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Antitumor Activity of the TLR-5 Ligand Flagellin in Mouse Models of Cancer[1]

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Flagellin, the structural protein subunit of the bacterial flagellum, is specifically recognized by TLR-5 and has potent immunomodulatory effects. The antitumor effects of purified *Salmonella typhimurium* flagellin were evaluated in mice transplanted s.c. with a weakly immunogenic murine tumor or with its variant stably transfected to express the highly antigenic human HER-2 oncoprotein. Peritumoral administration of flagellin 8–10 days after tumor implantation did not affect the growth rate of the weakly immunogenic tumor but significantly inhibited growth of the antigenic variant tumor. In contrast, flagellin administered at the time of implantation of the antigenic tumor led to accelerated tumor growth. These contrasting effects of flagellin on tumor growth correlated with the type of immune response induced; i.e., late flagellin administration was associated with an increased IFN-γ:IL-4 ratio and the decreased frequency of $CD4^+CD25^+$ T regulatory cells, whereas flagellin treatment at the time of tumor implantation decreased the IFN-γ:IL-4 ratio and increased $CD4^+CD25^+$ T cell frequency. When the early flagellin treatment was combined with administration of CpG-containing oligodeoxynucleotides, tumor growth was completely suppressed, indicating synergy between flagellin and CpG-containing oligodeoxynucleotides. Together, these data provide evidence that flagellin can have contrasting effects on tumor growth. *The Journal of Immunology,* **2006, 176: 6624–6630.**

The innate immune system detects infectious agents and distinguishes among different classes of pathogens by using pattern recognition receptors that are expressed in a cell-specific and compartmentalized manner on innate immune cells (1). The best characterized family of pattern recognition receptors, the TLRs, can sense different bacterial wall components as well as other microbial products such as CpG-DNA, dsRNA, and flagellin (2–4). Because TLRs can modulate both innate and adaptive immunity, stimulation of TLR pathways through their ligands represents a novel opportunity to activate an anticancer immune response. Indeed, different TLR ligands have been evaluated in various experimental tumor models (5–13), and some of these ligands are under investigation in human clinical trials (14–18). TLR-5 specifically recognizes the ligand flagellin, the structural protein subunit of the bacterial flagellum, and is expressed on innate immune cells, such as monocytes and immature dendritic cells, as well as on epithelial cells (19, 20). Thus, studies have shown that flagellin activates not only macrophages, monocytes, and dendritic cells but also intestinal and pulmonary epithelial cells, inducing the release of proinflammatory mediators (21, 22). However, although various vaccination studies have demonstrated the strong adjuvant activity of flagellin (23, 28), no studies to date have investigated its potential role in activating an immune response in cancer immunotherapy.

In the present study, we assessed the antitumor effect of flagellin treatment in tumor mouse models.

Materials and Methods

Cells and reagents

Mouse mammary tumor line D2F2, derived from a spontaneous mammary tumor that arose in BALB/c hyperplastic alveolar nodule line D2 (29), was provided by Dr. W. Z. Wei (Karmanos Cancer Institute, Detroit, MI). This NK cell-sensitive line was maintained in DMEM supplemented with 10% heat-inactivated FBS, 2.5 mM 2-ME, 0.5 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM MEM, nonessential amino acids, and antibiotics. The variant D2F2/E2 cell line expressing wild-type human ErbB-2 (provided by Dr. W. Z. Wei) was obtained as described (30) and maintained in medium as above but containing 0.8 mg/ml G418.

Endotoxin-free purified flagellin from *Salmonella typhimurium* was obtained from InvivoGen. Purified single-stranded phosphorothioated oligodeoxynucleotide (ODN)[4] 1668 (5′-TCCATGACGTTCCTGATGCT-3′) containing a CpG motif (31) was synthesized in endotoxin-free conditions by MWG Biotech.

Mice and in vivo experiments

BALB/c and athymic (nude) mice were purchased from Charles River Laboratories. All mice (6–8 wk of age) were maintained in laminar flow rooms at constant temperature and humidity with food and water ad libitum. All experiments were conducted in accordance with U.K. Coordinating Committee on Cancer Research guidelines and approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan. In experiments to evaluate therapeutic activity of flagellin in mice previously transplanted with the two different tumors (late administration), mice were inoculated s.c. with 3×10^5 (BALB/c mice) or 5×10^5 (athymic mice) D2F2/E2 or D2F2 tumor cells in the right flank and 8–10 days later were inoculated in the vicinity of the tumor with purified flagellin (5 μg/200 μl) or with 200 μl of saline daily for 4 days. Tumor growth was monitored twice weekly, and two perpendicular diameters of the tumor mass were recorded. Tumor volumes were calculated as $\pi/6 \times \text{length} \times \text{width}^2$. In experiments to evaluate antitumor activity of flagellin when administered immediately with tumor cells (early administration), BALB/c mice were inoculated s.c. with 3×10^5 D2F2/E2 or D2F2 tumor cells and treated at the site of tumor injection with 5 μg of flagellin or saline on the

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[4] Abbreviations used in this paper: ODN, oligodeoxynucleotide; SI, stimulation index; Treg, T regulatory cell.

