy was a third and exploratory endpoint during treatment, but the main objective during follow-up.

**Treatment**

The vaccine consisted of a 19 amino-acid long peptide derived from PD-L1 (IO103) and a 21 amino-acid long peptide from PD-L2 (IO120) with 100 µg of each peptide in each vaccine dissolved in water and 20% DMSO. The peptides were acquired from PolyPeptide laboratories, Strasbourg, France. 500 ul of the peptide solution were mixed with 500 ul of the adjuvant Montanide ISA-51 (Seppic Inc., Paris, France) immediately before vaccination giving a total injection volume of 1 mL. 6 bi-weekly injections followed by 9 monthly injections over the course of one year were scheduled for each patient. The first three doses of vaccine for each patient were without PD-L1, in order to get data exclusively for the PD-L2 peptide. The treatment schedule is illustrated in Supplemental Figure 1.

**Clinical evaluation**

Adverse events (AE) were defined and graded according to CTCAE v4.03 and systematically scored until the end of treatment. Vaccinations were stopped if patients showed sign of progression. Response evaluation was assessed by the Lugano criteria involving a PET-CT scan at baseline and after vaccinations. During the subsequent follow-up we focused on clinical outcome according to standard practice, and patients were censored when relapse therapy was initiated, at death, or at the data cutoff of February 12, 2021.

**Preparation of blood samples**

Blood samples were collected during vaccinations and Peripheral blood monocytes (PBMCs) were isolated using Lymphoprep (Stemcell Technologies, Vancouver, Canada) and Leucosep tubes (Greiner Bio-One, Kremsmünster, Austria). The PBMCs were freshly frozen in controlled rate Cool-Cell boxes (Brooks Life sciences, Chelmsford, MA, USA) in 90% human serum with 10% DMSO and stored in −140 degrees Celsius until needed for the following assays.

| Ann-Arbor stage | Histological grade | FLIPI2 Risk profile | t(14;18) Status | Number of prior treatments | Time from diagnosis to inclusion(years) | Time from last treatment to inclusion(months) | Time from last vaccine to data cutoff(months) |
|-----------------|--------------------|---------------------|----------------|---------------------------|----------------------------------------|---------------------------------------------|---------------------------------------------|
| Patient 1       | IV-B               | Intermediate        | Unknown        | 4                         | 11.5                                    | 9                                           | 16                                          |
| Patient 2       | IV-B               | Intermediate        | Unknown        | 3                         | 3.1                                    | 2                                           | 32                                          |
| Patient 3       | IV-B               | Intermediate        | Positive       | 3                         | 7.4                                    | 21                                          | 23                                          |
| Patient 4       | IV-B               | Intermediate        | Negative       | 3                         | 2.6                                    | 2                                           | 19                                          |
| Patient 5       | IV-B               | Low                 | Unknown        | 4                         | 12.0                                   | 5                                           | 18                                          |
| Patient 6       | IV-B               | High                | Positive       | 3                         | 7.7                                    | 14                                          | 13                                          |
| Patient 7       | III-A              | Low                 | Positive       | 1                         | 6.0                                    | 5                                           | 6                                           |
| Patient 8       | III-A              | High                | Unknown        | 1                         | 0.7                                    | 2                                           | 12                                          |

Mean age in years (range): 66.2 (56–74)  Gender M/F: 2/6
**Interferon gamma enzyme-linked immunospot (IFNγ-ELISPOT)**

T-cell responses toward IO103 and IO120 were assessed using IFNγ-ELISPOT, which measures the release of IFNγ from specific T-cells upon stimulation with the peptides. We followed the procedure described earlier. To improve sensitivity, we stimulated the peripheral blood mononuclear cells (PBMC) in one step. We used 96-well PVDF plates (MultiScreen, MAIP N45; Merck Millipore, Burlington, MA) coated with anti-IFNγ-mAb (Mabtech, Nacka Strand, Sweden). Secondary biotinylated anti-IFNγ-mAb, Streptavidin–enzyme conjugate and the enzyme substrate NBT/BCIP from Mabtech were used. Spots were counted using an ImmunoSpot Series 2.0 Analyzer (Cellular Technology Limited, Cleveland, OH), with an upper threshold of 500 spots/well. ELISPOT assays of PBMCs were done in triplicate with 2.5–3.0 × 10^5 cells/well. ELISPOT assays of SKILs were done with varying numbers of cells and primarily done in triplicate, with results in duplicates highlighted in figures.

**Flow cytometry**

Flow cytometry was performed on PBMCs collected during vaccination, freshly frozen and stored in −140 degrees celcius. Two panels characterizing T lymphocytes and other PBMCs including myeloid lineages were used (Supplemental Table 2). Fc receptors were blocked with human IgG at 50 μg/ml. The cells were stained in the dark at 4°C for 10 min, by use of a near-IR dead-cell stain kit (Thermo Fischer Scientific, Waltham, MA), and incubated for 20 minutes with antibodies. The cells were acquired using a NovoCyte Quanteon Flow Cytometer (Agilent, Santa Clara, CA). For T-cell subset analysis the CD3-positive, live singlet was gated out in NovoExpress 1.4.1 and exported to FlowJo v10.7.1. Further gating strategy details is provided in Supplemental Figure 2.

