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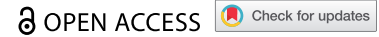
CCL22-based peptide vaccines induce anti-cancer immunity by modulating tumor microenvironment

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ABSTRACT

CCL22 is a macrophage-derived immunosuppressive chemokine that recruits regulatory T cells through the CCL22:CCR4 axis. CCL22 was shown to play a key role in suppressing anti-cancer immune responses in different cancer types. Recently, we showed that CCL22-specific T cells generated from cancer patients could kill CCL22-expressing tumor cells and directly influence the levels of CCL22 *in vitro*. The present study aimed to provide a rationale for developing a CCL22-targeting immunotherapy. Vaccination with CCL22-derived peptides induced CCL22-specific T-cell responses in both BALB/c and C57BL/6 mice, assessed by interferon-γ secretion *ex vivo*. Anti-tumor efficacy of the peptides was evaluated in mouse models engrafted with syngeneic tumor models showing a reduced tumor growth and prolonged survival of the treated mice. Vaccination induced changes in the cellular composition of immune cells that infiltrated the tumor microenvironment assessed with multicolor flow cytometry. In particular, the infiltration of CD8+ cells and M1 macrophages increased, which increased the CD8/Treg and the M1/M2 macrophage ratio. This study provided preclinical evidence that targeting CCL22 with CCL22 peptide vaccines modulated the immune milieu in the tumor microenvironment. This modulation led to an augmentation of anti-tumor responses. This study provided a rationale for developing a novel immunotherapeutic modality in cancer.

Background

Tumor cells have developed mechanisms to escape host immunosurveillance and maintain local conditions favorable to their own growth and proliferation. They achieve this by creating an immunosuppressive tumor microenvironment (TME) that prevents the recognition and destruction of tumor cells by effector T cells. Regulatory T cells (Tregs) play a crucial role in immunoregulation; they can suppress CD4+ and CD8+ T cells, dendritic cells (DCs), B cells, natural killer (NK) cells, and macrophages.[1] Tregs have been found to be increased in patients with various types of cancers,[2–4] and their presence has been associated with a poor prognosis.[5] Tregs contribute to immunosuppression through various cellular and molecular mechanisms. For example, Tregs secrete anti-inflammatory cytokines[6,7] and they impair T cell activation by depleting interleukin-2[8] or by suppressing antigen-presenting cell function through CTLA-4-mediated signaling.[9]

To attract Tregs to the tumor site, tumor cells secrete cytokines and chemokines, such as the C-C motif chemokine ligands (CCLs), CCL28,[10] CCL5,[11] CCL1,[12] and CCL22.[3] In particular, CCL22 binds to chemokine receptor 4 (CCR4), which is predominantly expressed on the surface of Tregs,

but it is also found on T-helper (th) 2 cells and cutaneous T cells.[13] CCL22 accumulation in solid tumors has been shown to lead to Treg infiltration in melanoma, ovarian, prostate, breast carcinomas, and glioblastomas.[3,14,15] In contrast, it was shown that tumors that lacked CCL22 expression were not infiltrated by Tregs, regardless of whether they produced other CCR4-binding chemokines, such as CCL17.[16] Those findings suggested that the recruitment of Tregs to the TME occurs via the CCL22:CCR4 axis, and it leads to an adverse clinical outcome.[16] Furthermore, studies have shown that increased CCL22 expression was associated with the presence of tumor-infiltrating macrophages, an increased presence of Tregs, and a subsequent suppression of host immune responses against cancer cells.[13]

Currently, several preclinical and clinical approaches are being evaluated that aim to disrupt Treg recruitment and immunosuppression by targeting the CCL22:CCR4 axis. The chemotactic recruitment of Tregs to the tumor site by CCL22 was inhibited *in vivo* by an anti-CCR4 antibody in a humanized murine model, leading to the restoration of anti-tumor immunity.[17] Another study showed that treatment with interferon α (IFNα) could suppress the *in vivo* secretion of CCL22, which was required for an effective CD8+ T-cell

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response.[18] These findings provided a rationale for targeting CCL22 to reduce immunosuppression and augment anti-tumor immune responses.

In this context, our research group previously described the presence of an immunogenic epitope derived from the signal sequence of CCL22 (which is cleaved prior to CCL22 secretion).[19] Importantly, T cells specific to the CCL22 epitope recognize and kill CCL22-expressing cancer cells, reducing CCL22 levels in *in vitro* culture. These T cells were found to be present in PBMCs from both cancer patients and healthy donors. To investigate further into the therapeutic potential of targeting CCL22, the present study evaluated the therapeutic efficacy of CCL22 peptide vaccines in mice. The treatment with CCL22 peptides expanded CCL22-reactive T cells *in vivo*, leading to an antitumor activity accompanied by a reduction in CCL22 production and Treg recruitment to the tumor site.

Methods

Cell culture

Tumor-derived murine cell lines, CT26 (colon carcinoma, RRID:CVCL_7256) and B16 (melanoma, RRID:CVCL_0159), were purchased from ATCC, and MC38 (colon carcinoma, RRID:CVCL_B288) cell line from Kerafast. The Pan02 (RRID:CVCL_D627) was obtained from CCIT cell bank. The 4T1 cell line (mammary carcinoma, RRID:CVCL_0125) was kindly donated by professor David Anz, Ludwig-Maximilians-Universität München. All cell lines used were mycoplasma tested. The cell lines were not authenticated. The cells were cultured in RPMI 1640 GlutaMax (Gibco, Cat: 61870–010) or Dulbecco's modified eagle GlutaMax (Gibco, Cat: 31966–021) medium, supplemented with penicillin, streptomycin (Gibco, Cat: 15140–122) and 10% fetal calf serum (Life technologies, Cat: 1020070–106).

Mouse strains

Female BALB/cJRj and C57BL/6NRj were purchased from Janvier labs, and C57BL/6NCrl mice were purchased from Scanbur/Charles River. The protocols and experimental procedures were approved by the Danish National Animal Experiments Inspectorate (Dyreforsøgstilsynet) and performed according to the national guidelines. All animal experiments were conducted according to the guidelines of Federation of European Laboratory Animal Science Association (FELASA). All mice were monitored and euthanized when displaying signs of discomfort.

