Tumor vaccine composed of CpG ODN class C and irradiated tumor cells up-regulates the expression of genes characteristic of mature dendritic cells and of memory cells

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Abstract. Only properly mature dendritic cells (DCs) in the presence of tumor antigens accomplish to activate all of the elements of the immune network and have the potential to induce tumor-specific effectors and memory T cells. In the current study, we firstly aimed to investigate the *in vivo* maturation of antigen presenting cells (APCs) at the molecular level by following the expression of *CD11c, CD86* and *MyD88* genes in the mixture of mononuclear cells after treatment of mice with a tumor vaccine composed of C-class CpG oligodeoxynucletides (CpG ODN) and irradiated melanoma B16F1 tumor cells. The second objective was to define whether the tumor vaccine induces generation of memory T cells ($CD44^{hi}CD62L^{lo/hi}CD27^{hi}$). Finally, based on gene expression pattern we aimed to determine the tissue distribution and homing of the (mature) APCs and memory cells after vaccination. We demonstrated that by tumor vaccine the APCs (including DCs) are manipulated *in vivo*. By this kind of vaccine, the differentiation and maturation of APCs is triggered primarily in the spleen and is subsequently followed by the migration of these APCs to the bone marrow. Once in the bone marrow, these APCs play a crucial role in the development and maintenance of long-lived memory T cells capable of preventing a relapse of malignant disease. In conclusion, our results provide insight into the nature and scope of the antitumor immune response elicited by this kind of tumor vaccine *in vivo*. We showed that the maturation of APCs is a prerequisite for the generation of effective long-term antitumor immunity.

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Introduction

With increased understanding of the requirements for initiating immunity, dendritic cells (DCs) have become attractive candidates to be exploited for vaccination against cancer (1-3). The DCs play a pivotal role in orchestrating the innate and adaptive components of the immune system so that the appropriate coordinated responses are mounted against tumors (4-6). The quality and quantity of induced immune responses are primarily dependent upon the activation state of the DCs (1-2,7). Immature DCs present antigens to T cells in the absence of appropriate co-stimulation leading to tolerance, while the mature DCs display unique properties that trigger the antigen-specific immunity (1,7-9). Maturation of DCs is associated with an enhanced expression of co-stimulatory molecules CD40, CD80, CD86 and several members of the tumor necrosis factor (TNF)/TNF-receptor family including the CD70 (ligand for CD27) as well as with an elevated expression of MHC I and II molecules on DCs which in turn acquire an increased ability to present tumor antigens to T and B effector cells (2,10). It is well recognized that 'danger' signals are essential pre-requisites for the maturation and activation of DCs into powerful antigen presenting cells (9-12). By mimicking bacterial DNA synthetic CpG oligodeoxynucletides (CpG ODN) act as danger signals and have been shown to stimulate maturation of DCs via the Toll-like receptor 9 (TLR9) (13-16). The activation of DCs by CpG ODN involves a signaling cascade culminating in the activation of transcription factors including nuclear factor-κB (NK-κB) and interferon (IFN)-regulatory factors (IRF7 and IRF5) (13) and subsequent up-regulation of co-stimulatory molecules and production of chemokines and cytokines including TNF-α, IL-6, IL-12, IL-15, IL-18 and IFNs (IFN-α, IFN-β) (10,17,18). As a result, the CpG ODN-stimulated DCs induce Th1 responses and are instrumental for the activation of cytotoxic T lymphocytes (CTL) and for enhancement of natural-killer (NK) cell effector functions (2,19,20). Additionally, the events occurring during primary exposure to antigen can impact not only the magnitude and quality of the initial CTL response but also the efficacy and longevity of the ensuing memory pool (21-23). Accumulating evidence suggests that naïve T cells are programmed to become memory cells in early phase of the

T cell response when appropriate signals from the T cell receptor (TCR), co-stimulatory molecules, and cytokines associated with inflammation are thought to be required (22,24,25). Besides, it was demonstrated that the depletion of DCs diminishes the generation of memory T cells, reiterating the pivotal role of DCs in primary and memory immune responses against pathogens and tumors (26,27).

However, the qualitative and quantitative effects of CpG ODN-activated DCs on the development of memory cells remain poorly characterized *in vivo*. For the development of effector and memory T cells during vaccination an interconnected multi-cellular network between antigen presenting cells (APCs), naive T lymphocytes and other cells of the innate immune system is required. In the current study, we therefore followed the expression of 7 selected genes in the mixture of mononuclear cells (MNCs) deriving from the assumption that this cell population reflects the extent of immunity induced by the tumor vaccine (composed of C-class CpG ODN and irradiated melanoma B16F1 tumor cells) *in vivo*. We addressed more precisely whether the tumor vaccine induces the generation of memory T cells ($CD44^{hi}CD62L^{lo/hi}CD27^{hi}$). In addition, based on gene expression pattern we aimed to determine the tissue distribution and homing of the (mature) APCs and memory cells after treatment.

Materials and methods

Cell lines. Murine B16F1 melanoma cells (American Type Culture Collection, ATCC, Rockville, MD) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FCS (Sigma, St. Louis, MO), penicillin (100 U/ml, Pfizer, New York, NY), streptomycin (100 μg/ml, Pfizer) and gentamycin (11 μg/ml, Invenex, Charing Falls, OH).

Animal tumor model. The experiments were performed on 8-10 weeks-old syngeneic female C57Bl/6 mice (Institute of Pathology, University of Ljubljana, SLO). Experimental animals were kept in standard animal colony at a natural day/night cycle. At least 10 healthy animals without signs of fungal or other infections, and with normal body weight, were included in each experimental group. Intraperitoneal (i.p.) B16F1 tumor model was employed. Tumors were induced by i.p. inoculation of $5x10^5$ viable B16F1 tumor cells in 0.2 ml EMEM supplemented with 2% FCS. The day of tumor cells inoculation was considered as day 0. The animals were monitored for the day of death and the proportion of survivors was noted. The average survival (AM) ± standard deviation (SD) ± standard error (SE) was calculated for the animals that ultimately developed tumors and consequently died of them. The experiments were repeated three times. The experiments were approved by the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401-35/2008/12; 323-02-725/2004/4).

