Nanosystem for Targeting T_{reg} *In Vivo*

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ABSTRACT

Due to their ability to suppress the activity of autoreactive cells, "natural" $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells (T_{reg}) are important drug targets for malignancies and autoimmune diseases. Immunological studies are revealing a growing subset of genes, which are able to affect T_{reg} function and could be targeted for novel therapies. However, one major obstacle to the development of T_{reg}-targeted therapies is the lack of approaches able to ensure selective delivery of drugs into these cells. Herein, we describe the fabrication of a nanosystem based on PEG-modified single-walled carbon nanotubes (PEG-SWCNTs) and aimed at targeting T_{reg} residing in specific tissues. PEG-SWCNTs conjugated with ligands against a T_{reg}-enriched marker were able to reach the nucleus of T_{reg} residing in the spleen of healthy mice or in the microenvironment of a B16 melanoma following systemic administration. Moreover, T_{reg}-targeted PEG-SWCNTs were able to deliver siRNA specifically into T_{reg} *in vivo*. Our data suggested that PEG-SWCNT-based nanosystems could open new avenues for the prevention/therapy of autoimmune diseases and malignancies based on the T_{reg}-specific tuning of molecular pathways.

Keywords: active targeting, regulatory T cells, carbon nanotubes, glucocorticoid-induced TNFR-related receptor.

1 INTRODUCTION

Pioneering solutions for cell-targeted drug delivery have recently come from nanotechnology. Using nanotechnology-derived particles (*nanoparticles*), several research groups have achieved *in vitro* and *in vivo* delivery of pharmaceutical and diagnostic agents into several types of cells and tissues [1]. Among the recently developed nanoparticles, polyethylene glycol (PEG)-modified single-walled carbon nanotubes (PEG-modified SWCNTs or PEG-SWCNTs) have gathered the highest attention in the biomedical community [2,3]. By using PEG-SWCNTs our research group has efficiently targeted all T cells *in vivo* and delivered antisense oligomers into T cells *in vitro* [4,5]. Our next objective is the targeting of "natural" $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells (T_{reg}) residing in specific tissues *in vivo*. T_{reg} are important drug targets for malignancies and autoimmune diseases and the T_{reg}-specific function manipulation is an highly desirable preventive and therapeutic approach [6,7]. Herein, we showed proof-of-principle evidence that *in vivo* PEG-SWCNT-based T_{reg}-specific targeting and cargo delivery is possible by using ligands against the glucocorticoid-induced TNFR-related protein (GITR), a known T_{reg} surface marker [8].

2 RESULTS AND DISCUSSION

To assess PEG-SWCNT-based T_{reg}-selective targeting and gene silencing we used GFP-FoxP3 and RFP/GFP-Foxp3 mice, respectively. GFP-FoxP3 mice were developed by the Rudensky group [9], RFP/GFP-FoxP3 mice were generated in our laboratory by breeding GFP-FoxP3 mice with mice ubiquitously expressing a RFP transgene under control of the β-actin promoter. All immune cells in RFP/GFP-Foxp3 mice expressed RFP, however T_{reg} in these mice were easily recognizable since they also expressed GFP. PEG-SWCNTs were targeted to T_{reg} by functionalizing them with monoclonal antibodies (mAbs) against GITR. Studies have shown that GITR is more highly expressed on the plasma membrane of T_{reg} than other cell subsets [$CD4^+$ $FoxP3^-$ (T_{eff}) and $CD4^-$ cells] in healthy tissues and become even more highly expressed on T_{reg} residing in inflamed tissues and tumor microenvironment with respect to other tissues (*i.e.* spleen), thereby potentially facilitating the targeting of T_{reg} residing in inflamed tissues and tumor microenvironment [10].

