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ORIGINAL ARTICLE

Salmonella-mediated tumor regression involves targeting of tumor myeloid suppressor cells causing a shift to M1-like phenotype and reduction in suppressive capacity

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Abstract The effectiveness of attenuated Salmonella in inhibiting tumor growth has been demonstrated in many therapeutic models, but the precise mechanisms remain incompletely understood. In this study, we show that the anti-tumor capacity of Salmonella depends on a functional MyD88-TLR pathway and is independent of adaptive immune responses. Since myeloid suppressor cells play a critical role in tumor growth, we investigated the consequences of Salmonella treatment on myeloid cell recruitment, phenotypic characteristics, and functional activation in spleen and tumor tissue of B16.F1 melanoma-bearing mice. Salmonella treatment led to increased accumulation of splenic and intratumoral $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells, exhibiting significantly increased expression of various activation markers such as MHC class II, costimulatory molecules, and Sca-1/Ly6A proteins. Gene expression analysis showed that Salmonella treatment induced expression of iNOS, arginase-1 (ARG1), and IFN-γ in the spleen, but down-regulated IL-4 and TGF-β. Within the tumor, expression of iNOS, IFN-γ, and S100A9 was markedly increased, but ARG1, IL-4, TGF-β, and VEGF were inhibited. Functionally, splenic $CD11b^{+}$ cells maintained their suppressive capacity following Salmonella treatment, but intratumoral myeloid cells had significantly reduced suppressive capacity. Our findings demonstrate that administration of attenuated Salmonella leads to phenotypic and functional maturation of intratumoral myeloid cells making them less suppressive and hence enhancing the host's anti-tumor immune response. Modalities that inhibit myeloid suppressor cells may be useful adjuncts in cancer immunotherapy.

Keywords Myeloid suppressor cells · Salmonella · Tumor immunity · Macrophages

Introduction

Tumor development is accompanied by a peculiar alteration of hematopoiesis that selectively expands myelomonocytic cells and leads to their progressive accumulation at the tumor site, bone marrow, blood, and peripheral lymphoid organs [1, 2]. The accrual of myeloid cells, including tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), within tumors leads to the suppression of anti-tumor activity of CTLs and NK cells, promotes angiogenesis and tumor cell metastasis and correlates with poor prognosis [1, 3–7]. These findings highlight the central role played by macrophages in tumor growth and metastasis and in regulating the host's anti-tumor responses, providing new targets for cancer immunotherapy. Interestingly, MDSCs cells share similar phenotypic and functional characteristics with myeloid

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suppressor cells first shown to be induced by Salmonella infection [8, 9] and subsequently observed in a variety of bacterial, fungal, and parasitic infections [10, 11].

Different subpopulations of myeloid cells are involved in tumor initiation, progression, and metastasis. Myeloid cells with a phenotype resembling classically activated, or M1, macrophages are associated with cancer promotion at the initial phases of development [12]. As tumors become gradually more established and progressively malignant, macrophage phenotype is changed to M2 trophic, or "alternatively activated" macrophages, a characteristic of TAMs. M2 macrophages have reduced inflammatory signature, which includes decreased iNOS and TNF-α expression, due to the dominant inhibition of NFκB [13]. Functionally, M2 macrophages play a central role in promoting angiogenesis and supporting the progressive growth and malignant transformation of tumors [14]. TAMs, which are recruited to tumors via hypoxia and growth factors such as CSF, are a major source of angiogenic molecules, including VEGF, and their depletion leads to retardation of tumor growth [15, 16]. Moreover, these macrophages are critical for the establishment of an immunoregulatory microenvironment that further protects against host anti-tumor immune attack.

MDSCs represent another immunosuppressive myeloid population within the tumor milieu. Phenotypically, murine MDSCs are characterized by expression of CD11b and Gr-1 surface proteins, relative immaturity, and reduced expression of mature myeloid markers. They include precursors of macrophages and granulocytes, and hence, the two recognized subpopulations of MDSCs are known as monocytic or granulocytic MDSCs. MDSCs secrete high levels of reactive oxygen and nitrogen species and suppress immune responses through the production of arginase-1 (ARG1), IL-10, and TGF-β [3]. Given their heterogeneity, many factors are known to influence the recruitment and activation of MDSCs, including products of activated Th1 and NK cells (IL-2 and IFN-γ), Th2 cells (IL-4 and IL-13), and tumor cells, such as IL-1β, IL-6, CSF, VEGF, TGF-β, and prostaglandin E2 (recently reviewed in [1]). Tumor-derived factors maintain the MDSC population in an undifferentiated state. Knockdown of STAT1 in breast tumors or enforced expression of a specific microRNA, miR-142-3p, in bone marrow impaired the generation of MDSCs and attenuated tumor progression [17, 18], thus validating the targeting of MDSCs as a viable strategy for immunotherapy.

Increased understanding of the tumor microenvironment and growth properties of facultative anaerobes, such as *Salmonella* species, has renewed interest in their potential use as anti-cancer agents [19, 20]. Administration of *Salmonella typhimurium* to tumor-bearing animals results in bacterial concentrations within tumors exceeding 10^9 CFUs per gram of tissue and leads to tumor regression [21, 22]. The chaotic vascularization and hypoxic conditions within tumors provide an ideal environment for the growth of anaerobic bacteria. Previously, we demonstrated the efficacy of a recombinant Salmonella strain in retarding the growth of B16.F1 tumors and enhancing host survival [23, 24]. Although the mechanism(s) by which Salmonella exert their effect remain incompletely understood, the treatment inhibited tumor angiogenesis and increased apoptosis [23].

Salmonella organisms utilize host macrophages as their primary niche for survival [25, 26]. Given the central role of macrophages in Salmonella infections and the demonstrated efficacy of Salmonella in inhibiting tumor growth, we hypothesized that this may be due to the targeting of myeloid cells within the tumors. In this report, we provide evidence that Salmonella-induced tumor retardation occurs concurrently with alterations in myeloid cells consistent with maturation to macrophage effectors and a reduction in their suppressive capacity.

Materials and methods

Cell lines, bacterial strains and mice

The melanoma cell line B16.F1 (H-2^b) was maintained in RPMI with 10 % FCS and supplements, as described [27]. BRD509E, an auxotrophic mutant of *S. typhimurium*, was prepared as described [28]. C57BL/6 mice (Harlan Olac, Bicester, UK) were bred in the animal facility of the College of Medicine and Health Sciences. MyD88-deficient [29] and CD154-deficient mice [30] have been described [31, 32]. Athymic NMRI/nude$^{nu/nu}$ mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed in filtered-air laminar flow cabinets. Male mice were used at 8–12 weeks of age for the present studies, which were carried out in accordance with, and after approval of, the Animal Research Ethics Committee of the College of Medicine and Health Sciences, UAE University.

Tumor implantation and treatment with live Salmonella

C57BL/6 or immune-deficient mice (8–10 mice per group unless otherwise indicated) were inoculated s.c. in the right flank with 2×10^5 B16.F1 cells and staged to day 12–14, as described [23]. Tumor growth was followed by quantitative determination of tumor volume, measured as the product of the perpendicular diameters using digital calipers, according to the formula: volume = $LxW^2/2$. Once tumors were established, mice were inoculated i.p. with $1–5 \times 10^5$ CFUs of BRD509E strain and followed for the subsequent 4 weeks. In some experiments, the bacterial load in spleen and tumor tissue of treated mice was quantitated as detailed previously.

