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Interleukin-6-mediated resistance to immunotherapy is linked to impaired myeloid cell function

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Abstract

High serum levels of interleukin-6 (IL-6) correlate with poor prognosis and chemotherapy resistance in several cancers. The underlying mechanisms and its effects on immunotherapy are largely unknown. To address this, we developed a human papillomavirus type 16 (HPV16)-associated tumor model expressing IL-6 to investigate the impact of tumor-expressed IL-6 during cisplatin chemotherapy and HPV16 synthetic long peptide vaccination as immunotherapy. The effects of tumor-produced IL-6 on tumor growth, survival and the tumor microenvironment were analyzed. Our data demonstrated that tumor-produced IL-6 conferred resistance to cisplatin and therapeutic vaccination. This was not caused by a changed in vitro or in vivo growth rate of tumor cells, or a changed sensitivity of tumor cells to chemotherapy or T-cell-mediated killing. Furthermore, no overt differences in the frequencies of tumor-infiltrating subsets of T cells or CD11b⁺ myeloid cells were observed. IL-6, however, affected the systemic and local function of myeloid cells, reflected by a strong reduction of major histocompatibility complex (MHC) class II expression on all major myeloid cell subtypes. Resistance to both therapies was associated with a changed intratumoral influx of MHC class II⁺ myeloid cells toward myeloid cells with no or lower MHC class II expression. Importantly, while these IL-6-mediated effects provided resistance to the immunotherapy and chemotherapy as single therapies, their combination still successfully mediated tumor control. In conclusion, IL-6-mediated therapy resistance is caused by an extrinsic mechanism involving an impaired function of intratumoral myeloid cells. The fact that resistance can be overcome by combination therapies provides direction to more effective therapies for cancer.

KEYWORDS

chemotherapy, immunotherapy, interleukin-6, tumor microenvironment

Abbreviations: BSA, bovine serum albumin; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; DPECs, double-positive effector cells; EFS, short elongation factor; FCS, fetal calf serum; GFP, green fluorescent protein; HPV, human papillomavirus; IC50, half-maximal inhibitory concentration; IFN γ , interferon gamma; IL-6, interleukin 6; IMDM, Iscove's modified Dulbecco's media; IRES, internal ribosome entry site; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MTD, maximum tolerated dose; PBS, phosphate-buffered saline; SLECs, short-lived effector cells; SLP, synthetic long peptide; Tregs, regulatory T cells.

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1 | INTRODUCTION

In many cancer types, increased serum concentrations of interleukin 6 (IL-6) are reported and this positively correlates with larger tumors, advanced disease stage, worse performance status and resistance to chemotherapy.¹⁻⁵ IL-6 is a pleiotropic cytokine with a major role in hematopoiesis, immune defense and oncogenesis. It is produced by tumor cells, various immune and non-immune cells and has a wide range of effects.⁶ Normal physiological serum concentrations of IL-6 are low and in the picogram per ml range, whereas in disease settings it may go up to micrograms per ml.⁷

Aberrant inflammatory signaling is a common feature in many gynecologic cancers where IL-6 may stimulate tumor cell proliferation directly^{8,9} as well as indirectly by increasing angiogenesis due to enhanced production of vascular endothelial growth factor (VEGF) by tumor cells or by the conversion of myeloid cells to M2 macrophages.^{4,10-13} In addition, autocrine IL-6 signaling was shown *in vitro* to provide cytotoxic resistance to tumor cells treated with platinum or paclitaxel chemotherapy, by stimulation of cell growth through the activation of the STAT3, Ras-ERK and PI3K-Akt signaling pathways,¹⁴⁻¹⁷ by upregulation of multidrug resistance genes and antiapoptotic proteins as well as by reduction of proteolytic activation of caspase-3 in tumor cells.^{18,19} Furthermore, we demonstrated that cisplatin and carboplatin as chemotherapy agents enhanced the production of IL-6 by tumor cells through stimulation of the DNA damage response pathway.¹⁰ However, no correlation existed between the levels of tumor-produced IL-6 and resistance to chemotherapy-induced cell death *in vitro*.¹⁰ Furthermore, the blockade of IL-6 did not have an impact on the response of a paclitaxel resistant tumor cell line to chemotherapy *in vivo*,²⁰ suggesting that other mechanisms may be in play.

It is now generally accepted that the composition of the tumor microenvironment (TME) is critical for the response to chemo-, radio- and immunotherapy.^{21,22} The presence of type-1 tumor-specific T cells, DCs and M1 macrophages has been positively correlated with outcome, whereas immunosuppressive cells such as Tregs, MDSCs and M2 macrophages in the TME can dampen tumor immunity and are related to a worse prognosis.²³ This suggests that IL-6-driven alteration of the TME may form a potential alternative indirect mechanism for chemoresistance. Along the same lines, it may also provide resistance to immunotherapy as this also depends on functional intratumoral myeloid cell populations and T cells.^{24,25} However, up to now, the distinct effects of IL-6 on tumor-infiltrating immune cells are largely unknown.

To investigate the role of tumor-expressed IL-6 in immunotherapy and chemotherapy *in vivo*, we engineered TC-1 tumor cells to express high levels of IL-6, as can be found in certain disease settings.¹¹ TC-1 has been used to study both chemotherapy and immunotherapy against human papillomavirus (HPV)-induced cancers.²⁶⁻²⁸ Increased serum IL-6 is found in HPV-related cancers²⁹⁻³¹ and when produced by cervical cancer cells³² or oropharyngeal cancer cells³³ it is related to a worse response to radiotherapy or chemotherapy, respectively. Here, we showed that tumor-produced IL-6 not only confers resistance to cisplatin chemotherapy but also to therapeutic

What's new?

Interleukin-6 (IL-6) cytokine has multiple effects on hematopoiesis and immune function and typically circulates at low levels. In cancer, however, IL-6 serum levels are significantly elevated, with suspected impacts on tumor behavior. In this study, using a mouse model of human papillomavirus-induced cancer with IL-6 expression, the authors show that tumor-produced IL-6 confers resistance to both chemotherapy and immunotherapy. Resistance was associated with impaired myeloid cell maturation, with no evidence of involvement of mechanisms intrinsic to tumor cells. Resistance was overcome by combining chemotherapy and immunotherapy, providing insight into a potentially effective therapeutic approach for cancers with IL-6-mediated resistance.

vaccination with synthetic long peptides (SLP). We did not observe a difference in tumor cell growth or sensitivity to cytotoxic mechanisms; however, a direct effect on antigen-presenting capacity of systemic and intratumoral myeloid cells, reflected by their lower levels of cell surface major histocompatibility complex (MHC) class II expression, was detected. Since control of TC-1 tumor growth by either therapy requires the presence of antigen-presenting cells,^{25,26} the effect of IL-6 on these cells may form a shared escape mechanism.

2 | MATERIALS AND METHODS

2.1 | Mice

Six- to eight-week-old wild-type female C57BL/6 mice were obtained from Charles River Laboratories. Mice were housed in individually ventilated cages under specific pathogen-free conditions in the animal facility of Leiden University Medical Center (LUMC, Leiden, the Netherlands). All animal experiments were approved by the national committee for animal experiments (CCD) under permit AVD116002015271 and were executed according to the animal experimentation guidelines of LUMC in compliance with the guidelines of Dutch and European laws.

2.2 | Tumor cell line and culture conditions

The tumor cell line TC-1 (RRID: CVCL_4699) (a kind gift from T.C. Wu, John Hopkins University, Baltimore, MD) was generated by retroviral transduction of C57BL/6 lung epithelial cells with the HPV16 E6/E7 and c-H-ras oncogenes.³⁴ This cell line was cultured as described previously.³⁵ TC-1 control and IL-6 tumor cell lines were made by plasmid DNA transfection of TC-1. The pcDNA3.1 vector

was first adapted by replacement of the cytomegalovirus (CMV) promoter with a short elongation factor (EFS) promoter. Next, a fragment was inserted containing the internal ribosome entry site (IRES) sequence and green fluorescent protein (GFP). The IL-6 gene was inserted between the EFS promoter and IRES sequence and GFP (TC-1.IL-6). For a control, no gene was inserted between EFS promoter and IRES sequence and GFP (TC-1 control). To confirm the transfection efficiency, cells were sorted based on GFP (TC-1 control) or GFP and IL-6 (TC-1.IL-6). Cells expressing >95% GFP⁺ or GFP⁺ and IL-6⁺ were used in the experiments. All the cell lines were cultured in Iscove's Modified Dulbecco's Media (IMDM) (BioWhittaker) supplemented with 8% fetal calf serum (FCS) (Greiner), 2 mM L-glutamine (Life Technologies), 50 IU/mL penicillin (Life Technologies) and 50 µg/mL streptomycin (Life Technologies). Cells were cultured in a humidified incubator at 37°C and 5% CO₂. Mycoplasma tests that were frequently performed for all cell lines by PCR were negative. All experiments were performed with mycoplasma-free cells.

