Targeting myeloid derived suppressor cells with all-trans retinoic acid is highly time-dependent in therapeutic tumor vaccination

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ABSTRACT
Tumor immune escape is a critical problem which frequently accounts for the failure of therapeutic tumor vaccines. Among the most potent suppressors of tumor immunity are myeloid derived suppressor cells (MDSCs). MDSCs can be targeted by all-trans-retinoic-acid (atRA), which reduced their numbers and increased response rates in several vaccination studies. However, not much is known about the optimal administration interval between atRA and the vaccine as well as about its mode of action.

Here we demonstrate in 2 different murine tumor models that mice unresponsive to a therapeutic vaccine harbored higher MDSC numbers than did responders. Application of atRA overcame MDSC-mediated immunosuppression and restored tumor control. Importantly, atRA was protective only when administered 3 d after vaccination (delayed treatment), whereas simultaneous administration even decreased the anti-tumor immune response and reduced survival.

When analyzing the underlying mechanisms, we found that delayed, but not simultaneous atRA treatment with vaccination abrogated the suppressive capacity in monocytic MDSCs and instead caused them to upregulate HLA-DR after TLR-ligation. Consistently, MDSCs from patients with colorectal carcinoma also failed to upregulate HLA-DR after ex vivo treatment with TLR-ligation.

Overall, we demonstrate that atRA can convert non-responders to responders to vaccination by suppressing MDSC function and not only by reducing their number. Moreover, we identify a novel, strictly time-dependent mode of action of atRA to be considered during immunotherapeutic protocols in the future.

Introduction
Therapeutic tumor vaccination represents a promising strategy to induce anti-tumor activity in cancer patients, while generally being well tolerated with only few relevant side effects.\textsuperscript{1} However, although strong \textit{in vitro} immune responses are detected in many individuals, significant clinical responses with evident tumor regression and prolonged survival appear to be induced only in a subgroup of patients.\textsuperscript{2,4}

Antigen-specific CD8\textsuperscript{+} cytotoxic T lymphocytes (CTLs) within the tumor are essential in anti-tumor immunity, and most tumor vaccines aim at improving CTL responses.\textsuperscript{5} To confer cytotoxic effector functions, CD8\textsuperscript{+} T cells need to be activated by professional antigen-presenting cells (APCs), in particular dendritic cells (DCs).\textsuperscript{6} For efficient CTL priming, DCs require several activation signals which, in principle, they can provide themselves, when activated by ligands of pattern recognition receptors. This process can be facilitated by CD4\textsuperscript{+} T helper cells and has been defined as classical DC-licensing.\textsuperscript{7,8} Alternatively, natural killer T (NKT) cells can license DCs; a process that can be initiated by the application of glycolipids.\textsuperscript{9}

It has been shown that combining CD4\textsuperscript{+} T helper and NKT-cell mediated DC-licensing - by applying TLR-9 ligands and NKT-cell activating ligands as adjuvants - results in even stronger, synergistically enhanced CTL responses,\textsuperscript{9} therefore providing an interesting tool for therapeutic tumor vaccination.

It is well established that certain blood cell populations counteract with T cell-based immunotherapy, such as regulatory T cells (T\textsubscript{reg}),\textsuperscript{10,11} and myeloid derived suppressor cells (MDSCs).\textsuperscript{12,13} MDSCs represent a heterogenic population of myeloid cells that, in mice, are defined as CD11b\textsuperscript{+}MHC-II\textsuperscript{−}Ly-6C\textsuperscript{−}Ly-6Chi (Gr-1\textsuperscript{low}) granulocytic MDSCs (G-MDSCs) and CD11b\textsuperscript{+}MHC-II\textsuperscript{−}Ly-6C\textsuperscript{−}Ly-6Chi (Gr-1\textsuperscript{low}) monocytic MDSCs (M-MDSCs) and can be detected under pathological conditions.\textsuperscript{14,15} MDSCs are found in the blood of cancer patients, often representing a major barrier to successful anti-tumor responses.\textsuperscript{16,17} MDSCs can be targeted by all-trans-retinoic-acid (atRA), which reduced their numbers and suppressed MDSC function in vitro.\textsuperscript{18,19}
patients\textsuperscript{16} and are associated with the suppression of effector T cell responses,\textsuperscript{17} the induction of T\textsubscript{reg},\textsuperscript{13} and most strikingly, a poor prognosis in cancer patients.\textsuperscript{18} Several reports on tumor immunotherapy have suggested that modulating frequencies of immunosuppressive T\textsubscript{reg} or MDSCs might improve the effects of tumor vaccination protocols.\textsuperscript{19}