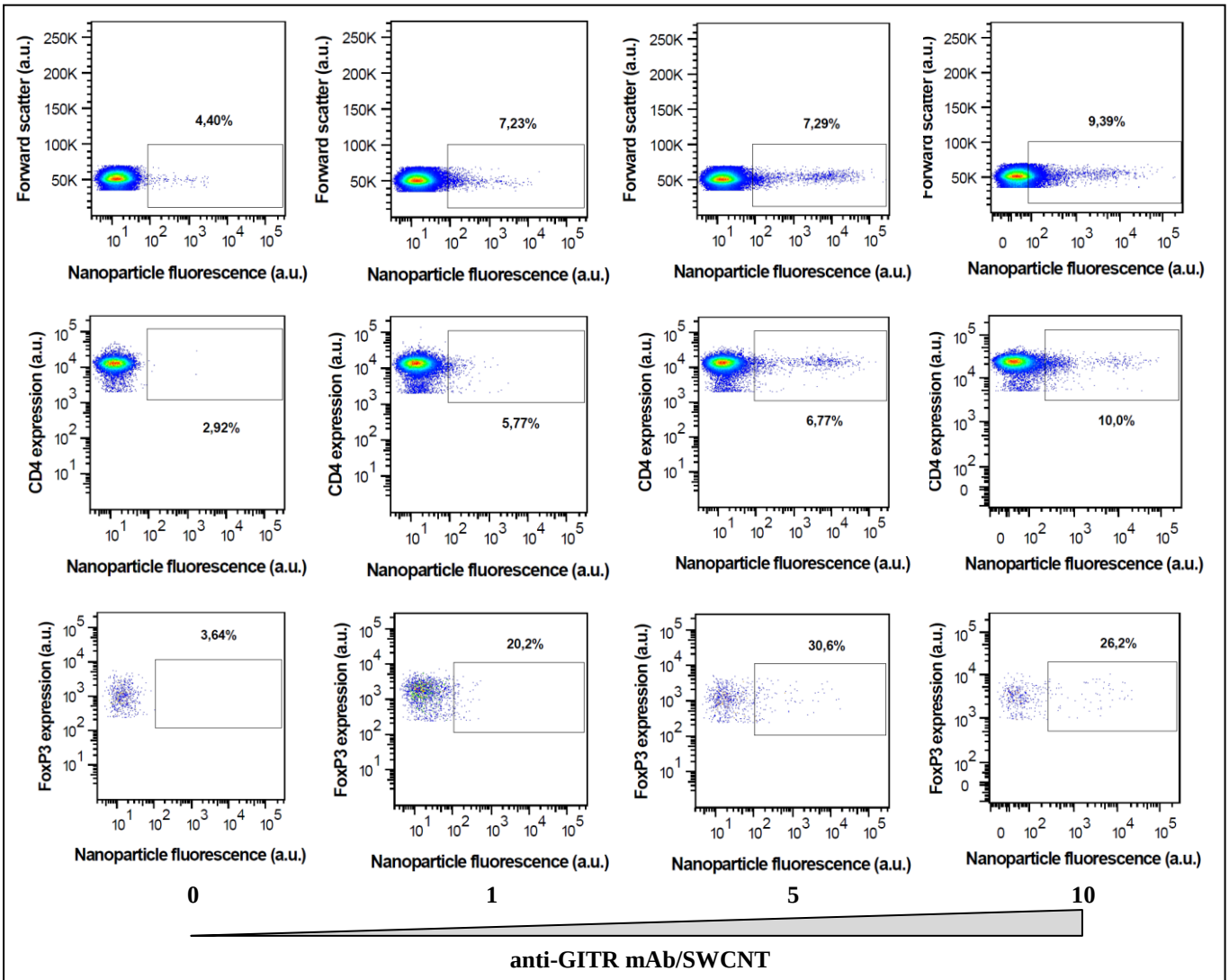

Figure 1. ***Ex vivo*** **effects of number of antibodies per PEG-SWCNT on T_{reg}-specific internalization of GITR-targeted PEG-SWCNTs.** Total splenocytes were isolated from 8 wk GFP-FoxP3 mice and incubated for 1 h with 300 pM of 650 nm-emitting fluorochrome-labeled PEG-SWCNTs carrying different amounts of anti-GITR mAb/SWCNT. Cells were stained with anti-CD4 and subjected to FACS analysis. Lower panels show FoxP3 *vs* PEG-SWCNT fluorescence of T_{reg} (gated as $CD4^+$ GFP^+), middle panels show anti-CD4 *vs* PEG-SWCNT fluorescence of $CD4^+$ T_{cells} (gated as $CD4^+$ GFP^- cells) and upper panels show forward scatter *vs* PEG-SWCNT fluorescence of remaining splenocytes (gated as $CD4^-$ GFP^- cells). Gates in the panels show the efficiency of PEG-SWCNT internalization (% of PEG-SWCNT-positive cells among the cells shown in the panel).

2.1 Fabrication of PEG-SWCNTs

PEG-SWCNTs loaded with targeting agents (mAbs) and cargo (fluorochromes and siRNA) were fabricated using our published protocol [4]. In particular, PEG-SWCNTs loaded with 650 nm- and near infrared (NIR)-emitting fluorochromes were used for *ex vivo* and *in vivo* investigations, respectively. Atomic force microscope images of PEG-SWCNTs showed the presence of individual particles composed of PEGylated SWCNTs having a narrow distribution of lengths centered at approximately 100 nm.

2.2 *Ex Vivo* T_{reg} Targeting by PEG-SWCNTs

First, we investigated the *ex vivo* effects of number of antibodies per PEG-SWCNT on the T_{reg} targeting efficiency and specificity of PEG-SWCNTs. Total splenocytes from GFP-FoxP3 mice were incubated with 300 pM of PEG-SWCNTs loaded with 1, 5 or 10 anti-GITR mAb/SWCNT for 1 h. FACS analysis showed that the T_{reg} targeting efficiency and specificity had a parabolic dependence with the number of targeting ligands and the maximum of T_{reg} targeting efficiency and specificity was obtained by PEG-SWCNTs carrying 5 anti-GITR mAb/SWCNT (Figure 1).