Vaccine preparation. Tumor vaccine was composed of $1x10^6$ B16F1 irradiated tumor cells and 30 μg of CpG ODN 2395 class C (Coley Pharmaceutical, Canada) per mouse.

Melanoma B16F1 tumor cells were trypsinized (0.25% trypsin, Sigma) and washed three times in the 10% serum-containing medium. The tumor pellets were then resuspended in the 2% serum containing EMEM (in concentration of $1x10^6$ cells/cm^2) and irradiated sublethally with 60 Gy on Darpac 2000x X-ray unit (Gulmay Medical Ltd., Shepperton, UK). Tumor cells, which were neither clonogenic *in vitro*, nor tumorigenic *in vivo* were taken as sublethally irradiated.

Vaccine and CpG ODN administration. The experimental mice were treated with the tumor vaccine or the vaccine components i.p. starting 7 days (day -7) before the injection of viable tumor cells (day 0). The vaccination was followed by two additional injections of 30 μg of CpG ODNs (i.p.) 5 and 3 days before (days -5 and -3) the injection of viable tumor cells (Fig. 1).

Evaluation of long-lasting immunity. Mice that have been preventively treated with the vaccine in combination with two additional doses of CpG ODN were challenged i.p. with $5x10^5$ viable B16F1 tumor cells. After 100 days, the survivors were re-challenged with $5x10^5$ viable tumor cells without additional pre-vaccination.

MNCs collection. The spleens, femurs and peritoneal lavages were collected 1, 3, 5 and 8 days after vaccine or the vaccine components administration (Fig. 2). The spleens were cut in small pieces and mechanically disrupted. The bone marrow was rinsed with EMEM from the femurs. The peritoneal cavity was rinsed two times with PBS and peritoneal lavages were then centrifuged at 1500 rpm for 5 min and cell pellets were resuspended in EMEM. MNCs were subsequently isolated by gradient centrifugation on Ficoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. The MNCs from three animals from the same group were pooled together. Isolated MNCs from treated and control (mock-treated) mice were used for gene expression analysis.

RNA isolation from MNCs. Total RNA was isolated from $3x10^6$ MNCs using RNAqueous-4PCR Kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and cDNA was synthesized from 400 ng of the total RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR). A set of 7 genes of interest (*CD11c, MyD88, CD86, CD27, CD62L, CD44, IL-15*) and 2 housekeeping genes (*GUSB, HPRT1*) were investigated using the TaqMan Gene Expression Assays (Table I). Assays were validated using serial dilutions of cDNA from control groups for confirmation of equal amplification efficiencies of the target and housekeeping genes. Individual qRT-PCR reactions were set up in triplicates in 96-well plates and were carried out in 10 μl reactions with 1x LightCycler480 Probes Master (Roche Applied Science, Mannheim, Germany), 1x TaqMan Gene Expression Assay (Applied Biosystems) and 200 ng cDNA. Thermocycling conditions using the Roche LightCycler480 instrument were as follows: 95˚C for 10 min and 45 cycles of 95˚C for 15 sec, 60˚C for 1 min and 72˚C for 1 sec. The data were analyzed by Roche LightCycler480 software and Ct values were extracted.

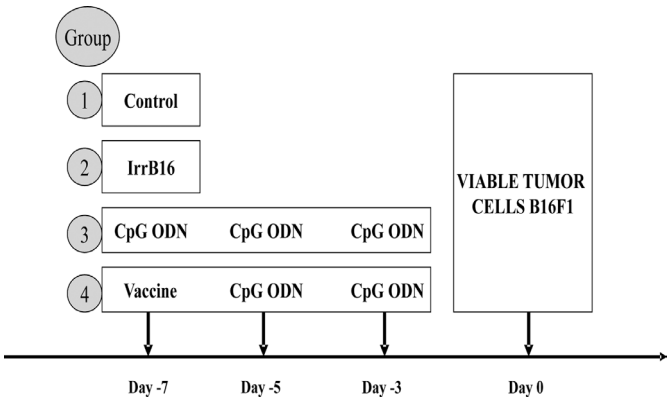

Figure 1. Time schedule of i.p. applications of vaccine or the vaccine components and viable tumor cells in experiments exploring the preventive effect. Control, mock-treated; IrrB16, irradiated B16F1 tumor cells; CpG ODN, CpG oligo-deoxynucletides; vaccine, combination of CpG ODN class C and irradiated tumor cells.

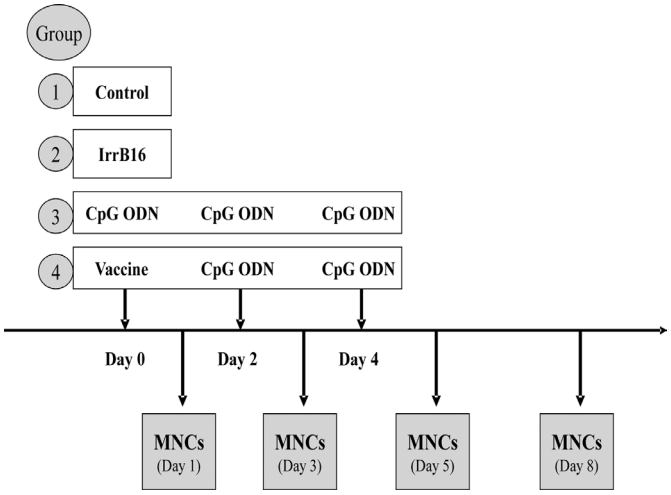

Figure 2. Time schedule of i.p. applications of vaccine or the vaccine components and of MNCs collection. MNCs were isolated from spleens, bone marrow and peritoneal lavages 1, 3, 5 and 8 days after the first application of vaccine or the vaccine components. Control, mock-treated; IrrB16, irradiated B16F1 tumor cells; CpG ODN, CpG oligodeoxynucleotides; vaccine, combination of CpG ODN class C and irradiated tumor cells.