In recent studies, all-trans-retinoic-acid (atRA) has been proven efficient to induce maturation and differentiation of various cell types, including haematopoietic progenitors, monocytes, DCs, and MDSCs \textit{in vitro} as well as \textit{in vivo}.\textsuperscript{21,20} Decreasing numbers of MDSCs and improved immune responses upon atRA treatment have been shown in mice and also suggested for tumor patients. Moreover, atRA is known to influence the effect of cancer vaccines,\textsuperscript{20} therefore potentially being a valuable compound for cancer immunotherapy. This has prompted the testing of atRA in ongoing clinical trials.\textsuperscript{21,22} In these trials, however, atRA was generally applied to the entire cohort without distinguishing between subjects that benefit from the vaccine itself and subjects that progress under treatment. In addition, atRA was applied together with the vaccine, not acknowledging a possible time-dependent efficacy.

Using our combined vaccination scheme comprising Ovalbumin (OVA) or carcinoembryonic antigen (CEA) as antigen, TLR9-ligand CpG as adjuvant for classical DC-licensing, and αGC as adjuvant for NKT-cell-mediated DC-licensing, we analyzed the response to tumor vaccination in 2 murine models (B16 melanoma and colorectal carcinoma). In addition, we sought to determine the optimal time point for atRA application and its mode of action.

**Results**

**Therapeutic tumor vaccination leads to tumor regression in a subgroup of mice**

It has previously been shown that combining CD4\textsuperscript{+}T-helper and NKT-cell mediated DC-licensing by applying both TLR-9-ligand CpG and NKT-cell-ligand αGC as adjuvants results in synergistically enhanced CTL responses.\textsuperscript{9} We inoculated mice subcutaneously with $5 \times 10^5$ B16-OVA melanoma cells and 10 d later performed therapeutic vaccination against OVA with this adjuvant combination to aim for efficient CTL induction. Vaccinated mice presented with a significantly reduced tumor growth compared with the untreated group; however, the tumors were not entirely rejected (Fig. 1A & B). Upon closer inspection, 2 types of vaccination responders could be discriminated: one group of mice showed a very slow tumor growth (responders, green), whereas tumors grew almost unimpeded after vaccination in the other group (non-responders, red) (Fig. 1A & B).

To clarify the underlying immune mechanism, we analyzed the numbers of immune cells in tumor and spleen tissues from responders and non-responders and compared these to non-vaccinated, tumor-bearing mice (CTRL) or naïve, tumor-free, untreated C57B/6J mice (w/o). We found that the tumors and the vaccination induced a general increase of immune cells in the spleen. Numbers of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells, B cells, NK cells, and T\textsubscript{reg}, on the other hand, did not differ in responders and non-responders, arguing against changes in these cells being the main responsible mechanism for the different tumor growth (Fig. 1 C & D, Fig. S1A & B). In contrast, we detected an upregulation of mRNA encoding for CCL20, TNF-α, IFN-γ, and LIGHT in responders, indicating that higher numbers of functional T cells might be present within the tumor (Fig. 1E).

**Non-responders have increased numbers of myeloid derived suppressor cells**

Focusing on immunosuppressive MDSCs by examining CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells via FACS, we detected a higher frequency of these cells in the tumors of non-responders (Fig. 1F). Accordingly, the histology of B16 melanomas showed a markedly increased infiltration of CD11b\textsuperscript{+} cells in tumors of non-responders supporting our FACS results (Fig. 1G). A more specific sub-classification using αLy6C and αLy6G showed that especially granulocytic MDSCs (G-MDSCs) were increased in the spleen, and monocytic MDSCs (M-MDSCs) were frequently found in the spleen and tumor of non-responders (Fig. 1 H & I, Fig. S1A & B). Comparing the proportion of regulatory cells and effector T cells, we found that the ratio of MDSCs, especially of M-MDSCs and CD8\textsuperscript{+} T cells, was significantly altered (Fig. 1J & K, Fig. S1C & D). However, the ratio of T\textsubscript{reg} and CD8\textsuperscript{+} T cells after vaccination was not changed in responders and non-responders (Fig. S1E), suggesting that the higher number of MDSCs abrogated the effect of the vaccination. To exclude that our vaccination scheme enhances or expands pre-existing immune cells, we next analyzed cells in the spleen and tumor tissue 10 d after tumor implementation but before vaccination. We detected no significant differences in the numbers of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, B cells, and NK cells in the individual mice of the entire cohort (Fig. S1H), excluding e.g. pre-existing CTL or higher amounts of MDSCs which might account for the tumor response after vaccination.