Isolation of spleen and tumor cells

Tumor and spleen cells were extracted 7 days post-Salmonella treatment. Spleen cell suspensions were prepared as detailed [32]. For tumor cell preparation, excised tumors were subjected to two 15-min enzymatic digestion cycles in HBSS containing collagenase type1 (50 μg/ml), hyaluronidase (25 μg/ml), DNase1 (10 μg/ml), and Soybean trypsin inhibitor (0.2 TIU/ml) at 37 °C and filtered through a 40 μm mesh. Myeloid cells were subsequently purified from spleen or tumor cell suspensions using $CD11b^+$ microbeads on the autoMACS separator, according to manufacturer's protocol (Miltenyi Biotec, Germany). The purity of cell populations was evaluated by flow cytometry and exceeded 80 % (for tumor) and 90 % (for spleen).

Antibodies and flow cytometry

Analysis of total spleen or tumor cells, or $CD11b^+$ myeloid cells was carried out using a 6-color FACS following a standard procedure [33]. Washed cells in staining buffer were incubated with anti-CD16/CD32-specific mAb for 30 min on ice to block Fc**γ**III/II receptor sites. Cells were stained with a combination of directly conjugated mAbs, washed and analyzed using FACSCanto II (BD Biosciences, Mountain View, CA). The antibodies used were CD11b-APC-Cy7, MHC class II (I-A/I-E)-APC, and Sca1-PE-Cy7 (eBioscience, San Diego, CA), Gr1-PE, CD80-FITC, CD86-APC (BD Biosciences), and CD40-FITC (SouthernBiotech, Birmingham, AL). Non-viable cells staining positive with 7AAD dye (eBioscience) were excluded from the analysis. Data collected on 50,000 cells were analyzed using FlowJo software (TreeStar Inc, Ashland, OR).

Quantitative RT-PCR analysis

RNA was extracted by TRIzol method and repurified on Qiagen columns (RNA easy mini kit, Qiagen, Valencia, CA). The quality and quantity of the RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) using manufacturer's protocol. Premade TaqMan primers and probes (Applied Biosystems) were used to study the expression of a set of genes (ARG1, iNOS, IFN-γ, IL-4, S100A9, TGF-β, IL-10, VEGF) associated with myeloid and tumor cell functions. Transcript levels of target genes were normalized according to the ΔCq method to respective mRNA levels of the housekeeping gene HPRT. The expression of the target gene is reported as the level of expression relative to HPRT.

In vitro T cell suppression assay

$CD11b^+$ myeloid cells were isolated from spleen and tumor tissues of tumor-bearing mice, with or without treatment with the attenuated Salmonella BRD509 strain and from the spleens of non-tumor-bearing mice (control). $CD4^+$ splenic T cells were isolated from normal spleens by magnetic sorting using anti-CD4 microbeads, and their purity confirmed to be >93 %. For the co-culture assay, $CD4^+$ T cells (1×10^5 cells/well) were stimulated with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (10 μg/ml) mAbs in 96-well round-bottom plate. T cells were co-cultured in the absence or presence of different $CD11b^+$ populations (either 1×10^4 or 1×10^3 per well) for a total of 24 h. Control cultures with myeloid cells cultured alone in the presence or absence of immobilized mAbs, and purified T cells cultured in the absence of mAbs were also setup. Culture supernatants were collected at 24 h and analyzed for IFN-γ using a specific ELISA (BD Biosciences) following manufacturer's instructions. Data are expressed as percentage suppression calculated as follows: % suppression = [1 − (IFNγ concentration of test cells/IFNγ concentration of control cells)] × 100.

Statistical analysis

Statistical significance between control and treated groups was analyzed using the unpaired, two-tailed Student's *t* test, using the statistical program of GraphPad Prism software (San Diego, CA). Survival analysis was performed by Kaplan–Meier survival curves and log-rank test, using the GraphPad Prism program. Differences between experimental groups were considered significant when p values were <0.05.

Results

Treatment with attenuated Salmonella inhibits the growth of B16.F1 tumors

B16.F1-preimplanted C57BL/6 mice with mean tumor volume of 200–300 mm^3 were injected with Salmonella strain BRD509E ("Treated" group), or with saline ("non-treated" group) and followed for 4 weeks. In agreement with previous findings [23, 24], Salmonella organisms reach significantly ($p = 0.023$) higher growth densities in tumors compared with their usual target organs, including spleen (Fig. 1a). Salmonella treatment resulted in a significant inhibition of tumor growth with mean tumor volumes in treated mice reaching 48.3 ($p = 0.044$) and 34.6 % ($p = 0.007$) of corresponding means of non-treated groups on days 3 and 8 post-treatment, respectively (Fig. 1b). On

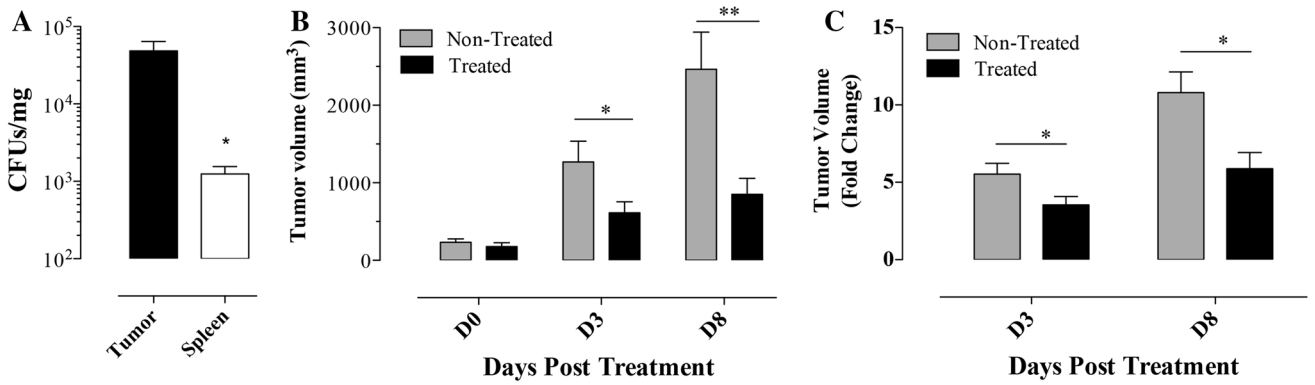

Fig. 1 Inhibition of tumor growth following treatment with attenuated *Salmonella* organisms. **a** Tumor-bearing C57BL/6 mice were inoculated with Salmonella and 8 days later spleen and tumor bacterial loads were determined. Each data point represents the mean ± SEM of 4 mice per group. **b** Changes in tumor volumes at the indicated days post-treatment with Salmonella (Treated group) or saline (non-treated group). **c** The data in graph **b** is represented as fold change in volumes in comparison with the means for each group prior to start of treatment. Results are representative of four independent experiments. *Asterisks* denote statistically significant differences between tumor and spleen CFUs (**$p < 0.01$; *$p < 0.05$)

average, tumors in non-treated mice grew 5.5- and 10.8-fold by days 3 and 8, respectively, while the corresponding growth in BRD509E-treated animals was approximately 3.5- and 5.9-fold (p values of 0.042 and 0.013 between treated and non-treated groups on days 3 and 8, respectively) (Fig. 1c).