Tumor engraftment and measurement

CT26 (0.2×10^6), B16 (0.5×10^6) or Pan02 (1×10^6) tumor cells were suspended in 200 μL phosphate buffered saline, and injected subcutaneously into the right flank of recipient mice. For orthotopic modeling, we inoculated 0.1×10^6 4T1 cells into the fat pad of the abdominal mammary gland. Mice were randomized to different treatment arms, with tumor sizes distributed evenly between the treatment groups. Tumor size was measured with a Vernier caliper, and tumor volume was calculated based on the perpendicular diameters of individual tumors ($\frac{length \times width^2}{2}$). Only mice that reached the endpoint, defined as a maximal tumor volume of 1500 mm^3, were included in the survival analysis.

For flow cytometry and gene expression analysis, tumors were harvested 6 days after the last treatment dose. The harvested tissue was dissociated through incubation for 45 min at 37°C and 650 rpm in Hanks's Balanced salt solution (Sigma Aldrich, Cat: 55021C) containing 75 μg/mL DNase (Sigma Aldrich, Cat: DN25) and 2 mg/mL of collagenase IV (Millipore, Cat: C5138).

Peptide vaccination

Murine CCL22 peptide sequences were selected with the online epitope prediction database, SYFPEITHI.[20] Sequences were selected based on high-affinity binding to MHC class I in the H2L^d for BALB/c or H2D^b for C57BL/6 strain (Table 1). Peptides were purchased from Pepscan, and purity was ≥80%. Lyophilized peptides were mixed to a concentration of 10 mM in dimethyl sulfoxide (DMSO, Fisher Scientific, Cat: D12345). Dissolved peptides were emulsified at 1:1 in Montanide ISA 51 VG water-in oil-adjuvant (SEPPIC, Cat: 36362ZFL2R3), with a double female luer lock connector (Promepla, Cat: ODG0015ST) and 1-ml Inject-F syringes (BRAUN, Cat: 9166017 V). Peptides were administered to mice via a subcutaneous injection of 100 μg peptide in a volume of 100 μL. Injections were delivered at the base of the tail with a 27-gauge needle (BD Bioscience, Cat: 302200). Animals were vaccinated once per week and up to 2 times per experiment. Control mice received DMSO diluted in water and emulsified in Montanide.

ELISpot

Spleens and lymph nodes were processed through 70 μm cell strainers to obtain a single cell suspension, followed by a red blood cell lysis with RBC lysis solution (Quiagen, Cat: 158904). 9×10^5 splenocytes or lymph node cells were seeded in triplicate into 96-well MSIPN4W multiscreen plates (Millipore, Cat: MSIPN4W50) coated with 6 μg/mL anti-mouse interferon gamma (IFNγ) antibody (AN18 monoclonal capture antibody, Mabtech, Cat: 3321-2-1000). The cells were stimulated with 5 μM of peptide (treatment), DMSO (negative control), or concanavalin A (positive control, Sigma-Aldrich, Cat: C5275). Incubation with the presence of the antigen lasted from 16 to 18 h after which, they were washed off and secondary antibody was added (monoclonal anti-mouse IFNγ R4-8A2 biotinylated antibody, Mabtech, Cat: 3321-6-10), followed by streptavidin-ALP (Mabtech, Cat: 3321–10) for detection. The assay was developed with BCIP/NBT plus substrate (Mabtech, Cat:

Table 1. Sequences of mouse CCL22-derived peptides.

Peptide	Peptide name	Length (amino acids)	Predicted haplotype binding	Sequence
CCL22	CCL22$_{6-14}$	9	H2L^d	VPLLVALVL
	CCL22$_{10-19}$	10	H2D^b	VALVLLAVAI

3650–10). Spots of IFNγ secreted from activated cells were counted with the ELISpot reader, CTL ImmunoSpot S6 Ultimate analyzer (Cellular Technology Limited), and Immunospot Software (Cellular Technology Limited).

Flow cytometry

For flow cytometry analysis, cells were stained and fixed using standard protocols. Acquisition was performed with a BD LSRFortessa III (BD Biosciences), and data were analyzed with FlowLogic 7.2.1 software (inivai Technologies). Details on the used antibody panels are given in Supplementary Tables S1 and S2.

Quantitative PCR

Total RNA was purified from tumors with the RNeasy Plus Mini Kit (Qiagen, Cat: 74136), according to the manufacturer's guidelines. Then, cDNA was synthesized from 1 μg RNA with the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Cat: 4368814), according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with the Light Cycler 480 TaqMan Master mix (Roche Diagnostics, Cat: 04887301001) and TaqMan Expression Assay probes (Applied Biosystems). Thermocycling was performed on a LightCycler 480 II system (Roche). Expression of the gene of interest was normalized to the expression of hypoxanthine phosphoribosyltransferase (HPRT), and differential expression was calculated with the $\Delta\Delta CT$ method. A list of probes used in this study can be found in Supplementary Table S3.

Gene expression analysis

RNA from CT26 tumor tissue was purified with the RNeasy Plus Mini Kit (Qiagen, Cat: 74136), according to the manufacturer's guidelines. Gene expression was analyzed with the nCounter Mouse PanCancer Immune Profiling panel (Nanostring technologies), which included an array of 770 immuno-oncology-related genes.[21] Data were normalized to the expression of endogenous genes. Batch effects were corrected with the empirical Bayes method, ComBat.[22] Differential expression analyses were performed with a quasi-likelihood F test (edgeR package).[23] Genes that showed significantly differential expression (p-values <0.05) were considered differentially expressed (note that changes were generally subtle and p-values were not corrected for multiple testing). Rank-based gene-set enrichment analyses[24] were performed with the fgsea package,[25] and gene ontology terms enriched with a p value < 0.05 were considered (likewise, these p-values were not corrected for multiple testing, as only immunological terms can be expected to be enriched from the Mouse PanCancer Immune Profiling panel, while the test defaults to all testing 7,573 biological process terms). These analyses were performed in R.

Statistical analysis

All statistical analyses, including the Student's t-test, Log-Rank test, and mixed-model analysis, were performed with GraphPad Prism 9 (GraphPad Software). Unless otherwise noted, statistical significance was defined as a P-value <0.05.

Data availability

The data that supports the findings described in this study were generated by the authors and are available upon reasonable request.

RESULTS

CCL22 peptide therapy induces expansion of CCL22-specific T cells

We have previously demonstrated that, stimulating PBMCs from either healthy donors or cancer patients with a decamer peptide derived from the CCL22-signal sequence predicted to bind to HLA-A2, led to the activation of CCL22-specific T cells.[19] To evaluate whether CCL22-specific T cells could be activated in vivo by vaccination with CCL22 peptide, murine CCL22 peptides that were predicted to bind to MHC class I in C57BL/6 and BALB/c mice were identified.