To substantiate the hypothesis that the number of MDSCs is responsible for the tumor progression, we analyzed the function of MDSCs. FACS-Sorted M- and G-MDSCs (gating strategy in Fig. S1F) from vaccinated mice (both, responders and non-responders), non-vaccinated tumor-bearing animals, and healthy control mice were co-cultured with activated CD8\textsuperscript{+} T-cells and anti-CD3/CD28 beads (Fig. S1G). Notably, the observed T cell suppression in these experiments was similar in non-responders, responders, and non-vaccinated tumor-bearing mice, indicating that the absolute number of MDSCs rather than their suppressive capacity per cell was important. Of note, M-MDSCs were more potently inhibiting T cells, since T cell suppression was already detected after being co-cultured at a 1:1 ratio. M-MDSCs and G-MDSCs cells isolated from untreated healthy animals did not possess suppressive capacity (Fig. S1G, last row).

Taken together, the vaccination scheme induced an increase of immune cells in spleen as well as tumor tissue. Non-responders displayed an elevated number of MDSCs, especially M-MDSCs, resulting in a diminished immune response.
Delayed atRA treatment improves the effect of tumor vaccination in non-responders in a time-dependent manner

It has been shown, that atRA reduces the numbers of MDSCs in vitro and in vivo. To test whether additional application of atRA could affect the vaccination effect in mice initially not responding to vaccination, we treated tumor-bearing non-responders with atRA starting either on the day of vaccination (day 0, simultaneous treatment, orange line) or delayed 3 d after the vaccination was performed (delayed treatment, blue line). While tumors progressed in untreated animals, delayed atRA (day 3) application dramatically reduced B16 tumor growth (Fig. 2A & C, blue line). Remarkably, even established tumors shrunk during delayed atRA treatment after vaccination in previously non-responding mice (Fig. 2A & C, blue line). Surprisingly, the anti-tumor effects of delayed atRA treatment could not be detected when atRA-treatment was initiated simultaneously to vaccination on day 0. This group of animals displayed an unimpeded tumor growth.
which was similar to the one in control groups (Fig. 2A & C, orange line). Control atRA-treated animals without therapeutic vaccination showed an aggressive tumor growth comparable to non-vaccinated mice (black dotted line), excluding a direct effect of atRA on the tumor.

We next reproduced the in vivo experiments with the same adjuvant combination in a second murine tumor model using the colorectal carcinoma cell line MC38, which expresses the tumor associated carcinoembryonic antigen (CEA). In this model, vaccination alone without atRA-treatment did not influence the tumor growth (Fig. 2B, black line). This is most likely due to the poor immunogenicity of CEA in contrast to the highly immunogenic OVA. However, mice receiving vaccination and delayed atRA-treatment were able to control tumor growth (Fig. 2B & D, red line), whereas, again, simultaneous initiation of atRA and vaccination yielded unimpeded, massive tumor growth (Fig. 2B & D, blue line). When we checked for survival of mice inoculated with MC38-CEA, we detected a longer survival of vaccinated mice having received delayed atRA treatment (Fig. 2E, blue line). Moreover, in vitro treatment of B16-melanoma or MC38 tumor cells with atRA caused no differences in an impedance-based cell assay, indicating that atRA does not act per se on tumor cells (Fig. S3).
**AtRA treatment inhibits the suppressive capacity of monocytic MDSCs**

To clarify which suppressive immune cells were affected by atRA, we enumerated T_{reg} and MDSCs in tumor and spleen tissue. We found no differences in T_{reg} frequencies between atRA-treated versus untreated mice of tumor-bearing animals (Fig. S2A). Also, numbers of CD11b^{+}Gr-1^{+} MDSCs and, more specifically, Ly-6C/G cells were similar in atRA-treated vs. non-treated tumor-bearing mice (Fig. 2F & G, Fig. S2B-E), indicating that atRA, no matter when applied (starting d0 or d3), did not act by reducing the quantity of regulatory immune cells in our vaccination model. Therefore, we examined the suppressive capacity of these cells. While the suppressive capacity of G-MDSCs and T_{reg} was unchanged (Fig. S2F & G), M-MDSCs from delayed atRA-treated tumor-bearing mice showed no suppressive function toward T cells in vitro (Fig. 2H, Fig. S2F). In contrast, when vaccination and atRA-treatment (d0) were initiated simultaneously, M-MDSCs showed an unaffected suppressive effect on T cells (Fig. 2H, Fig. S2F). Lastly, we detected significantly elevated frequencies of antigen-specific and functional CTL after delayed atRA treatment in tumors (Fig. 2I), indicating that reduced M-MDSCs frequencies indeed led to more functional T effector cells. These findings demonstrate that atRA markedly impacts M-MDSCs with a distinct, differential effect that depends on the exact time interval to vaccination.