Salmonella-mediated inhibition of tumor growth and enhanced host survival are independent of adaptive immune responses

To map the immune system requirements for the observed Salmonella-mediated tumor suppression, we used three different mouse strains with varying genetic deficiencies. Athymic NMRI/nude mice are deficient in T lymphocytes. CD154$^{-/-}$ mice have a disrupted costimulatory pathway required for T-B cell interactions and immunoglobulin isotype switching and MyD88$^{-/-}$ mice lack a critical adaptor molecule required for most TLR signaling pathways. As shown in Fig. 2a–d, identical tumor inhibition was observed in C57BL/6 (Fig. 2a), NMRI/nude (Fig. 2b) and CD154$^{-/-}$ (Fig. 2c) mice, suggesting that Salmonella-mediated tumor inhibition is independent of T lymphocytes and T-dependent antibody responses. In contrast, tumor growth was unimpeded in MyD88$^{-/-}$ mice (Fig. 2d), indicating that Salmonella-induced inhibition is dependent on TLR signaling pathways, most likely in the myeloid cell compartment. Animal survival was also assessed in tumor-bearing wild-type and NMRI/nude mice following Salmonella inoculation. The data presented in Fig. 2e, f demonstrate that significant improvement in survival was equally evident in wild-type C57BL/6 ($p < 0.0001$) and NMRI/nude mice ($p = 0.002$), further confirming that this response is independent of T lymphocytes.

Accumulation of myeloid cells in the spleens of tumor-bearing mice following Salmonella treatment

The phenotypic and functional alterations in myeloid spleen populations of tumor-bearing mice were analyzed 8 days after treatment with Salmonella. Based on staining with mAbs specific to CD11b and Gr-1, three subpopulations of myeloid cells were identified, CD11b^{+}Gr-1^{H}, CD11b^{+}Gr-1^{Int} and CD11b^{+}Gr-1$^{-/L}$ (Fig. 3). The co-expression of CD11b and Gr-1 proteins defines the population of MDSCs within spleen and tumor tissues. In the spleens of non-treated animals, the mean percentages of each of these populations were 16.3 ± 1.8, 2.6 ± 0.1, and 6.2 ± 0.1, respectively. In the spleens of treated mice, the respective percentages were 29.3 ± 0.3, 6.7 ± 0.2 and 3.2 ± 0.2, which represents ~2-fold increase in the two Gr-1^{H} and Gr-1^{Int} myeloid populations (Supplementary Fig. S1*A-C*). In non-tumor-bearing animals, the corresponding percentages of the three myeloid populations were 3.3 ± 0.1, 1.4 ± 0.3, and 4.3 ± 0.3, respectively (control mice). The differences between non-treated and Salmonella-treated mice were also evident when the groups were compared in terms of absolute cell numbers. As shown in Supplementary Fig. S1*D-F*, the fold increase in cell number of each myeloid subpopulation in the treated group was sevenfold, tenfold, and 1.9-fold for CD11b^{+}Gr-1^{H}, CD11b^{+}Gr-1^{Int} and CD11b^{+}Gr-1$^{-/L}$ cells, respectively. The Gr-1^{H} and Gr-1^{Int} subpopulations correspond to granulocytic and monocytic MDSCs, respectively [34].

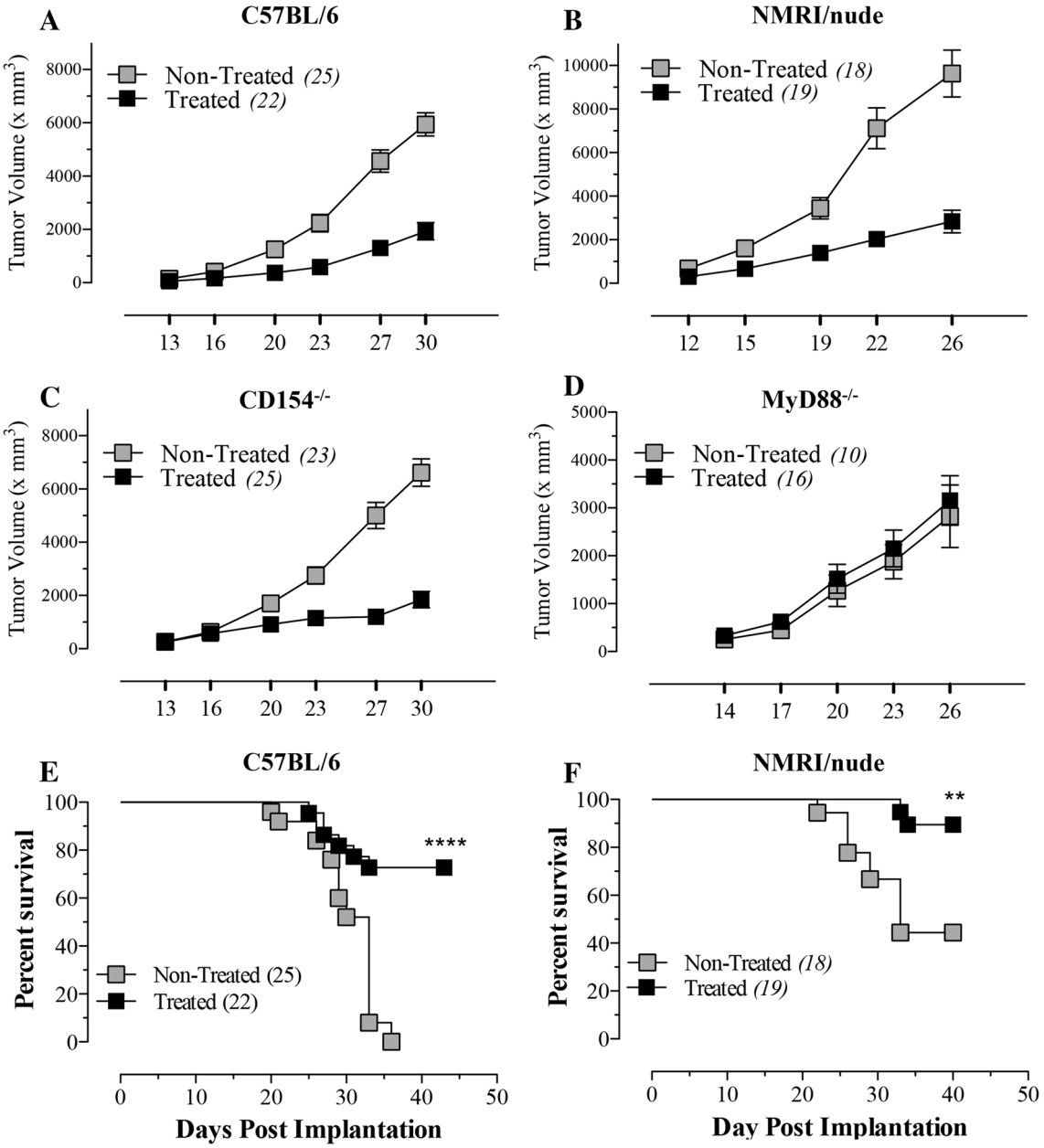

Fig. 2 Salmonella-mediated inhibition of tumor growth and enhanced host survival are independent of adaptive immune responses. B16.F1 tumor growth was followed in C57BL/6 (**a**), athymic NMRI$^{nu/nu}$ (**b**), CD154$^{-/-}$ (**c**), or MyD88$^{-/-}$ (**d**) mice either non-treated or treated with Salmonella. **e**, **f** Animal survival was also followed in Salmonella-treated C57BL/6 (**e**) and NMRI$^{nu/nu}$ (**f**) mice for up to 6 weeks. *Numbers* in parenthesis denote the number of mice per group and each data point represents the mean ± SEM in the respective group, pooled from 2 to 3 individual experiments. *Asterisks* denote statistically significant differences between indicated groups (****$p < 0.0001$; **$p < 0.01$)