2.3 | Tumor experiments and treatments

Mice were inoculated subcutaneously with 1×10^5 tumor cells in 200 µL phosphate-buffered saline (PBS) containing 0.2% BSA on day 0. Tumor size (horizontal dimension \times vertical dimension) was measured two times a week using a caliper. When a palpable tumor was present (day 8), mice were divided into groups with comparable tumor sizes. On day 8 post tumor challenge, mice were treated with an SLP (prime) vaccine or cisplatin. Boost vaccine was given on day 22 post tumor challenge. Mice were vaccinated with the SLP vaccine subcutaneously in the contralateral flank (suboptimal setting) or tail base (optimal setting). The SLP vaccine contains 100 µg HPV16 E7₄₃₋₆₃ (GQAEPDRAHYNIVTFCKCDS) covering both Th epitope and the cytotoxic T lymphocyte (CTL) epitope with 20 µg CpG (ODN1826, InvivoGen) dissolved in 200 µL (flank) or 50 µL (tail base) PBS and emulsified with incomplete Freund's adjuvant (IFA; flank). We have shown that under optimal conditions, therapeutic vaccination results in full tumor regression and cure of all mice with an established TC-1 tumor, whereas under suboptimal vaccine conditions TC-1 tumors do regress but after a period regrow.^{27,36} Maximum tolerated dose (MTD) of 10 mg/kg cisplatin was provided intraperitoneally in 300 µL in NaCl. Mice were routinely weighed 2 to 3 times per week. After cisplatin administration, mice were weighed 3 to 4 times per week until mice recovered. Exclusion criteria were ulceration of tumors and insusceptibility for cisplatin treatment as evidenced by complete lack of weight loss. Mice were euthanized when tumor size reached >2000 mm³ in volume or when mice lost >20% of their total body weight (relative to initial body mass).

2.4 | Flow cytometry

For analysis of (tumor-infiltrating) immune populations, tumors were disrupted in small pieces and incubated with 0.4 mg/mL Liberase TL

Research grade (Roche) in IMDM for 15 minutes at 37°C. Spleens were digested by incubating with 0.02 mg/mL DNase (deoxyribonuclease I from bovine pancreas, Sigma-Aldrich) and 1 mg/mL collagenase D (Roche) for 10 minutes at room temperature. Single-cell suspensions were prepared by mincing spleen and tumor pieces through a 70 µm cell strainer (BD Biosciences). Cells were resuspended in staining buffer (PBS + 2% FCS + 0.05% sodium azide) and incubated with various fluorescently labeled antibodies against: CD8a (clone 53-6.7), CD3 (clone 145-2C11), CD11b (clone M1/70), CD11c (clone N418), CD45.2 (clone 104), F4/80 (clone BM8), Ly6C (clone HK1.4), Ly6G (clone 1A8), class II (clone M5/114.15.2) and IL-6 (clone MP5-20F3). Antibodies were obtained from eBioscience and Biolegend. APC labeled-H-2D^b tetramers containing HPV16 E7₄₉₋₅₇ peptide (RAHYNIVTF) were used as E7 tetramer (E7 Tm). For dead cell exclusion, 7-aminoactinomycin D (7-AAD; Invitrogen), Zombie Aqua (Biolegend) and Zombie NIR (Biolegend) were used. To measure IL-6 production, tumor cells (10 000 cells/well) were plated in 96-well cell culture flat-bottom plates in the presence of brefeldin A (4 µg/mL). After 24 hours incubation, cells were fixed in 4% paraformaldehyde for 30 minutes. Samples were analyzed with a BD LSR II or LSRFortessa flow cytometer, and results were analyzed using the FlowJo software (Tree Star).

2.5 | Live-cell analysis

To measure the live cell proliferation and growth rate of the tumor cells, 5000 cells per well in F12-K medium were seeded into 96-well flat bottom plate. After overnight incubation, 50 µL Annexin V reagent (InCucyte Annexin V Red Reagent for Apoptosis, Essen Biosciences) was added to the plate and incubated in IncuCyte system (IncuCyte live-cell analysis system, Essen BioScience, Michigan) for 5 days. Data were analyzed using the IncuCyte software (Essen BioScience, Michigan).

2.6 | MTT assay

Tumor cells (7000 cells per well) were plated in a 96-well culture flat-bottom plate. After 24 hours, cells were treated with escalating dosages of cisplatin. Cells were extensively washed after overnight incubation and grown for an additional 24 hours in fresh medium. Cell viability assay was determined using a standard colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay. Absorbance was measured at a test wavelength of 570 nm, and the reference wavelength was 655 nm.

2.7 | IL-6 ELISA

Mouse IL-6 ELISA Ready-SET-Go! Kit (Invitrogen) was used to measure the amount of IL-6. Serum of the mice or supernatant of cultured cells were obtained and proceeded as described in the protocol of the kit.

2.8 | CTL assay

Naïve C57BL/6 mice were vaccinated with SLP vaccine as described earlier on day 0 and day 14. Splenocytes were isolated 8 days after the last vaccination and restimulated *in vitro* with DCs loaded with SLP. After 6 days restimulation, splenocytes were harvested by using EDTA. Next, live cells were enriched by high-density solution of Ficoll and centrifugation and used as effector cells in CTL assay. TC-1 control and TC-1 IL-6 tumor cells were exposed to IFN γ (10 IU, Prospec) 1 day before CTL assay and were used as target cells. Target cells were labeled with 100 μ L 51 Cr for 1 hour, washed and plated into a 96-well round-bottom plate at a density of 2000 tumor cells/well with different ratios of effector cells. After 4 hours incubation, supernatant of the cells was harvested and the percentage of 51 Cr release was measured by a gamma counter.

2.9 | Statistical analysis

Survival for differentially treated mice was compared using the Kaplan-Meier method and the log-rank (Mantel-Cox) test. Additional statistical methods are stated in the legends. All *P* values <.05 were considered significant.

3 | RESULTS

3.1 | IL-6 produced by tumor cells does not affect tumor intrinsic growth and apoptosis

To investigate the impact of tumor-expressed IL-6 on immunotherapy and chemotherapy, we used TC-1 tumor cells, expressing the HPV-16 oncoproteins E6 and E7, and engineered these cells to express only GFP (TC-1 control) or GFP and IL-6 (TC-1.IL-6) (Figure 1A,B). IL-6 was secreted in the supernatant of TC-1.IL-6 cell cultures and at the higher picogram/ml range level in the serum of mice injected with TC-1.IL-6 cells when compared to TC-1 control cells (Figure 1C-E). Considering the growth of stimulatory effect of IL-6,^{8,9} we measured the growth rate of TC-1.IL-6 cell line and compared it to TC-1 control by cell count. Both cell lines displayed a similar growth and apoptosis rate *in vitro* (Figure 1F-G). To confirm this in a preclinical setting, we injected these tumor cells into naïve mice and followed the tumor outgrowth over time. TC-1 control and TC-1.IL-6 tumor-bearing mice displayed comparable tumor outgrowth (Figure 1H). Thus, tumor-produced IL-6 does not induce intrinsic differences in the growth rate or apoptosis of tumor cells.

3.2 | IL-6 produced by tumor cells hampers the therapeutic effect induced by immunotherapy and chemotherapy

To assess the impact of tumor-produced IL-6 on the efficacy of chemotherapy and immunotherapy, mice were challenged with either

TC-1 control or TC-1.IL-6 tumor cells. When tumors were palpable on day 8, mice were treated either with a therapeutic peptide vaccine in the tail base (optimal setting) or with the MTD of cisplatin, reflecting settings delivering 100% cure of mice bearing TC-1 control tumors.^{26,36} Following peptide vaccination, all the tumors regressed albeit that half of the tumors recur in mice with IL-6 producing tumors. Similarly, cisplatin treatment induced full tumor regression in mice, but TC-1.IL-6 tumor recurrence was observed in 5 of 18 treated mice (Figure 2A,B). The efficacy of these individual treatments was high with 100% survival for mice challenged with TC-1 control, whereas a strong and significant reduction to 50% survival after vaccination and 75% survival after chemotherapy was observed in mice challenged with TC-1.IL-6 (Figure 2C,D). This demonstrates that tumor expressed IL-6 hampers the therapeutic effect of chemotherapy and shows that the effect on immunotherapy is even more evident. To exclude that this difference in clinical reactivity was due to an intrinsic difference in sensitivity to cytotoxic mechanisms, *in vitro* cultured TC-1 control and TC-1.IL-6 tumor cells were tested in cytotoxicity assays with effector T cells from naïve or peptide vaccinated mice. T-cell-mediated killing was similar to both cell lines (Figure 2E). Likewise, the viability of TC-1 control and TC-1.IL-6 tumor cells in the presence of escalating doses of cisplatin was identical, as indicated by the similar dose of cisplatin (3.36 μ g/mL) to kill 50% of the cells (IC50 [half-maximal inhibitory concentration]; dashed line; Figure 2F). Taken together, these data showed that tumor-expressed IL-6 hampers the antitumor effect of therapeutic vaccination and cisplatin chemotherapy and this was not caused by intrinsic differences in tumor cell growth, apoptosis or sensitivity to (immunomediated) cytotoxic mechanisms.