Relative gene expression data analysis. Fold-differences in expression were calculated using the comparative Ct method (ΔΔCt method) as described previously (28). Briefly, the Ct values of both the control (mock-treated) groups and treated groups were first normalized to endogenous housekeeping genes (*GUSB* and *HPRT1*). Subsequently, the obtained ΔCt values of the treated groups were normalized to the ΔCt values of the control groups.

Statistical analysis. The survival curves of *in vivo* experiments were plotted by the method of Kaplan and Meier using GraphPad Prism 3.0 software. Survival curves were compared using the log-rank test. $P<0.05$ was considered as statistically significant. Fold-changes in gene expression were analyzed using SigmaStat 3.0 software. Statistical significance was determined by the Student's t-test. $P<0.05$ was considered as statistically significant.

Results

A significant preventive antitumor immunity is achieved with the vaccine composed of irradiated tumor cells and CpG ODN class C. According to the results of our previous studies neither CpG ODN alone nor the irradiated tumor cells alone substantially triggered the antitumor preventive immunity (27). Convincing antitumor immunity was not achieved until experimental mice were injected with CpG ODN in combination with irradiated tumor cells (tumor vaccine) followed by two additional injections of CpG ODN (Fig. 3A). The proportion of animals totally protected from the development of aggressive i.p. B16F1 tumors after such treatment was 75.0%. In this group of mice, the average survival was statistically significantly improved compared to control mock-treated mice ($p<0.0001$). The average survival was also statistically significantly changed (compared to control) after three applications of CpG ODN ($p=0.0012$), while the treatment with irradiated tumor cells only did not affect the average survival of experimental mice ($p=0.2667$).

Additionally, tumor vaccine followed by two applications of CpG ODN induced long-lasting protective antitumor immunity (Fig. 3B). Mice which had been preventively

Table I. TaqMan gene expression assays used for qRT-PCR.

Gene symbol	Assay ID	Gene name
GUSB[a]	Mm00446957_m1	Glucuronidase β
HPRT1[a]	Mm01545399_m1	Hypoxanthine guanine phosphoribosyl transferase 1
CD11c (Itgax)	Mm01271275_m1	Integrin α x
MyD88	Mm00440338_m1	Myeloid differentiation primary response gene 88
CD86 (B7-2)	Mm00444543_m1	CD86 antigen
CD27 (Tnfrsf7)	Mm01185210_m1	CD27 antigen (TRAF-linked TNF receptor)
CD62L	Mm01284453_m1	L-selectin
CD44 (Ly-24)	Mm01277164_m1	CD44 antigen
IL-15	Mm00434210_m1	Interleukin 15

The TaqMan assays were obtained from Applied Biosystems (Foster City, CA). [a]Endogenous control, housekeeping gene.

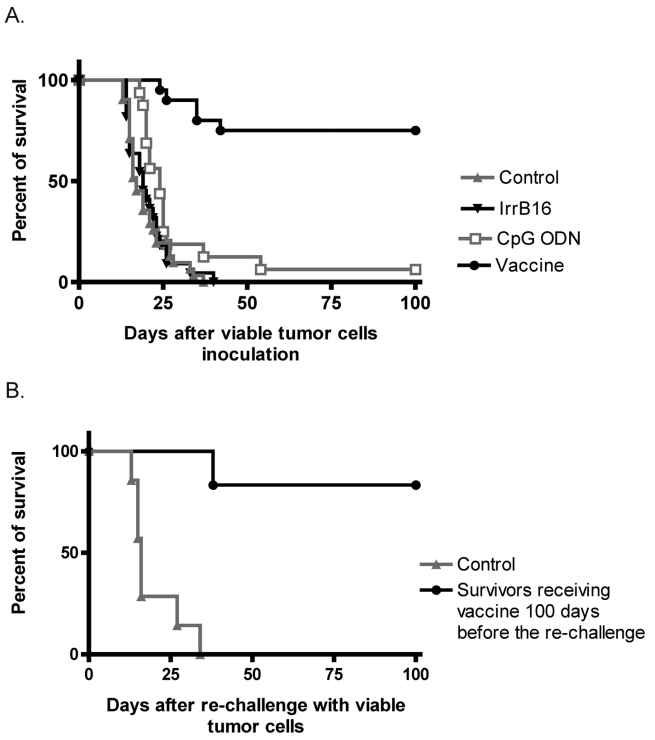

Figure 3. Survival of pre-vaccinated mice challenged i.p. with $5x10^5$ viable B16F1 tumor cells. (A) Survival curves after the first tumor challenge. (B) Survival curves after the second tumor challenge, pre-vaccinated mice surviving the first tumor challenge were without any additional treatments re-challenged 100 days after the first tumor challenge. Control, mock-treated mice; IrrB16, mice treated with irradiated tumor cells only on day 7 before inoculation of viable tumor cells; CpG ODN, mice injected three times with sole CpG ODN on days 7, 5 and 3 before inoculation of viable tumor cells; vaccine, mice treated with the tumor vaccine (combination of CpG ODN and irradiated tumor cells) on day 7, followed by two additional injections of CpG ODN on days 5 and 3 before inoculation of viable tumor cells.

vaccinated and had already survived one tumor challenge were re-challenged with tumor cells 100 days after the first tumor challenge, without any additional treatments. Fig. 3B that comprises the survival data of these experimental mice shows that in 83.3% of mice that had survived the first tumor challenge a long-lasting immunity was triggered ($p<0.0001$ compared to control group).