Enhanced expression of costimulatory molecules and activation markers on splenic macrophages following Salmonella treatment

Analysis of the expression of activation/maturations markers on CD11b^{+}Gr-1^{H} and CD11b^{+}Gr-1^{Int} cells from non-treated and Salmonella-treated animals revealed a significant upregulation in MHC class II, CD80, CD86, CD40 and Sca-1 proteins in treated mice (Fig. 3 and Supplementary Fig. S2*A*-*E*). Although the increased expression of these proteins was evident in both CD11b^{+}Gr-1^{H} and CD11b^{+}Gr-1^{Int} populations, the extent of this upregulation was greater in CD11b^{+}Gr-1^{Int} cells. In CD11b^{+}Gr-1^{H} cells, Salmonella treatment led to increased expression of MHC class II (6.6-fold), CD80 (2.6-fold), CD86 (1.7-fold), CD40 (3.2-fold) and Sca-1 (61.1-fold). By contrast, the

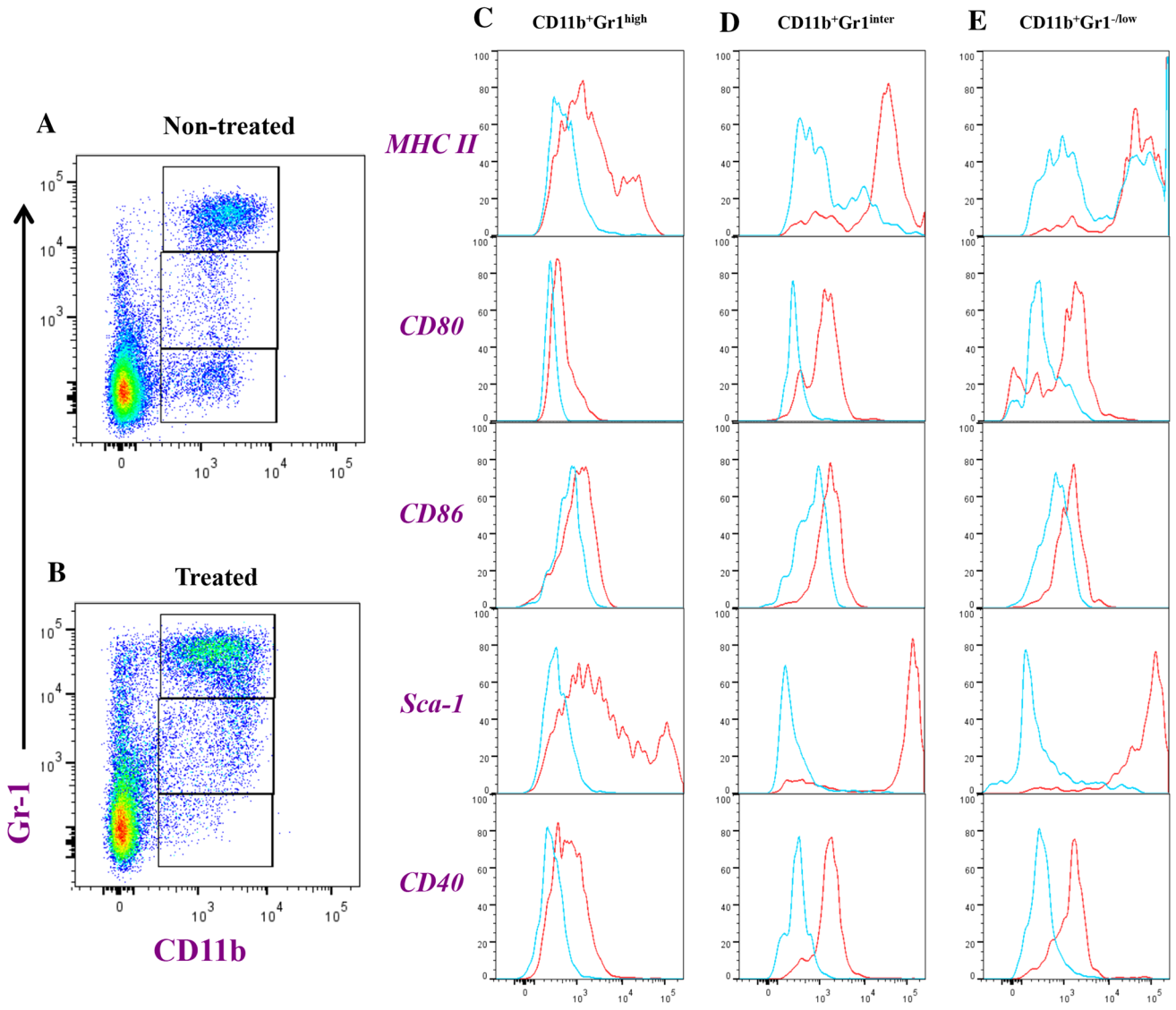

Fig. 3 Flowcytometric analysis of splenic myeloid cells in tumor-bearing mice. Splenocytes were prepared from saline (non-treated; plot **a**) or Salmonella-injected mice (treated; plot **b**) and analyzed for CD11b and Gr-1 positivity. $CD11b^{+}Gr\text{-}1^{high}$ (panel **c**), $CD11b^{+}Gr\text{-}1^{Inter}$ (**d**), and $CD11b^{+}Gr\text{-}1^{-/low}$ (**e**) were further analyzed for expression of MHC class II, CD80, CD86, Sca-1 and CD40, as indicated. The histograms depict extent of staining in cells of non-treated (*blue line*) or Salmonella-treated mice (*red line*). Each experimental group included 3 mice. Results of individual mice are shown and are representative of three independent experiments

corresponding increases in $CD11b^{+}Gr\text{-}1^{Int}$ cells were 9.2, 5.0, 2.2, 5.9, and 140.3-fold, respectively (Supplementary Fig. S2).