3.3 | Tumor-produced IL-6 alters the composition and phenotype of local and systemic immune cells

Our previous work showed that tumor-expressed IL-6 skews the phenotype of myeloid cells toward immunosuppressive cells *in vitro*.^{10,12} To test the hypothesis that the observed resistance of IL-6-producing tumors to immunotherapy and chemotherapy was based on an altered immune microenvironment, we examined the impact on the systemic (spleen) and local (tumor) composition and phenotype of immune cells. Comparison of the T-cell infiltrate in TC-1 control and TC-1.IL-6 tumors (Supplementary Figure 1A,B) showed no significant differences in the percentages of tumor-infiltrating CD45⁺ immune cells, CD8⁺ cells, CD4⁺ cells and CD4⁺Tregs (Figure 3A,B). In addition, no alteration was found in the percentage of CD8⁺CD127⁻KLRG-1⁺ and CD8⁺CD127⁺KLRG-1⁺ effector cells, although the population of CD4⁺ CD127⁻KLRG-1⁺ was decreased in TC-1.IL-6 tumors (Figure 3C and Supplementary Figure 1C). To determine the systemic effect of tumor-produced IL-6 on T cells, we measured the percentage of different types of immune cells in the spleen. The percentages of total CD8⁺ and CD4⁺ were decreased in the spleen (Figure 3D). However, the percentage of KLRG-1⁺ cells within CD4⁺ and CD8⁺ T cells increased, suggesting a higher percentage of systemic effector-

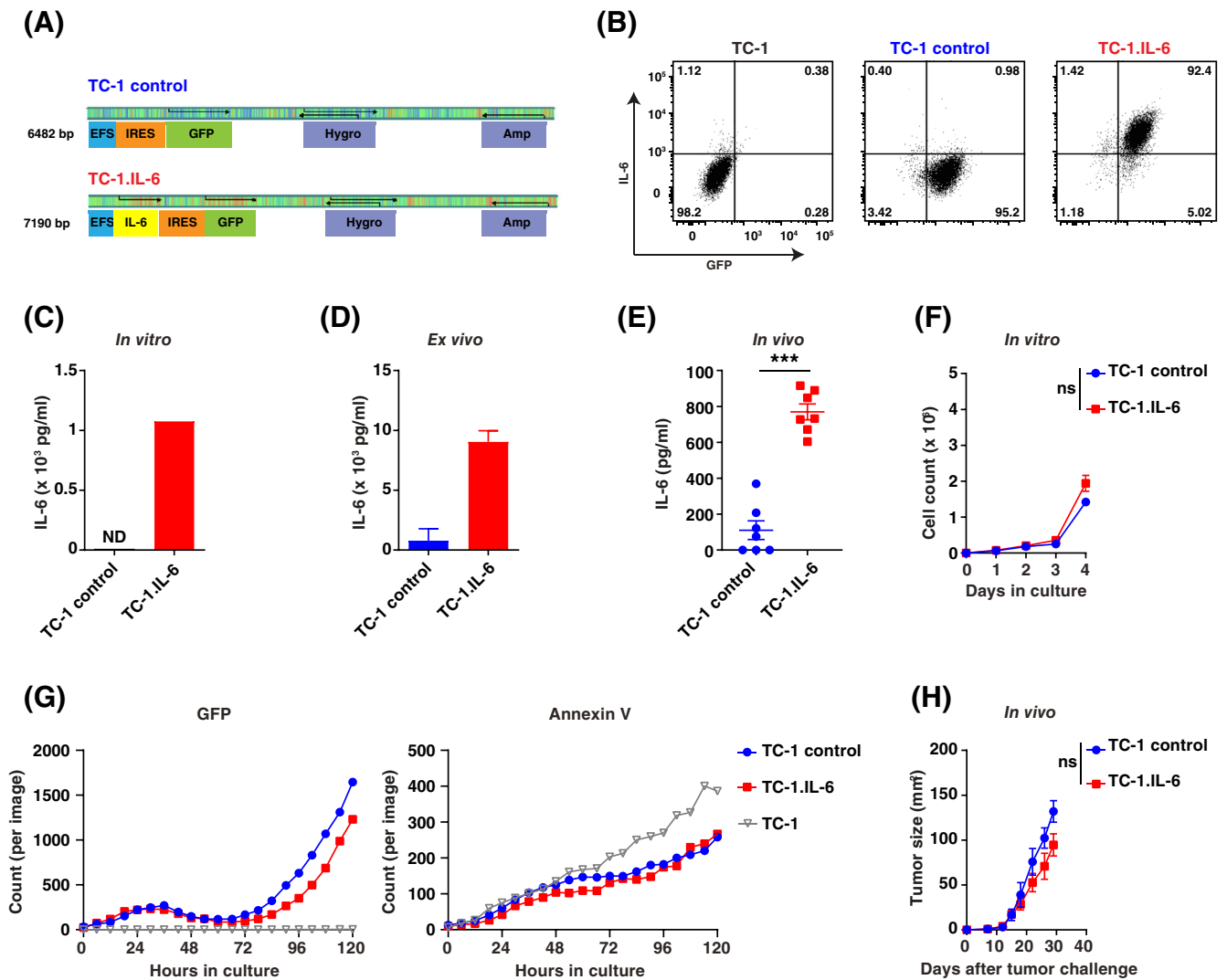


FIGURE 1 TC-1 control and TC-1 IL-6 constructs, IL-6 production and growth rate. A, Schematic figure of pcDNA3.1 vector that was used for TC-1 transfection as described in Section 2. These vectors express only GFP (TC-1 control) or GFP and IL-6 under EFS promoter (TC-1.IL-6). B, Representative flow cytometry plots of GFP and IL-6 production in TC-1, TC-1 control and TC-1.IL-6 tumor cells as measured by intracellular staining. C-E, IL-6 measurement in the supernatant of TC-1 control and TC-1.IL-6 cultured *in vitro* cell lines (representative of three independent experiments). C, Supernatant of TC-1 control and TC-1.IL-6 tumor microenvironment isolated from TC-1 control and TC-1.IL-6 tumor-bearing mice (4 mice per group) (D) and serum of the TC-1 control and TC-1.IL-6 tumor-bearing mice (7 mice per group) at day 17 post tumor inoculation measured by ELISA (E). F, Cell count of cultured TC-1 control and TC-1.IL-6 tumor cells at the initial time point and 1, 2, 3 and 4 days after culturing (performed in duplicates). G, Live cell analysis of cultured TC-1 control and TC-1.IL-6 by counting GFP⁺ and annexin V⁺ cells measured by IncuCytes as described in Section 2. H, Tumor outgrowth of TC-1 control and TC-1.IL-6 tumor bearing mice (5-6 mice per group). Graphs indicate mean values with standard error of mean. Experiments expected in G were performed three times with similar outcomes. Significance was determined by Mann-Whitney test. *P < .05; **P < .01; ***P < .001. EFS, short elongation factor; GFP, green fluorescent protein; IL-6, interleukin 6 [Color figure can be viewed at wileyonlinelibrary.com]

memory T cells in mice with IL-6 producing tumors (Figure 3E and Supplementary Figure 1D).

3.4 | Tumor-produced IL-6 induces a less mature phenotype in myeloid cells

Next, we analyzed the intratumoral and splenic myeloid compartments. The percentage of total CD11b⁺ myeloid cells was slightly

increased in TC-1.IL-6 tumors (Figure 3B). The myeloid cells were subdivided into three subsets based on CD11b and CD11c expression; CD11b⁺ CD11c⁻, CD11b⁺ CD11c⁺ and CD11b⁻ CD11c⁺ myeloid cells (Figure 4A), shown in the gating strategy provided in Supplementary Figure 2A. Although the population of CD11b⁺CD11c⁺ cells was increased, the CD11b⁻CD11c⁺ cells (DCs) declined in TC-1.IL-6 tumors (Figure 4A). Subsequently, we examined the antigen-presenting capacity of the macrophages and maturation level of DCs using MHC class II expression as a read out.³⁷ Strikingly, the overall