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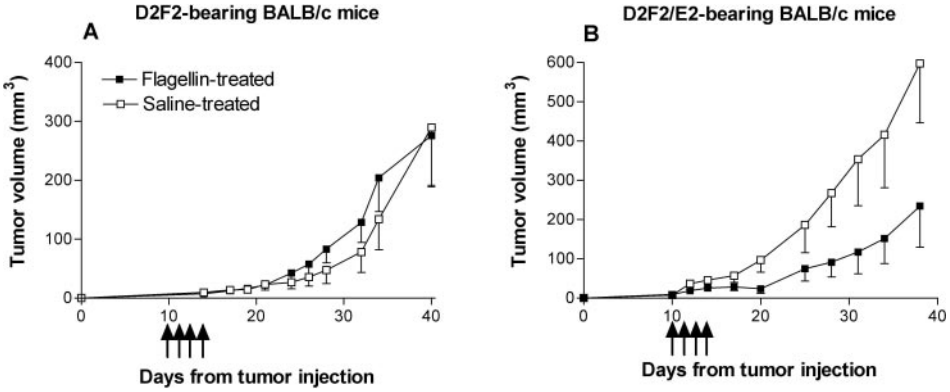

FIGURE 1. Antitumor activity of flagellin (late treatment) in D2F2- and D2F2/E2-bearing mice. Tumor volumes (mean ± SE) in BALB/c mice (10 mice per group) treated with flagellin (■) or saline (□) for 4 days (arrows) at the tumor site (late administration) 10 days after injection of D2F2 (*A*) or D2F2/E2 (*B*) tumor cells.

day of tumor injection and on the following 3 days. In experiments to evaluate antitumor activity of flagellin and CpG-ODN, BALB/c mice were injected s.c. with D2F2/E2 tumor cells and treated with flagellin (5 μg) and CpG-ODN (40 μg) at the tumor site on the day of tumor injection and on the following 3 days (early administration) or 8–10 days later for 4 days (late administration). Tumor growth was monitored and evaluated as above.

Proliferation assay

BALB/c mice (four mice per group) were injected s.c. with 3×10^5 D2F2/E2 tumor cells and 10 days later were treated in the vicinity of the tumor with purified flagellin (5 μg/200 μl) or with 200 μl of saline daily for 4 days. Fifteen days later, splenocytes were collected and plated at 1×10^5 cells/well in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2.5 mM 2-ME, 0.5 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM MEM, nonessential amino acids, 2 mM HEPES buffer (complete medium), and antibiotics in a 96-well flat-bottom plates. Stimulator cells (D2F2/E2 and D2F2) were irradiated at 20,000 rad and plated at 1×10^4 cells/well. Each sample was performed in six replicates. Cells were incubated at 37°C in 5% CO_2 for 5 days and pulsed with [^{3}H]thymidine at 1 μCi/well for 18 h. Thymidine incorporation was measured with a Trilux β-scintillation counter (Wallac). Control wells were cultured with medium alone to determine background proliferation. Results were reported as the stimulation index (SI; mean cpm of stimulated cells/mean cpm of medium-cultured cells).

Cytokine production by lymph node cells and splenocytes

The Th1- or Th2-type cytokine pattern induced by flagellin treatment was evaluated in groups of mice (three to four mice per group) injected s.c. in the right hind footpad with 5 μg of flagellin alone, with 3×10^5 viable tumor cells and 5 μg of flagellin administered immediately, with 3×10^5 viable tumor cells and 5 μg of flagellin plus 40 μg of CpG-ODN administered immediately, or with 3×10^5 viable tumor cells and 5 μg of flagellin administered at the site of injection 3 days after tumor cell injection. Mice were sacrificed at day 6 after flagellin treatment, and popliteal lymph nodes were aseptically removed. Mice injected with tumor cells alone were sacrificed at day 6 (as a control for early flagellin administration) or 9 (as a control for late flagellin administration) from tumor implantation. Lymphocytes were mechanically dissociated and cultured (2×10^5 cells/well) in 96-well flat-bottom plates precoated or not with anti-CD3 mAb (1 μg/well) (BD Pharmingen) at 37°C for 18 h. Supernatant was collected and tested for IFN-γ and IL-4 production by ELISA (Endogen).