Expression of inflammatory genes is upregulated in splenic macrophages following Salmonella treatment

RNA was extracted from total splenocytes or $CD11b^{+}$ splenic myeloid cells (>90 % purity) of non-treated or Salmonella-treated mice to assess the expression levels of iNOS, IL-4, IL-10, ARG1, IFN-γ, and TGF-β genes by qRT-PCR (Fig. 4a–f). Salmonella treatment led to a significant upregulation in iNOS (15-fold; $p = 0.005$) and ARG1 (4.8-fold; $p = 0.017$) in $CD11b^{+}$ cells. In contrast, the expression of IL-4 and TGF-β was markedly reduced following Salmonella treatment (p values 0.018 and 0.0008 between non-treated and treated groups, respectively) but no significant change in IL-10 levels was observed. At the level of the whole spleen, the expression level of IL-4 was reduced by 21-fold in tumor-bearing mice compared with wild-type controls ($p \leq 0.0001$) and was further inhibited (by 22-fold) after Salmonella treatment ($p \leq 0.0001$). In sharp contrast, expression of IFN-γ increased by 19-fold in tumor-bearing mice compared

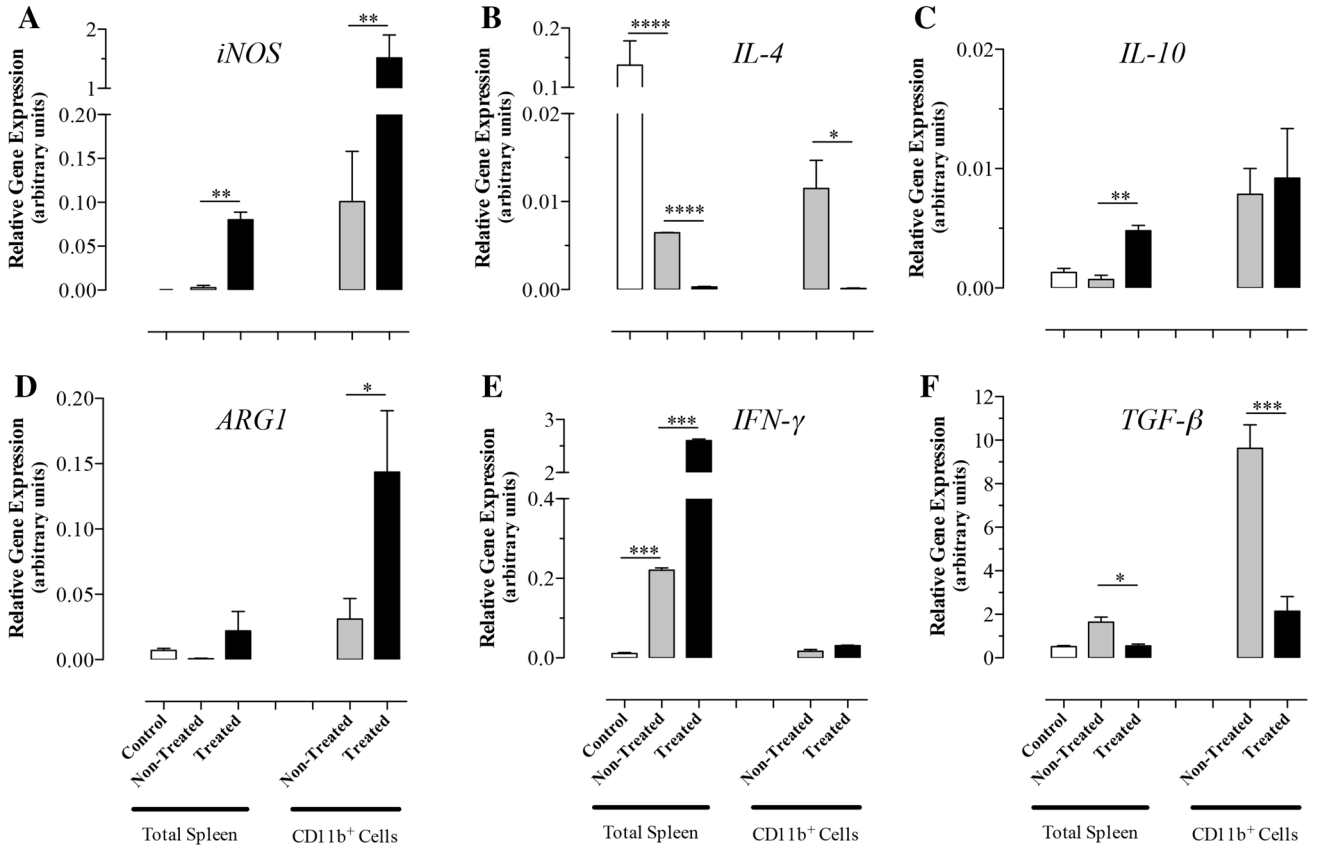

Fig. 4 Relative expression levels of iNOS (**a**), IL-4 (**b**), IL-10 (**c**), ARG1 (**d**), IFN-γ (**e**), and TGF-β (**f**) mRNA isolated from whole spleen or splenic myeloid cells. Cells were obtained from normal (control group) or B16.F1-bearing mice at day 8 post-injection with Salmonella (treated group) or saline (non-treated group). Each data point represents the mean ± SEM of 6 (total spleens) or 3 ($CD11b^+$ cells) mice per group. *Asterisks* denote statistically significant differences between the indicated groups (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). The results are representative of three independent experiments

with controls ($p = 0.0008$) and was further increased (by ~12-fold) upon Salmonella treatment ($p = 0.0002$). The induced changes in gene expression are consistent with the induction of an inflammatory response involving activated macrophages in the spleen of Salmonella-inoculated animals.

Salmonella treatment induces myeloid cell recruitment and activation within the tumor tissue

The next series of experiments were focused on studying the phenotypic and functional characteristics of tumor-infiltrating cells. Staining with mAbs specific to CD11b and Gr-1 identified two myeloid populations, $CD11b^+Gr\text{-}1^+$ and $CD11b^+Gr\text{-}1^-$ cells (Fig. 5a). Treatment with Salmonella resulted in >3-fold expansion of the $CD11b^+Gr\text{-}1^+$ cells but not $CD11b^+Gr\text{-}1^-$ population (Fig. 5a, b and Supplementary Fig. S3*A*-*B*). Analysis of the relative expression of activation markers on intratumoral cell populations demonstrated that Salmonella treatment led to an enhancement in the expression of several proteins, particularly on $CD11b^+Gr\text{-}1^+$ cells, including MHC class II (4.8-fold increase), CD80 (twofold) and Sca-1 (53.6-fold) (Fig. 5c, d and Supplementary Fig. S3*C*-*E*).