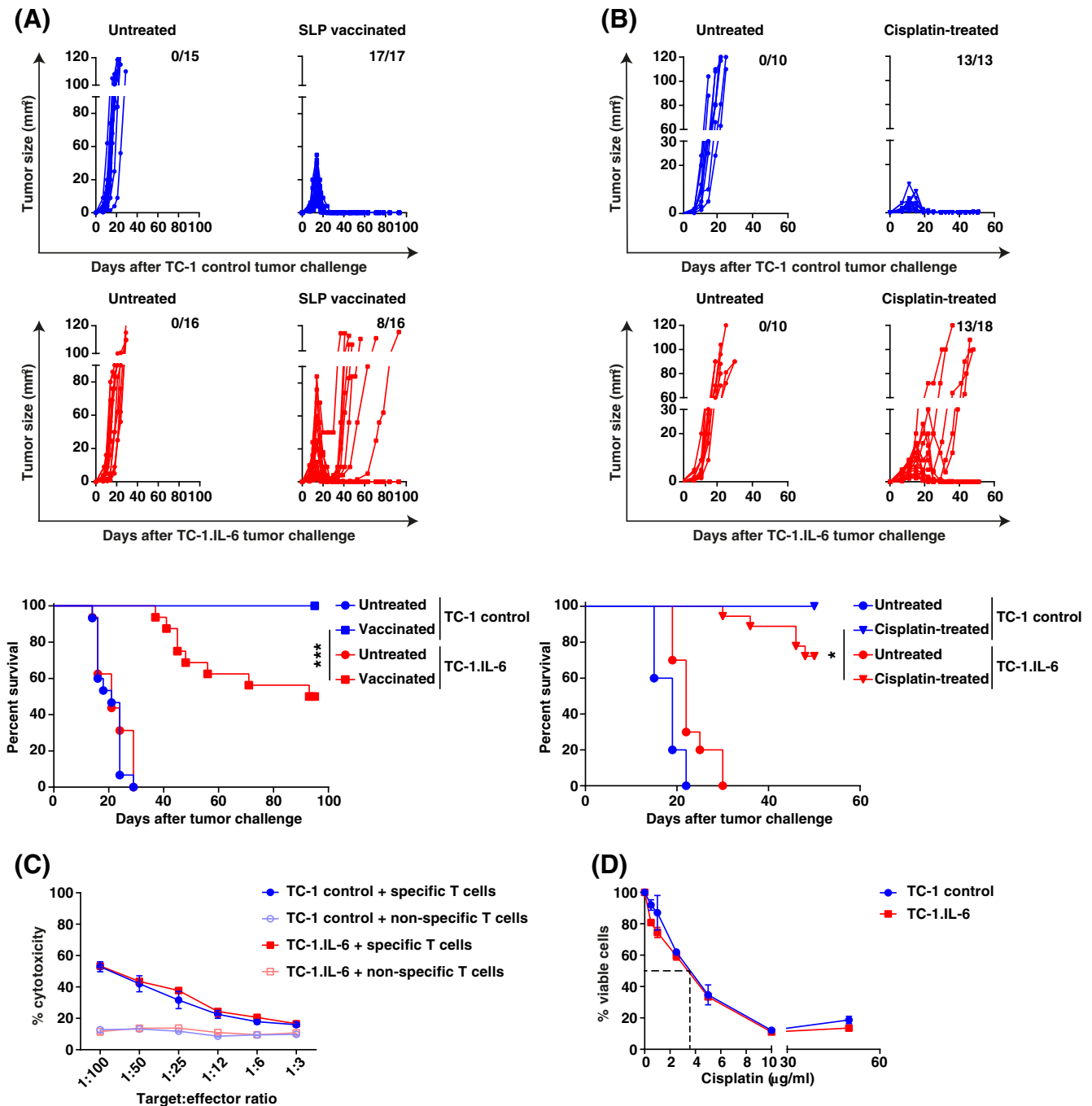


FIGURE 2 IL-6 produced by tumor cells induces resistance to immunotherapy and chemotherapy. A,B, Tumor outgrowth and survival graph of the TC-1 control and TC-1.IL-6 tumor bearing mice that received SLP vaccine in the optimal setting (A) or maximal tolerated dose of cisplatin (B) as described in Section 2. The number shown above the tumor outgrowth is the number of mice alive from the total number of mice. Data are pooled from two independent experiments (5–9 mice per group), yielding similar results. Significance was determined by a log-rank (Mantel–Cox) test (* $P < .05$; ** $P < .01$; *** $P < .001$). C, The percentage of TC-1 control and TC-1.IL-6 tumor cells (target cells) killed by splenocytes isolated from SLP vaccinated or naïve mice as control (effector cells) at different ratios as measured by CTL assay as described in Section 2. Experiment was performed once. D, The percentage of viable TC-1 control and IL-6 tumor cells in the presence of the different concentrations of cisplatin measured by MTT assay. Dashed line shows the IC₅₀ concentration for the tumor cells. Experiments were performed twice with similar outcomes. CTL, cytotoxic T lymphocyte; IL-6, interleukin 6; IC₅₀, half-maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; SLP, synthetic long peptide [Color figure can be viewed at wileyonlinelibrary.com]

expression of MHC class II on intratumoral CD11b⁺ myeloid cells was strongly reduced (Figure 4B). Detailed analysis of the three defined subsets of myeloid cells demonstrated significantly lower expression

of MHC class II (Figure 4C). Moreover, although the population of CD11b⁺CD11c⁺ cells was increased, more than half of this population also did not display MHC class II (Figure 4C). The CD11b⁺CD11c⁺ cell

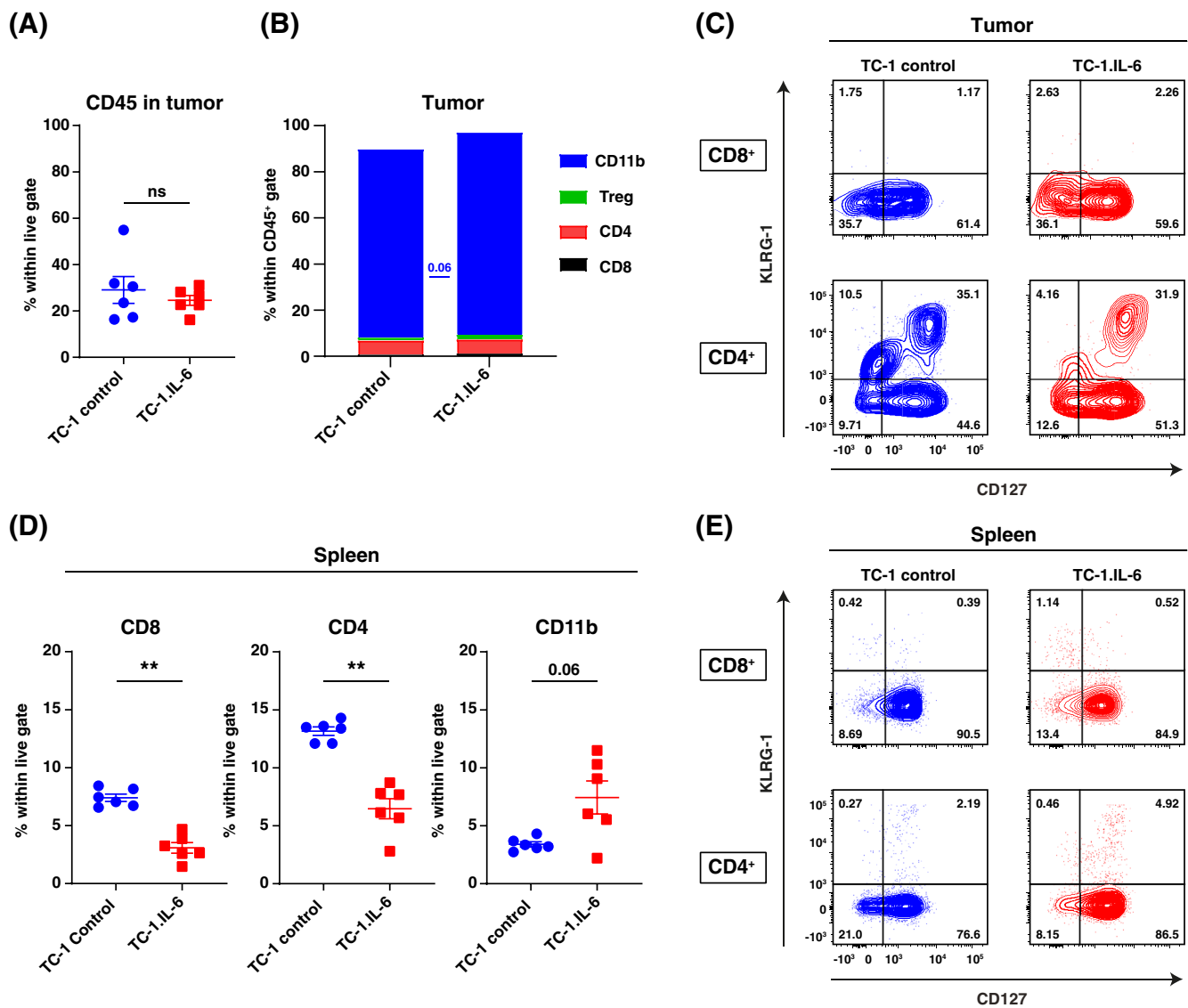


FIGURE 3 IL-6 produced by tumor cells alters the composition of intratumoral and systemic immune cells. A-E, Mice were injected with TC-1 control and TC-1.IL-6 cells on day 0. Tumors and spleens were analyzed on day 16 post tumor challenge. A, The percentage of intratumoral CD45⁺ leukocytes within live cells. B, The percentage of intratumoral total CD8⁺, total CD4⁺, and Tregs and CD11b⁺ cells within CD45⁺ cells. C, The representative flow cytometry plots of intratumoral CD8⁺ and CD4⁺ cells based on CD127 and KLRG-1 in TC-1 control and TC-1.IL-6 tumor-bearing mice analyzed 16 days post tumor challenge. D, The percentage of total CD8⁺, total CD4⁺ and CD11b⁺ cells within live cells in spleen. E, The representative flow cytometry plots of CD8⁺ and CD4⁺ cells based on CD127 and KLRG-1 in spleens of TC-1 control and TC-1.IL-6 tumor-bearing mice analyzed 16 days post tumor challenge. Each dot represents data from an individual mouse (6 mice per group). Graphs indicate mean values with standard error of mean. Significance was determined by Mann-Whitney test. * $P < .05$; ** $P < .01$; *** $P < .001$. Experiments were performed twice with similar outcomes. IL-6, interleukin 6; Tregs, regulatory T cells

population was further dissected on the basis of F4/80 and MHC class II expression, showing a clear decrease in the percentage of class II⁺F4/80⁺ cells (macrophages) in IL-6 producing tumors while the percentage of MHC class II negative F4/80⁺ macrophages was increased (Supplementary Figure 2B). Further analyses of CD11b⁺CD11c⁻ subset, based on the granulocytic marker Ly6G, demonstrated no significant difference in the percentage of Ly6G⁺ neutrophils in TC-1.IL-6 tumors compared to controls (Supplementary Figure 2C).

