SUPPLEMENTARY INFORMATION FOR

Title: STING ligand c-di-GMP improves cancer vaccination against metastatic breast cancer

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Running title: c-di-GMP improves Listeria-Mage-b against metastatic breast cancer

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Figure S1: Various immunization protocols for LM-Mb and c-di-GMP. Various immunization protocols have been tested in a metastatic breast tumor model 4T1, as described previously (1). Briefly, c-di-GMP and LM-Mb was tested at a high dose in a semi-therapeutic and a therapeutic setting. For the semi-therapeutic immunizations (protocol A) (A), mice were immunized ip with c-di-GMP (150 nmol) on days 0, 7, and 14, and with LM-Mb ip on days 1, 8, and 15, while 4T1 tumor cells ($10^5$) were injected into the mammary fat pad on day 3. For the therapeutic immunizations (protocol B)(B), mice were injected with 4T1 tumor cells ($10^5$) in the mammary fat pad on day 0, immunized intraperitoneally (ip) with c-di-GMP (150 nmol) on days 3, 10, and 17, and with LM-Mb ($10^7$ CFU) (ip) on days 4, 11, and 18. We also tested low dose of c-di-GMP and LM-Mb in a therapeutic setting. Briefly, 4T1 tumor cells ($10^4$) were injected into the mammary fat pad on day 0, and c-di-GMP (0.01 nmol) was administered every day ip, starting on day 3, while LM-Mb ($10^4$ CFU) was administered ip on days 4, 7, 10, 13, and 16 (protocol C)(C). Finally, we tested one high dose of c-di-GMP (150 nmol), followed by multiple low doses of c-di-GMP. Briefly, 4T1 tumor cells ($10^4$) were injected into the mammary fat pad on day 0, and one high dose of c-di-GMP (150 nmol) was administered on day 3, followed by low dose c-di-GMP (0.01 nmol) every day ip for 16 days (protocol D)(D).

Figure S2: LM-Mb and c-di-GMP is effective against metastatic breast cancer in a therapeutic setting. BALB/c mice received three therapeutic immunizations with high doses of c-di-GMP and LM-Mb according Immunization protocol B (see Fig S1B). All mice were euthanized nineteen days after the first immunization and analyzed for the
number of metastases (A) and tumor weight (B). All groups were compared to LM-Mb+c-di-GMP. The results shown here is the average of three experiments with n=5 mice per group. Mann-Whitney test. *p<0.05, **<0.01, ***<0.001, ****<0.0001. Values p<0.05 were considered statistically significant.

**Figure S3: High dose c-di-GMP killed 4T1 tumor cells directly.** 4T1 tumor cells (2000 cells in 0.1 ml) were incubated with 150 nmol of c-di-GMP for 24h. The viability of the 4T1 tumor cells was assayed by MTT assay. The results shown here is the average of two experiments. Unpaired t test. *p<0.05, **<0.01, ***<0.001, ****<0.0001. Values p<0.05 were considered statistically significant.

**Figure S4: Effect of c-di-GMP on cell viability and cell death.** The STING-positive 4T1 cells (A) and STING-negative HEK293T cells (B) (2000 cells in 0.1 ml) were cultured with various doses of c-di-GMP (15, 7.5, 3.75, 1.87, 0 nmol) for 24h, and then cell viability was analyzed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) method at a wave length of 570 nm. In addition, cell death of 4T1 tumor cells (C) and HEK293T cells (D) in c-di-GMP-treated cultures was determined by subtracting % live cells from non-treated cells (100%). The results shown here is a representative of two experiments. Statistical differences in viability were found between 4T1 and HEK293T cells; Unpaired t test at 15 nmol (♦) p<0.0001, 7.5 nmol (*) p<0.0001, and in cell death between 4T1 and HEK293T cells; Unpaired t test at 15 nmol ($) p<0.0001, 7.5 nmol (#) p=0.0002. p<0.05 is significant.
Figure S5: Developing of a therapeutic protocol with c-di-GMP and Listeria for clinical application. Mice injected with $0.5 \times 10^5$ 4T1 tumors in mammary fat pad and on day 4 after tumor challenge mice were injected with lower doses i.e. 100, 50 or 25 nmol was administered every three days on day, and results were compared to our normal treatment dose of 150 nmol once per week (A). Various doses of Listeria ie; $10^7$, $10^6$, $10^5$ or $10^4$ CFU every three days and compared this to our normal immunization protocol $10^7$ CFU one time per week (B). Fourteen days after tumor challenge mice were euthanized and analyzed for the number of metastases and tumor weight. The results shown here are the averages of three independent experiments. In each experiment n= 5 mice per group. The error bars represent the SEM.