Intratumoral myeloid cells up-regulate inflammatory genes and downregulate expression of suppressive phenotype following bacterial treatment

Gene expression analysis was carried out in whole tumor cells and intratumoral $CD11b^+$ myeloid cells (Fig. 6a–h). Treatment with Salmonella led to a significant increase in the expression of iNOS, both at the level of the whole tumor (8.9-fold; $p = 0.034$) and, more importantly, in $CD11b^+$ cells (3.3-fold) (Fig. 6a). In contrast, expression of ARG1 was reduced by 2.4- and 3.2-fold in whole tumor ($p = 0.031$) and myeloid cells, respectively (Fig. 6e). These alterations were accompanied by a pronounced inhibition (41.7-fold) in IL-4 expression in myeloid cells and an increase (tenfold; $p < 0.0001$) in IFN-γ expression in tumors of Salmonella-treated animals (Fig. 6b, f). The expression of S100A9, a proinflammatory product of

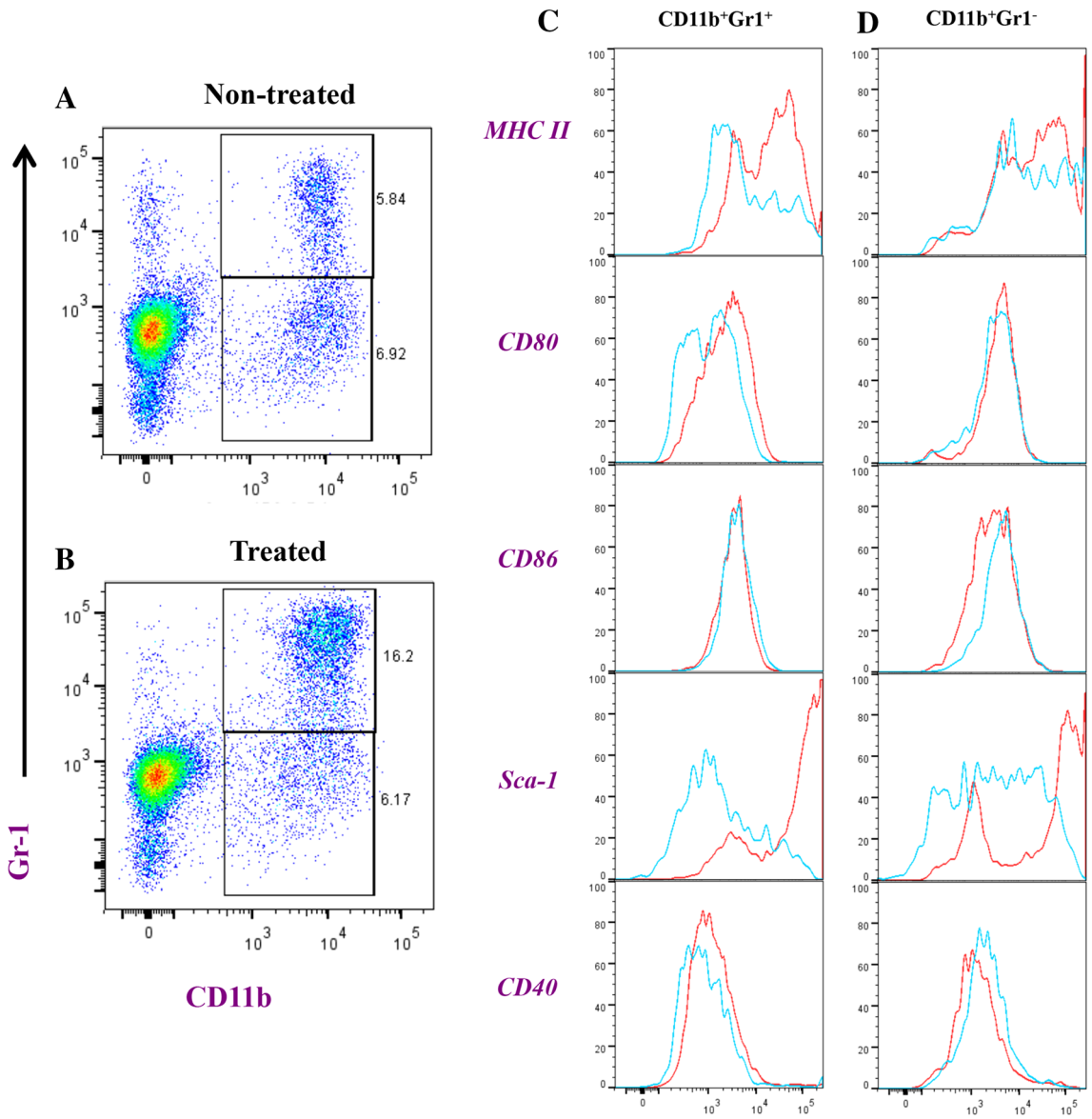

Fig. 5 Flowcytometric analysis of intratumoral myeloid cells in B16.F1-bearing mice. Tumor-infiltrating cells were prepared 8 days post-inoculation of Salmonella (treated; plot **b**) or saline (non-treated; plot **a**) and analyzed for CD11b and Gr-1 positivity. $CD11b^{+}Gr\text{-}1^{+}$ (**c**) and $CD11b^{+}Gr\text{-}1^{-}$ (**d**) were further analyzed as described in the legend of Fig. 3. Results are representative of two independent experiments

macrophages [35], was greatly enhanced in intratumoral $CD11b^{+}$ cells of Salmonella-treated mice (Fig. 6d). Interestingly, expression of VEGF by myeloid cells was reduced by threefold following Salmonella treatment (Fig. 6h), consistent with the previously reported decrease in angiogenesis that was observed in tumors of treated animals [23]. Finally, analysis of the expression of IL-10 and TGF-β, two immunomodulatory mediators of macrophages, revealed that while IL-10 was not altered in intratumoral myeloid cells from Salmonella-treated mice (Fig. 6c), expression of TGF-β was reduced by approximately twofold, though not reaching statistical significance (Fig. 6g). This is perhaps a reflection of the complex nature of gene regulation seen in tumor tissue in the presence of viable Salmonella organisms.

Reduced T cell suppressive capacity of intratumoral myeloid cells, but not splenic macrophages, following treatment with Salmonella

Purified myeloid cells from tumors or spleens of non-treated or Salmonella-treated mice were co-cultured at various ratios with normal splenic $CD4^{+}$ T cells and stimulated with anti-CD3/CD28 mAbs. T cell responsiveness was

