Protein Cell 2013, 4(12): 887–889
DOI 10.1007/s13238-013-3916-x

Protein & Cell

LETTER

Enhancement of DC-mediated anti-leukemic immunity *in vitro* by WT1 antigen and CpG co-encapsulated in PLGA microparticles

Dear Editor,

Leukemia is an important hematological malignancy characterized by uncontrolled proliferation of hematopoietic tumor cells (Schrappe et al., 2000). It is the most common cancer in children and threatens the health of adults as well. Although the treatment of leukemia has improved in recent years, the prognosis of many types of leukemia remains poor (Jarnjak-Jankovic et al., 2005). Currently, new therapeutic methods are needed to further improve the outcome of leukemia treatment. Preclinical research and clinical trials indicate that immunotherapeutic strategy has potent value in treating leukemia (Jarnjak-Jankovic et al., 2005). Immunotherapy, as an adjuvant cancer treatment after chemotherapy and radiation, may destroy the residual tumor cells and lower the risk of relapse (Zindl and Chaplin, 2010). Dendritic cell (DC)-based immunotherapy has been recognized as a promising approach of cancer treatment in recent years (Figdor et al., 2004). DC-based immunotherapy has already shown promise in follicular non-Hodgkin's lymphoma and other hematological malignancies (Jarnjak-Jankovic et al., 2005). In order to implement DC-based immunotherapy against leukemia, a tumor antigen that is highly expressed in most hematological malignancies is warranted. Wilms' tumor protein (WT1) may serve as a promising leukemic antigen. It is over-expressed in many types of leukemia and solid tumors, but under-expressed in normal tissue (Oka et al., 2004). Using WT1 peptide as the tumor antigen, reduction of leukemic blast cells was observed in early stage clinical trials (Oka et al., 2004). However, the immunogenicity of WT1 peptide is relatively weak, and the WT1-specific immune response is usually limited (Oka et al., 2004). At present, there is urgent need to explore new technologies that can improve the immunogenicity of WT1 antigens. Prior research has shown that poly (lactide-co-glycolide) (PLGA) nano/microparticles (NPs/MPs) can enhance the immunogenicity of antigens. In animal studies, tumor antigens carried by PLGA particles significantly improved the anticancer immune response (Zhang et al., 2011; Ma et al., 2012). So far, however, there is no report on utilizing PLGA particles to enhance the anti-leukemic immunity induced by WT1 antigen. In this study, we investigated whether PLGA MPs encapsulating a WT1 peptide would affect human DC-mediated anti-leukemic immune response.

PLGA MPs carrying WT1 and/or CpG were formulated using the standard w/o/w approach (Ma et al., 2012). Confocal images revealed that the particles were spherical in shape (Fig. 1A). Dynamic light scattering (DLS) data showed that the size distribution of the particles was in the range of 0.84–1.60 μm, with a mean radius of 0.61 μm (Fig. 1B), and a polydispersity index (PDI) of 0.247. In order to trigger an immune response, antigen-carrying MPs need to be endocytosed by DCs. To study this issue, FITC-loaded MPs were incubated with immature human DCs. Subsequent confocal microscopy revealed that the MPs were well engulfed by the DCs and mainly localized in the cytoplasm (Fig. 1C). This result was in agreement with extensive prior studies, which showed that PLGA particles from 50 nm to 3 μm in size could be efficiently taken up by DCs (Kovacsovics-Bankowski and Rock, 1995; Zhang et al., 2011; Ma et al., 2012).

To investigate whether the PLGA carriers would influence the immunogenicity of WT1 peptide, human DCs were stimulated separately with saline, MP, soluble WT1 peptide, or MP-encapsulated peptide (termed MP (WT1)). DC-mediated anti-leukemic immune cytotoxicity was evaluated. As shown in Fig. 1D, MP (WT1) induced the most potent anti-leukemic response among all stimulating paradigms ($P < 0.05$), indicating that PLGA antigen carriers could moderately enhance the immunogenicity of WT1 peptide. To further enhance the anti-leukemic immunity, WT1 and CpG were co-encapsulated in PLGA MP to fabricate MP (WT1 + CpG), which was subsequently compared with other immune stimulating formulations in cytotoxicity experiments. As shown in Fig. 1E, MP (WT1 + CpG) induced an anti-leukemic cytotoxicity that was significantly higher than the other treatments ($P < 0.01$). The results also indicated that co-encapsulation of WT1 and CpG in the same particle could trigger a stronger DC-mediated anti-leukemic response than separate encapsulation of the two agents.