To assess the ability of each epitope to generate CCL22-specific T cells in vivo, mice were immunized with each CCL22 peptide twice, and one week after the second immunization, CCL22 peptide-specific T-cell responses were evaluated by IFNγ Elispot assay. Two immunogenic CCL22 peptide sequences were identified: (i) for C57Bl/6 mice with predicted affinity for MHC class I H2D^b (referred to as CCL22$_{10-19}$) and (ii) for BALB/c mice predicted to bind to MHC class I H2L^d (referred to as CCL22$_{6-14}$). A significant CCL22-specific immune response was detected in both the spleen (CCL22$_{10-19}$: Figure 1a-b,=0.0007; CCL22$_{6-14}$: Figure 2a-b, =0.0021) and the lymph nodes (CCL22$_{10-19}$: Figure 1a,=0.0043; CCL22$_{6-14}$: Figure 2a,<0.0001).

CCL22 peptide therapy delays tumor growth

Having established that CCL22-specific T cells can be expanded by peptide vaccination, we hypothesized that vaccine-induced CCL22-specific T cells may traffic to the tumor site and target CCL22-expressing cells, leading to anti-tumor response. To identify a relevant tumor model for assessing the immunotherapeutic effect of CCL22 peptide vaccines, CCL22 expression in different engrafted syngeneic tumors was evaluated by qPCR (**supplementary figure S1a**). All tested models (CT26, 4T1, B16 and Pan02) confirmed expression of CCL22, and therefore examined in the subsequent experiments. To evaluate whether the immune infiltrate in the TME are the main intratumoral source of CCL22 in vivo, we analyzed CCL22 expression in the CD45 positive infiltrate and CD45 negative negative fraction of engrafted syngeneic tumors. We confirmed that CCL22 expression is limited to the CD45 positive fraction in all the analyzed models (**supplementary figure 1B**).