**Table 2. Adverse reactions and serious adverse events.**

| Adverse events (AE) with possible relation to the vaccine | Number of events | Grade | Number of patients, n = 8 (%) |
|----------------------------------------------------------|------------------|-------|------------------------------|
| Injection site reaction                                  | 11               | 1−2   | 5 (63)                       |
| Flu-like symptoms                                         | 8                | 1     | 6 (75)                       |
| Fatigue                                                  | 3                | 1     | 3 (38)                       |
| Arthralgia                                                | 1                | 1     | 1 (13)                       |
| Chills                                                   | 1                | 1     | 1 (13)                       |
| Myalgia                                                  | 2                | 1     | 2 (25)                       |
| Diarrhea                                                 | 3                | 1     | 1 (13)                       |
| Bursitis                                                 | 1                | 2     | 1 (13)                       |
| Neutropenia†                                              | 1                | 3     | 1 (13)                       |
| AE grade 3+ and Serious Adverse Events (SAE) not related to the vaccine (AE) Neutropenia† | 4 | 3−4 | 3 (25) |
| AE Lung embolism                                          | 1                | 3     | 1 (13)                       |
| (SAE) Febrile neutropenia                                | 1                | 3     | 1 (13)                       |
| (SAE) Admission due to atrial fibrillation               | 2                | 3     | 2 (25)                       |
| (SAE) Cholecystitis                                       | 1                | 3     | 1 (13)                       |

**Minimal residual disease by qPCR**

PBMC samples from each time point were screened for minimal residual disease (MRD) using qPCR with primers targeting the hallmark translocation t(14;18). This was performed at the EuroMRD validated CLL-laboratory at Rigshospitalet, and sequences for the primers and probes were provided by Professor Christiane Pott, Kiel, as part of the EuroMRD consortium.25,26 Primers with the specified sequences were acquired from Integrated DNA Technologies (Coralville, IA) and the TaqMan probes marked with FAM and TAMRA were acquired from Thermo Fisher Scientific. Two plasmids containing the most frequent breakpoints were used for standard curves and as positive control also provided by Prof. C. Pott.25 As part of the validated assay, DNA from healthy donors was used as a negative control. MRD for patients without detectable translocation were assessed by nested PCR with individual primers targeting the clonal Ig heavy-chain variable region.25 Samples from all time points were run in triplicate with 500 ng DNA/well on a Quantstudio 7 (Thermo Fisher Scientific). DNA quality and

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**Figure 1.** Individual peptide-specific T-cell responses in PBMCs. A) T-cell interferon gamma (IFN-γ) release after stimulation with either PD-L1 or PD-L2 peptides in individual patients during vaccination. (*)DFR<1x, **DFRx2) B) Comparison between PD-L1 and PD-L2 responses from all timepoints. Means with 95% CI, tested with the unpaired t-test C) PD-L1 and PD-L2 responses over time with bars indicating means. Note that PD-L1 was added to the vaccine after three vaccinations with the PD-L2 peptide alone. The Wilcoxon matched-pairs test was used to test significance between time points.
quantity were checked with quantifiable albumin amplification and results were corrected accordingly. Interpretation and determination of sensitive and quantitative range were done according to guidelines published by the EuroMRD consortium. Positive MRD was defined as cycle threshold values in the sensitive range of the assay and was quantifiable in the range specified in the guidelines. Negative MRD was defined as cycle threshold values outside the sensitive range.

**Statistics**

The primary endpoint was on descriptive basis using the CTCAE scoring system. To determine the significance of ELISPOT results, we used distribution-free sampling (DFR) for triplicates and for duplicates we defined the empirical responses threshold of $2 \times$ background spot count. Changes in ELISPOT results over time for individual peptides were...
tested using the Wilcoxon matched-pairs signed-rank test, and for comparison of ELISpot responses between PD-L1 and PD-L2 we used the unpaired t-test. Changes over time in immune populations identified by flow cytometry were tested using one-way ANOVA. The Kruskal–Wallis test was used for MRD changes over time with results outside the quantitative range set at $5 \times 10^{-5}$. Calculations and visual presentation of data were done in GraphPad Prism 9.0.0 except DFR which was calculated in R v 3.6.1.

Results

Patients

Eight patients were included from January 2018 to January 2019. Six patients were included in a relapse setting and had a minimum of three prior treatments (range 3–4). Two patients were included after first-line treatment and received bimonthly anti-CD20-antibody maintenance along with the vaccinations. Individual patient characteristics are shown in Table 1. Six patients completed the vaccination schedule, while treatment was discontinued in patient 1 (Pt1) and Pt2 after six and seven doses, respectively, due to signs of progression. They were followed clinically onwards. At data cutoff, February 12, 2021, the median follow-up from last vaccination was 17 months for all patients (range 6–32).