Figure S6: Kinetic curve of 4T1 primary tumors in c-di-GMP-treated and saline mice. BALB/c mice were immunized with one high followed by multiple low doses c-di-GMP and challenged with 4T1 tumor cells according Immunization protocol D. The tumor sizes were determined by measuring the two largest perpendicular diameters (D1 and D2) of the primary tumors using a caliper during treatment with c-di-GMP every other day until euthanasia (Day 18). The tumor size is expressed in mm$^2$ (D1xD2). The results shown here is the average of 5 mice per group.

Figure S7: Maturation of DC and MDSC in spleens of 4T1 tumor-bearing mice by c-di-GMP in vitro. Spleen cells (pooled of 3 mice per group) were isolated from 4T1 tumor-bearing mice and treated in vitro (2000 cells in 0.1 ml) with various doses of c-di-GMP (0.5 and 1 nmol). 24 hrs (A) and 72 hrs (B) later, DC (CD11b$^+CD11c^+$) and MDSC
(CD11b⁺Gr1⁺) were analyzed for expression levels of markers involved in maturation of APC, such as CD80, CD86, and MHC class II, by flow cytometry. The MDSC and DC were gated within the total live cell population.

**Figure S8: The effect of c-di-GMP on CD4 and CD8 T cell populations in the lymph nodes of 4T1 tumor-bearing mice.** BALB/c mice were immunized with one high followed by multiple low doses c-di-GMP and challenged with 4T1 tumor cells according *Immunization protocol D*. At the end of treatment (day 19), cells were isolated from their inguinal lymph nodes (pooled of 3 mice per group) and analyzed for subsets of CD4 and CD8 T cells by flow cytometry. The CD4 and CD8 T cells were gated within the live CD3⁺ cell populations.

**Figure S9: Activation of CD8 T cells by c-di-GMP-treated MDSC compared to non-treated MDSC.** Spleen cells of naïve mice (pooled from 3 mice per group) were activated with CD3/CD28 (1 µg/2 µg) in the presence and absence of MDSC of 4T1 tumor-bearing mice at a one-to-one ratio (0.5x 10⁶ spleen cells and 0.5x10⁶ MDSC per 500 µl serum-free medium(2)) for 72 hrs. BrdU (1 mM, BD Biosciences) was added to the medium 18 hrs before harvesting the cells. The CD8 (Percp-cy5.5) T cells were gated within the CD3 (Alexa-488) population and analyzed by flow cytometry. The amount of BrdU was used as activation marker. CD3/CD28 strongly activated the CD8 T cells, while the MDSC strongly inhibited the CD3/CD28 activation (A). In a parallel experiment, the spleen cultures with CD3/CD28 were incubated with MDSC of 4T1 tumor-bearing mice that were pretreated with c-di-GMP at various concentrations (0,
0.25, 0.5, 1.0 nmol for 24 hrs) and cultured for 72 hrs. The c-di-GMP-treated MDSC reduced immune suppression by MDSC and partly restored CD8 T cell activation by CD3/CD28 compared to non-treated MDSC (BCD).

**Figure S10: Gating of MDSC in blood of 4T1 tumor-bearing mice.** BALB/c mice were immunized with low doses c-di-GMP and LM-Mb and challenged with 4T1 tumor cells according Immunization protocol C. Nineteen days after tumor challenge mice were euthanized and the percentage of MDSC (CD11b⁺Gr1⁺) was determined in blood in vivo by flow cytometry. The CD11b⁺Gr1⁺ cells were gated in the total live white blood cell population. The results shown here are a representative of 3 independent experiments with n=5 mice per group.

**References**