These nanoparticles (hereafter named as PEG-SWCNT-GITR) were used for successive *ex vivo* and *in vivo* investigations.

Next, we investigated the *ex vivo* effects of dose and incubation time on the T_{reg} targeting efficiency and specificity of PEG-SWCNT-GITR. Total splenocytes from GFP-FoxP3 mice were incubated with 300 pM of PEG-SWCNT-GITR for 1 h or 6 h, or cultured for additional 5 h after 1 h of nanotube incubation. FACS analysis showed that increasing the incubation time from 1 h to 6 h led to lower T_{reg} *vs* T_{eff} and T_{reg} *vs* $CD4^-$ cell targeting specificities, whereas cells cultured for an additional 5 h after 1 h of nanotube incubation showed no statistical difference between T_{reg} and T_{eff} targeting efficiencies and between T_{reg} and $CD4^-$ cell targeting efficiencies with respect to cells analyzed immediately after 1 h of nanotube incubation. Moreover, total splenocytes from GFP-FoxP3 mice were incubated with 100 pM, 300 pM or 1nM of PEG-SWCNT-GITR. FACS analysis showed that increasing the concentration from 100 pM to 300 pM led to an increase in T_{reg} targeting efficiency and specificity, whereas a further increase to 1 nM led to no increase in T_{reg} targeting efficiency and a decrease in T_{reg} targeting specificity.

Finally, we investigated the mechanism of T_{reg} internalization and trafficking of PEG-SWCNT-GITR. Total splenocytes were isolated from GFP-Foxp3 mice, incubated with PEG-SWCNT-GITR or PBS alone (control) at 4 ºC or 37 ºC for 6 h, then stained with fluorochrome-labeled goat anti-mouse IgG mAbs – in order to probe the presence of nanotubes bound to the plasma membrane of T_{reg} – and subjected to FACS analysis. The almost identical low fluorescence intensity from control cells and T_{reg} treated with PEG-SWCNT-GITR at 37 ºC and the intense fluorescence from T_{reg} treated with PEG-SWCNT-GITR at 4 ºC confirmed that PEG-SWCNT-GITR were internalized by T_{reg} through an energy-dependent mechanism (GITR-mediated endocytosis). In order to investigate the trafficking of PEG-SWCNT-GITR, T_{reg} were sorted from total splenocytes, incubated with nanoparticles for 1 h, 6 h or cultured for additional 5 h after 1 h incubation and analyzed by confocal microscopy. Confocal images of T_{reg} central planes showed punctuate features in proximity of the plasma membrane in cells incubated for 1 h, whereas a diffused fluorescence in the nucleus of T_{reg} was observed in cells incubated for additional 5 h after 1 h incubation and in those incubated for 6 h with PEG-SWCNT-GITR. Taken together these results suggested that PEG-SWCNT-GITR were internalized by T_{reg} through GITR-mediated endocytosis and entrapped into endosomes, traslocated into the cytoplasm, released into the cytoplasm and finally transported into the nucleus of T_{reg}.

2.3 *In Vivo* T_{reg} Targeting by PEG-SWCNTs

First, we investigated the pharmacokinetic profile of PEG-SWCNT-GITR. Groups of three C57BL/6J mice were administered with a single injection of 20 μg of PEG-SWCNT-GITR (labeled with NIR-emitting fluorochromes). Blood samples (approximately 0.1 mL) were collected at different time points, their NIR emission recorded and used to calculate the blood concentration and pharmacokinetic parameters of PEG-SWCNT-GITR. We found that the blood concentration of PEG-SWCNT-GITR was best described by a two-compartmental model and that PEG-SWCNT-GITR reached the distribution balance among blood and tissues in ~20 min and were eliminated with a time constant of ~2 h.

Next, we investigated the *in vivo* T_{reg}-targeting dose-dependence of PEG-SWCNT-GITR. GFP-Foxp3 mice were administered with a single injection of different amounts of PEG-SWCNT-GITR and sacrificed 24 h after the administration. Splenocytes were isolated and subjected to FACS analysis. We found the best combination of T_{reg}-targeting efficiency and specifity for mice treated with 5 μg. This dose of PEG-SWCNT-GITR was used for successive *in vivo* investigations. No non-specific T_{reg}-targeting was observed when mice were administered with PEG-SWCNTs loaded with 5 anti-HA mAb/SWCNT.