Adverse events

The most common adverse reaction in the study was injection site reaction, experienced by five patients (Table 2 and Supplemental Table 3). Some skin reactions were long-lasting subcutaneous lumps possibly granulomas which has been seen in relation to the adjuvant Montanide.28 Six patients experienced mild flu-like symptoms such as myalgia, arthralgia, and fatigue within a day or two of the vaccination. Five patients had one or more infections of common origin as expected for this patient population, with details provided in supplemental table 3. We observed grade 3–4 neutropenia in four patients. Of these, one had a history of recurrent neutropenia since a treatment of obinutuzumab and bendamustine with an occasional need of G-CSF stimulation.29 Another patient had declining neutrophils entering the trial with nadir and normalization shortly after inclusion and one patient had neutropenia during a major infection alongside with rituximab treatment. One patient (Pt2) experienced short-lived neutropenia and thrombocytopenia during progression, but as the disease regressed spontaneously during the follow-up, we reclassified the progression as pseudo-progression, and retrospectively marked these adverse events as possibly related to the vaccine. Three serious adverse events occurred all based on the admission criteria and were considered related to known health conditions (Table 2). One patient was admitted with cholecystitis due to an obstructive gall bladder stone, which was visible at the baseline PET-CT. The gall bladder was removed and shortly after the surgery the patient had a lung embolus. This patient had a recent history of deep vein thrombosis and active lymphoma as risk factors. Two patients had frequent paroxysmal atrial fibrillation prior to inclusion, and both were admitted briefly during the trial. The last patient had recurrent neutropenia as described above and were shortly admitted with febrile neutropenia.

Vaccine responses assessed by ELISpot and flowcytometry

To investigate the immune response toward the vaccine, PBMCs were tested for reactivity against the PD-L1 and PD-L2 peptides. Six out of eight patients had responses toward PD-L1 prior to PD-L1 vaccination in contrast to one out of eight with baseline responses toward PD-L2. (Figure 1a, Supplemental Figure 3). In general, the PD-L1 peptide gave stronger and more frequent immune responses than PD-L2 ($P = .0024$) (Figure 1b). Initially, we detected a significant increase in PD-L2-specific T-cell responses in blood ($P = .031$) that reached a plateau after 3 vaccinations, and a similar trend for PD-L1 specific T-cell responses ($P = .078$) (Figure 1c).

To test the migration capabilities of the vaccine induced T-cells we performed a DTH skin test after 6 vaccines. After 48 hours, Pt3 and Pt7 had strong visual skin infiltration and the remaining patients had minor indeterminable infiltration (data not included). We succeeded in expanding SKILs from the biopsies from five patients as described in the supplemental methods.21,30 In Pt3-Pt7 we detected responses to at least one of the vaccine peptides. (Supplemental Figure 4) Instead of DTH, Pt1 and Pt2 had a biopsy taken from involved lymph nodes at progression. From these we expanded tumor-infiltrating lymphocytes (TILs) by stimulation with high-dose IL-2 and tested their reactivity toward the peptides. (Supplemental methods) Interestingly, both patients displayed a response toward the PD-L2 peptide in the tumors, in contrast to no detectable PD-L2 responses in the blood. (Supplemental Figure 4). To further characterize the systemic impact of the vaccine, we investigated changes in the circulating immune composition by flow cytometry during the vaccinations.31 In general, we observed fewer Tregs over time, along with an increase in senescent CD8+ populations, although not significant. (Supplemental Figure 5) Individually, Pt3 and Pt4, who had signs of clinical response as presented below, had the most prominent decline in Treg numbers (Figure 2a) and a consistent decline in both CD4+ and CD8+ naive T-cells throughout the vaccinations (Figure 2b and 2c). They also had the most prominent increase in both CD4+ and CD8+ senescent populations (Figure 2d and 2e), and Pt3 had a shift from CD8 + T effector memory cells re-expressing CD45RA (TEMRA) to more CD8 + effector memory T-cells (figure 2f and 2g). We saw no significant changes in the PD-1 expression of effector T cells (Figure 2h). Overall, we did not observe any noteworthy changes in the other lymphocyte populations as well as in the myeloid cell subsets (Supplemental Figure 5).

Clinical response evaluation

Patients were included at varying time points after standard treatment with immunotherapy or radiation therapy as depicted individually in Figure 3. The mean time between last treatment and first vaccination was 7 months (range 2–21). At inclusion, five patients were in complete remission (CR) and three patients still
had measurable disease (MD), with PR after the latest treatment. Disease status for each patient at baseline, end of treatment (EOT) and follow-up is illustrated in the Sankey graph (Figure 4).