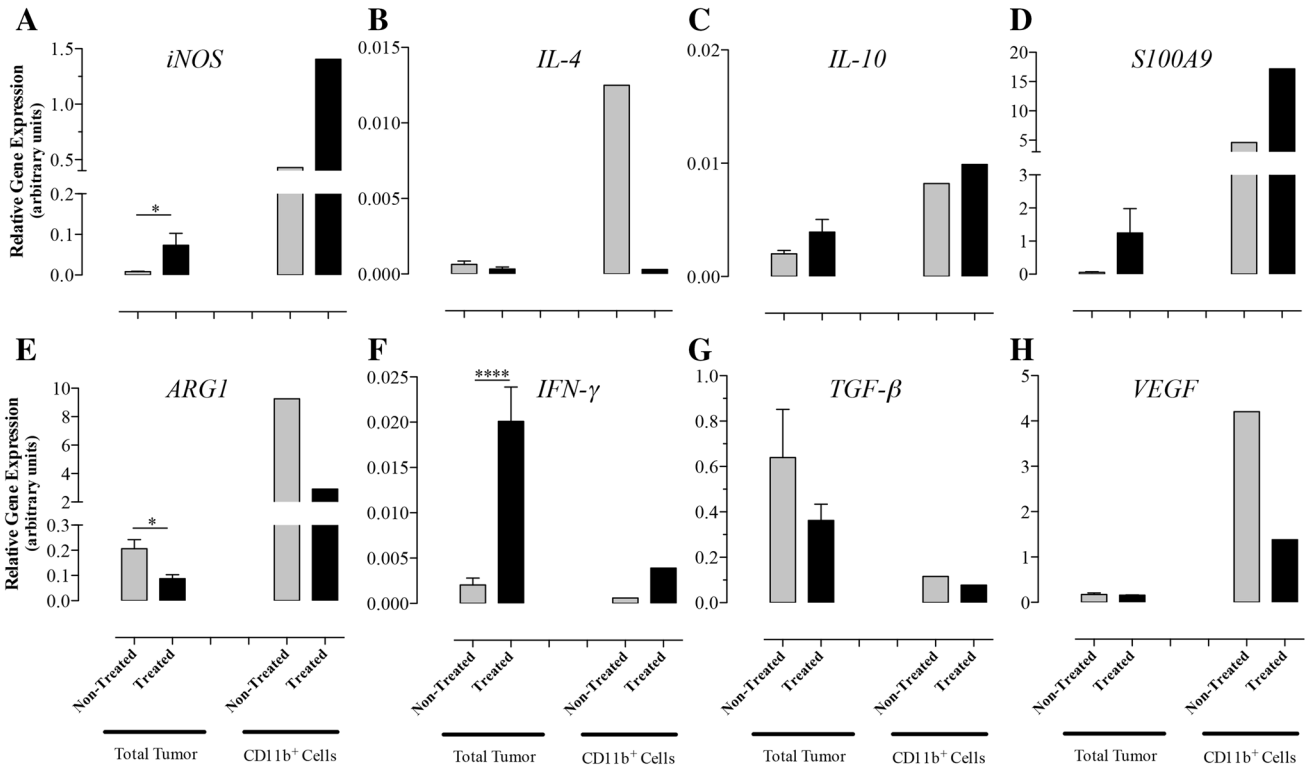

Fig. 6 Relative expression levels of iNOS (**a**), IL-4 (**b**), IL-10 (**c**), S100A9 (**d**), ARG1 (**e**), IFN-γ (**f**), and TGF-β (**g**) and VEGF (**h**) mRNA isolated from tumor-infiltrating cells or purified intratumoral myeloid cells. Tumor cells were isolated at day 8 post-inoculation with Salmonella (treated group) or saline (non-treated group). For total tumor groups, each data point represents the mean ± SEM of 3 mice. For the purified CD11b$^+$ groups, mRNA was isolated from a pool of cells obtained from 5 to 6 tumors per group. *Asterisks* denote statistically significant differences between the indicated groups (***$p < 0.001$; *$p < 0.05$). The results are representative of two independent experiments

assessed by determining the level of IFN-γ released into the culture supernatant 24 h later. As shown in Fig. 7a, addition of CD11b$^+$ cells isolated from 7 day-infected spleens to normal T cells (at 1:10 ratio, respectively) suppressed IFN-γ production (mean of 34.2 ± 6.1 %; "infected control" group), which is in agreement with earlier studies [8, 36, 37]. As expected, no suppression of the response was observed when splenic CD11b$^+$ cells from non-infected animals were used (Fig. 7a; "saline control" group). Next, splenic CD11b$^+$ cells isolated from tumor-bearing mice 7 days following inoculation with Salmonella (treated or "T" groups) or saline (non-treated or "NT" groups) were used (Fig. 7b). Co-culture with splenic myeloid cells from non-treated, tumor-bearing mice at a final ratio of 10 or 1 % resulted in significant inhibition of IFN-γ secretion (41.7 ± 2.0 and 31.3 ± 4.6 %, respectively; Fig. 7b, "NT" groups). Equivalent suppression of the T cell response was observed in co-cultures with splenic CD11b$^+$ cells isolated from Salmonella-treated, tumor-bearing animals (40.3 ± 1.3 and 28.2 ± 8.3 %, respectively; Fig. 7b, "T" groups). Finally, CD11b$^+$ cells were isolated from the tumors of animals with or without treatment with Salmonella organisms (Fig. 7c). Co-culture with CD11b$^+$ cells from non-treated tumors resulted in marked inhibition of T cell responsiveness (38.1 ± 3.0 and 27.3 ± 1.8 % at 10 and 1 % ratio, respectively; "NT" groups). Interestingly, however, significant loss of suppressive capacity was observed when T cells were co-cultured with intratumoral CD11b$^+$ cells of Salmonella-treated mice (Fig. 7c; "T" groups). The degree of T cell suppression observed in these co-cultures was reduced to 19.7 ± 2.0 and 9.2 ± 5.8 % at final 10 and 1 % cell ratios, respectively. This represents a significant reduction in suppressive capacity (48.3 %; $p = 0.002$ and 66.3 %; $p = 0.041$, respectively) compared with myeloid cells of non-treated tumors (Fig. 7c, compares corresponding "NT" and "T" groups).

Discussion

Systemic administration of facultative anaerobic bacteria, such as *S. typhimurium*, to tumor-bearing mice can effectively retard tumor growth. The capacity of Salmonella strains to achieve this appears to be dependent on their

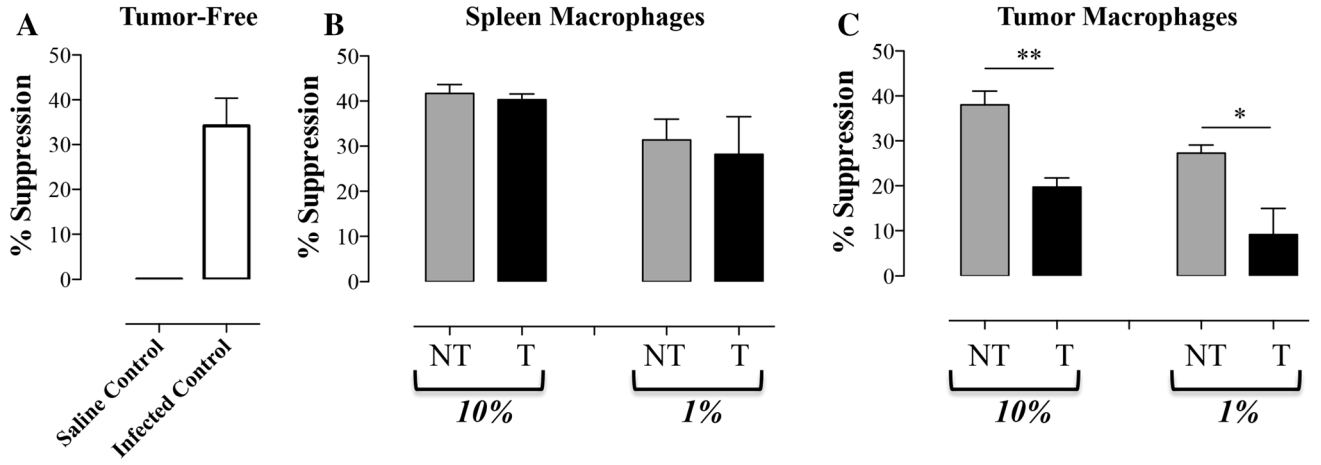

Fig. 7 Reduced suppressive capacity of intratumoral myeloid cells following Salmonella treatment. **a** $CD11b^+$ cells were isolated from the spleens of normal or Salmonella-infected mice and added at a final concentration of 10 % to cultures of $CD4^+$ T cells. Alternatively, $CD11b^+$ cells were isolated from the spleens (**b**) or tumors (**c**) of tumor-bearing mice, with or without Salmonella treatment ("NT": non-treated, "T": treated), and added at a final concentration of 1 or 10 % to CD4 T cells. All cultures were set up in triplicates and IFN-γ production was assayed in supernatants collected after 24 h. The data are expressed as percentage suppression in IFN-γ production compared with cultures containing stimulated T cells alone. *Asterisks* denote statistically significant differences between the indicated groups (**$p < 0.01$; *$p < 0.05$)

ability to stimulate the host's innate immune system. Bacterial strains with mutated lipid A or defective purine synthesis are unable to inhibit tumor growth due to poor tissue colonization [38]. The preferential targeting of Salmonella organisms to tumors is facilitated by TNF-α-mediated increase in intratumoral blood vessel permeability and the ability of Salmonella to respond to chemoattractants released by dying tumor cells [39, 40]. Being motile, Salmonella organisms invade tumor tissue efficiently and are able to reach deep into poorly vascularized regions. Moreover, the hypoxic conditions, which represent a major hindrance to conventional radiotherapy and chemotherapy, provide ample environment for the growth and proliferation of Salmonella organisms.