Next, we measured the systemic effect of IL-6 produced by tumor cells on splenic myeloid cells. The percentage of total CD11b⁺ myeloid cells slightly increased in the spleen (Figure 3D). However, the percentage of MHC class II⁺ CD11b⁺ cells was lower and the MHC class II expression level on the remaining MHC class II⁺ cells was decreased (Figure 4D). In addition, detailed analysis of myeloid cells based on CD11b and CD11c markers displayed significant decrease in the percentage of CD11b⁻CD11c⁺ cells while the percentage of CD11b⁺CD11c⁺ cells remained unchanged (Figure 4E). Interestingly,

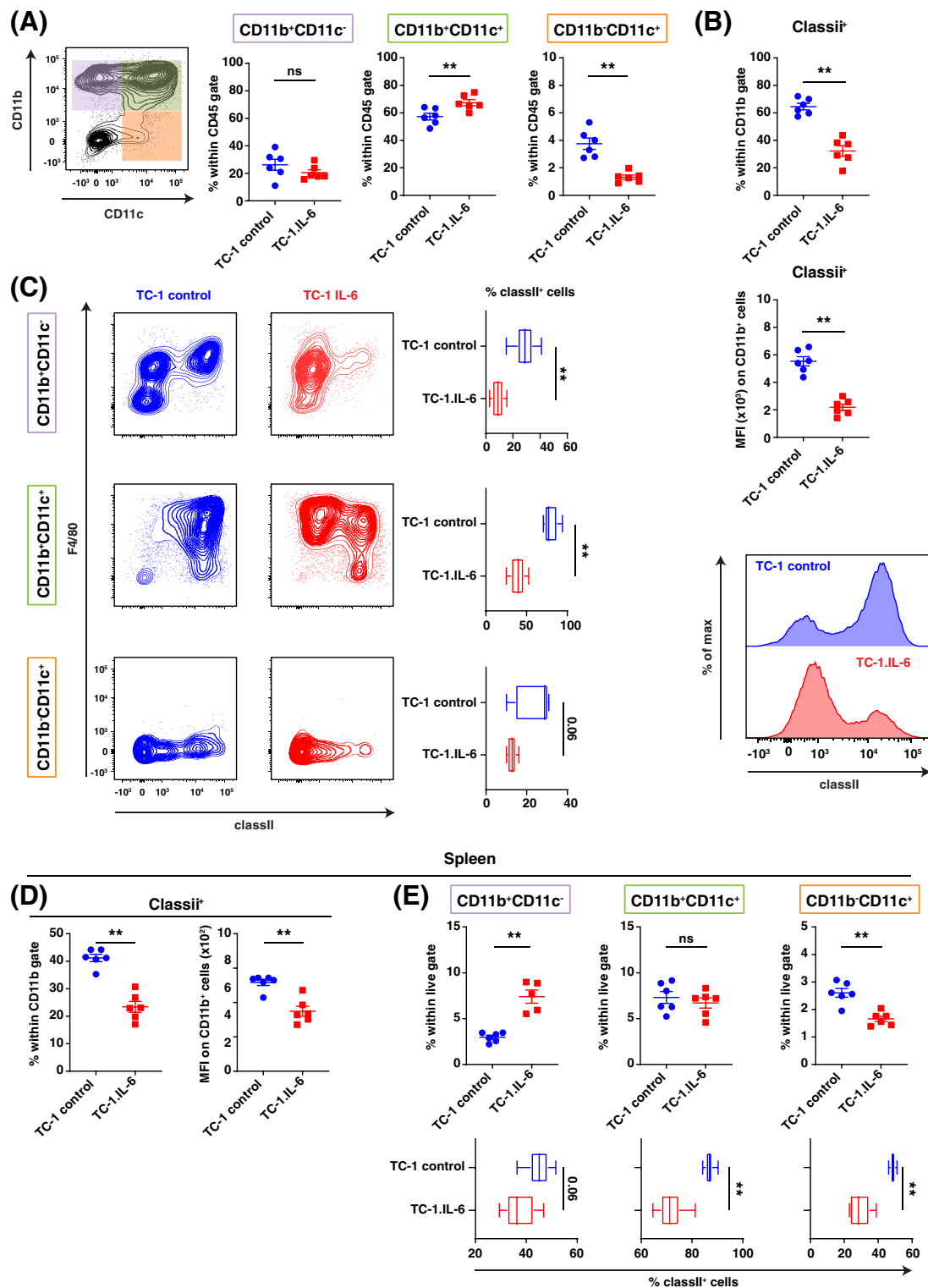


FIGURE 4 IL-6 produced by tumor cells induces MHC class II^{negative/low} myeloid cells in the tumor and spleen. A, The percentage of intratumoral CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ myeloid cells within CD45⁺ cells. A representative flow cytometry plot of myeloid cells based on CD11b and CD11c (gated on 7AAD⁻CD3⁻CD19⁻ cells) is displayed in the left. B, The percentage (top) and expression in MFI (middle) of MHC class II within intratumoral CD11b⁺ cells. A representative flow cytometry plot of MHC class II on intratumoral myeloid cells in TC-1 control and TC-1.IL-6 tumor-bearing mice (bottom). C, The percentage of MHC class II⁺ within CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ intratumoral myeloid cells. Representative flow cytometry plots of these subsets based on MHC class II and F4/80 are displayed at the left. D, The percentage and expression in MFI of MHC class II within splenic CD11b⁺ cells. E, The percentage of splenic CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ myeloid cells within the live gate (top) and the percentage of MHC class II⁺ cells within CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ splenic myeloid cells (bottom). Each dot represents data from an individual mouse (5-6 mice per group). Dot plot and box plot graphs indicate mean values with standard error of mean and min to max, respectively. Significance was determined by Mann-Whitney test. **P* < .05; ***P* < .01; ****P* < .001. Experiments were performed twice with similar outcomes. IL-6, interleukin 6; MHC, major histocompatibility complex [Color figure can be viewed at wileyonlinelibrary.com]

although the populations of CD11b⁺CD11c⁻ and Ly6G⁺CD11b⁺ cells were increased in the spleen, all the three defined subsets based on CD11b and CD11c expression comprised a lower percentage of MHC class II⁺ cells (Figure 4E and Supplementary Figure 4D). Taken together, these data show that tumor cell-produced IL-6 results in the systemic and local accumulation of myeloid cells with impaired antigen-presenting capacity (macrophages) and of an immature phenotype (DCs). The fact that the systemic myeloid cell population is a phenocopy of those found in the tumor was also observed by us in other studies.²⁸

3.5 | IL-6 produced by tumor cells hampers tumor-specific T cell reactivity during immunotherapy

To dissect the mechanism underlying a less effective response of TC-1.IL-6 to therapeutic vaccination, we measured the effect of optimal peptide vaccination on the systemic and intratumoral immune composition. Previously, we demonstrated that peptide vaccination significantly increases the percentage of intratumoral CD8⁺ and CD11b⁺ in TC-1 tumors.²⁵ IL-6 production only slightly hampered this vaccine-induced influx of immune (CD45⁺) cells (Figure 5A), CD8⁺ cells (Figure 5B) and tumor-specific (E7 Tm⁺) CD8⁺ T cells (Figure 5C), whereas the influx of CD11b⁺ cells, CD4⁺ T cells or Tregs was not affected at all (Figure 5B). Upon peptide vaccination, the percentage of CD8⁺CD127⁺KLRG-1⁺ was significantly increased but IL-6 had no impact on the intratumoral percentages of these cells (Figure 5D and Supplementary Figure 3A). Next, we investigated the systemic effect of IL-6 during therapeutic vaccination. Similar to the untreated condition (Figure 3D), the IL-6-mediated reductions in CD4⁺, CD8⁺ and regulatory T cells as well as slightly increased CD11b⁺ myeloid cells in the spleen were retained after vaccination (Figure 5E). No difference in the phenotype of T cells, based on CD127 and KLRG-1, was found in the spleen of vaccinated TC-1.IL-6 tumor-bearing mice compared to the TC-1 control tumor-bearing mice (Figure 5F and Supplementary Figure 3B). In addition, the percentage of tumor-specific CD8⁺ T cells increased by peptide vaccination and did not differ between TC-1 control and TC-1.IL-6 tumor-bearing mice (Supplementary Figure 3C). These data suggest that tumor-produced IL-6 did not grossly affect the tumor-specific CD8⁺T cell response.

3.6 | Immunotherapy fails to enhance MHC class II expression on myeloid cells in IL-6-producing tumors

Previously, we showed that therapeutic vaccination increases the expression of MHC class II on intratumoral myeloid cells.²⁵ Therefore, we investigated the capacity of therapeutic vaccination to restore MHC class II expression in the TC-1.IL-6 tumor cell setting. Optimal vaccination did not lower the fraction of CD11b⁺CD11c⁻ cells which was increased in TC-1.IL-6, whereas it increased the percentages of CD11b⁺CD11c⁺ myeloid cells and CD11b⁻CD11c⁺ cells, but to a similar level as in TC-1 control tumors (Figure 6A). Changes in the

percentage of CD11b⁺CD11c⁻ was not due the changes in the percentage of Ly6G⁺CD11b⁺CD11c⁻ neutrophils as the percentage of these cells were not affected by vaccination or IL-6 produced by tumor (Supplementary Figure 4A). The percentage of MHC class II⁺ myeloid cells increased in TC-1.IL-6 tumors after vaccination, but with much greater variability between TC-1.IL-6 tumor-bearing mice. Furthermore, the overall percentage of CD11b⁺ MHC class II⁺ myeloid cells remained significantly lower in TC-1.IL-6 tumors (Figure 6B). Moreover, the expression level of MHC class II on the remaining MHC class II-positive myeloid cells was also significantly lower in TC-1.IL-6 compared to TC-1 control tumors (Figure 6B). Detailed analysis of three defined subsets demonstrated no significant differences in the percentage of MHC class II⁺ cells within CD11b⁺CD11c⁻ and CD11b⁺CD11c⁺ cells after vaccination. However, the percentage of MHC class II⁺ cells within CD11b⁻CD11c⁺ cells as well as the expression of MHC class II on these cells was much lower in TC-1.IL-6 after vaccination (Figure 6C and Supplementary Figure 4B). In addition, the three defined population of MHC class II and/or F4/80-positive CD11b⁺CD11c⁺ cells did not differ between SLP-vaccinated TC-1 IL-6 tumor-bearing mice and TC-1 control mice (Supplementary Figure 4C).