At the EOT evaluation one patient (Pt3) had improved remission status from PR to CR (Figure 5, A). Three patients maintained a CR and one patient had SD. Three patients seemingly progressed during vaccinations, however two of these patients (Pt2 and Pt4) had spontaneous regression of disease during the following seven to 8 months (Figure 5b and 5c) indicating pseudo-progression as seen with checkpoint inhibitors and addressed in the refined Lugano response classifications.32,33 None of the patients in CR at EOT showed signs of progression during follow-up, however one deceased 6 months after last vaccine, cause not reported. Pt3 achieving CR during vaccinations were in FU for 21 months from latest treatment to inclusion without signs of progression. The patient had no symptoms or signs of infection at inclusion; however, we cannot rule out other causes of lymph node activity as we did not take a biopsy. MRD measurements for this patient were negative at all time points.

**Figure 3.** Swimmers graph. Course of disease for each patient since diagnosis, with responses to treatment indicated by symbols. Patients in follow-up were censored on February 12, 2021, at death, or at initiation of relapse therapy.B, bendamustine; CVP, cyclophosphamide, vincristine/oncovin, prednisone; CHOP, CVP with adriamycin; G, obinutuzumab; ICE, ifosfamide, carboplatin, etoposide; MTX, methotrexate; R, rituximab RT, radiation therapy. HD-C: High Dose Cyclophosphamide.CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PDp, Pseudo-progression.

**Figure 4.** Sankey graph following disease status from baseline (BL) through end of treatment (EOT) and follow-up (FU). During follow-up patients in CR were only clinically assessed. CR, complete response; cCR, clinically assessed CR; MD, measurable disease; PR, partial response; SD, stable disease; PD, progressive disease; PDp, Pseudo-progression.
(Supplemental table 4). Pt2 and Pt4 with pseudo-progression both patients had a short history of aggressive disease with three chemotherapeutic regimens applied within 36 months. During these treatments Pt4 achieved PR as best response and MRD was detected at all time points, confirming residual disease at trial entry (Supplemental table 4). The EOT scan for this patient showed metabolic activity in initial lymphoma sites including abdominal bulk and predominately retroperitoneal disease (Figure 5c). A biopsy was taken, and follicular lymphoma was confirmed by standard pathology assessment. The patient was followed with no further therapy and 8 months later had 66% regression in target lesions (Figure 5 C). Pt2 experienced neutropenia and B-symptoms along with gastritis during vaccination and a biopsy confirmed progression. As the neutrophil count normalized, and the symptoms diminished after the vaccinations were stopped we followed the patient without further FL therapy and seven months later the disease had regressed to near CR, with only a small metabolically active lymph node present in the right axilla (Figure 5b). We also did an interesting observation in Pt6 where we detected a significant drop in MRD during vaccination (P = .0004) (Figure 6). This patient achieved CR on the treatment prior to vaccination, but slowly progressed during rituximab maintenance prior to inclusion. Thus, the patient had been in rituximab for 14 months at inclusion and continued the treatment during the vaccinations.

**T-cell infiltration in tumor biopsies**

We observed a high number of T cells in the progression biopsy of Pt4. Therefore, we quantified the T-cell infiltration with the QuPath software\(^{34}\) in the current progression biopsies and biopsies from previous progressions from Pt1, Pt2 and Pt4. This quantitative analysis showed more than twice as dense CD3+ T-cell infiltration after vaccination in Pt4 (Supplemental Figure 6a and Supplemental Fig. 7). In Pt1 and Pt2 we saw an increased number of CD3+ cells/mm\(^2\) in the malignant follicles (Supplemental Figure 6b). In Pt1, this was despite of a drop in the general CD3+ density in the whole lymph node (supplemental Figure 6a).

**Discussion**

Regulatory feedback mechanisms, such as the upregulation of PD-L1 and PD-L2, are crucial for restraining the immune system for the controlling of auto-immune reactions. However, in the context of cancer immunotherapy these are harmful as they prevent immunological elimination of the cancer cells. Indeed, therapeutic blockade of immune checkpoint pathways, particularly the PD-1 pathway, has shown to be an effective treatment in many solid and hematological malignancies.\(^6\) The last years we have described an alternative approach for targeting the PD1/PD-L1/PDL2 pathway with specific T-cells. We have characterized the existence of spontaneous immunity toward PD-L1 and PD-L2 in patients with different malignancies and showed that the activation of such T cells boosted both anti-viral and anti-cancer T-cell responses.\(^{13,18}\) Hence, the activation of PD-L1/PD-L2- specific T cells by vaccination may both directly, by the targeting of malignant cells, and indirectly, by the modulation of the tumor microenvironment, support the anti-leukemic immune response.