IFN-γ and IL-4 production was also evaluated in mice injected s.c. with D2F2/E2 tumor cells (3×10^5) and treated with 5 μg of flagellin or saline on the day of tumor injection and in the following 3 days. After 15 days, spleens were aseptically removed from all mice and plated at 8×10^5 cells/well in complete medium (as described above). Stimulator cells (D2F2/E2 and D2F2) were irradiated (20,000 rad) and plated at 8×10^4 cells/well. Each sample was plated in four replicates. Splenocytes and stimulator cells were incubated at 37^{0}C in 5% of CO_2 for 5 days and IFN-γ and IL-4 release into the supernatant was evaluated by ELISA.

Flow cytometry

The percentage of $CD4^+CD25^+$ T cells in blood and in spleen was evaluated in tumor-free mice injected i.p. with flagellin (5 μg) or not injected, and in tumor-bearing mice not treated or treated with flagellin (5 μg) at day 0 (early treatment) or at day 10 after tumor inoculation (late treatment) (four mice per group). Flagellin treatment was repeated on the following 3 days. Peripheral blood lymphocytes and splenocytes were placed in 50 μl of PBS containing 2% FCS and stained with FITC-conjugated anti-CD4 (H129.19; BD Pharmingen) and PE-conjugated anti-CD25 (PC61 5.3) (Caltag) Abs at concentrations recommended by the manufacturer. After incubation of the samples for 25 min at 4°C, erythrocytes were lysed by the addition of 2 ml of ammonium chloride lysing solution (1.5 M NH_4Cl, 100 nM $KHCO_3$, 10 nM Na_4EDTA, pH 7.2) and incubation at 4°C for 15 min. For intracellular detection of Foxp3, after staining with anti-CD4 and anti-CD25 Abs, cells were fixed and permeabilized (BD Cytofix/Cytoperm PLUS KIT; BD Pharmingen) and then blocked with anti-Fc receptor Ab 2.4G2 (BD Pharmingen) for 15 min at 4°C. After incubation with PE-Cy5-conjugated anti-mouse/rat Foxp3 (FJK-16s) Ab (eBioscience) for 25 min at 4°C, erythrocytes were lysed as above. Lymphocytes and splenocytes were

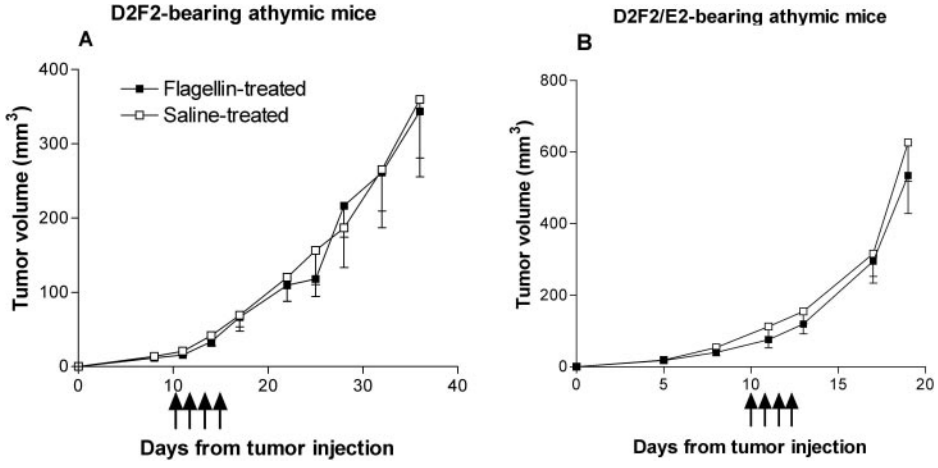

FIGURE 2. Antitumor activity of flagellin (late treatment) in D2F2- and D2F2/E2-bearing athymic mice. Tumor volumes (mean ± SE) in athymic (nude) mice (10 mice per group) treated with flagellin (■) or saline (□) for 4 days (arrows) at the tumor site (late administration) 10 days after injection of D2F2 (*A*) or D2F2/E2 (*B*) tumor cells.