The vaccine composed of CpG ODN and irradiated tumor cells acts through the augmentation of maturation of APCs and induces their migration to bone marrow and peritoneal cavity. With the intention to determine the capability of the vaccine to induce the maturation of APCs at the molecular level, the expression of maturation markers CD11c and CD86 in MNCs isolated from spleens, bone marrow and peritoneal lavages 1, 3, 5 and 8 days after the first treatment was followed (Fig. 2). Additionally, we followed the expression of the *MyD88* gene which encodes the central adaptor protein in TLR9-mediated signaling. The main function of this protein is the induction of transcription of cytokines and co-stimulatory molecules (26). Results presented in Fig. 4 show a significant up-regulation of *CD11c, CD86* and *MyD88* genes determined in splenic MNCs of experimental mice 1 day after the first treatment. Compared to control, a significant increase of *CD11c* gene expression was determined after the treatment with tumor vaccine or its components (Fig. 4A). In all treated groups (treated with irradiated tumor cells only, sole CpG ODN, or combination of both - tumor vaccine), the expression of *CD11c* gene was increased compared to mock-treated group ($p=0.018$, $p<0.001$, $p<0.001$, respectively). The up-regulation of *CD11c* gene was furthermore enhanced after the treatment with CpG ODN or tumor vaccine in comparison with the group treated with irradiated tumor cells only ($p<0.001$, $p=0.001$, respectively). Similarly, an augmented up-regulation of *CD86* gene was determined in mice treated with sole CpG ODN and tumor vaccine compared to the group treated with irradiated tumor cells only ($p=0.002$, $p=0.035$, respectively) (Fig. 4D). The enhanced expression of *CD11c* and *CD86* genes correlated with the increased expression of *MyD88* gene. Also the expression of *MyD88* gene was additionally amplified after the treatment with sole CpG ODN ($p=0.001$) or tumor vaccine ($p=0.029$) in comparison with the treatment with irradiated tumor cells only (Fig. 4G).

In MNCs isolated from the bone marrow of experimental mice, a significant up-regulation of *CD11c* and *CD86* genes was observed 3 and 8 days after the first treatment (Fig. 4B and E). Compared to mock-treated mice, a significantly increased expression of *CD11c* and *CD86* genes was determined in all treated mice 3 days after the first treatment. The p-values for *CD11c* and *CD86* were 0.011 and 0.048 after the treatment with irradiated tumor cells only, 0.007 and 0.023 after the treatment with sole CpG ODN and <0.001 and 0.015 after the treatment with tumor vaccine, respectively. Eight days after the first treatment the enhanced expression of these two genes was retained only in MNCs obtained from mice treated with sole CpG ODN and in the group of mice treated with tumor vaccine. In the group treated with irradiated tumor cells only, the expression of *CD11c* and *CD86* genes was no longer up-regulated. In bone marrow MNCs no changes in *MyD88* gene expression were detected (Fig. 4H).

In MNCs isolated from peritoneal cavities of experimental mice, an increased expression of *CD11c* and *CD86* genes was detected 3 days after the first treatment (Fig. 4C and F). The enhanced expression of these two genes was determined in MNCs from mice treated with irradiated tumor cells only and tumor vaccine ($p=0.022$; 0.021 for *CD11c* and $p=0.013$; 0.006 for *CD86*, respectively). Furthermore, 5 days after the first treatment the up-regulation of *CD11c* gene was detected only in mice treated with the tumor vaccine. The significantly increased expression in mice treated with the vaccine was determined even when compared to mice treated only with irradiated tumor cells ($p=0.016$) and to mice injected solely with CpG ODN ($p=0.009$). In group treated with tumor vaccine, the expression of *CD86* gene was also increased 5 days after vaccination, but the difference was not statistically significant ($p=0.062$). No changes in the expression of *MyD88* gene were observed in MNCs isolated from peritoneal lavages (Fig. 4I).

Tumor vaccine composed of CpG ODN and irradiated tumor cells triggers the formation of memory cells that primary reside in bone marrow. Since it has been demonstrated that in >80.0% of survivors the long-lasting antitumor immunity was triggered with vaccination (Fig. 3B), we also aimed to determine whether the expression intensity of selected genes (*CD62L, CD44* and *CD27*) reflects the development of memory