In this study we show that vaccination with the peptides PD-L2 (IO120) and PD-L1 (IO103) are safe, with manageable and mild adverse events. The vaccines can induce mild flu-like symptoms and cause local reactions at the injection site. Several patients were bothered by long-lasting granulomas, which is frequently seen with the use of the adjuvant Montanide.\(^{28}\) There was a high frequency of isolated neutropenia in the study, which is not unusual for patients recently treated with chemotherapy or rituximab.\(^{25}\) However, Pt2 experienced short-lived neutropenia during the period with pseudo-
progression. Two previous attempts on autologous stem cell harvests had been unsuccessful due to insufficient mobilization of stem cells, indicating an exhausted bone marrow in this patient. Thus, although the observed neutropenia is a result of the underlying conditions of the patients, the initiated immune reactions might briefly have exhausted the neutrophil reserve similar to the mechanisms seen in infection induced neutropenia. Of note, we have not seen neutropenia in other trials containing the PD-L1 peptide.

The vaccine was broadly immunogenic, as we detected peptide-specific immune responses in all patients. We observed stronger and more frequent responses against the PD-L1 peptide, which could be due to a broader epitope processing across HLA types or could indicate that these patients harbor a larger pool of naturally occurring PD-L1 experienced T cells. The latter is supported by the high baseline recognition of PD-L1. In addition, the PD-L1 responses at baseline were more frequent than what we have seen in other studies. So these patients might already have a decent anti-regulatory T-cell activity at this stage of disease. We were not able to detect PD-L1 responses in the tumor biopsies, but recall that the PD-L1 peptide was added to the vaccine after three doses of PD-L2 alone, as an explanation to an inferior infiltration compared to PD-L2. Another important factor is that TILs were cultured with high dose IL2 for several weeks which might exhaust some populations of T-cells preventing them to show up in the ELISpot assay. The largest increase in immune responses against both peptides were observed at the start of vaccination, suggesting that the peripheral immune response met an early threshold of activity and/or exhaustion of the T cell pool. Indeed, we did see a systemic trending increase in a senescent CD57high CD27low population which is typical for reduced proliferation capacity and antigen-induced apoptosis. On the other hand, we observed a decreasing trend in the number of Tregs, which could indicate an immune modulatory effect of the vaccine. A decrease in Tregs have been associated to a favorable prognosis for prostate cancer treated with a cancer vaccine. Otherwise, we observed a rather stable immune composition in the periphery. Overall, any such changes may be more evident in infiltrated sites such as tumors and lymph nodes.

The study was not powered to conclude on the clinical efficacy of the vaccines considering the small number of heavily treated patients in the study and the capricious nature of FL. However, Pt3 achieved CR during vaccinations. This patient had a small tumor burden with 21 months of stable disease prior to inclusion. There was a discrepancy between the baseline scan and MRD status, raising the question of whether the lymph node was metabolically active by other means or if the disease burden was below the sensitivity of the qPCR assay. The patient had no signs of infection when the scan was performed, but we did not take a biopsy disallowing histological confirmation. A large study investigating the concordance between PET-CT and MRD detection found that metabolic active disease on PET-CT maintained clinical relevance despite MRD-negativity. All in all we cannot entirely dedicate this result to the vaccine. Another interesting clinical observation was the two cases of pseudo-progression of Pt2 and Pt4, which is a known phenomenon of immunotherapy, where immune infiltration of tumors cause and initial swelling with subsequent regression. We did see an unusually high number of T-cells in the progression biopsy of Pt4 which might have caused the temporary swelling and metabolic activity of the lymph nodes. Unfortunately, we did not have live cells from the tumor biopsy of Pt4 to investigate reactivity toward the vaccine. In the progression biopsies of Pt1 and Pt2 we noted a significant increase in intrafollicular T-cells compared to previous biopsies, but with simple immune histochemistry available we were unable to further characterize the phenotype of these cells. However, in subsequent studies with larger data sets this should be explored with spatial immune profiling techniques. However, in Pt2, we detected TILs reactive toward