To investigate the potential anti-tumor effect of a CCL22$_{10-19}$ peptide, the immunotherapeutic effect was evaluated in mice subcutaneously engrafted with pancreatic cancer (Pan02) or melanoma (B16) cells (Figure 1c–). Treatment with CCL22 vaccine led to significant reductions in tumor growth in
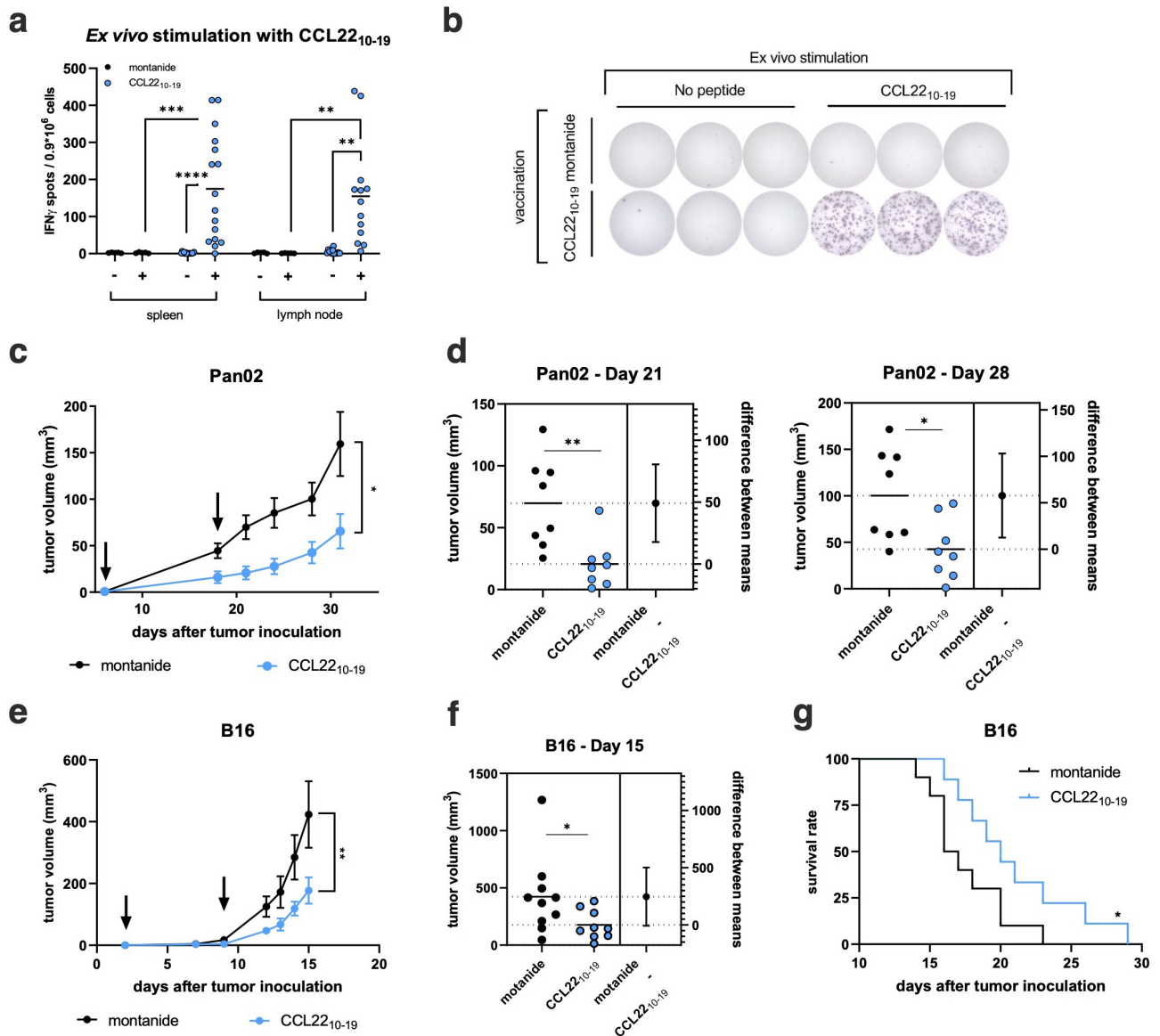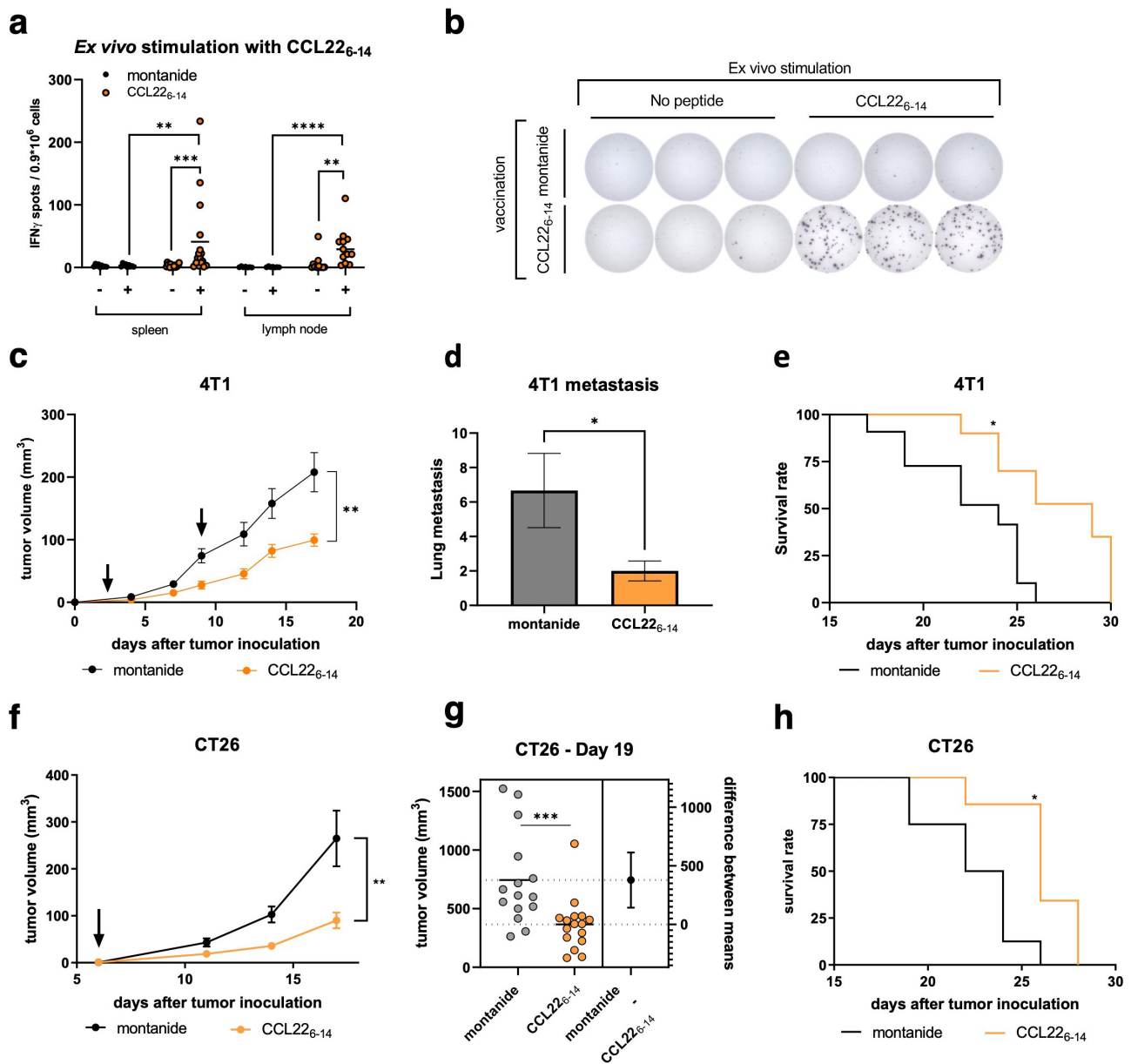
Figure 1. Effects of CCL22 peptide vaccination in C57BL/6 mice. (a-b) H2D^b-restricted CCL22-derived CCL22$_{10-19}$ epitope was administered subcutaneously (n = 13). A peptide-free treatment (Montanide) served as a negative control (n = 5). (a) IFNγ ELISpot assay results show *ex vivo* cell responses to no stimulation (–) or stimulation with 5 µg/ml CCL22-peptide (+) in splenocytes (*left*) and lymph node cells (*right*) prepared from treated C57BL/6 mice. Significant differences were determined with the non-parametric Mann Whitney t-test. (b) Representative images of ELISpot wells show positive responses to CCL22 stimulation (*bottom right*) in splenocytes from CCL22-immunized C57BL/6 mice. (c-g) C57BL/6 mice were inoculated subcutaneously with Pan02 (C and D, n = 8) or B16 (E-G, n = 10) tumor cells, then treated with CCL22$_{10-19}$ peptide on post-inoculation days 7 and 18 (C, black arrows) or on days 2 and 9 (E, black arrows). (c,e) Mean tumor growth ±standard error of the mean (SEM). Differences in tumor growth were evaluated with a mixed model analysis. (d) Differences in Pan02 tumor growth on post-inoculation days 21 (*left*) and 28 (*right*). (f) Differences in B16 tumor growth 15 days after inoculation. (g) Survival curves show survival of B16F mice, with or without a CCL22$_{10-19}$ administration. Survival difference was assessed with the Gehan-Breslow-Wilcoxon test. *p ≤ 0.05, **p ≤ 0.01 ***p ≤ 0.001, ****p ≤ 0.0001.

the Pan02 (Figure 1c p = 0.0116, and **Figure D** day 21 p - = 0.0030, day 28 p = 0.0207) and B16 (Figure 1e p = 0.0013 and **Figure E** p = 0.035) models compared to control mice. The differences in growth were already present at 18 days after inoculation in the Pan02 model (p = 0.0143) and at 12 days in B16 (p = 0.046). Furthermore, the treatment significantly increased the survival of B16-bearing mice, which further confirmed the anti-tumor activity of CCL22-peptide vaccine (Figure 1g, = 0.0475).