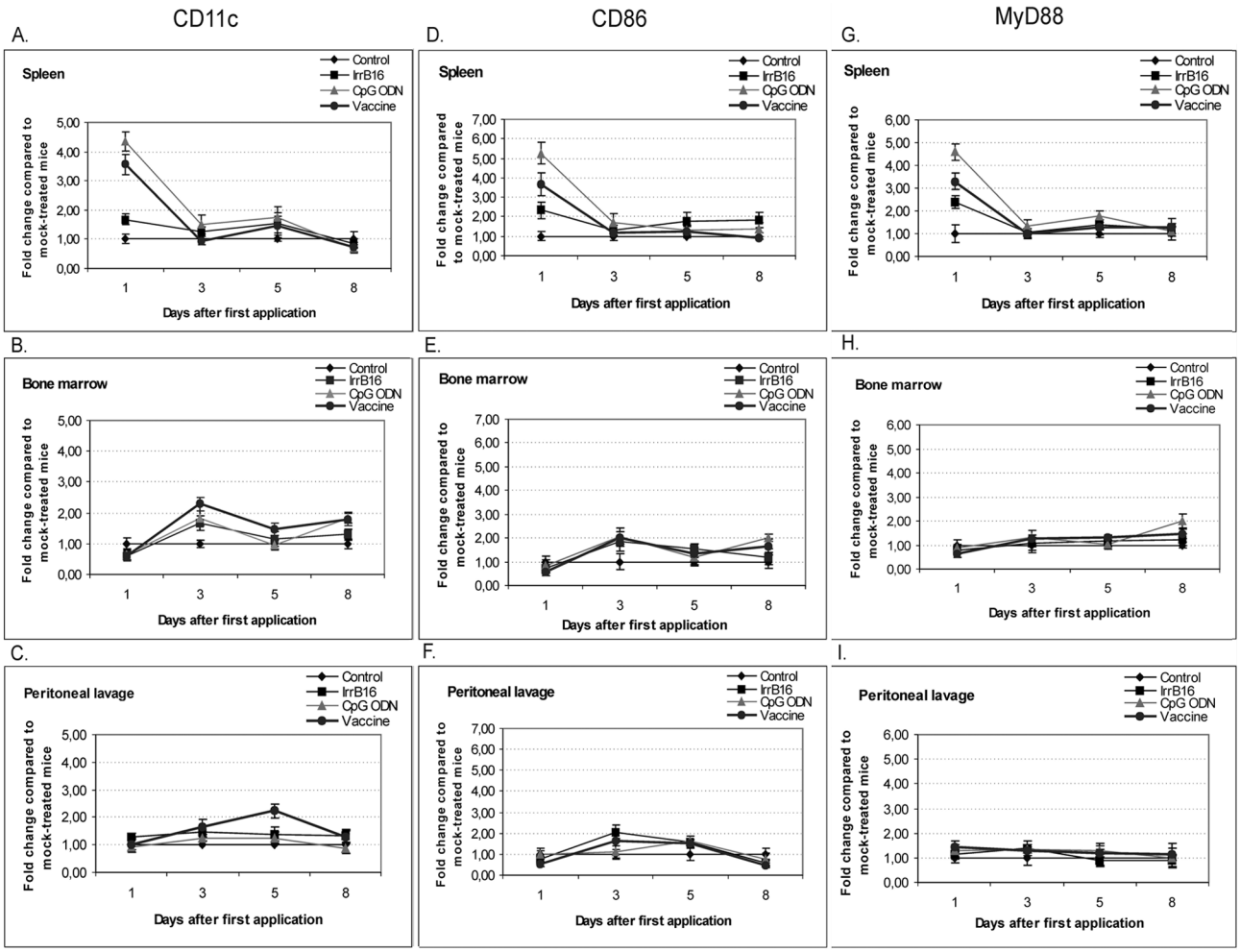

Figure 4. An enhanced maturation of APCs among the splenic MNCs was determined one day after the first treatment in mice treated with sole CpG ODN or tumor vaccine and was followed by their migration to bone marrow in the next days after treatment. Control, mock-treated mice; IrrB16, mice treated with irradiated tumor cells only on day 0; CpG ODN, mice injected three times with sole CpG ODN on days 0, and 2 and 4 after the first application; vaccine, mice treated with the tumor vaccine (combination of CpG ODN and irradiated tumor cells) on day 0, followed by two additional injections of CpG ODN on days 2 and 4 after vaccine application; day 0, day of the first application. MNCs were isolated from spleens, bone marrow and peritoneal lavages of treated mice 1, 3, 5 and 8 days after the first application of vaccine or the vaccine components. The MNCs from three animals from the same group were pooled together. Expression of *CD11c, CD86* and *MyD88* genes was determined using the qRT-PCR.

cells after vaccination. Simultaneously, the distribution of memory cells after vaccination was followed by the determination of the expression of the very same genes over time.

CD62L. Fig. 5A shows that 1 day after the first treatment the expression of *CD62L* gene is markedly enhanced in splenic MNCs obtained from all treated groups. Compared to control, the p-value for mice receiving only irradiated tumor cells was p=0.003, for mice treated with sole CpG ODN p<0.001 and for mice treated with tumor vaccine p<0.001. However, the highest expression of *CD62L* gene was detected in groups treated with sole CpG ODN or tumor vaccine (p=0.004 and 0.005 compared to group treated with irradiated tumor cells only). The expression of *CD62L* gene decreased in the next days after the first treatment. In the group treated with irradiated tumor cells only, the expression of *CD62L* declined to the level similar to the one detected in the control group. In spite of the evidential decrease of expression levels after day 1 in mice treated with sole CpG ODN or tumor vaccine, these expression levels still remained significantly higher than in control mice (p=0.011; 0.009, respectively).

In bone marrow MNCs, an elevated expression of *CD62L* was detected 3 days after the first treatment in all treated groups in comparison with the mock-treated group (p=0.003 for mice that received irradiated tumor cells only; p=0.001 for mice treated with sole CpG ODN and p=0.001 for mice treated with tumor vaccine). Subsequently, the fold-change of *CD62L* gene expression increased from day 3 to day 8 only in mice treated with tumor vaccine (Fig. 5B). In this group, the expression of *CD62L* was significantly increased even when compared to the group treated with irradiated tumor cells only (p=0.009) and to the group treated with sole CpG ODN (p=0.003).

In case of MNCs isolated from peritoneal lavages, the elevated expression of *CD62L* was detected 1 and 5 days after the first treatment (Fig. 5C). Most evident was the increase in the level of expression on day 5 after the first treatment, as the *CD62L* gene was markedly up-regulated in MNCs obtained