**Delayed atRA treatment is associated with increased expression of MHC-class-II on myeloid cells and prevents induction of tumor-specific CTL**

Having identified M-MDSCs to be pre-dominantly affected by atRA in our vaccination model, we aimed to better understand the functional changes in these cells mediated by atRA. Firstly, we phenotypically characterized tumor-infiltrating MDSCs using flow cytometry. We could observe a slight expression of MDSC-associated markers, such as IFN-γR6, IL-4Rα, and B7-H1, but could not detect any differences for the expression of CD11c, F4/80, CD34, CD80, IL-4Rα, and B7-H1 on CD11b^{+}Ly6C^{+} cells (or M-MDSCs) (Fig. 3A). However, we detected a marked upregulation of MHC-class-II (I-A) expression on CD11b^{+}Ly6C^{+} myeloid cells in mice treated with delayed atRA compared with vaccinated mice without or with concomitant atRA treatment (Fig. 3A). Next, we performed histopathological analyses and examined the expression of MHC-class-II on tumor tissue, which in general is absent on MDSCs.24 In line with our phenotypical results, we were able to detect a co-localized expression of CD11b and I-A in time-delayed treated animals, which was absent in the other groups (Fig. 3B).

Since function is one of the most essential markers for MDSCs, we examined the immunosuppressive as well as cross-presenting capacity in correlation with the expression of I-A on CD11b^{+}Ly6C^{+} cells. MDSCs of tumor-bearing animals were FACS-sorted either before or after vaccination (3 or 6 days) and treated with atRA time-delayed or simultaneously. MDSCs were co-cultured with activated CD8^{+} T cells (test for suppression) or the CD8^{+} T cell hybridoma cell line (test for cross-presentation). The proliferation of CD8^{+} T cells and the IL-2 production of B3Z cells was correlated with the I-A expression on MDSCs for different time points and treatments. MDSCs before vaccination (0 days) did not induce IL-2 production in B3Z cells or express I-A but had a strong suppressive capacity toward CD8^{+} T cells. The vaccination reversed the suppressive and cross-presenting abilities, and the cells also upregulated I-A. Three days later, the cells were suppressive again, did not cross-present, and lacked I-A (black bars). In the case of simultaneous atRA treatment, the vaccination showed no effect on cross-presentation or I-A expression (blue bars), indicating that these cells stayed suppressive. In the case of delayed atRA treatment, we observed an increase of cross-presentation, I-A expression, and a lack of suppression. However, after 6 days, these cells did not reverse to the suppressive phenotype (orange bars, Fig. 3C).

This indicates that the vaccination induced a phenotypic and, in particular, functional change of myeloid cells from a suppressive to a more immune-stimulatory phenotype and leads to a recruitment of new infiltrating immune cells. Furthermore, atRA stabilizes the phenotype but does not induce maturation. In the case of delayed treatment, atRA conserves the inflammatory phenotype, whereas delayed atRA treatment stabilizes the suppressive phenotype.

**AtRA treatment suspends the activation of human CD14^{+}HLA-DR^{+} MDSCs**

Finally, we wanted to determine whether the observed effects also apply to human MDSCs. To accomplish this, we isolated MDSCs from colorectal cancer patients (see clinicopathological parameters in Table S1) and discriminated between CD14^{+}HLA-DR^{high} and CD14^{+}HLA-DR^{low} cells. Both groups were treated with LPS ex vivo, which resulted in an upregulation of HLA-DR. Additional treatment with atRA prevented this LPS-induced upregulation of HLA-DR (Fig. 4A and B), in all 6 analyzed patients.

Hence, atRA does not induce maturation in human myeloid cells but can prevent the conversion to an activated or inflammatory state when administered simultaneously with TLR-ligands.

**Discussion**

Vaccination strategies strive to reach a maximally effective CD8^{+} T cell response,10,25 which is crucial for the elimination of tumors. We found that therapeutic vaccination with our adjuvant scheme combining CpG and αGC results in robust antitumor immune responses with strong cytotoxicity leading to significantly reduced tumor growth compared with non-vaccinated animals. However, a subgroup of mice did not respond to therapeutic vaccination (non-responders), although displaying similarly increased frequencies of T cells, B cells, and NK cells in spleen and tumor tissue. This finding reflects frequent clinical observations8 and motivated us to elucidate the underlying mechanisms of non-response to tumor immunotherapy.