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**Figure 6.** Minimal residual disease by qPCR. Quantitative PCR measurements of Minimal residual disease (MRD) in Pt6. The upper dotted line indicate the threshold for the quantitative range and the lower dotted line indicate the threshold for the sensitive range as defined under methods. Results between the lines are thus MRD positive but not quantifiable (NQ)
PD-L2 and the metabolic activity in both Pt2 and Pt4 was colocalized with the previous lymphoma sites, which might indicate a selective homing of the T-cells toward tumors, a hallmark of successful immunotherapy.\textsuperscript{31} Further, immune-infiltrated tumors are associated with favorable outcome in FL patients, and pseudo-progression has been correlated to better overall survival comparable to regression in other cancers treated with immunotherapy.\textsuperscript{3,32} Thus we might have witnessed a recapture of immunological control over the lymphoma cells, as seen in spontaneous regressions in this disease.\textsuperscript{10} In the case of Pt2 who had B-symptoms along with neutropenia and thrombocytopenia, we speculate if the patient had real progression followed by a late onset effect of the vaccine. Nonetheless, for both Pt2 and Pt4, the fact that malignant cells were present after vaccination without deteriorating disease indicates that the disease is subject to some restriction and possibly immunological control. We are obliged to mention that Pt4 did not carry the t(14;18) translocation, as seen in 15\% of FL cases. T(14;18)-negative cases have distinct genetic and molecular expression patterns, but in general, they do not seem to differ in regard to prognosis and response to established treatments.\textsuperscript{42,43} However, we cannot say if these differences influence the more intricate mode of action of cancer vaccines in comparison to chemotherapy. As our last case we found a significant reduction in circulating tumor DNA in Pt6. This patient received bi-monthly rituximab along with the vaccinations and we were not able to distinguish between the effects of the two treatments. However, the patient had been in rituximab treatment for 14 months prior to inclusion, favoring the vaccine as the possible cause. In addition to these cases, several patients had long-lasting remissions despite aggressive and high-risk disease and the majority of patients had good peptide recognition by SKILs, which has been correlated with clinical benefit in other vaccination trials.\textsuperscript{44,45} Looking collectively at the clinical results, the vaccine might have enhanced immunological surveillance for several patients, however we are not able to entirely able to distinguish between the effects of the vaccine and the initial treatments. We combined the treatments in this way based on an assumption that the vaccine would perform better in patients with low tumor burden. To further explore the efficacy of the vaccine we are currently testing it as monotherapy for untreated CLL patients in a phase II trial. For indolent lymphoma, the optimal timing, sequence, and choice of regimens remain a matter of debate. It is standard practice to postpone treatment until symptomatic disease or large tumor burden as treatment with neither rituximab or chemotherapy of asymptomatic patients have improved overall survival.\textsuperscript{46,47} Considering the low toxicity of the vaccine it would be interesting to exploring the efficacy in asymptomatic patients in an attempt to initiate early immunological control, mimicking spontaneous regression. Nonetheless, as FL patients often require multiple treatments and cycle through available regimen, additional treatment options and possibilities of combinations, are welcome in this palette.

**Conclusion**

In conclusion, PD-L1/PD-L2 dual vaccination is safe in follicular lymphoma, with a favorable safety profile compared with chemotherapy. We were able to measure peptide-specific T-cells in blood, skin, and tumor biopsies. One patient achieved CR and two patients have long-lasting disease control after temporary pseudo-progression. These early signs of efficacy compel further investigation in a larger trial.

**Acknowledgments**

We would like to acknowledge the tremendous technical support from Merete Jonassen, Sandra Ullitz Færch, Betina Saxild, Susanne Wendt, and Christina Granhøj. We would also like to thank Eva Ehrnrooth and Mai-Britt Zocca for their help and good discussions. Further we greatly acknowledge the EuroMRD consortium and the work by Professor Christiane Pott, Kiel, for developing a validated MRD assays in FL and providing the plasmids and primer sequences for the MRD analyses.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

The work was funded through a research agreement between IO Biotech ApS and the National Center for Cancer Immune Therapy (CCIT-DK), Herlev Hospital, Capital Region, Denmark; National Center for Cancer Immune Therapy, Herlev Hospital [Basic support from Center]; Region Hovedstadene [Research agreement between the capital region of Denmark and IO Biotech].

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**References**

1. Klausen U, Jørgensen NGD, Graulund JH, Holmstrøm MO, Andersen MH. Cancer immune therapy for lymphoid malignancies: recent advances. Semin Immunopathol. 2019;41(1):111–124.
2. Dreyling M, Ghieimini M, Rule S, Salles G, Ladetto M, Tonino SH, et al. Newly diagnosed and relapsed follicular lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2021;32(3):298–308.
3. Conconi A, Ponzi C, Lobetti-Bodoni C, Motta M, Rancoita PMV, Stathis A, et al. Incidence, risk factors and outcome of histological transformation in follicular lymphoma. Br J Haematol. 2012;157 (2):188–196.
4. Kriedel R, Sehn LH, Gascoyne RD. Pathogenesis of follicular lymphoma. J Clin Invest. 2012;122(10):3424–3431.
5. Casulo C, Byttebier M, Dawson KL, Zhou X, Farber CM, Flowers CR, et al. Early relapse of follicular lymphoma after rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone defines patients at high risk for death: An analysis from the National LymphoCare Study. J Clin Oncol. 2015;33(23):2516–2522.
6. Sun L, Zhang L, Yu J, Zhang Y, Pang X, Ma C, et al. Clinical efficacy and safety of anti-PD-1/PD-L1 inhibitors for the treatment of advanced or metastatic cancer: a systematic review and meta-analysis. Sci Rep. 2020; 10(1): (article number 2083).

7. Myklebust JH, Irish JM, Brody J, Czerwinski DK, Houot R, Kohrt HE, et al. High PD-1 expression and suppressed cytokine signaling distinguish T cells infiltrating follicular lymphoma tumors from peripheral T cells. Blood. 2013;121(8):1367–1376.

8. Laurent C, Charmpi K, Gravelle P, Tosolini M, Franchet C, Yseaart L, et al. Several immune escape patterns in non-Hodgkin's lymphomas. Oncoimmunology. 2015;4(8):e1026530.