To further evidence the results of the above cytotoxicity experiments, IFN-γ release was also measured from lymphocytes stimulated with various immunogenic paradigms. ELISA assays revealed that MP (WT1) was superior than soluble WT1 peptide in inducing IFN-γ production, and MP (WT1 + CpG) was even more efficacious than MP (WT1) (Fig. 1F, $P < 0.01$). Thus, the

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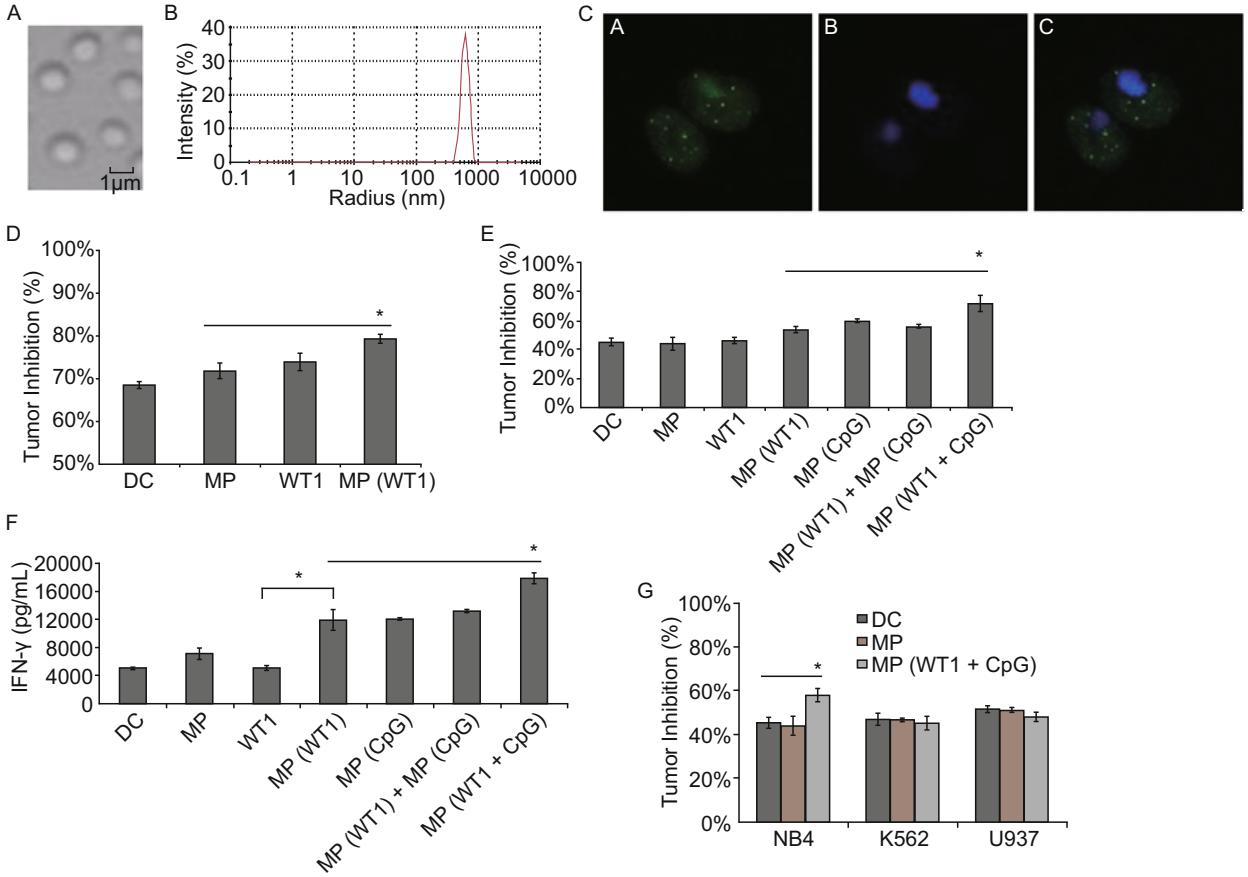

Figure 1. Effects of encapsulation of WT1 peptide by PLGA MP on DC-mediated anti-leukemic immunity. (A) Confocal microscopic image of PLGA particles. (B) Size distribution of the particles per DLS analysis. (C) Confocal microscopy images of the DCs incubated with FITC-loaded MPs. Left panel: DCs incubated with PLGA-MPs containing FITC-labeled protein (green). Middle panel: the nuclei of the DCs were stained by DAPI (blue). Right panel: superimposed images of A and B are shown. (D) Effects of MP, WT1, or MP (WT1) on anti-leukemic response. Tumor inhibition produced by lymphocytes mixed with DCs that had been exposed to empty MP, WT1 of 1 μg/mL, or MP (WT1) containing 1 μg/mL peptide. The DC group was treated by saline and used as the control. The stimulated DCs were co-cultured with lymphocytes, which were subsequently mixed with the target cells (NB4 leukemic cells) to induce immune cytotoxicity. The tumor inhibition was measured using the 5- or 6-(N-Succinimidyloxycarbonyl)- 3',6'-O,O'-diacetyl-fluorescein (CFSE) fluorescence-based cytotoxicity assay (n = 6, ± SD). (E) Comparison of tumor inhibition induced by various forms of MPs, using NB4 as the target cells. The WT1 concentration of 1μg/mL was used in experiment groups of WT1, MP (WT1), MP (WT1) + MP (CpG), and MP (WT1 + CpG). The CpG concentration of 2 μg/mL was used in experiment groups of MP (CpG), MP (WT1) + MP (CpG), and MP (WT1 + CpG) (n = 6, ± SD). (F) Production of IFN-γ by lymphocytes stimulated with various immunogenic treatments. Secreted IFN-γ in supernatant of culture medium was measured by ELISA (n = 6, ± SD). (G) Immune cytotoxicity triggered by MP or MP (WT1 + CpG) on target cells of NB4, K562, and U937 (n = 6, ± SD, E:T = 20:1). The star indicates a statistically significant difference.