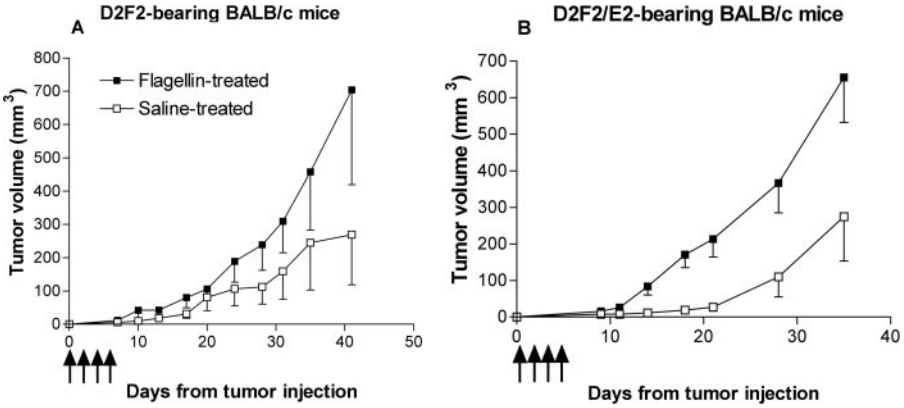

FIGURE 3. Antitumor activity of flagellin (early treatment) in D2F2- and D2F2/E2-bearing mice. Tumor volumes (mean ± SE) in BALB/c mice injected with D2F2 (nine mice per group) (*A*) or D2F2/E2 (10 mice per group) (*B*) tumor cells and treated with flagellin (■) or saline (□) at the time of tumor implantation and in the following 3 days (arrows).

washed twice and analyzed by FACSCalibur flow cytometry. Data analysis was performed using CellQuest software (BD Biosciences).

Statistical analysis

Differences in mean tumor volume, cytokine levels, cell proliferation, and percentage of $CD4^+CD25^+$ T cells in the different experimental groups were compared using an unpaired *t* test. Values of $p < 0.05$ (two-tailed) were considered significant.

Results

Flagellin activity on tumor growth

The antitumor immune response induced by flagellin was evaluated in mice bearing the weakly immunogenic, NK cell-sensitive murine breast carcinoma D2F2 or its antigenic variant D2F2/E2 stably transfected with the human *HER-2* oncogene. Two groups of 20 BALB/c mice were injected s.c. with D2F2 cells or D2F2/E2 cells. Ten days later, each group was randomly divided into two and treated daily at the tumor site for 4 days with purified flagellin or with saline. In D2F2 tumor-bearing mice, flagellin treatment did not modify tumor growth rate (Fig. 1*A*), whereas in D2F2/E2 tumor-bearing mice, flagellin treatment significantly reduced tumor growth as compared with control mice ($p = 0.04$) (Fig. 1*B*). No differences in tumor growth rate were detected between flagellin-treated and saline-treated groups in athymic nude mice bearing D2F2 or D2F2/E2 tumors (Fig. 2). These findings indicate that in our models, flagellin induces antitumor activity against the antigenic tumor but not against the parental tumor and that, consistent with the data of Wei et al. (32), T cells play a role in the protection against the D2F2/E2 tumor. To confirm that the antitumor flagellin effect was T cell mediated, splenocytes obtained from mice injected with the antigenic D2F2/E2 tumor and treated 10 days later with flagellin or saline were incubated with irradiated D2F2/E2 or D2F2 cells for 5 days at an effector-stimulator ratio of 10:1. Cell proliferation was measured by $[^3H]$thymidine incorporation, and the SI was calculated using splenocytes incubated in medium alone. Flagellin treatment increased cell proliferation significantly in response to D2F2/E2 cells (SI 18.0 ± 5.6 vs 1.6 ± 1.0 in saline-treated mice; $p = 0.0012$). T cell flagellin-induced proliferation was not confined to the HER-2 Ag because splenocytes from flagellin-treated mice also proliferated in response to D2F2 cells (SI 13.6 ± 4.6 in flagellin-treated vs 2.0 ± 3.0 in saline-treated mice; $p = 0.014$).

To determine whether an earlier administration of flagellin might improve its antitumor activity, s.c. D2F2/E2-injected BALB/c mice were treated with flagellin or saline on the day of tumor injection and on the following 3 days; tumor growth rate was unexpectedly increased in flagellin-treated mice compared with saline-treated mice ($p = 0.03$; Fig. 3*B*). No significant increase in tumor growth rate was induced by flagellin treatment in mice bearing D2F2 tumors (Fig. 3*A*).

Antitumor immune response induced by flagellin

The contrasting effects of flagellin on tumor growth might reflect a shift in the Th1/Th2 response or a change in the frequency of the $CD4^+CD25^+$ T regulatory cell subset (Tregs) or both, as suggested by previous reports of a flagellin-induced Th1/Th2 shift (23, 24, 33–36); TLR-5 expression on T regulatory cells (Tregs) might also play a role (37). We analyzed the Th1 and Th2 cytokine secretion pattern in mice injected intrafootpad with D2F2/E2 cells and treated with flagellin at the same site either immediately or 3 days later. On day 6 after flagellin injection, popliteal lymph nodes draining the injection site were harvested and seeded in plates precoated or not with anti-CD3 mAb. Cytokine assay of supernatants from anti-CD3 mAb-coated wells revealed low production of