The reasons why some individuals succeed or fail to immunotherapy are manifold. Some of the factors are a concomitant immune activation, the vascularization of the tumor site, the presence of immunosuppressive cells and, as recently described,
Figure 3. Delayed atRA treatment is associated with increased expression of MHC-class-II on myeloid cells and prevents inhibition of tumor-specific CTL. CD11b<sup>+</sup>Ly6C<sup>+</sup> cells isolated from B16-melanomas were analyzed by flow cytometry for the expression of the markers CD11c, F4/80, CD34, I-A, CD80, IFN-γRβ, IL-4Rα, and B7-H1 (A) or by immunohistochemistry for the expression of CD11b and MHC-class-II (B) from vaccinated mice without atRA treatment and with simultaneous or with delayed atRA treatment. CD11b<sup>+</sup>Ly6C<sup>+</sup> cells from tumor-bearing mice were analyzed for the expression of I-A and FACS-sorted 6 d after vaccination with simultaneous or delayed atRA treatment. These cells were then co-cultured with CFSE-labeled, stimulated CD8<sup>+</sup> T cells (suppression) for 3 d or with the cell line B3Z (IL-2 secretion) for 24 hours (C). Shown are representative results (A & B) or cumulative results (C) from 3 independent experiments.

Figure 4. AtRA treatment suspends the activation of human CD14<sup>+</sup>HLA-DR<sup>+</sup> MDSCs. Human monocytes or induced human MDSCs were stimulated in the absence or presence of atRA for 4h with LPS, and the expression of HLA-DR on CD14<sup>+</sup> cells was analyzed by flow cytometry (A). HLA-DR<sup>+</sup> monocytes and HLA-DR<sup>−/low</sup> MDSCs from 6 patients with colorectal carcinoma were isolated using FACS-Sort. In line with A, cells were also stimulated with LPS for 4h in the absence or presence of atRA. The expression of HLA-DR on CD14<sup>+</sup> cells was analyzed by flow cytometry (B). Shown are representative results (A) or cumulative results from 6 experiments (B).
the influence of the microbiota. Anticancer immune responses seem to be profoundly shaped by the microbiome.26-28 It has been shown that oxaliplatin and cyclophosphamide both reduce tumor growth less efficiently in mice kept under germ-free conditions or treated with broad-spectrum antibiotics. In line, the combination of oxaliplatin and CpG oligonucleotides is not efficiently capable of recruiting CD11b+ MHC-class-II+ myeloid cells producing TNF into the tumor which are essential for antitumor immunity.29,30 Moreover, it has been described that the microbiota can also influence the IL-12 dependent Th1 response and by this, affects the outcome of immunotherapeutic approaches.7

Besides the influence of vaccination and chemotherapeutic approaches, the microbiome can also affect the therapeutic efficacy of checkpoint inhibitors. Altogether, the success of anticancer therapies is essentially associated with the gut microflora.21,31,32

The recruitment of leukocytes into the tumor is mediated by adhesion molecules and chemokines expressed by endothelial cells in the vessels. As the microbiome can be influenced by a specific vaccination protocol, also endothelial cells might be modulated and determine the outcome of the treatment, respectively.33

In this study, we were especially interested in the analysis of the contribution of immune-regulatory and immunosuppressive cells, although we cannot exclude (and due to structural limitations cannot perform experiments to exclude) an influence of the vessel architecture or microbiome on the vaccination outcome.

As described earlier, we had not detected numeric differences in immune effector cell populations in responders and non-responders. Transplantation of tumors as well as vaccination per se can induce an increase of Tregs in tumor tissues and the spleen34,40; an observation that we confirmed in our experiments. However, there was neither a difference in Treg frequencies nor in the per cell suppressive capacity of Tregs in vaccinated responders compared with non-responders. This made it rather unlikely that Tregs are responsible for the failure of therapeutic vaccination in our experimental setting.

We then focused on MDSCs and found non-responders to have significantly increased numbers of CD11b+Ly6C+ M-MDSCs in contrast to responders. A recent study reported that frequencies of circulating MDSCs directly correlate with the clinical outcome in melanoma patients treated with the CTLA-4-antibody ipilimumab, and that M-MDSCs are mainly responsible for MDSC-induced immunosuppression.18 In contrast, G-MDSCs, which are also increased in patients with melanoma, do not correlate with clinical outcome.18 Although we found both M- and G-MDSCs to be increased in non-responders in our model, in vitro suppression assays with FACS-sorted MDSCs identified M-MDSCs to be more suppressive. We observed no differences in vaccinated and non-vaccinated mice regarding the suppressive capacity per cell of isolated MDSCs, indicating that the rather increased numbers of MDSCs contributed to the prominent inhibition of anti-tumor CTL effector function than their suppressive potential. However, we did not observe any differences in the number or function of Tregs between responders and non-responders, suggesting that M-MDSCs are most likely responsible for the ineffective clearance of tumor cells in some mice in our experimental model. It is obvious that additional factors might account for the non-response to tumor vaccination as discussed above and we are aware of the fact that we here focused only on one small piece of the puzzle.