The production of IFN γ by the tumor infiltrating T cells may result in the upregulation of PD-L1 on CD11b⁺ myeloid cells. Indeed, the expression of PD-L1 was upregulated in vaccinated mice bearing TC-1 control tumors. However, in SLP-vaccinated TC-1.IL-6 tumor-bearing mice the CD11b⁺ myeloid cells hardly upregulated PD-L1 expression (Supplementary Figure 4D), indicating that tumor-produced IL-6 may also hamper adaptive PD-L1 expression.

The systemic effects were less pronounced but a phenocopy of what was seen in the tumor but with a slight increased percentage of CD11b⁺CD11c⁻ cells (Supplementary Figure 5A), a lower percentage of MHC class II⁺ CD11b⁺ cells and a decreased expression level of MHC class II on CD11b⁺ myeloid cells (Supplementary Figure 5B) and in particular in the CD11b⁻CD11c⁺ DC subset (Supplementary Figure 5C). Cumulatively, our experiments suggested that the IL-6-mediated change in the maturation of systemic and intratumoral DC subsets and the antigen-presenting capacity of macrophages after therapeutic vaccination results in incomplete eradication of tumor cells, reflected by the rapid recurrence of TC-1.IL-6 tumors.

3.7 | Increasing the strength of the initial tumoricidal hit decreases tumor escape

We previously showed that cisplatin-mediated control of TC-1 tumors depended on the influx of tumors with inflammatory phagocytic Ly6C^{hi} myeloid cells expressing higher levels of MHC class II and costimulatory molecules.²⁶ Therefore, we determined how IL-6 affected the cisplatin-induced myeloid cell infiltration. The tumor-produced IL-6 did not alter cisplatin-induced total CD45⁺ immune cell infiltration (Figure 7A) and percentage of total CD11b⁺ cells was even higher in TC-1.IL-6 tumors after treatment with cisplatin (Figure 7B). Almost all cells retained the expression of MHC class II (Figure 7C,

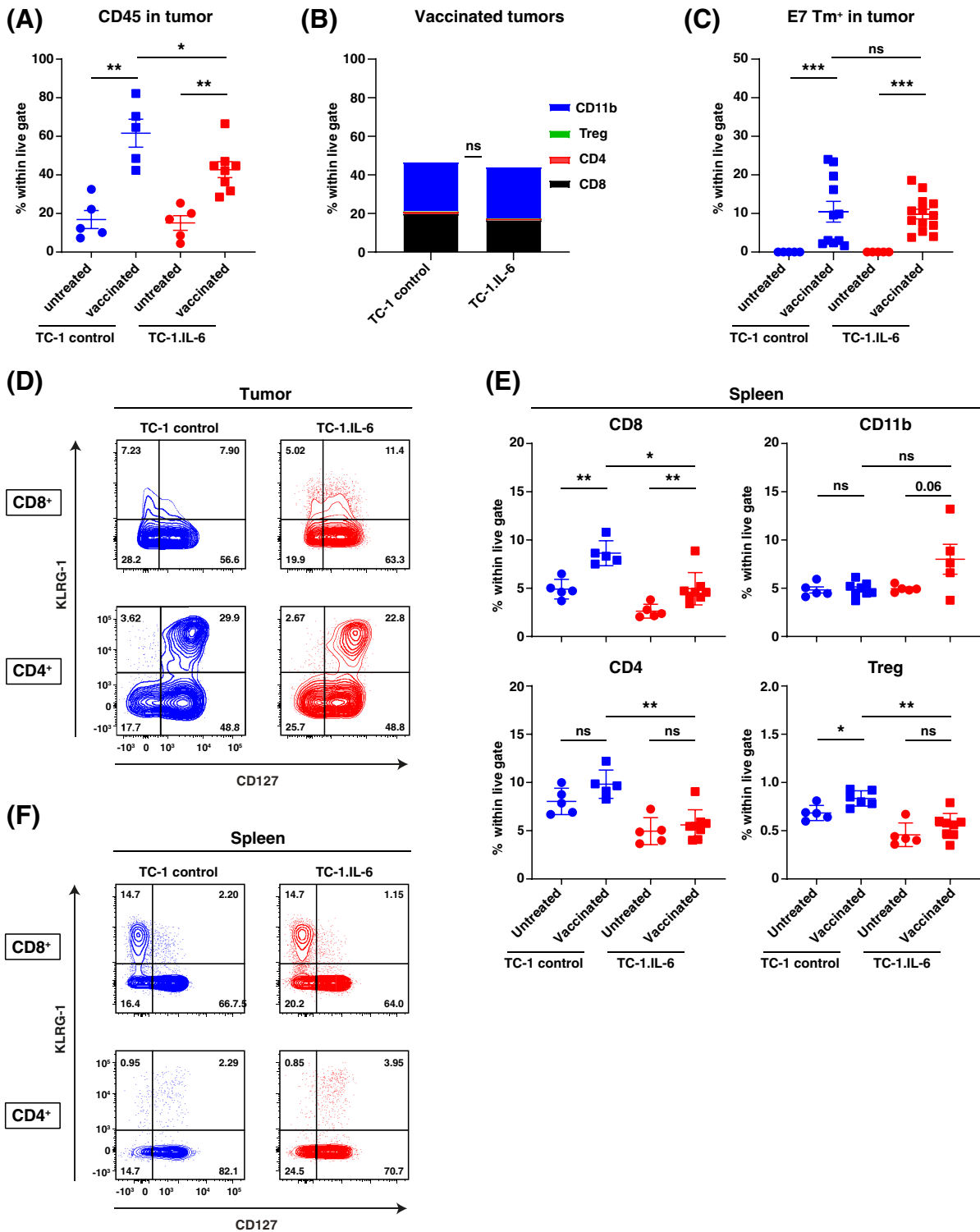
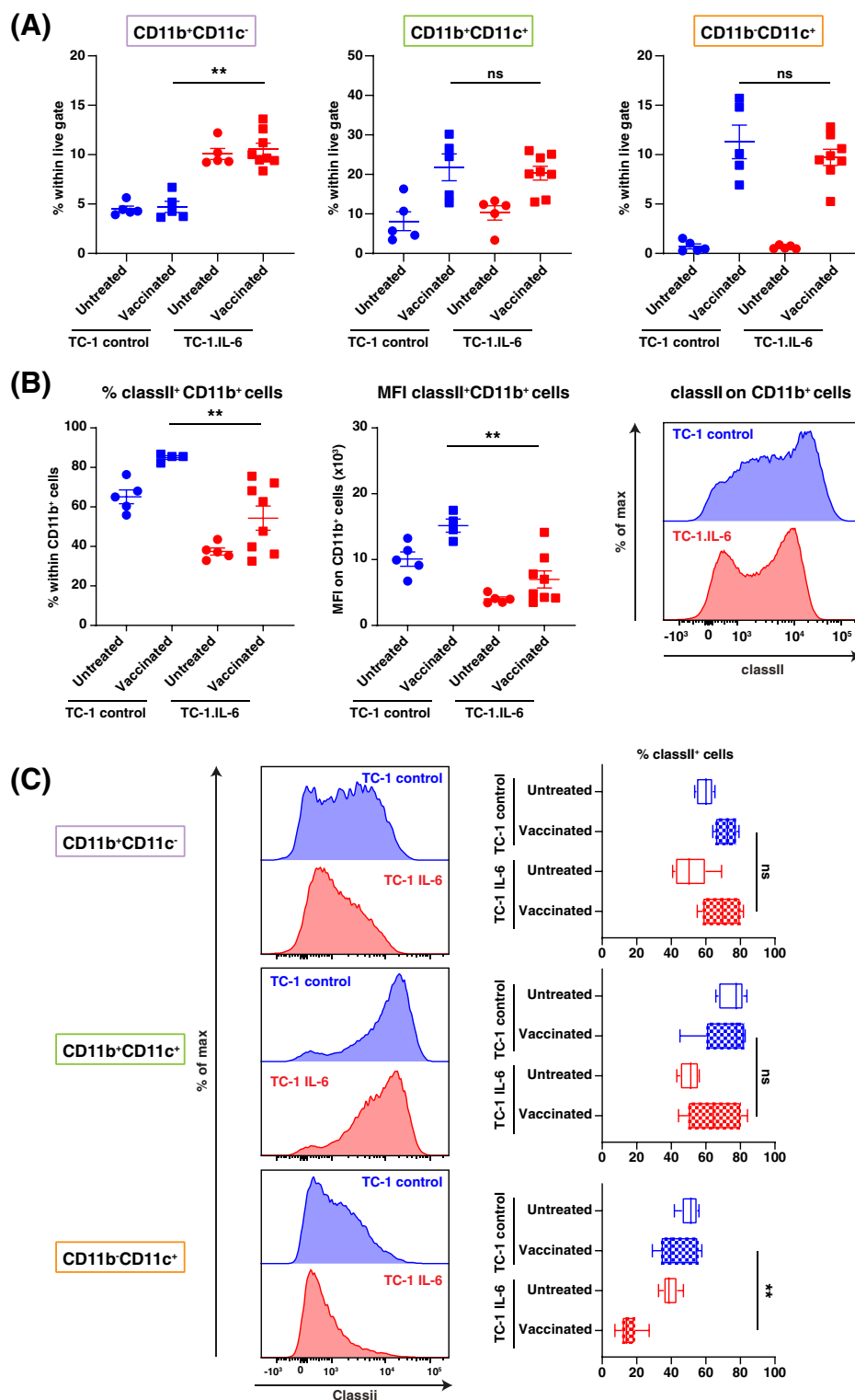