Similarly, the immunotherapeutic effects of CCL22$_{6-14}$ peptide was assessed in an orthotopic model of breast cancer (4T1) and a subcutaneous model of colon carcinoma (CT26) in BALB/c mice (Figure 2c-g). In mice bearing 4T1

tumors vaccination led to significant reduction in tumor growth at day 9 after tumor inoculation (p = 00018). In this model, the CCL22 vaccine induced overall reduction in tumor growth in 4T1 model (Figure 2c; p = 0.0017), associated with significantly lower metastasis burden in the lungs (Figure 2d; p = 0.0219) and improved survival (Figure 2e; p=0.0240), compared to untreated controls. CT26-bearing mice also displayed reduced tumor growth, which was detectable after a single treatment with the CCL22$_{6-14}$ peptide (figure 2f p = 0.0055 and **2g**; p = 0.0006). In addition, the treated mice showed significantly better survival compared to controls (Figure 2h; p=0.0372). Thus, the anti-tumor activity of CCL22 peptide

Figure 2. Effects of CCL22 peptide vaccination in BALB/c mice. BALB/c mice were immunized subcutaneously with a H2L^d-restricted CCL22-derived epitope CCL22$_{6-14}$ (at least 7 mice per group). A peptide-free treatment (montanide) served as a negative control (n ≥ 7). (a) IFNγ ELISpot assay results show *ex vivo* cell responses to no stimulation (−) or stimulation with 5 μg/ml CCL22-peptide (+) in splenocytes (*left*) and lymph node cells (*right*). Significant differences were determined with the non-parametric Mann Whitney t-test. (b) Representative images of ELISpot wells show positive responses to CCL22 stimulation (*bottom right*) in splenocytes from CCL22-immunized BALB/c mice. (c-h) BALB/c mice were inoculated orthotopically with 4T1 cells (C-E, n = 11) or subcutaneously with CT26 (F-H, n = 12) tumor cells, then treated with CCL22$_{6-14}$ on post-inoculation days 2 and 9 (C, black arrows) or 6 (F, black arrow). (c) Average growth of 4T1 tumors ± SEM for CCL22$_{6-14}$-treated mice (Orange), compared to the Montanide control group (black). (d) Difference between groups in metastasis burdens in the lungs, on post-inoculation day 20. (e) Survival of mice bearing 4T1 tumors. The CCL22$_{6-14}$ immunization improved survival, from a median of 24 to 29 days. (f) Average growth of CT26 tumors ± SEM. (g) Difference between groups in the mean CT26 tumor growth on post-inoculation day 19 (n = 20). (h) Survival of CT26-bearing mice. The CCL22$_{6-14}$ treatment improved survival, from a median of 23 to 26 days. Differences in tumor growth were tested with a mixed model test; differences in survival were assessed with the Gehan-Breslow-Wilcoxon test. *$p ≤ 0.05$, **$p ≤ 0.01$) ***$p ≤ 0.001$, ****$p ≤ 0.0001$.

vaccines was confirmed in all the syngeneic tumor models tested.

CCL22 peptide vaccine impacts myeloid infiltration in CT26 tumors

Given the hypothesized mechanisms of action of CCL22 vaccines, that the treatment may impact on tumor-promoting Treg cells via activation of CCL22-specific T cells, the CT26 model was chosen for subsequent experiments based on previous studies demonstrating that the proportion of Tregs in the CT26 tumor TME was higher than the proportions observed in other tumor models.[23] Hence, CT26-tumor-bearing mice were treated with CCL22$_{6-14}$ peptide and tumor-infiltrating leukocytes were analyzed to evaluate treatment-induced changes in the TME (Figure 3). Given that M2 macrophages and dendritic
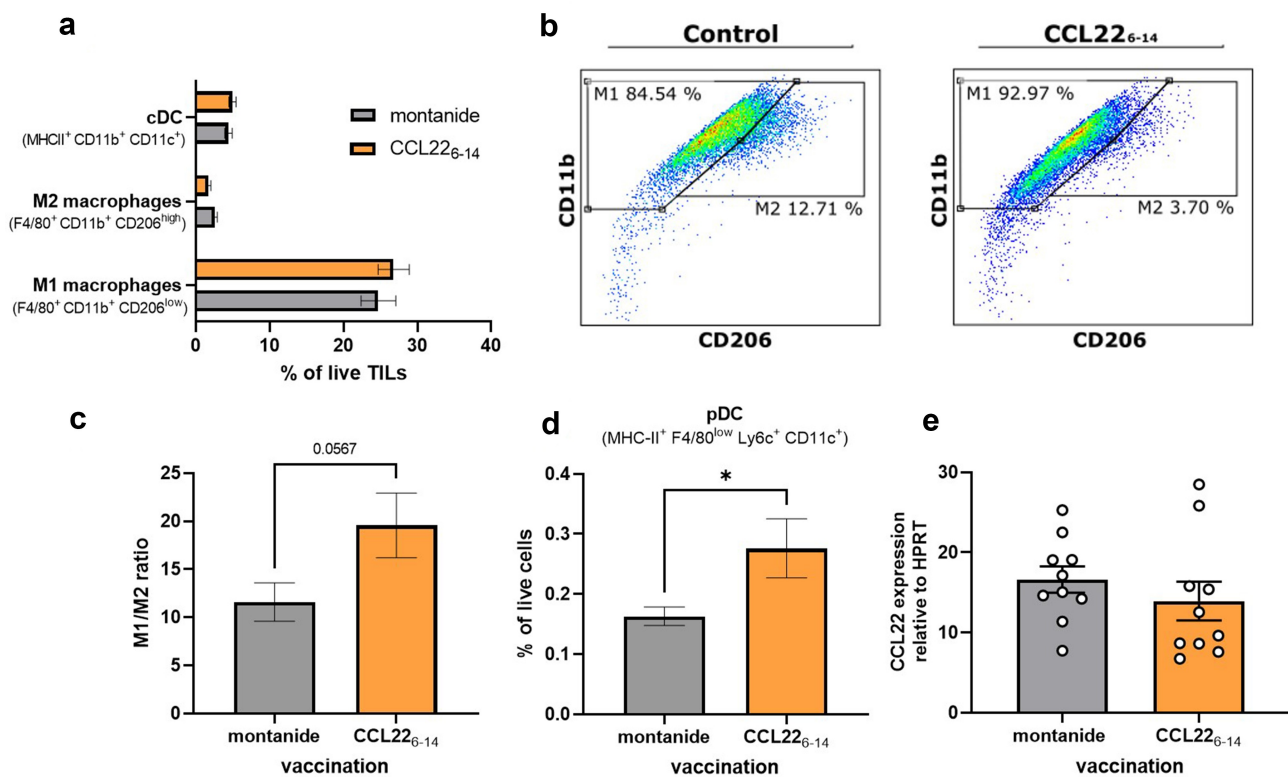
Figure 3. CCL22 peptide immunization leads to alterations in myeloid infiltration of CT26 tumors. (a-d) Flow cytometry analysis of myeloid subsets that infiltrated CT26 tumors performed on day 6 after a CCL22$_{6-14}$ immunization (n = 10) or control peptide free treatment (n = 10; also refered to as montanide in figures). (a) Percentages of M1 macrophages (MHC-II$^+$, CD11b$^+$, F4/80$^+$, and CD206^{low}), M2 macrophages (MHC-II$^+$, CD11b$^+$, F4/80$^+$, and CD206^{high}), and classical dendritic cells (cDC: MHC-II$^+$, CD11c$^+$, and CD11b$^+$) among the total live infiltrating leukocytes (TILs: CD45$^+$). (b) Representative example of dot plots for macrophague populations for the CCL22$_{6-14}$-immunized mice and control mice (peptide free treatment refered to as montanide in figure) (c) M1 to M2 ratio of tumor infiltrating macrophages and (d) Plasmatocytoid dendritic cell infiltration out of total live infiltrating cells (e) CCL22 expression determined by qPCR analysis on tumor samples resected 6 days after administering the CCL22$_{6-14}$ treatment. Expression was evaluated relative to hypoxanthine phosphoribosyltransferase (HPRT) expression. Data are shown as an average value ± SEM. Differences were evaluated with the unpaired t-test; *p ≤ 0.05.