Table I. *Cytokine secretion pattern produced by popliteal lymph nodes*

Treatment	IFN-γ (pg/ml)	IL-4 (pg/ml)	IFN-γ:IL-4 Ratio
None	5,994 ± 1,246[a]	7.2 ± 2.4	873 ± 125
Flagellin alone	1,899 ± 1,161	8.0 ± 5.0	240 ± 5.7
D2F2 + flagelllin early	31,565 ± 4,613	305 ± 49	104 ± 8.7
D2F2 alone[b]	30,783 ± 6,696	247 ± 71	133 ± 23
D2F2 + flagellin late	13,038 ± 4,103	87 ± 4.0	118 ± 48
D2F2 alone[c]	12,988 ± 2,567	114 ± 10	112 ± 26
D2F2/E2 + flagellin early	55,469 ± 621	581 ± 150	109 ± 27
D2F2/E2 alone[b]	69,310 ± 2,552	298 ± 41	242 ± 36
D2F2/E2 + flagellin late	50,286 ± 5,584	266 ± 29	192 ± 3.4
D2F2/E2 alone[c]	39,269 ± 7,111	337 ± 32	115 ± 12

[a] Data represent means ± SE ($n = 3$–4).
[b] Mice sacrificed at day 6 from tumor implantation (as control for early flagellin administration).
[c] Mice sacrificed at day 9 from tumor implantation (as control for late flagellin administration).