The effect of CpG - or more general - vaccination, is under debate in the literature. On the one hand, it has been shown that CpG is able to overcome the immune-suppressive effect of MDSCs and convert these cells into pro-inflammatory, anti-gens-presenting DCs.35,36 On the other hand, it has also been shown that vaccination with CpG is able to induce MDSCs.37,38 With our model, we are at least partly able to explain this controversy. An inflammatory stimulus seems to induce the expansion of myeloid cells which are stimulatory in the beginning. Furthermore, already existing MDSCs are deleted or transformed into functional antigen-presenting cells. In a second step, stimulatory cells are transdifferentiated into immune-regulatory MDSCs, typically after 6-7 d. This is in line with previous work, that myofibroblasts induce MDSCs from monocytes within 3 d after an activation period of 3 d.39 This hypothesis is also supported by the fact that especially immunogenic cancers induce MDSCs.40,41,42

We next questioned whether non-responders could become sensitive toward tumor vaccination after modulation of MDSCs with atRA. This compound has been described to modulate MDSCs in vivo by reducing their number.20 In addition, atRA is a well-known compound in hematology where it is routinely applied to treat acute promyelocytic leukemia (AML-M3), a subtype which is characterized by the presence of promyelocytic blasts. AtRA is considered to abrogate the differentiation block of promyelocytic blasts in AML-M3 and consequently enables the differentiation of immature promyelocytic blasts into mature granulocytes.

Since MDSCs represent another myeloid cell population with a high plastic potential, it has been speculated that atRA treatment might have similar effects on the differentiation process of MDSCs.

In 2 different murine tumor models, we found that non-responders receiving atRA-treatment 3 d after vaccination showed significant shrinkage of established tumors. Unexpectedly, vaccinated non-responders treated with atRA starting on the day of vaccination displayed advanced tumor growth similar to the unvaccinated control group. Treatment of tumor-bearing mice with atRA alone in the absence of vaccination did not lead to any tumor shrinkage, indicating a synergistic, particularly vaccination-dependent effect of the therapeutic vaccination combined with delayed atRA. However, we did not observe any changes in the number of MDSCs in contrast to other studies.20

Upon analysis of the suppressive per cell capacity of MDSCs, we found that M-MDSCs isolated from animals treated with delayed atRA did not show suppressive potential but, in contrast, cross-presented antigens and stimulated T cells. On the other hand, MDSCs isolated from simultaneously-atRA-treated mice had strong suppressive effects on T cell proliferation. IFNγ production of CD8+ T cells isolated from tumor tissue and the spleen was markedly higher in delayed atRA-treated mice compared with untreated mice or simultaneously treated animals. This indicates that delayed atRA-treatment induced a decreased suppressive potential of MDSCs, leading to improved
MDSCs are considered to represent cells with high plastic potential that are able to transdifferentiate quickly, according to particular factors they encounter.\textsuperscript{43} We hypothesized that the application of our vaccine in mice or LPS-treatment of human MDSCs \textit{ex vivo} might induce specific factors that modulate the phenotype or the functional, activatory status of MDSCs. Depending on the exact time interval between atRA-application and vaccination, atRA might then affect the MDSCs under different conditions and therefore exert specific effects.

To check our hypothesis, we performed a detailed phenotypic and functional analysis of myeloid cells under different conditions. We detected a significant upregulation of MHC-class-II-molecules on CD11b\textsuperscript{+}Ly6C\textsuperscript{+}cells in the delayed group that had received atRA starting 3 d after vaccination, whereas MHC-class-II remained negative in the group that was treated with atRA starting from the day of vaccination. Hence, application of the antigen/CpG\textsubscript{α}GC vaccine induced a functional change from CD11b\textsuperscript{+}Ly6C\textsuperscript{+}MHC-class-II – negative cells to a “DC-like,” stimulatory CD11b\textsuperscript{+}Ly6C\textsuperscript{+}MHC-class-II – positive status which was present 3 d after vaccination. The application of atRA conserved this time-dependent, particular functional activity and inhibited a further transdifferentiation (Fig. 5). Similar results have been shown for the differentiation of T\textsubscript{regs} and Th17 T cells, where atRA inhibits Th17 T cells by blocking IL-6 signaling.\textsuperscript{44} However, our study is limited due to structural and facility-based confinements to exclude a direct influence of atRA or the vaccination on e.g., the microbiome or vessel architecture.