9. Tobin JWD, Keane C, Gunawardana J, Mollee P, Birch S, Hoang T, et al. Progression of disease within 24 months in follicular lymphoma is associated with reduced intratumoral immune infiltration. J Clin Oncol. 2019; 37(34):3300–3309.

10. Drobsky WR, Qazi R. Spontaneous regression in non-hodgkin's lymphoma: Clinical and pathogenetic considerations. Am J Hematol. 1989; 31(2):138–141.

11. Andersen MH. Immune regulation by self-recognition: Novel possibilities for Anticancer Immunotherapy. J Natl Cancer Inst. 2015;107(9):djv154.

12. Ahmad SM, Martinenaite E, Holmström M, Jørgensen MA, Met Ö, Nastasi C, et al. The inhibitory checkpoint, PD-L2, is a target for effector T cells: novel possibilities for immune therapy. Oncoimmunology. 2018;7(2):e1390641.

13. Ahmad SM, Larsen SK, Svane IM, Andersen MH. Harnessing PD-L1-specific cytotoxic T cells for anti-leukemia immunotherapy to defeat mechanisms of immune escape mediated by the PD-1 pathway. Leukemia. 2014;28(1):236–238.

14. Munir S, Andersen GH, Met Ö, Donia M, Frosig TM, Larsen SK, et al. HLA-restricted CTLs that are specific for the immune checkpoint ligand PD-L1 occur with high frequency in cancer patients. Cancer Res. 2013;73(6):1764–1767.

15. Munir S, Andersen GH, Svane IM, Andersen MH. The immune checkpoint regulator PD-L1 is a specific target for naturally occurring CD4(+) T cells. Oncoimmunology. 2013;2(4):e239911.

16. Munir S, Andersen GH, Woettmann A, Ödum N, Becker JC, Andersen MH. Cutaneous T cell lymphoma cells are targets for immune checkpoint ligand PD-L1-specific, cytotoxic T cells. Leukemia. 2013;27(11):2251–2253.

17. Ahmad SM, Martinenaite E, Holmström M, Jørgensen MA, Met Ö, Nastasi C, et al. The inhibitory checkpoint, PD-L2, is a target for effector T cells: Novel possibilities for immune therapy. Oncoimmunology. 2018;7(2):e1390641.

18. Ahmad SM, Svane IM, Andersen MH. The stimulation of PD-L1-specific cytotoxic T lymphocytes can both directly and indirectly enhance antileukemic immunity. Vol. 4. Blood Cancer Journal. Nature Publishing Group; 2014. p. e230.

19. Iversen TZ, Engell-Noerregaard L, Ellebaek E, Andersen R, Larsen SK, Bjorn J, et al. Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2,3 dioxygenase. Clin Cancer Res. 2014;20(1):221–232.

20. Kjeldsen JW, Iversen TZ, Engell-Noerregaard L, Mellemgård A, Andersen MH, Svane IM. Durable clinical responses and long-term follow-up of stage III–IV Non-Small-Cell Lung Cancer (NSCLC) patients treated with IDO peptide vaccine in a phase i study—A brief research report. Front Immunol. 2018;9: (article number 2145).

21. Jørgensen NG, Klausen U, Graslund JH, Helleberg C, Aagaard TG, Do TH, et al. Peptide Vaccination Against PD-L1 with IO103 a Novel Immune Modulatory Vaccine in Multiple Myeloma: A Phase I First-in-Human Trial. Front Immunol. 2020;11: (article number 359035).

22. Svane I-M, Kjeldsen JW, Lorentzen CL, Martinenaite E, Andersen MH. Clinical efficacy and immunity of combination therapy with nivolumab and IDO/PD-L1 peptide vaccine in patients with metastatic melanoma: A Phase I/II trial. Ann Oncol. 2020; 31(5):S1176 (abstract LBA48).

23. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, et al. Recommendations for Initial Evaluation, Staging, and Response Assessment of Hodgkin and Non-Hodgkin Lymphomas: The Lugano Classification. J Clin Oncol. 2014;32(27):3059–3067.

24. McCutcheon M, Wehner N, Wensky A, Kushner M, Doan S, Hsiao L, et al. A sensitive ELISPOT assay to detect low-frequency human T lymphocytes. J Immunol Methods. 1997;210(2):149–160.

25. Pott C, Brüggemann M, Rügen M, van der Velden VH, van Dongen JM, Kneba M. MRD detection in B-cell non-Hodgkin lymphomas using Ig gene rearrangements and chromosomal translocations as targets for real-time quantitative PCR. In: Kükpers R, editor. Methods in Molecular Biology. (Humana Press, New York, NY); 2019. p. 199–228.

26. van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: Guidelines for interpretation of real-time quantitative PCR data. Leukemia. 2007;21(4):604–611.