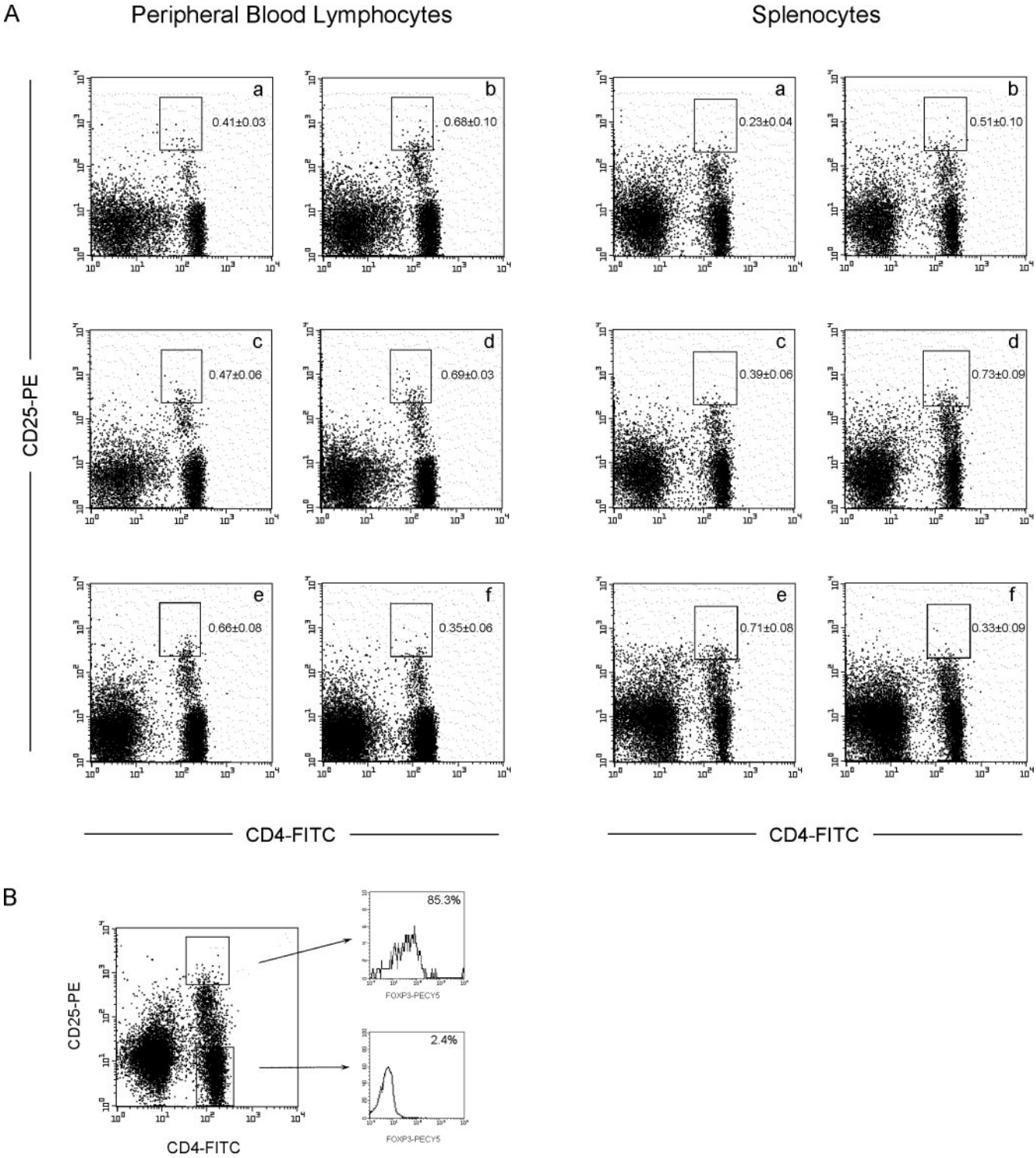

FIGURE 4. Effect of flagellin treatment on $CD4^+CD25^+$T cell percentage in mouse blood and spleen. *A,* Percentage of $CD4^+CD25^+$ T cells in blood and spleen evaluated by flow cytometry: Mice were untreated or treated i.p. with flagellin (*a* and *b,* respectively); mice injected with D2F2/E2 tumor cells untreated or treated with flagellin at time of tumor injection (early treatment) (*c* and *d,* respectively) or 10 days later (late treatment) (*e* and *f,* respectively). Blood and spleens were collected 7 days after the first flagellin treatment. Data represent the percentage (mean ± SE) of $CD4^+CD25^+$ T cells among $CD4^+$ cells. *B,* Intracellular staining of Foxp3 transcriptional factor. Percentage of $Foxp3^+$ cells among $CD4^+CD25^+$ and $CD4^+CD25^-$ gated cells are indicated. PECy5, Phycoerythrin-Cy 5.

IFN-γ and IL-4 in non-tumor-bearing mice treated or not with flagellin (Table I). In mice injected with tumor cells and 3 days later with flagellin, the IFN-γ:IL-4 ratio was significantly higher than that in mice injected with tumor cells alone ($p = 0.004$), whereas in mice injected with flagellin and tumor cells contemporaneously, the IFN-γ:IL-4 ratio was significantly reduced as compared with the group treated with tumor cells alone ($p = 0.045$; Table I).

The weakly immunogenic D2F2 tumor was associated with lower IFN-γ:IL-4 ratios than those observed in mice injected with

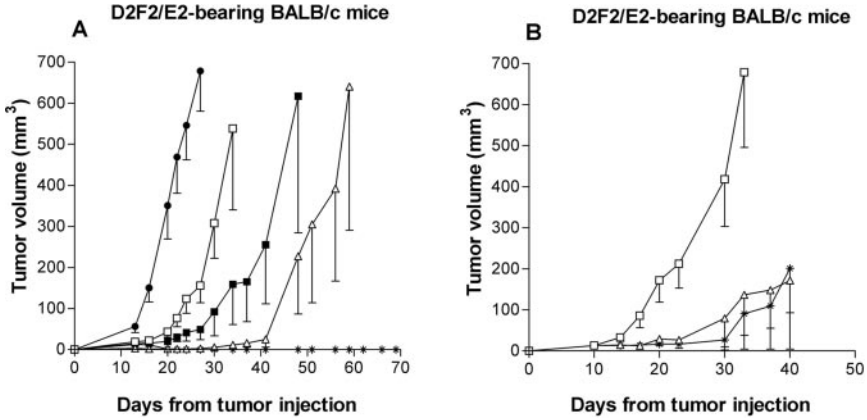

FIGURE 5. Therapeutic synergy of flagellin and CpG-ODN. Tumor volumes (mean ± SE) in BALB/c mice injected with D2F2/E2 tumor cells (nine mice per group) (*A*) and treated early with flagellin (●), late with flagellin (■), early with CpG-ODN (△), early with flagellin plus CpG-ODN (∗), or with saline (□). *B,* BALB/c mice injected with D2F2/E2 tumor cells (seven mice per group) and treated late with CpG-ODN (△), late with flagellin plus CpG-ODN (∗), or with saline (□).

immunogenic D2F2/E2 tumor ($p = 0.048$). Early administration of flagellin with D2F2 cells led to a reduction, although not significant, in the IFN-γ:IL-4 ratio, whereas no increase of this ratio was observed in late flagellin-treated mice (Table I).

To determine whether the loss of protection observed in D2F2/E2 tumor-bearing mice treated with flagellin immediately after tumor injection was related to a shift in the Th profile of HER2-specific T cells, splenocytes from mice injected with D2F2/E2 tumor cells and treated contemporaneously with flagellin or saline were stimulated in vitro for 5 days with irradiated D2F2/E2. Decreased production of IFN-γ was observed in flagellin-compared with saline-treated mice (mean ± SE, IFN-γ 495 ± 138 pg/ml vs 6185 ± 669 pg/ml; $p = 0.0002$). A reduction, although not significant, in IFN-γ production was also observed when splenocytes from flagellin-treated mice were stimulated with irradiated D2F2 tumor cells (mean ± SE, IFN-γ 2373 ± 918 pg/ml and 4371 ± 816 pg/ml flagellin and saline-treated, respectively; $p = 0.1$). IL-4 production was undetectable in all samples.