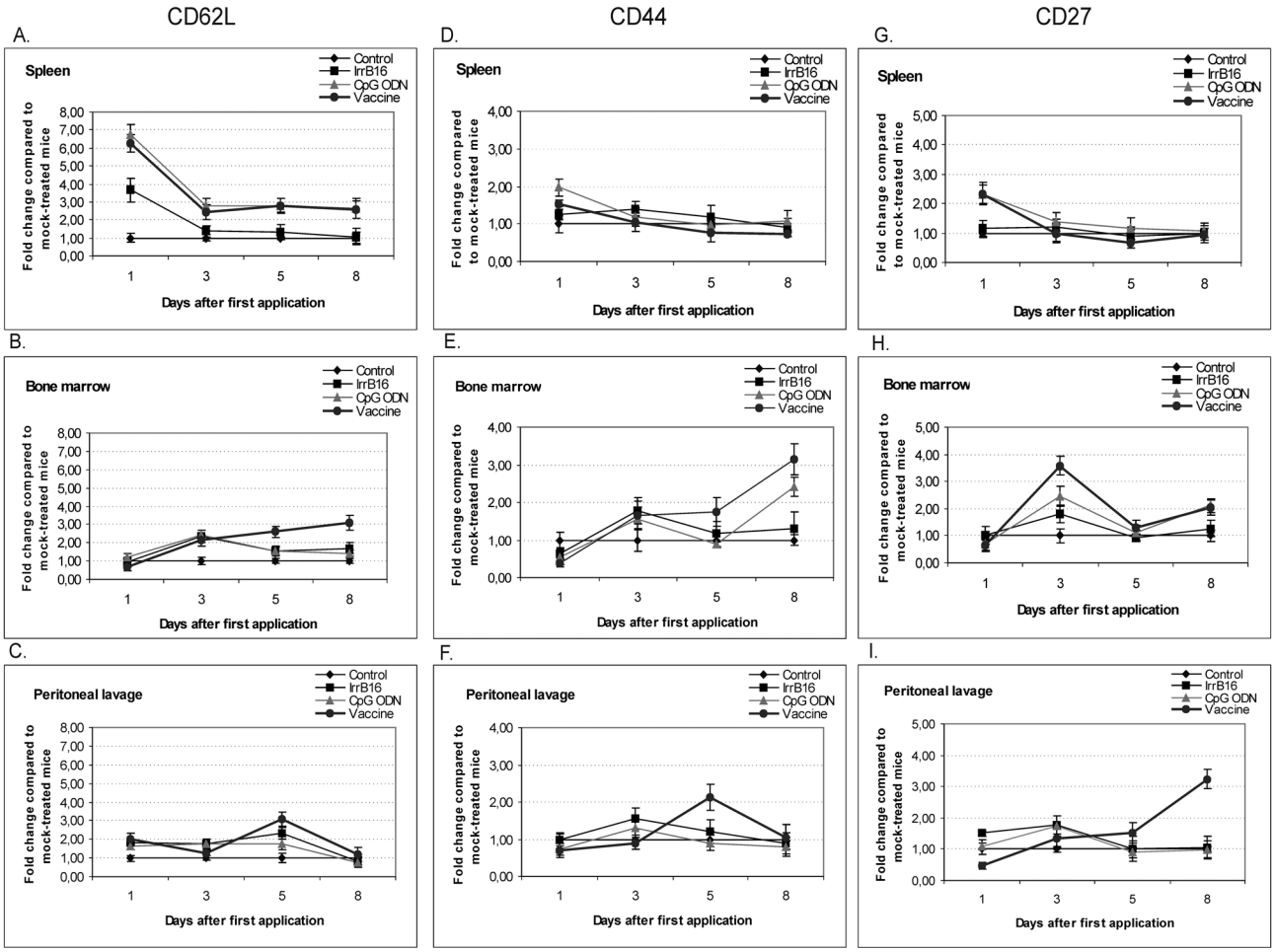

Figure 5. Tumor vaccine followed by two additional applications of CpG ODN induced the development of memory cells that preferentially reside in the bone marrow of treated mice. Control, mock-treated mice; IrrB16, mice treated with irradiated tumor cells only on day 0; CpG ODN, mice injected three times with sole CpG ODN on days 0, and 2 and 4 after the first application; vaccine, mice treated with the tumor vaccine (combination of CpG ODN and irradiated tumor cells) on day 0, followed by two additional injections of CpG ODN on days 2 and 4 after vaccine application; day 0, day of the first application. MNCs were isolated from spleens, bone marrow and peritoneal lavages of treated mice 1, 3, 5 and 8 days after the first application of vaccine or the vaccine components. The MNCs from three animals from the same group were pooled together. Expression of *CD62L*, *CD44* and *CD27* genes was determined using the qRT-PCR.

from mice treated with tumor vaccine (p=0.001 compared to control group).

CD44. Additionally, the *CD44* gene expression was followed in MNCs from differently treated mice. In splenic MNCs, the expression of *CD44* gene was elevated 1 day after vaccination in mice that were treated with sole CpG ODN or tumor vaccine (p=0.006; 0.016, respectively). In mice that received irradiated tumor cells only, the expression of this gene remained unchanged compared to the control group (p=0.186). In the next days after the first treatment, the expression of *CD44* in splenic MNCs from treated mice no longer differed from the one detected in control mice (Fig. 5D).

Opposed to splenic MNCs, the up-regulation of *CD44* gene in bone marrow MNCs was not detected until day 5 after the first treatment (Fig. 5E). A significantly increased expression of the *CD44* gene was on day 5 determined in mice treated with the tumor vaccine (p=0.035). The expression of *CD44* in this group continuously increased up to 8 days after the first treatment (p=0.001 compared to control group). Similarly, in mice that were injected with three applications of sole CpG ODN the expression of *CD44* was markedly increased 8 days after the first injection (p=0.009). The up-regulation of the *CD44* gene in these two groups was also more pronounced in comparison to the mice treated with irradiated tumor cells only (p=0.018 and 0.020, respectively).

In MNCs isolated from peritoneal lavages, the increased expression of *CD44* gene was determined 5 days after the first treatment only in mice treated with the tumor vaccine (p=0.009) (Fig. 5F). The increase in the expression of *CD44* was statistically significant also in comparison with other two treated groups (p=0.028 compared to group that received irradiated tumor cells only and p=0.006 compared to mice injected with sole CpG ODN). However, the up-regulation of the *CD44* gene was not sustained in the following days after vaccination.

CD27. The up-regulation of the *CD27* gene, coding for a TRAF-linked TNF receptor, was determined in splenic MNCs 1 day after the first treatment in mice treated with sole CpG ODN or tumor vaccine (p=0.005 and 0.002, respectively). The expression of *CD27* declined in the next days after the first