27. Moodie Z, Price L, Janetzki S, Britten CM. Response determination criteria for ELISPOT: Toward a standard that can be applied across laboratories. Methods Mol Biol. 2012; 792 185–196.

28. Van Doorn E, Liu H, Huckriede A, Hak E. Safety and tolerability evaluation of the use of Montanide ISA51 as vaccine adjuvant: A systemic review. Vol. 12. Human Vaccines and Immunotherapeutics. Taylor and Francis Inc; 2016. p. 159–169.

29. Verriere B, Gastaud L, Chamoery E, Peyrade F, Deletie E, Bourjel K, et al. Description of late onset neutropenia in indolent lymphoma patients treated with bendamustine plus rituximab. Hematol Oncol. 2018;36(1):144–149.

30. De Vries JIM, Bernsen MR, Lesteerhuij JD, Scharenborg NM, Strijk SP, Gerritsen MJP, et al. Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. J Clin Oncol. 2005;23(24):5779–5787.

31. Milcent B, Josseaume N, Petitprez F, Riller Q, Amorim S, Loiseau P, et al. Recovery of central memory and naive peripheral T cells in Follicular Lymphoma patients receiving rituximab-chemotherapy based regimen. Sci Rep. 2019;9(1) (article number 13471).

32. Jia W, Gao Q, Han A, Zhu H, Yu J. The potential mechanism, recognition and clinical significance of tumor pseudoprogression after immunotherapy. Cancer Biol Med. 2019;16:655–670.

33. Cheson BD, Ansell S, Schwartz L, Gordon LI, Advani R, Jacene HA, et al. Refinement of the Lugano classification response criteria for lymphoma in the era of immunomodulatory therapy. Blood. 2016; 128(21):2489–2496.

34. Bankhead P, Loughrey MB, Fernández JA, Dombrovsky Y, McArt DG, Dunne PD, et al. QuPath: open source software for digital pathology image analysis. Sci Rep. 2017;7: (article number 16887).

35. Tesfa D, Palmblad J. Late-onset neutropenia following rituximab therapy: incidence, clinical features and possible mechanisms. Vol. 4. Expert Review of Hematology. Taylor & Francis; 2011. p. 619–625.

36. Lerman Y, Kim M. Neutrophil Migration Under Normal and Seaps Conditions. Cardiovasc Hematol Disord Targets. 2015;15(1):19–28.

37. Jørgensen NG, Kae J, Graslund JH, Ö M, Nielsen SL, Pedersen AW, et al. Vaccination against PD-L1 with IO103 a novel immune modulatory vaccine in basal cell carcinoma: a phase iia study. Cancers (Basel). 2021;13(4):911.

38. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood. 2003;101(7):2711–2720. doi:10.1182/blood-2002-09-0782-8.

39. Gulley JL, Arlen PM, Madan RA, Tsang KY, Pazdur MR, Skarupa L, et al. Immunologic and prognostic factors associated with overall survival employing a pokviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. Cancer Immunol Immunother. 2010;59 (5):663–674.

40. Trotman J, Davies A, Hiddemann W, Hoster E, Marcus R, Schmidt C, et al. Relationship between MRD and PET responses and PFS in previously untreated follicular lymphoma in the GALLIUM trial. J Clin Oncol. 2018;36(15_suppl): (abstract 7557).
41. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1–10.

42. Leich E, Salaverria I, Bea S, Zettl A, Wright G, Moreno V, et al. Follicular lymphomas with and without translocation t (14;18) differ in gene expression profiles and genetic alterations. Blood. 2009;114(4):826–834.

43. Nann D, Ramis-Zaldivar JE, Müller I, Gonzalez-Farre B, Schmidt J, Egan C, et al. Follicular lymphoma t(14;18)-negative is genetically a heterogeneous disease. Blood Adv. 2020;4(22):5652–5665.

44. de Vries IJM, Bernsen MR, Lesterhuis WJ, Scharenborg NM, Strijk SP, Gerritsen M-JP, et al. Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. J Clin Oncol. 2005;23(24):5779–5787.

45. Aarnitzen EHJG, Bol K, Schreibelt G, Jacobs JFM, Lesterhuis WJ, Van Rossum MM, et al. Skin-test infiltrating lymphocytes early predict clinical outcome of dendritic cell-based vaccination in metastatic melanoma. Cancer Res. 2012;72(23):6102–6110.

46. Ardeshna K, Smith P, Norton A, Hancock B, Hoskin P, et al. Long-term effect of a watch and wait policy versus immediate systemic treatment for asymptomatic advanced-stage non-Hodgkin lymphoma: a randomised controlled trial. Lancet. 2003;362(9383):516–522.

47. Ardeshna KM, Qian W, Smith P, Braganca N, Lowry L, Patrick P, et al. Rituximab versus a watch-and-wait approach in patients with advanced-stage, asymptomatic, non-bulky follicular lymphoma: an open-label randomised phase 3 trial. Lancet Oncol. 2014;15(4):424–435.