The effect of flagellin on $CD4^+CD25^+$ T cell frequency was evaluated by FACScan analysis of peripheral blood cells and splenocytes harvested from tumor-bearing and non-tumor-bearing mice. Blood and spleen from non-tumor-bearing mice injected with flagellin revealed an increase in the percentage of $CD4^+$ $CD25^+$ T cells (from 0.41 ± 0.03 to 0.68 ± 0.1, $p = 0.049$, in blood; from 0.23 ± 0.04 to 0.51 ± 0.1, $p = 0.047$, in spleen) (Fig. 4*A*). Intracellular staining for the Foxp3 transcription factor, a further specific marker for Treg cells, demonstrated that >85% of $CD4^+CD25^+$ gated cells were $Foxp3^+$ (Fig. 4*B*). Analysis of D2F2/E2 tumor-bearing mice untreated or treated with flagellin immediately after tumor injection (early administration) revealed a significant expansion of Treg cells in blood and spleen as compared with mice injected with tumor cells alone (from 0.47 ± 0.06 to 0.69 ± 0.03, $p = 0.025$, in blood; from 0.39 ± 0.06 to 0.73 ± 0.09, $p = 0.024$, in spleen). By contrast, the percentage of Treg cells in mice treated with flagellin at day 10 after tumor inoculation (late administration) was significantly reduced as compared with that observed in mice injected with tumor cells alone (from 0.66 ± 0.08 to 0.35 ± 0.06, $p = 0.029$, in blood; from 0.71 ± 0.08 to 0.33 ± 0.09, $p = 0.023$, in spleen) (Fig. 4*A*). Similar analysis in blood of D2F2 tumor-bearing mice treated with flagellin immediately after tumor injection also revealed an expansion of Treg cells as compared with untreated mice (from 0.78 ± 0.15 to 0.51 ± 0.14, $p = 0.043$), whereas no reduction in the percentage of these cells was observed when flagellin was administered late (from 0.86 ± 0.20 to 0.79 ± 0.16).

Therapeutic synergy of flagellin and CpG-ODN

Based on our findings above indicating that administration of flagellin when the immune system is already activated vs a Th1 response favoring this polarization, we analyzed the antitumor activity of flagellin combined with the TLR-9 ligand CpG-ODN, which promotes Th1 polarization (38) and exerts an antitumor effect in different experimental models (9–11). Five groups of BALB/c mice (nine mice per group) were injected s.c. with D2F2/E2 cells and treated as follows: group 1 received an early administration of flagellin; group 2 received late administration of flagellin; group 3 received CpG-ODN alone; group 4 received early administration of flagellin plus CpG-ODN; and group 5 received saline. Consistent with our results above, tumor growth rate was significantly ($p = 0.03$) increased in mice treated with flagellin at the time of tumor injection vs the saline group, whereas a significant reduction of tumor growth was observed in mice receiving late administration of flagellin as compared with the saline group ($p = 0.04$; Fig. 5*A*). Tumor growth rate was reduced in mice treated with CpG-ODN alone ($p = 0.002$) as compared with the saline group, and this antitumor effect of CpG-ODN was significantly increased ($p = 0.006$) by flagellin treatment as compared with the CpG-ODN-treated group, because a complete inhibition of tumor growth was observed in mice receiving the combined therapy.

Analysis of the antitumor effect of combined treatment with flagellin and CpG-ODN at 10 days after D2F2/E2 tumor implantation revealed no significant increase by flagellin over the effect observed with CpG-ODN alone (Fig. 5*B*).

Discussion

In the present study, we explored the effect of the TLR-5 ligand flagellin on tumor growth and show that flagellin treatment in mice bearing tumors rendered antigenic by transfection with the human HER2 gene induced significant inhibition of tumor growth, which was not observed in mice bearing the weakly antigenic, NK cell-sensitive parental tumor (39). Unlike other TLR ligands, flagellin does not appear to involve the recruitment of innate effectors in its antitumor activity, as suggested by the complete absence of tumor inhibition in athymic mice. This absence is not due to an intrinsic resistance of the tumor because inhibition was observed when D2F2/E2 tumor-bearing athymic mice were treated with CpG-ODN (data not shown). The findings that flagellin, when presented as a purified soluble molecule, promotes mainly an adaptive immune response might rest in its protein nature, which distinguishes it from most other TLR ligands. In contrast, tumor growth was accelerated when flagellin was administered at the time of tumor