IFN-γ production profile was largely in agreement with the immune cytotoxicity experiments, verifying the finding that encapsulation of WT1/CpG could enhance the anti-leukemic immunity.

To reduce the probability of autoimmune disease and improve the potential of clinical application, it is important that the immune cytotoxicity enhanced by MP (WT1 + CpG) is relatively specific against the WT1-positive leukemic cells. To evaluate this issue, several target cells were employed in immune cytotoxicity study, including NB4 (HLA-A2$^+$ and WT1$^+$), K562 (HLA-A2$^-$ and WT1$^+$), and U937 (HLA-A2$^+$ and WT1$^-$). The enhancement of DC-mediated cytotoxicity was only observed in NB4 cells, but not in K562 and U937 cells (Fig. 1G) The results indicated that the immunity enhanced by MP (WT1 + CpG) was relatively specific against the tumor cells that expressed both HLA-A2 and WT1, but not the cells that were either HLA-A2 or WT1 negative.

This study was the first attempt to evaluate whether the encapsulation of WT1 antigen by PLGA particles would influence DC-mediated anti-leukemic immunity. Extensive previous studies have shown that antigens carried by PLGA particles can overcome immune tolerance and boost immunogenicity

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(Kovacsovics-Bankowski and Rock, 1995; Zhang et al., 2011; Ma et al., 2012). PLGA particles can protect the antigen from enzymatic degradation (Kovacsovics-Bankowski and Rock, 1995), increase the efficiency of antigen-loading (Hanlon et al., 2011; Ma et al., 2012), facilitate cross-presentation of the antigen (Ma et al., 2011), and co-deliver adjuvant into DCs (Zhang et al., 2007). In agreement with these immune-modulating mechanisms of PLGA carriers, we also observed that PLGA MPs could be effectively taken into the cytoplasm of DCs (Fig. 1C). Moreover, MP (WT1) moderately enhanced the DC-mediated anti-leukemic immune response compared to soluble WT1 peptide (Fig. 1D). Additionally, MP (WT1 + CpG) further augmented the anti-leukemic reaction (Fig. 1E and 1F). These findings extend the application of PLGA-based immune-modulating technique to an important tumor antigen WT1, which is widely expressed in hematological malignancies (Bergmann et al., 1997; Oka et al., 2004). Immunotherapy holds promise for improving the outcome of leukemia treatment, and DC-mediated treatment is an essential strategy of modern immunotherapy (Figdor et al., 2004). For typical DC therapy, autologous DCs are usually pulsed by soluble tumor antigens *in vitro*, before being administered to patients to trigger the desired immune response (Schreiber et al., 2010). In this study, we developed a novel DC-based WT1 vaccine for inducing anti-leukemic response. We propose that MP (WT1 + CpG) may be used to stimulate patient-derived DCs instead of soluble WT1 antigen, since MP (WT1 + CpG) can significantly enhance the anti-leukemic immunity. This approach has reasonable feasibility, since both WT1 peptide and CpG have been approved for clinical trials (Cooper et al., 2004) and PLGA is a FDA-approved excipient for human use (Rokkanen et al., 1985). It should be noted that the present study was conducted using human DCs with *in vitro* experiments. Future research may focus on testing the idea *in vivo* with animal leukemic models.

Taken together, we observed that encapsulation of WT1 antigen in PLGA MPs enhanced the anti-leukemia immune response mediated by human DCs *in vitro*. In addition, when CpG was co-encapsulated with WT1 antigen in the MPs, the immune cytotoxicity against leukemic cells was further augmented. These results provide new perspectives for development of high-efficacy anti-leukemic immunotherapy and improvement of the current WT1-DC vaccines.

FOOTNOTES

This work was supported by the National Natural Science Foundation of China (Grant No. 81071870), the National Basic Research Program (973 Program) (No. 2011CB933504), and the 111 project from the Chinese Ministry of Education.

Liang Zhang, Sun Zhao, Yan Hu, Jinhong Duan, Ning Gu, Haiyan Xu, Xian-Da Yang declare that they have no conflict of interest. Additional informed consent was obtained from all volunteers for which idetifying information is included in this artcle. All institutional and national guidelines for the care and use of laboratory animals were followed.

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