FIGURE 5 IL-6 produced by tumor decreases the T-cell response induced by peptide vaccination. A-F, Mice were injected with TC-1 control and TC-1.IL-6 cells on day 0. On day 8 post tumor challenge, mice were vaccinated with SLP vaccine according to the optimal setting or kept untreated. Tumors and spleens were analyzed on day 16 post tumor challenge. A, The percentage of intratumoral CD45⁺ leukocytes within live cells. B, The percentage of intratumoral total CD8⁺, total CD4⁺, Tregs and CD11b⁺ cells within live cells. C, The percentage of intratumoral tumor-specific (E7 Tm⁺) CD8⁺ T cells within live cells (5-11 mice per group). The data are pooled from two experiments. D, Representative flow cytometry plots of intratumoral CD8⁺ and CD4⁺ cells based on CD127 and KLRG-1 in SLP-vaccinated TC-1 control and TC-1.IL-6 tumor-bearing mice. E, The percentage of total CD8⁺, CD11b⁺, total CD4⁺ and Tregs within live cells in the spleen of untreated or SLP-vaccinated TC-1 control and TC-1.IL-6 tumor-bearing mice. F, Representative flow cytometry plots of splenic CD8⁺ and CD4⁺ cells based on CD127 and KLRG-1 in SLP-vaccinated TC-1 control and TC-1.IL-6 tumor-bearing mice. Each dot represents data from an individual mouse (5-8 mice per group). Graphs indicate mean values with standard error of mean. Significance was determined by Mann-Whitney test. **P* < .05; ***P* < .01; ****P* < .001. Experiments were performed twice with similar outcomes. IL-6, interleukin 6; MHC, major histocompatibility complex; SLP, synthetic long peptide; Tregs, regulatory T cells

FIGURE 6 Intratumoral myeloid cells display less MHC class II in IL-6 producing tumors treated with peptide vaccination. A-C, Mice were injected with TC-1 control and TC-1.IL-6 cells on day 0. On day 8 post tumor challenge, mice were vaccinated with SLP vaccine according to the optimal setting or kept untreated. Tumors were analyzed on day 16 post tumor challenge. A, The percentage of intratumoral CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁺ myeloid cells within the live gate. B, The percentage of MHC class II⁺ cells and expression in MFI of MHC class II of intratumoral CD11b⁺ cells. A representative flow cytometry plot of MHC class II expression by intratumoral myeloid cells in peptide-vaccinated TC-1 control and TC-1.IL-6 tumor-bearing mice is shown at the right. C, The percentage of MHC class II⁺ within CD11b⁺CD11c⁻, CD11b⁺CD11c⁺ and CD11b⁻CD11c⁻ intratumoral myeloid cells in peptide-vaccinated TC-1 control and TC-1.IL-6 tumor-bearing mice. Representative flow cytometry plots of these subsets based on MHC class II are displayed at the left. Each dot represents data from an individual mouse (5-8 mice per group). Dot plot and box plot graphs indicate mean values with standard error of mean and minimum to maximum, respectively. Significance was determined by Mann-Whitney test. **P* < .05; ***P* < .01; ****P* < .001. Experiments were performed twice with similar outcomes. IL-6, interleukin 6; MHC, major histocompatibility complex; SLP, synthetic long peptide [Color figure can be viewed at wileyonlinelibrary.com]



upper panel), whereas the expression levels of MHC class II on TC1.IL-6 infiltrating CD11b⁺ cells were in fact much lower than in TC1 control tumors.(Figure 7C, lower panel), suggesting that cisplatin just tapped into the systemic repertoire of immature and mature leukocytes for recruitment into IL-6 producing tumors. Subsequently, we focused on the cisplatin-mediated influx of inflammatory Ly6C^{hi} cells. Again, the percentage of these cells did increase substantially in both

TC-1.IL-6 and TC-1 control after treatment with cisplatin, but their levels remained significantly lower in TC-1.IL-6 tumors and they displayed a lower level of MHC class II expression (Figure 7D). Analysis of the different subsets of CD11b⁺ and/or CD11c⁺ myeloid cells (Supplementary Figure 6A) revealed that the CD11b⁺CD11c⁺ cell population, which was the most abundant population (about 40% of the total live cells) in cisplatin-treated tumors, remained unchanged in

TC-1 control and TC-1.IL-6 tumors after cisplatin treatment (Supplementary Figure 6B). This cell population retained the expression of MHC class II, albeit at significantly lower levels in TC-1.IL-6

tumors than in TC-1 control tumors (Supplementary Figure 6B), consistent with the decrease in MHC class II observed for the total CD11b⁺ cell population (Figure 7C). No overt change in differences

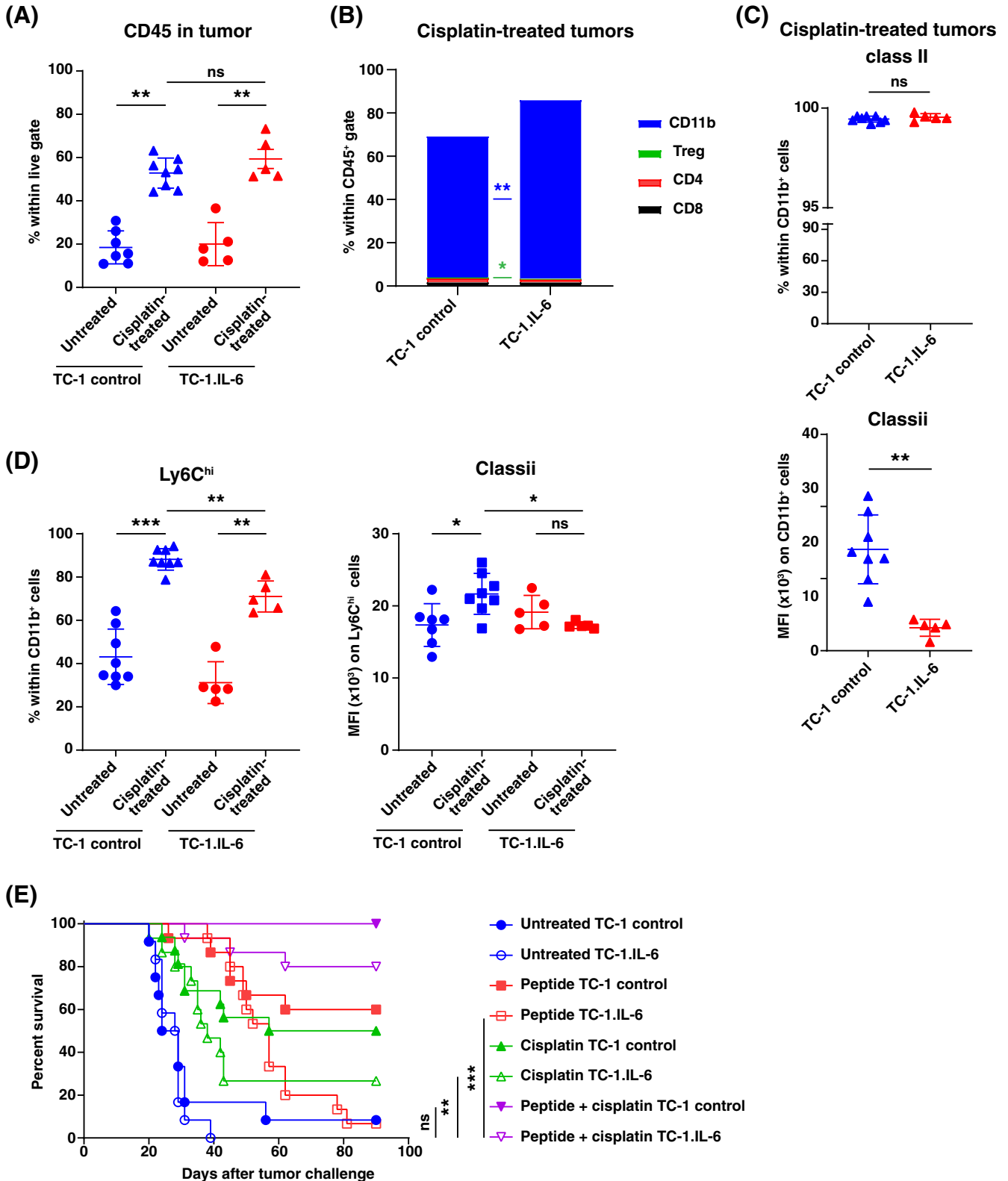


FIGURE 7 Legend on next page.