implantation. The type of immune response induced correlated with the timing of flagellin treatment and with a Th1 cytokine secretion pattern observed when flagellin was injected into palpable tumor-bearing mice, whereas a Th2 cytokine response was detected in mice receiving flagellin at the time of tumor implantation. Thus, it appears that flagellin can maintain and enhance the Th1 response, normally elicited by an antigenic tumor, whereas Th2 polarization prevails when flagellin is introduced during T cell sensitization. Indeed, increased IFN-γ:IL-4 ratios were observed in mice injected with the D2F2/E2 immunogenic tumor as compared with the D2F2 weakly immunogenic tumor. When flagellin was injected late in mice with the immunogenic tumor, it might have further activated a preactivated response to result in tumor growth inhibition. Thus, the antigenicity of the tumor might underlie the changes in the balance of the Th1 vs Th2 response. Two recent studies support the conclusion that flagellin can induce a Th2 response. In one study (24), highly purified monomeric flagellin isolated from *Salmonella* was shown to act as a Th2 adjuvant to polarize the response to OVA; a second study (36) showed that soluble recombinant flagellin is Th2-polarizing whereas native surface-bound flagellin on live *Salmonella* favors a switch to a Th1 phenotype. Thus, the context of flagellin treatment is also an important determinant of the direction of Th polarization.

TLR ligands have recently been shown to influence proliferation and suppressive activity of $CD4^+CD25^+$ Tregs through an indirect mechanism mediated by dendritic cell activity (40) and also through a direct interaction with TLRs specifically expressed on Tregs (37). Expression of TLR-4, -5, -7, and -8 on Tregs has been observed, and the TLR-4 ligand LPS was shown to elicit Treg proliferation and strengthen their suppressive activity in vitro (37). Moreover, Crellin et al. (41) have recently demonstrated in a human system that flagellin directly enhances the suppressive capacity of Treg cells and increases their expression of Foxp3. Our data indicate a time-dependent component of the effect of flagellin on $CD4^+CD25^+$ T cells. Indeed, we detected an increased frequency of Tregs in mice injected with flagellin simultaneously with immunogenic or low immunogenic tumor cells, but a reduced expansion of these cells, which naturally increase in the presence of a growing tumor (42–45), in mice bearing the immunogenic tumor and treated with flagellin several days after implantation. By contrast, no reduction in the percentage of these cells was observed in mice bearing the low immunogenic tumor and injected with flagellin later. Consistent with the observations above, this result indicates that the preactivated response produced by the immunogenic tumor is critical for the flagellin to further enhance proinflammatory response resulting in the blocking of Tregs.

$CD4^+CD25^+$ T cell activity might also influence the cytokine response to flagellin. Several findings demonstrate that Tregs can contribute to Th2 polarization by suppressing the Th1 response during parasitic infection (46, 47). $CD4^+CD25^+$ T cells might also be modulated by IL-4 produced in response to flagellin administration. Accordingly, Maerten (48) recently reported that IL-4 prevents spontaneous apoptosis of in vitro-cultured Treg cells and increases their suppressive activity on naive $CD4^+$ T cells.

The natural property of purified flagellin to induce a predominantly Th2 response, together with its ability to induce a protective response only against antigenic tumors through T cell-mediated mechanisms and independently through innate immune effectors might argue against the usefulness of flagellin in oncology. However, the combination of flagellin with other activators of the immune response via TLR might lead to an enhanced immune response and, in turn, more effective tumor immunotherapy. In an established murine tumor model, it was recently shown that the TLR-9 ligand CpG-DNA and the TLR-3 ligand dsRNA synergize in their antitumor activity (42). Moreover, in vitro-selected TLR agonist combinations have been reported to increase production of IL-12 and IL-23, leading to enhanced Th1-polarizing capacity of dendritic cells (49).

Our evaluation of the potential usefulness of flagellin in the oncological context in association with the Th1-promoting CpG-ODN revealed that administration of CpG-ODN at the time of early flagellin treatment abrogated the negative effects of flagellin on tumor growth and enhanced the CpG-ODN antitumor activity, ensuring complete tumor protection. However, no significant synergy was observed in mice receiving the combined treatment 10 days after D2F2/E2 or D2F2 (data not shown) tumor implantation. Together, our data indicate that the TLR-5 ligand flagellin has contrasting effects on tumor growth rate. Thus, although the discovery of TLRs and their ligands has enabled analyses that no longer involve the use of whole bacterial extracts, clinical application of these ligands in cancer immunotherapy must be preceded by a thorough understanding of their immunological properties.

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