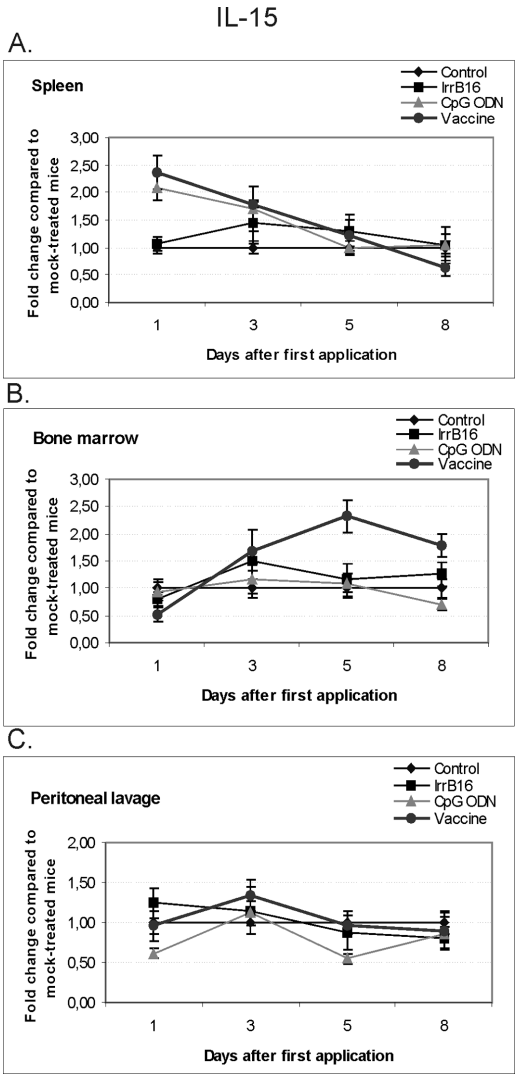

Figure 6. Expression levels of *IL-15* gene were enhanced after treatment with tumor vaccine and sustained enhanced mainly in the bone marrow. Control, mock-treated mice; IrrB16, mice treated with irradiated tumor cells only on day 0; CpG ODN, mice injected three times with sole CpG ODN on days 0, and 2 and 4 after the first application; vaccine, mice treated with the tumor vaccine (combination of CpG ODN and irradiated tumor cells) on day 0, followed by two additional injections of CpG ODN on days 2 and 4 after vaccine application; day 0, day of the first application. MNCs were isolated from spleens, bone marrow and peritoneal lavages of treated mice 1, 3, 5 and 8 days after the first application of vaccine or the vaccine components. The MNCs from three animals from the same group were pooled together. Expression of *IL-15* gene was determined using the qRT-PCR.

treatment to the levels comparable with the ones observed in the control group (Fig. 5G).

In MNCs isolated from the bone marrow of mice treated with the tumor vaccine, the up-regulation of *CD27* gene was marked 3 days after the first treatment, even in comparison with the *CD27* expression determined in MNCs obtained from mice treated with irradiated tumor cells only (p=0.003) and from mice treated with sole CpG ODN (p=0.017) (Fig. 5H). Subsequently, the elevated expression of *CD27* persisted up to 8 days after the first treatment in bone marrow MNCs obtained from the mice treated with sole CpG ODN or tumor vaccine (p=0.003 and 0.005, respectively), while in mice treated with irradiated tumor cells only, the expression of *CD27* was similar to the level detected in control mice.

The expression of *CD27* in MNCs isolated from peritoneal lavages of mice treated with tumor vaccine was increasing with time after the first treatment. The marked increase in the expression of *CD27* gene was observed 8 days after the first application (p<0.001) (Fig. 5I).

Based on gene expression pattern, we can conclude that tumor vaccine composed of irradiated tumor cells and CpG ODN induced an up-regulation of *CD62L, CD44* and *CD27* genes, which encode for markers characteristic for memory cells.

Expression of IL-15 is enhanced after treatment with tumor vaccine composed of CpG ODN and irradiated tumor cells primarily in bone marrow MNCs. The IL-15 represents a crucial signal in generation and maintenance of memory T cells (29). The up-regulation of *IL-15* in our experiments was significant 1 and 3 days after the first treatment in splenic MNCs isolated from mice treated with CpG ODN or tumor vaccine (Fig. 6A). In bone marrow MNCs, the elevated expression of *IL-15* gene was for the first time detected 3 days after the first treatment yet only in mice treated with the tumor vaccine (p=0.048) (Fig. 6B). In this group of mice, the increase in *IL-15* expression was even more evident 5 days after the first treatment (p=0.002) and was sustained also 8 days after the first treatment (p=0.006). No changes in the expression of *IL-15* gene were observed in MNCs isolated from peritoneal lavages (Fig. 6C).

Discussion

Cells of innate immunity (including the DCs) play a prominent role in the processes that control effector T cell expansion, differentiation and memory cell formation (1-3,30). It is obvious from the results of various studies in humans and in experimental animals that only properly maturated DCs in the presence of tumor antigens are efficient for the induction of antitumor immunity network (1,27,30-33).

In our previous studies (27,33,34) we have demonstrated that the DCs could be efficiently maturated *in vivo* through vaccination of mice with irradiated tumor cells in combination with CpG ODN class C (tumor vaccine). We have also confirmed that processes triggered by this vaccination included the activation of the effectors of native immunity (phagocytes) as well as the activation of CTL. However, the main cells influenced by the vaccine appeared to be the phagocytes (including the DCs) and the main process their maturation (27). In the current study we aimed to explore further the mechanism of action of the vaccine composed of irradiated tumor cells and CpG ODN at the level of gene expression of chosen genes *in vivo*. Changes in gene expression after treatment were assessed in MNCs deriving from the assumption that this cell population reflects the extent of immunity induced by the tumor vaccine *in vivo*. MNCs were isolated from spleens, bone marrow and peritoneal lavages of treated mice 1, 3, 5 and 8 days after the first application of the vaccine or components of the vaccine. Our objectives were firstly to confirm the maturation of APCs (including the DCs) after treatment and secondly to determine the homing of mature APCs (DCs) after vaccination. Since the expression of CD11c and CD86 markers is enhanced on maturated APCs (DCs,