were found when this subset of CD11b⁺CD11c⁺ myeloid cells was divided on the basis of F4/80 expression (Supplementary Figure 7A). Furthermore, the percentage of CD11b⁻CD11c⁺ DCs was significantly lower in cisplatin-treated TC-1.IL-6 tumors, but no change in the expression of MHC class II was found (Supplementary Figure 6B). Interestingly, the percentage of MHC class II expressing CD11b⁺CD11c⁻ cells was higher in cisplatin-treated TC-1.IL-6 tumor-bearing mice compared to TC-1 control tumors (Supplementary Figure 6B). Furthermore, while the percentage of Ly6G⁺ and classII⁺F4/80⁺ CD11b⁺CD11c⁻ cells remained unchanged, the percentage of classII⁻F4/80⁻ cells decreased significantly (Supplementary Figure 7B-C). Based on our earlier data showing the synergistic effects of T-cell produced TNF α and cisplatin on tumor cell death,²⁷ we hypothesized that the use of cisplatin in combination with vaccination would result in enhanced tumor cell control of IL-6 producing tumors. In order to show the synergy of the combination in TC-1 control tumors, the mice were suboptimal vaccinated with SLP as this would lead to recurrences.^{27,36} Indeed, now half of the mice challenged with TC-1 control tumors succumbed to disease when treated with either vaccination or chemotherapy as monotherapy. The combination, however, led to the survival of all mice (Figure 7E). Interestingly, almost all mice with TC-1.IL-6 tumors displayed resistance to each of the two therapies as single treatment; however, their combined use proved to be a much more effective treatment (Figure 7E). Of note, while the impact of cisplatin treatment on TC-1 control and TC-1.IL-6 tumor outgrowth was somewhat lower than observed before this was not unexpected based on our previous studies.²⁶ Importantly, the difference in survival between the two different cisplatin-treated tumors was retained in each experiment. Thus, the combination of different types of cytotoxic mechanisms as provided by T cells and cisplatin to kill TC-1.IL-6 tumor cells is likely to substitute for the tumor cell killing mechanisms employed by inflammatory myeloid cells,^{25,38} which are impaired in the TC-1.IL-6 mouse model.

4 | DISCUSSION

In our study, we have shown that IL-6 produced by tumor cells induces resistance to immunotherapy and chemotherapy in an HPV-induced tumor model expressing picogram levels of IL-6. We excluded

the previously mentioned intrinsic factors of IL-6 on tumor cells as an escape mechanism and demonstrated that the systemic and local skewing of myeloid cells toward an antigen presentation impaired phenotype functions as an extrinsic escape mechanism. Importantly, we here show that the IL-6-mediated therapy resistance can effectively be overcome by combination of two therapies which are unsuccessful on as single agent therapy.

In the past, the tumor-intrinsic effects of IL-6 to stimulate tumor cell proliferation and survival pathways were suggested to mediate therapy resistance, but in our tumor model we did not detect any difference in growth or apoptosis rate between these two cell lines in vitro or in vivo. Injection of the same numbers of TC-1 control or TC-1 IL-6 cells into naïve mice resulted in comparable tumor outgrowth curves and all the mice succumbed at day 30 after tumor challenge. Although it has been shown that both exogenous and endogenous IL-6 induce resistance to cisplatin and paclitaxel by decreasing proteolytic activation of caspase-3 and increasing the expression of multidrug resistance-related genes and apoptotic inhibitory proteins,¹⁴⁻¹⁹ there was no difference in cisplatin sensitivity between TC-1 control and TC-1.IL-6 tumor cells. Moreover, both cell lines displayed a comparable sensitivity to the killing activity of E7-specific CD8⁺ T cells present in the splenocytes of vaccinated mice. Furthermore, both TC-1 and TC-1.IL-6 tumors regressed after peptide vaccine and cisplatin treatment although IL-6 producing tumors recurred quickly. Thus, despite the IL-6 production, the tumor cell lines displayed no intrinsic properties that would explain the difference in clinical response when treated with therapeutic vaccination or cisplatin chemotherapy.

IL-6 influences the recruitment, functional activation, differentiation and survival of leukocytes as well as sustains adaptive immunity.³⁹ Our in-depth analyses of systemic and local immunity revealed that tumor-produced IL-6 affected the composition and phenotype of immune cells. Without any treatment, IL-6 produced by tumor cells enhanced the percentage of CD11b⁺ myeloid cells in both tumor and spleen potentially due to IL-6-mediated increased proliferation⁴⁰ and lowered apoptosis⁴¹ of these cells. However, our phenotypic analysis showed that these myeloid cells are less matured (DCs) and comprises macrophages with an impaired antigen presenting capacity as concluded from the decreased percentage in MHC class II⁺ cells and the reduced expression levels of this molecule at the cell surface of the

FIGURE 7 Combination therapy of peptide vaccination and chemotherapy improves the survival of mice in IL-6 producing tumors. A-D, Mice were injected with TC-1 control and TC-1.IL-6 cells at day 0. On day 8 post tumor challenge, mice were treated with the maximal tolerated dose of cisplatin or kept untreated. Tumors were analyzed on day 16 post tumor challenge. A, The percentage of intratumoral CD45⁺ within live cells. B, The percentage of CD11b⁺, total CD8⁺, total CD4⁺ and Tregs within live gate in TC-1 control and TC-1.IL-6 tumor-bearing mice treated with cisplatin. C, The percentage of MHC class II⁺ cells (top) and MHC class II expression in MFI (bottom) on intratumoral CD11b⁺ myeloid cells. D, The percentage of intratumoral inflammatory Ly6C^{hi} cells within total CD11b⁺ myeloid cells and the expression level of MHC class II in MFI on these cells. Each dot represents data from an individual mouse (5-8 mice per group). Graphs indicate mean values with standard error of mean. Significance was determined by Mann-Whitney test. *P < .05; **P < .01; ***P < .001. E, The survival of TC-1 control and TC-1.IL-6 tumor-bearing mice after treatment with cisplatin, peptide vaccination in the suboptimal setting or the combination treatments (12-16 mice per group). The data shown in A to D are obtained from one experiment. The data shown in E are pooled from independent experiments with similar outcomes. Significance was determined by a log-rank (Mantel-Cox) or Mann-Whitney test (*P < .05; **P < .01; ***P < .001). IL-6, interleukin 6; MHC, major histocompatibility complex; Treg, regulatory T cells

remaining positive myeloid cells. This fits with earlier data showing that the IL-6-STAT3 pathway reduces intracellular MHC II $\alpha\beta$ -dimers through enhanced cathepsin S activity³⁹ and block DC maturation.⁴² In addition, no gross alterations in T cells were observed when tumor cells expressed IL-6, except that the percentage of spontaneously tumor-infiltrating CD4⁺CD127⁻KLRG1⁺ effector T cells was slightly lower in TC-1.IL-6 tumors. This corresponds with an earlier report on the effect of IL-6 in the TME on CD4⁺ effector T-cell differentiation.⁴³ Potentially, this is a reflection of the suppressive effect of IL-6 on MHC class II, costimulatory molecules and IL-12 production⁴⁴ by professional APC.

The IL-6-induced change in the TME toward a higher percentage of tumor-infiltrating immature myeloid cells is likely to explain why IL-6-producing tumors are refractory to cisplatin treatment and to immunotherapy. Cisplatin-induced cure of TC-1 tumor-bearing mice required the influx of both tumor-specific CD8⁺ T cells and MHC class II⁺ inflammatory phagocytic Ly6C^{hi} myeloid cells.²⁶ Similarly, the therapeutic vaccine-induced regression of TC-1 tumors requires the intratumoral presence of phagocytic MHC class II⁺ myeloid cells,²⁵ which were shown to kill tumor cells by phagocytosis and TNF.³⁸ However, none of the used therapies fully corrects the effects of IL-6 on myeloid cells and as such their tumor-killing capacity. Substituting for this via the combination of cytotoxic mechanisms employed by T cells and cisplatin, however, can correct for this. The IL-6-mediated increase in myeloid cells with no to low MHC class II expression confirms recent reports indicating that IL-6 fosters the generation of MDSC, and decreased the expression of MHC class II, CD80/86 and IL-12 in DC.^{39,45,46} In addition, its contribution to therapy resistance is in line with our data showing the increased systemic and local presence of immature myeloid cells suppressed tumor-specific T-cell reactivity and functions as a vaccine resistance mechanism in mice and patients with progressively growing HPV16⁺ tumors.^{28,47}

Finally, our data extend prior observations that upregulation of IL-6 by tumor or increased level of this cytokine in the serum negatively correlates with the induced antitumor response and tumor regression, proposing this cytokine as a predictive biomarker.^{48,49} In addition, our data clearly demonstrated the negative effect of IL-6 on myeloid cells in both immunotherapy and chemotherapy settings. This suggests that IL-6 is a negative immune modulator that should be taken into consideration during the development and testing of anti-cancer therapeutic strategies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be made available upon reasonable request.

ETHICS STATEMENT

All animal experiments were approved by the national committee for animal experiments (CCD) under permit AVD116002015271 and were executed according to the animal experimentation guidelines of LUMC in compliance with the guidelines of Dutch and European laws.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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