cells are the primary source of CCL22 in the TME of murine tumors,[18] we first evaluated the myeloid cell infiltration in CT26-bearing mice. Flow cytometry analysis of tumor samples showed a trend toward a decline in M2 macrophage infiltration and an increase in M1 macrophage infiltration (Figure 3a,b), accounting for an average two-fold increase in the M1/M2 ratio (p = 0.056; Figure 3c). The treatment also led to an increased tumor infiltration of plasmatocytoid dendritic cells (p = 0.041; Figure 3d). In the majority of treated mice, the decline in M2 macrophages was associated with a reduction in the CCL22 expression levels in the TME, however this was not statistically significant (p = 0.36; Figure 3e).

CCL22 peptide immunization leads to reduction in tumor Treg recruitment and increase in CD8 + T cells

Since the immunomodulatory function of CCL22 in cancer is attributed to its ability to recruit Tregs to the TME, the infiltration of lymphoid populations in CT26 tumors was then assessed. CCL22 peptide treatment resulted in a significant increase in CD8$^+$ T cell infiltration (p = 0.0230; Figure 4a,d) and a significant reduction in Treg infiltration (p = 0.0203; Figure 4a,d). These changes resulted in a higher CD8/Treg ratio in treated animals (p = 0.0402; Figure 4b). Furthermore, the infiltration of PD-1$^+$ CD8$^+$ T cell subset also increased after treatment with CCL22$_{6-14}$

(p = 00407; Figure 4c,d) while the PD-1 $^-$ CD8$^+$ T cell fraction remained the same (data not shown). Taken together, these data suggests that the anti-tumor activity of CCL22 peptide vaccines is associated with a lower infiltration of immunosuppressive cells and a higher infiltration of pro-inflammatory cells, thus supporting our hypothesis that CCL22 vaccine modulates the immunosuppressive TME.

CCL22 peptide vaccine alters immune gene signature in the TME

To further elucidate the molecular mechanism underlying the therapeutic effect of the CCL22 peptide vaccine, gene expression of pan-immune markers from CCL22 peptide-treated CT26 tumors were analyzed with a Mouse PanCancer Immune Profiling Panel (NanoString technologies), which contained 770 immuno-oncology-related mouse genes.[21] Cluster classification was employed to find key immune and cellular processes, which identified a set of 20 differentially expressed genes (Supplementary figure 2). Next, a gene set enrichment analysis was performed to describe key signaling pathways affected by the CCL22 peptide treatment (Figure 5a) .

The gene set enrichment analysis revealed a significant upregulation of cytotoxicity in the TME (p = 0.005, Figure 5ba), an increase in toll-like-receptor pathway signaling (p = 0.024, Figure
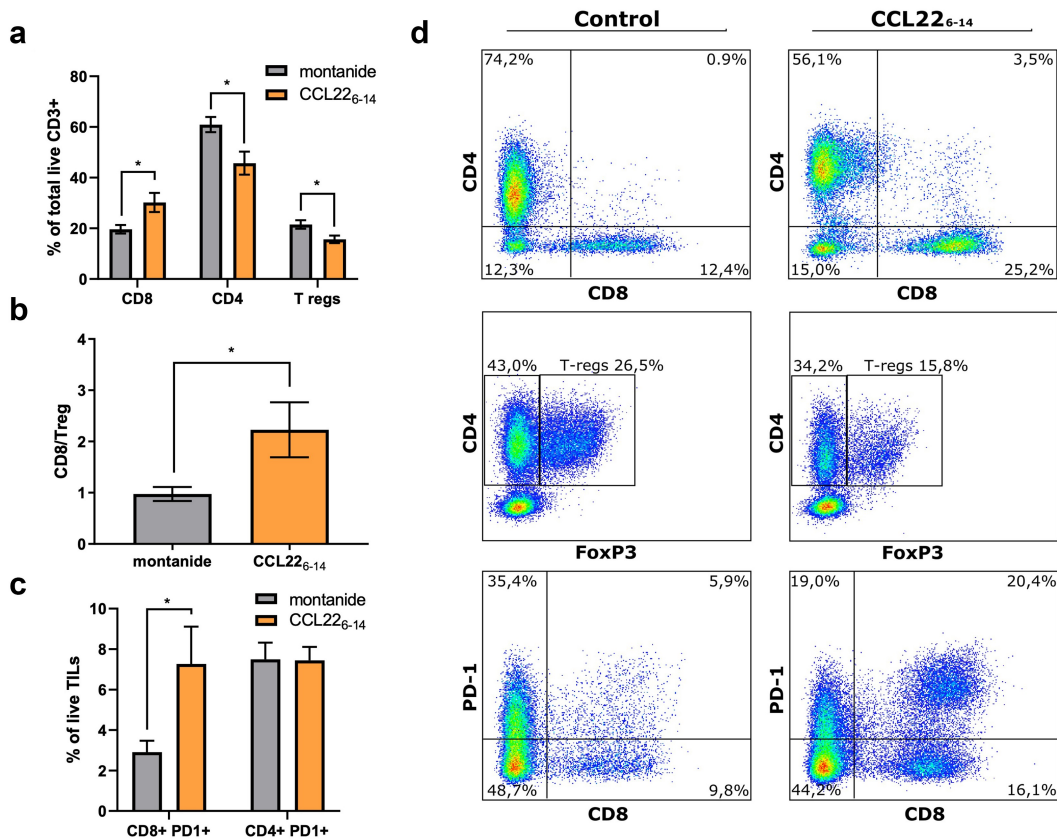
Figure 4. CCL22 peptide vaccination-induced changes in the lymphoid infiltration of CT26 tumors. Flow cytometry analysis of T-cell subsets that infiltrated CT26 tumors resected 6 days after CCL22-immunization (n = 8) or peptide free control treatment (montanide, n = 8). (a) The populations are described as percentages of CD4$^+$ T cells, CD8$^+$ T cells, and Tregs (CD4+, Foxp3+), out of the total T-cell population (CD45$^+$ CD3$^+$); (b) ratio of CD8$^+$ T cells to Tregs after CCL22 and control (Montanide) treatment. (c) Differences in populations of CD8$^+$ PD1$^+$ and CD4$^+$ PD1$^+$ subpopulations of activated tumor infiltrating T cells (d) Representative examples of dot plots for each population analyzed. Data are shown as average value per group ± SEM; differences between groups were evaluated with the unpaired t-test; *p ≤ 0.05.