Once in the tumor, Salmonella organisms were shown to be inherently cytotoxic to a variety of tumor cell types, causing the tumors to regress [19]. The capacity of Salmonella to induce the host's innate immune responses, such as infiltration of tumor tissue by inflammatory cells and secretion of proinflammatory cytokines, is partly responsible for their anti-cancer properties [41, 42]. Salmonella could also increase accessibility of tumor tissue to innate immune cells, including DCs, by upregulating the expression of connexin 43 by tumor cells, a key cellular protein involved in the formation of gap junctions [43]. The increase in gap junction formation leads to enhanced cross-presentation of tumor antigens and hence more effective anti-tumor T cell responses. Thus, cancer treatment with oncolytic Salmonella organisms relies not only on the inherent anti-tumor properties of the bacteria but also is greatly aided by cooperation with the host's immune system. Our findings with a number of different immune-deficient mouse strains suggest that the effectiveness of Salmonella anti-tumor treatment relies mostly on the induction of innate immune responses through the TLR-MyD88 signaling pathway. This is consistent with a previous report demonstrating that Salmonella treatment of tumor-bearing mice is rather ineffective in the absence of TLR4, a receptor for the innate recognition of Gram-negative bacterial LPS by macrophages and neutrophils [42].

Since immune responses within the tumor microenvironment are largely regulated by myeloid suppressor cells, we reasoned that Salmonella treatment may also target these cells. In the present study, we evaluated the capacity of attenuated Salmonella to influence the phenotype and functional activity of myeloid suppressor cells in mice with established melanoma. Our findings demonstrate an apparent dichotomy in the effect of treatment on myeloid cells in the spleen and tumor tissue. Thus, while the systemic treatment of tumor-bearing hosts with Salmonella leads to increased frequency of classically activated, proinflammatory, $CD11b^+/Gr\text{-}1^+$ myeloid cells in both spleen and tumor, reversal of the suppressive functional capacity was only observed in intratumoral myeloid cells. This suggests that splenic and intratumoral myeloid cells are differentially regulated by Salmonella organisms.

Infection with attenuated Salmonella induces a transient state of splenomegaly that normalizes over a period of 4 weeks as the infection is resolved by the host's immune system [32, 44]. Splenomegaly is associated with a large influx of inflammatory cells, largely made up of neutrophils and macrophages, into the spleen of infected animals.

Systemic administration of attenuated Salmonella is also associated with the induction of IL-12, IL-18, IFN-γ, TNF-α, iNOS and reactive oxygen intermediates within 24–48 h of inoculation, a process that helps to limit intracellular bacterial proliferation and ultimately leads to the development of a protective Th1-mediated immune response [32, 45]. Curiously, however, Salmonella infection also leads to the emergence of nitric oxide-secreting myeloid cells with suppressive capacity [8, 36, 37]. Given these effects on the immune system, the influence of Salmonella organisms on anti-tumor immune responses is likely to be quite complex.

Treatment with attenuated Salmonella was associated with increased cell ratios of both granulocytic ($CD11b^{+}/Gr\text{-}1^{H}$) and monocytic ($CD11b^{+}/Gr\text{-}1^{Int}$) MDSCs within the spleen as well as an increase in $CD11b^{+}/Gr\text{-}1^{+}$ myeloid cells within the tumor tissue. Salmonella treatment led to an increase in the expression of several IFNγ-regulated proteins, such as MHC class II and costimulatory molecules, which are known to enhance antigen-presentation of myeloid cells. These alterations correlated with a significant enhancement in IFN-γ expression in both spleen and tumor tissue. By contrast, expression of IL-4 was inhibited in both splenic and intratumoral myeloid cells following treatment. A notable exception to this co-regulation was the differential effect of Salmonella treatment on ARG1 expression. While the expression of ARG1 was substantially decreased in intratumoral myeloid cells after Salmonella treatment, it was increased in splenic myeloid cells (compare Figs. 4d, 6e). ARG1 acts to hydrolyze L-arginine to urea and ornithine, ultimately leading to the production of reactive nitrogen intermediates, such as peroxynitrites, which is a potent inhibitor of T cell responses [46]. Given the fact that, within the tumor microenvironment, ARG1 expression is coordinated by IL-4/IL-13 [47], it is perhaps not surprising that Salmonella treatment also resulted in a dramatic loss of IL-4 expression in intratumoral myeloid cells. In contrast, expression of iNOS in intratumoral myeloid cells was increased after Salmonella treatment. Therefore, our current findings suggest that Salmonella-mediated inhibition of ARG1 expression in tumor myeloid cells appears to be mainly responsible for the loss of their T cell suppressive capacity. This is consistent with the view that ARG1 expression is the prototypical marker for defining tumor-associated suppressor macrophages [4]. Importantly, our findings also highlight the IL-4-IL-13/ARG1 axis as a target of Salmonella treatment in cancer immunotherapy. A recent report demonstrated that intratumoral injection of attenuated Salmonella bacteria also results in increased percentage of $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells [48]. These cells were shown to secrete TNF-α, but their functional suppressive activity was not directly assessed.

A previous report demonstrated that treatment with attenuated *Salmonella choleraesuis* led to an inhibition of tumor growth that was associated with an upregulation in the expression of IFN-γ and IFN-γ-dependent chemokines CXCL9 and CXCL10 [42]. This response was dependent on the expression of a functional TLR4 protein, suggesting that the host immune response to LPS plays a critical role in this process. Moreover, inhibition of tumor growth correlated with decreased angiogenesis and increased level of apoptosis within the tumor tissue. Importantly, CXCL9 and CXCL10 chemokines are known to induce cell recruitment into tumor tissue and to exert potent anti-angiogenic activity [49, 50]. The previously reported reduction in microvessel density [23] and the current findings of decreased VEGF expression in macrophages of Salmonella-treated animals suggest an inhibition in the activity of tumor-associated angiogenic macrophages. Taken together, our data identify MDSCs and TAMs as targets of treatment with Salmonella, thereby providing another mechanism by which Salmonella organisms mediate their anti-tumor effects. The data also highlight the potential of utilizing this pathway in boosting immune responses to tumors.

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Conflict of interest The authors declare no competing interests.

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