monocytes) (2,10), we followed their gene expression in MNCs isolated from differently treated mice. Additionally, the expression of *MyD88* gene was followed as the myeloid differentiation factor 88 (MyD88) is associated with signaling via the TLR9 (16). The fold-change in expression of *CD11c* and *CD86* genes determined in splenic MNCs demonstrated the significant up-regulation of these genes 1 day after the first treatment. The up-regulation of the two genes was more pronounced after the treatment with sole CpG ODN ($p<0.001$; $p=0.002$, respectively) or tumor vaccine ($p=0.001$; $p=0.035$, respectively) compared to the treatment with irradiated tumor cells only. Since the expression of CD11c and CD86 markers is increased in maturated APCs (DCs, monocytes), it can be concluded that in treated mice an enhanced maturation of the APCs among splenic MNCs was triggered as the result of vaccination. This is in accordance with our previous results where a significant increase in the portion of $CD86^{+}CD11c^{+}$ cells among splenic MNCs was determined in mice treated with the same vaccine (27). In addition, the expression of the gene coding for MyD88, that functions as the key adaptor molecule required for signaling through TLR9 (16) was detected to be up-regulated in splenic MNCs 1 day after the first treatment. In this context, the MyD88 served to translate the recognition of CpG ODN by TLR9 that consequently involved the activation of several mitogen-activated kinases (MAPK) and transcription factors (such as NF-κB and IRF7) culminating in the transcription of genes encoding inflammatory cytokines such as IL-12, IL-15, IL-18 and IFNα/β, chemokines and co-stimulatory molecules in APCs (10,35,36). The up-regulation of the *MyD88* gene expression correlated with the increased expression of *CD11c* and *CD86* genes in splenic MNCs isolated from mice treated with sole CpG ODN or tumor vaccine suggesting again that the maturation of APCs (including the DCs) was triggered after the injection of tumor vaccine (37). Unlike in the splenic MNCs, a significant up-regulation of *CD11c* and *CD86* in MNCs isolated from the bone marrow of treated mice was not detected earlier than 3 days after the first application of the vaccine or the vaccine components. The up-regulation of these two genes was also not accompanied with the up-regulation of *MyD88* alluding that after the treatment fully maturated DCs (APCs) migrated from the spleen to bone marrow. This situation persisted for some time since in bone marrow MNCs, the enhanced expression of *CD11c* and *CD86* genes was noticeable even 8 days after the treatment of mice with sole CpG ODN or tumor vaccine. Our observation is quite in accordance with other reports that the circulating DCs have a considerable bone marrow tropism and that the bone marrow retains the DCs even better than the spleen after 24 h (38). The homing of the DCs to the bone marrow could contribute to the generation and maintenance of memory T cells after and already during the vaccination. Multiple mechanisms that involve both the early programming of memory T-cell precursors as well as the continuous supply of external signals are important for the development of the long-lived memory pool (39). Among signals that program memory precursors, the IL-15 *trans*-presented by myeloid cells (especially by the DCs) has clearly been shown to drive the generation and maintenance of memory T cells (29,39). In line with these findings were also our observations of the up-regulation of *IL-15* detected firstly 3 days after vaccination in bone marrow MNCs obtained from mice treated with the tumor vaccine. Moreover, the higher expression of *IL-15* in this group was sustained also 8 days after the first treatment indicating that some agents (e.g., CpG ODN) up-regulating the *IL-15* in DCs are, as supposed, enhancing maintenance and survival of memory T cells (15,29).

Regarding the above results and the fact that the creation of T-cell memory against tumor antigens is crucial for the generation of a durable antitumor protection especially in patients with an increased risk of relapse (4,40), the next objectives of our study were to prove the production of memory T cells and to determine where the memory cells preferentially reside after the treatment. Therefore, we followed the expression of *CD62L, CD44* and *CD27* genes in MNC populations after treatment of mice with tumor vaccine or the vaccine components as the $CD44^{hi}CD62L^{hi/lo}CD27^{hi}$ phenotype is consistent with the memory T cell development (29,30). In splenic MNCs, the expression levels of these three markers were increased 1 day after the first treatment. This might be due to the activation of naïve T cells by APCs as all these markers are consequently up-regulated in these cells (41). Notably, in the following days after the first treatment these genes were up-regulated in MNCs obtained from bone marrow and peritoneal lavages of mice injected with sole CpG ODN or tumor vaccine. The expression levels of *CD62L* and *CD44* genes gradually increased with time after the first treatment. Furthermore, in mice treated with the tumor vaccine the up-regulation of these memory markers in bone marrow MNCs was even more pronounced compared to mice treated with irradiated tumor cells only or with sole CpG ODN. This could be explained with the formation of a long-lived memory T cell population that preferentially resides in the bone marrow but only after treatment with the tumor vaccine. This is consistent with reports by others that $CD44^{hi}CD62L^{hi}CD27^{hi}$ memory T cells reside primarily in the bone marrow where they acquire all necessary signals/factors for their survival and proliferation (42-44). Beside the IL-15, also the CD70/CD27 interactions are important in this maintenance suggesting that the CD27 and IL-15 signals work together to optimize this response (29,39). In the presence of bone marrow DCs, the CD8 T cells have increased levels of CD127, CD27 and CD62L consistent with that of the central memory phenotype and/or memory precursor effectors cells (29).

Finally, the gene expression analysis also confirmed our results from the previous study, where it was demonstrated that after the re-challenge of survivors with viable tumor cells one hundred days after the first tumor challenge without additional treatments, in more than 80.0% of these survivors that had previously received the tumor vaccine and two additional applications of CpG ODN a long-lasting immunity was triggered (27). This indicates the significance of a more durable presence of the CpG ODN for the activation of a sufficient number of APCs capable of inducing the memory cell development as none of the survivors from the groups pre-vaccinated with tumor vaccine only (without additional treatments with CpG ODN) survived the second challenge with viable tumor cells (27).

In conclusion, we demonstrated that by the tumor vaccine composed of CpG ODN and irradiated tumor cell the APCs

(including the DCs) are manipulated *in vivo*. By this kind of vaccine, the differentiation and maturation of APCs is triggered primarily in the spleen and is subsequently followed by the migration of these APCs to the bone marrow. Once in the bone marrow, these APCs (especially the DCs) play a crucial role in the development and maintenance of long-lived memory T cells capable of preventing a relapse of malignant disease.

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