5ca) and an upregulation of NK-mediated immunity (p = 0.025, Figure 5da). Conversely, processes such as growth-factor binding (p = 0.007, Supplementary figure 3) and tissue morphogenesis (p = 0.028, Supplementary figure 3) were downregulated, which suggested a reduction in the activation of cells and processes associated with tumor progression. This reduction was illustrated by an individual gene expression analysis (Figure 5eb), which showed significant declines in the expression of hypoxia-inducible factor 1α[26,27] (p = 0.0166) and CD276[28,29] (p = 0.0403), after a CCL22-peptide treatment. Both have been associated with tumor growth and progression and with a poor clinical prognosis.[26,27,29] The CCL22-vaccine treatment also caused a reduction of the expression of S100a8 which has been related with a macrophage promotion of tumor invasion and migration.[30] In addition, the treatment lead to an increase in the expression of interferon regulatory factor 1[31] (p = 0.0156) and CD160[32,33] (p = 0.0347), suggesting an enhancement in T-cell and NK-cell activity. Consistently with qPCR data, a reduction in the expression of CCL22 in treated CT26 tumors was also observed in the nanostring analysis (Figure 5fc)

Discussion

Accumulating evidence suggests that CCL22 expression is a key factor in the recruitment of Tregs into the TME of different types of human cancers; hence, CCL22 can promote an immunosuppressive microenvironment.[16] This provided the rationale for targeting CCL22 to augment anti-tumor immune responses. Moreover, pro-inflammatory CCL22-specific T cells were found to be present in cancer patients and in healthy subjects, and they targeted CCL22-expressing cells *in vitro*.[19] Accordingly, we postulated that a treatment with CCL22-derived peptide vaccines might potentially stimulate CCL22-specific T cells *in vivo* and lead to a beneficial anti-tumor effect.

In this study, we demonstrated that, as previously observed in humans, it is also possible to activate and expand CCL22-specific T-cell responses in mice with CCL22-derived peptides. Strikingly, simple treatment with the peptide leads to a significant delay in tumor growth in several murine syngeneic tumor models. Based on our observations of CCL22-specific T cells in humans and mice, we hypothesized that CCL22-specific T cells were an integral part of the immune system, which could be readily activated to modulate immune suppression in the TME, and thus, impair tumor development.

To our knowledge, CCL22 was the first chemokine to be described as a T-cell target. However, a recent study also identified T cells that targeted TGF-β-expressing cells in a cancer setting.[34] Hence, this phenomenon of T cells targeting immunoregulatory antigens may be more general than originally thought. Indeed, in other studies, pro-inflammatory T cells were identified that recognized peptide sequences derived from
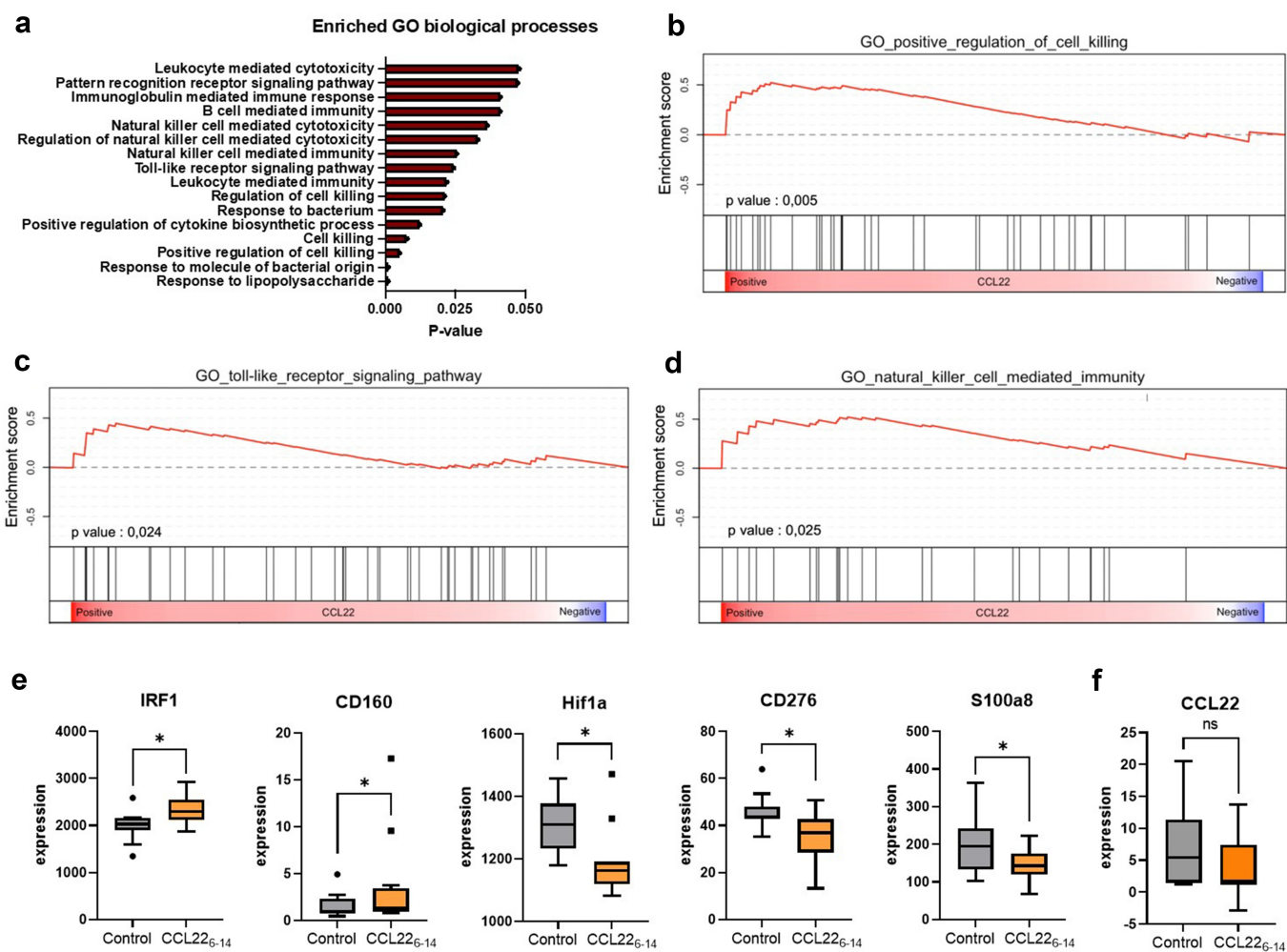
Figure 5. Gene expression in CT26 tumors after a CCL22-peptide vaccination. BALB/c mice were inoculated subcutaneously with CT26 tumor cells. At 6 days after inoculation, mice received a subcutaneous injection of either a control solution or CCL22$_{6-14}$ (n = 12 mice per group). Tumors were harvested on day 11, RNA was extracted, and gene expression was analyzed with the nCounter Mouse PanCancer Immune Profiling Panel. (a) Overview of major enriched GO biological processes that showed a statistically significant difference with immunization. (b-d) Examples of gene Set Enrichment (GSEA) analyses identified the biological processes that were upregulated upon treatment: (b) cell killing, (c) toll-like receptor signaling pathway and (d) natural killer cell mediated immunity. (e) Boxplots show significantly differentially expressed genes in CCL22-treated samples, compared to controls. IRF1: interferon regulatory factor 1; Hif1α: hypoxia-inducible factor 1α. (f) Boxlot showing CCL22 expression in nanostring sample readout. On box plots, the line indicates the median value and the box limits the first and third quartiles, while the bars indicate the maximum and minimum values.