We also investigated the *in vivo* intracellular trafficking of PEG-SWCNT-GITR. T_{reg} were sorted from splenocytes of GFP-FoxP3 mice administered with PEG-SWCNT-GITR and sacrificed 24 h after the administration. Confocal microscopy revealed that PEG-SWCNT-GITR localized within the cytosol and nucleus of T_{reg} of treated mice.

Finally, we investigated the *in vivo* targeting of T_{reg} residing in the tumor microenvironment. B16 melanoma-bearing GFP-Foxp3 mice were administered with a single injection of PEG-SWCNT-GITR and sacrificed 24 h after the administration. We found that the nanoparticles targeted T_{reg} residing in the tumor microenvironment with higher efficiency and specificity with respect to those residing in the spleen.

2.4 Delivery of siRNA Duplexes into T_{reg} *In Vivo*

Our previous results showed that PEG-SWCNTs armed with ligands against a T_{reg}-enriched marker were able to reach the cytoplasm and nucleus of T_{reg} residing in different tissues (i.e. spleen of healthy mice of B16 melanoma). Based on these results, we investigated the possibility to deliver genetic inhibitors into T_{reg} *in vivo*. We used RFP/GFP-Foxp3 mice and siRNA against DsRed2 to inhibit expression of RFP in living animals.

Small interfering (si) RNA were designed as 27-mer asymmetric duplexes with one 2-base 3' overhang on the antisense strand in combination with 2 DNA residues to the 3' end of the sense strand. These structural features have been described to limit heterogeneity of the cleaved siRNA products and increase siRNA potency. Furthermore, it is well known that the potency of an siRNA can change by an order of magnitude or more shifting the predicted (21-mer) Dicer cleavage product by few bases along the mRNA

target sequence. Four anti-DsRed2 [indicated as siRNA(1)-(4)] and one scramble RNA (siRNAScr) duplexes were designed and HEK293 cells were lipofected with pDsRed2-N1 plasmid and different concentrations of siRNA duplexes. After 24 h the cells were analyzed by FACS and by quantitative RT-QPCR. Our data showed that 50 nM siRNA(3) elicited the highest decrease in RFP expression (approximately 70%) than the other sequences and the scramble one and it was chosen for the successive *in vivo* investigations. RFP/GFP-Foxp3 mice were administered a single retro-orbital injection of PEG-SWCNT-GITR carrying siRNA(3) and were sacrificed 24 h after the administration. Control animals were administered a single retro-orbital injection of PEG-SWCNT-GITR. RNA duplexes were linked to the nanotubes through a disulfide bond in order to enable their release once the nanoparticles were internalized by T_{reg}. FACS analysis of splenocytes isolated from animals injected with nanoparticles carrying siRNA(3) showed a T_{reg}-specific decrease in RFP signal of approximately 50% with respect to that from splenocytes isolated from animals injected with siRNA-devoid nanoparticles. The decrease in RFP signal in T_{eff} was lower than that exhibited by T_{reg} (Figure 2).

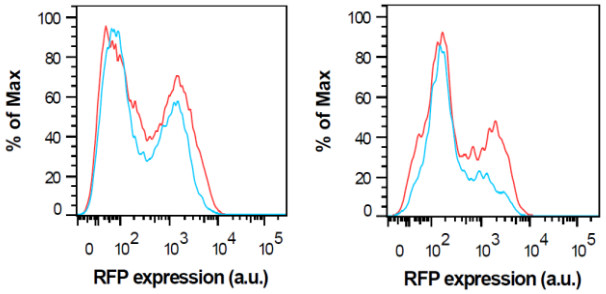

Figure 2. *In vivo* T_{reg}-specific silencing by GITR-targeted PEG-SWCNTs. 8 wk RFP/GFP-Foxp3 mice were retro-orbitally administered with 5 μg of fluorochrome-labeled PEG-SWCNTs carrying 5 anti-GITR mAb/SWCNT and either anti-RFP siRNA or siRNA-devoid in PBS. Total splenocytes were isolated after 24 h, stained with anti-CD4 and subjected to FACS analysis. RFP expression in T_{eff} (left panel) and T_{reg} (right panel) T cells isolated from animals treated with siRNA-loaded (blue line) or siRNA-devoid (red line) PEG-SWCNTs are reported.

3 CONCLUSIONS

Herein, we showed that PEG-modified SWCNTs loaded with ligands for a T_{reg}-enriched receptor can be targeted to T_{reg} residing in specific tissues (*i.e.* spleen of healthy mice or tumor microenvironment of B16 melanoma-bearing mice) and deliver siRNA specifically into T_{reg} *in vivo*. To the best of our knowledge there are no reports about the specific delivery of cargo in T_{reg} (and other T cell subsets) both *ex vivo* and *in vivo*. Although small-molecule drugs will undoubtedly continue to play a dominant role in the near future, our work demonstrates that the combination of immunology and nanotechnology is an exciting track that will open new avenues for the development of novel drugs against autoimmune diseases and malignancies.

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