In conclusion, the combination of therapeutic vaccination with delayed atRA administration significantly enhanced the anti-tumor effect in those animals that initially did not respond to therapeutic tumor vaccination. However, the time interval between vaccination and atRA application is highly critical; whereas an early, simultaneous application yields unimpeded tumor growth, a later, deferred treatment with atRA significantly improves the success of tumor vaccination.

Hence, the treatment of cancer patients with therapeutic tumor vaccination and atRA needs to follow a rationally designed, well-timed treatment protocol. This novel additional option may therefore be of benefit for cancer patients initially failing to respond to tumor immunotherapy.

**Material and methods**

**Mice**

All animal experiments were performed according to the federal German law regarding the protection of animals. C57Bl/6j mice and H2-KbSIINFEKL restricted TCR-transgenic animals (OT-I) were bred and maintained under specific pathogen-free conditions according to the FELASA guidelines at the central animal facility at the University Hospital Bonn. All experiments were performed with permission of the local authorities (AZ: 84–02.04.2013.A014). All experiments were performed with male mice, with a weight of 20 g and an age of 8–11 weeks.

For tumor implantation, 5 × 10\textsuperscript{5}B16 melanoma or MC38-CEA colorectal carcinoma cells were injected subcutaneously into the left flank. After 10 days, vaccination was performed using 200 μg Ovalbumin for B16 (Sigma-Aldrich, Seelze, Germany) or CEA for MC38 with 20 μg of CpG-Oligonucleotide 1668 (TBI Mol Berlin) and 0.2 μg α-Galactosylceramide (Axxora, Lörrach, Germany) in 100μl PBS. A dose of 500 ng/day/mouse retinoic acid (Tretinoïn, Roche, Grenzach-Wyhlen, Germany) was administered orally.

**Tumor models**

B16-OVA (expressing plasmid derived full length OVA) cells were obtained from Prof. Dr. Dominik Wolf, Bonn, formerly Innsbruck, MC38-CEA from Prof. Dr. Mathias Heikenwalder. B16-OVA or MC38-CEA cells were maintained in complete DMEM containing 10% FCS, 200 μg/ml penicillin, 200 U/ml streptomycin and 4 mM L-Glutamine, (all from Invitrogen-Gibco). To maintain OVA expression, B16-OVA cell cultures contained G418 (400 μg/ml). Cell lines were tested routinely for the presence of mycoplasma using the Gen-Probe detection system (Gen-Probe, San Diego, CA). Only cell lines tested negative for mycoplasma were used in our experiments. For

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**Figure 5.** Schematic hypothesis of functional transformation of myeloid cells during inflammation. The stimulatory index of inflammatory monocytes is plotted over time under steady-state (A) or chronic inflammatory conditions (B). Inflammatory monocytes are activated by vaccination and show a DC-like, pro-inflammatory phenotype with immune stimulatory properties. After approximately one week, monocytic cells have changed their functional phenotype and down-regulate effector functions (A). Retinoic acid blocks the development of these cells, independent of their status. Pro-inflammatory cells will keep their status prolonged equally to regulatory cells. However, in this case, atRA does not induce a maturation of “immature” monocytic MDSCs. Under pathological conditions, this picture is more complex. When atRA is used without any further immunomodulatory treatments, it has no effect on MDSCs, but can block the development of newly immigrating monocytes into MDSCs within the tumor environment. However, also the activation of monocytes is blocked by atRA. Here, we have shown that atRA in combination with an optimally synchronized strong inflammatory stimulus converts MDSCs and newly entering monocytes into antigen-presenting, immune stimulatory cells. In a second, strictly time-dependent step, we prevented the further development of monocytes into MDSCs.
tumor implantation, 5 × 10^5 B16 melanoma or MC38-CEA colorectal carcinoma cells were injected subcutaneously to the left flank. Tumor size was measured using digital caliper and tumor volume was calculated using the ellipsoid formula: \( V = \frac{4}{3} \pi r^3 \).

Cell isolation
Splenic and tumor infiltrating cells were isolated as described previously. Briefly, spleen and tumor tissues were mechanically disrupted and further digested with 0.1% collagenase (Sigma-Aldrich, Seelze, Germany) in RPMI for 10 minutes at 37°C. Single cells were further isolated using 40% percoll and ficoll separation solution in a density gradient centrifugation. For proliferation assays, CD8⁺ T cells and CD11c⁺ dendritic cells were isolated by immuno-magnetic separation with anti-CD8 or anti-CD11c antibody labeled microbeads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Monocytic and granulocytic MDSCs were isolated using FACS-Aria III cell sorter (BD) or SH800 (Sony) gating on CD11b⁺Ly6C⁻Ly6G⁻ or CD11b⁺Ly6C⁰Ly6G⁰high cells. Regulatory T cells were isolated gating on CD4⁺CD25highCD127low cells. Purity of isolated cells was > 95%.