immunoregulatory antigens, such as PDL1,[35] IDO,[36] arginase 1[37] and arginase 2.[38] Moreover, promising results have been found in murine models with a peptide-based immunotherapy directed against an IDO[39] and Arginase 1-expressing cells[40] combined with checkpoint inhibitors.

The present study demonstrated that a treatment with CCL22 peptides led to a reduction in the expression of CCL22 within the tumor. As anticipated, this reduction in CCL22 levels correlated with a reduction in Treg recruitment to the tumor site. In parallel, we observed an increase in the infiltration of pro-inflammatory cells to the TME of CT26 tumors and an increase in the CD8/Treg ratio upon treatment. Furthermore, we also observed an increased activation of the infiltrated CD8 + T cells. These observations were previously associated with a favorable prognosis.[41] The observed increase in the infiltration of CD8+ PD-1 + T cells in the tumor also raises the possibility of exploring the combination of the vaccination with CCL22-derived peptide with checkpoint inhibitors targeting PD-1.

These findings were also supported by an RNA multiplex analysis, which revealed an upregulation of genes related to biological and molecular processes involved in immune responses in the TME and a downregulation in the expression of genes associated with tumor progression, such as hypoxia-inducible factor 1α and CD276. We also observed an increase in the expression of interferon regulatory factor 1, which is activated during inflammatory responses to IFNγ. Interestingly, interferon regulatory factor 1 is known to induce the production of type I IFN,[42] which has been shown to suppress the intratumoral secretion of CCL22.[18]

The CCL22:CCR4 axis has been shown to function as an immune checkpoint; this axis is crucial for controlling T-cell immunity, particularly in the TME context, where a CCL22 deficiency was correlated with prolonged survival.[43] Consequently, different approaches to disrupt this axis are currently under investigation. A CCR4 antagonist (mogamulizumab), was recently developed to disrupt CCL22:CCR4-mediated Treg recruitment to the tumor.[44] Anti-CCR4 antibodies showed promising results

in vivo. These antibodies effectively inhibited chemotactic Treg recruitment to the tumor in a CCL22-dependent manner, which led to the restoration of anti-tumor immunity in a humanized murine model.[17] Furthermore, those findings were reproduced in a clinical setting, and in 2018, mogamulizumab was approved by the FDA for the treatment of cutaneous T-cell lymphoma. Subsequently, a phase Ia trial tested a combination therapy of mogalinumab and nivolumab (anti-PDL1) in multicancer solid tumors and found that effector Tregs were depleted in peripheral blood and in the TME.[45] In some cases, the Treg depletion induced by mogamulizumab was associated with serious autoimmune side effects.[46,47] Its application in combination with checkpoint inhibitors was tested in a phase I[48] and a phaseI/II[49] study. However, the combination did not results in a potent antitumor efficacy in patients with advanced solid tumors therefore, leaving an open door for other approaches aiming at targeting Treg recruitment through the CCL22:CCR4 axis.

In comparison, in the present study, we aimed to activate T cells that specifically targeted CCL22-producing cells in the TME. This approach led to a significant reduction in Treg recruitment to the tumor site correlating with a reduction of tumor growth. We speculated that CCL22-specific T cells could directly eliminate CCL22-expressing cells, but further research is needed to address this hypothesis. The targeting of CCL22-expressing cells may raise the question of a potential undesired alteration of peripheral tolerance induced by vaccination with CCL22-derived peptides. However, self-reactive cells against CCL22 have been identified in healthy individuals who do not suffer from any identified autoimmune disorder.[19] Moreover, similar vaccination strategies targeting other self-antigens have been tested in clinical trials without identifying severe adverse reactions to the treatment or long-term toxicity.[50,51] Additionally, a phase 1/2 clinical trial recently showed that the combination of an immune-modulatory vaccine against IDO and PD-L1 with nivolumab improved the clinical efficacy of the checkpoint inhibitors for the treatment of metastatic melanoma without an increased toxicity.[52] The exploration of the combination of the vaccination approach described in this paper with checkpoint inhibitors will be of high relevance for the translation of this therapeutic approach to the clinic.

In conclusion, we demonstrated that a CCL22-derived peptide vaccines effectively inhibited tumor progression in vivo. This novel approach showed favorable therapeutic effects, and thus, it could potentially provide a basis for future cancer immunotherapy.

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Disclosure statement

ILM, KLK, MC, EM and AWP are employed by IO Biotech. M-BZ is an employee and a shareholder in IO Biotech. MHA is Founder, a shareholder and a Scientific Advisory Board member in IO Biotech. The other authors declare no direct conflicts of interest.

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Panagiotis Mantas http://orcid.org/0000-0001-6106-409X
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