Flow cytometry and FACS-Sort
The phenotype of MDSCs was determined by multi-color flow cytometry using the following antibodies: anti-CD11b (M1/70), anti-Ly6C (4K1.4), anti-Ly6G (1A8), anti-CD11c (N418), anti-F4/80 (BM8), anti-I-A/I-E (M5/114.15.2), anti-CD80 (16–10A1), anti-CD3 (145–2C11), anti-CD4 (RM4–5), anti-CD8 (53–6.7), anti-CD8α (53–6.7), anti-CD127 (PK136), anti-CD34 (HM34), anti-B7-H1 (10F.9G2), anti-IFN-γRB (MOB-47), anti-IL-4RB (I015F8), anti-IFNγ (XM61.2), anti-CD25 (PC61), anti-Foxp3 (FJK16S), anti-I-A/I-E (M5/114.15.2) anti-CD14 (M5E2), and anti-HLA-DR (MEM-12). Dead cells were excluded with 7AAD or propidium iodide. Isotype matching fluorochrome-labeled antibodies were used as indicated. Intracellular staining was performed using the Foxp3 fixation and lysis buffer (BD Bioscience, Heidelberg, Germany). Absolute cell numbers were determined with Absolute Counting Beads (Life Technologies, Darmstadt, Germany). Acquisition was performed using LSR-Fortessa (BD Bioscience, Heidelberg, Germany). Absolute cell numbers were determined using the Foxp3 measurement.

Proliferation / suppression assay
Isolated CD8⁺ T cells (wildtype or OT-I) were carboxyfluorescein-succinimidyl-ester (CFSE) labeled and either stimulated with Dynabeads Mouse T-activator CD3/CD28 for T-Cell expansion and activation (life technologies, Darmstadt, Germany) or with isolated and Ovalbumin-loaded CD11c⁺ dendritic cells. Cells were co-cultured with MDSCs at the ratio 1:1 if not indicated differently. Proliferation was determined by the dilution of CFSE on CD8⁺ T cells.

Histology
Freshly cut sections (2 mm) of tumor tissue were stained with Hematoxylin/Eosin or antibodies against CD11b (M1/70) or I-A (11B11). Incubation and staining was performed on a Bond MAX (Leica). Slides were scanned using a SCN400 slide scanner (Leica).

Impedance-based cell assay
B16-OVA or MC38-CEA were plated out in E-plates (2 × 10⁴ well in 96-well format). After 3 hours, atRA was added at different concentrations (1–30 μM). The Cell index was calculated according to \( CI = \max_{i=1...N} \left( \frac{R_{cell(f)}}{R_{cell(0)}} - 1 \right) \), where \( R_0(f) \) and \( R_{cell(0)} \) are the electrode resistance without or with cells.

Isolation and treatment of CD14⁺ HLA-DRlow MDSC
Pre- or intra-surgical blood samples were obtained from colorectal cancer patients that underwent tumor resection at the Dept. of Surgery, TUM or Bonn. Patients with neoadjuvant pre-treatment were excluded. CD14⁺ HLA-DRlow MDSC were sorted out of peripheral blood using a Sony SH800 after staining for CD14 and HLA-DR. Approval was obtained from the Institutional Ethics Committee of the University of Munich, Germany (No. 1926/7) or Bonn (No 173/09 from 09/09/09). Informed, written consent was obtained from participants in accordance with the Declaration of Helsinki. Myeloid cells were stimulated with either LPS (100 ng/ml, Sigma) or atRA (500 nM, Sigma) for 4 hours followed by HLA-DR measurement.

Quantitative PCR
RNA was purified using RNeasy Kit (Quiagen) followed by reverse transcription with the Vilo Kit (ThermoFischer Scientific) according to the manufacturer’s protocol. The expression analysis was performed using SYBR-Green (Roche) and the PCR was performed on a ABI PRISM7900 HT Fast Real-Time PCR System (AB). The expression levels of mRNA were normalized to the housekeeping gene GAPDH.

Statistics
All experiments were performed with 4–6 mice per group and repeated at least 3 times. Results are shown as SEM. Statistical significance was calculated using ANOVA, Logrank/Mantel-Cox test or Student T-test (\( *p \leq 0.05; **p \leq 0.01; ***p \leq 0.001 \)).

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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Author contributions
Conception and design of study: BH, AH. Performed the experiments: BH, CL and AH. Analyzed the data: BH and AH. Wrote the paper: BH, AH and CK. Critical reading of manuscript: HG, LD, NG, PK and PB.

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