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Differential Immunostimulatory Effects of EGFP and +36 GFP on Immune Cells

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ABSTRACT

Introduction: Green fluorescent protein (GFP) and its variants are pivotal in tracking gene expression across various gene delivery systems. While GFP is typically employed for intracellular reporting, it can be modified to display on cell surfaces for labeling. Previous research indicates GFP might have immunogenic effects, notably enhancing tumor-specific T cell responses. This study explores the immunostimulatory differences between enhanced GFP (EGFP) and the supercharged variant, +36 GFP. **Methods:** Recombinant EGFP and +36 GFP proteins were generated using an *Escherichia coli* expression system. Murine bone marrow-derived dendritic cells (BMDCs) were generated using established protocols. Splenocytes were isolated from murine spleens via mechanical disruption and red blood cell lysis. The RAW 264.7 macrophage cell line was cultured in complete DMEM medium. Immune cells were then incubated with varying concentrations of EGFP and +36 GFP, separately, for 48 h. Cytokine levels (IFN-γ, TNF-α, IL-10) were quantified using sandwich ELISA. **Results:** Purified EGFP and +36 GFP migrated as a single band of ~27 kDa on SDS-PAGE, consistent with their predicted molecular weights. Both EGFP and +36 GFP elicited cytokine production from T cells and macrophages. EGFP significantly increased TNF-α secretion and decreased IL-10 secretion compared to +36 GFP in both T cells and macrophages. **Conclusion:** Our findings demonstrate that EGFP and +36 GFP differentially stimulate immune cells, suggesting that the selection of GFP variant could influence the immunological outcomes in therapeutic applications. This necessitates careful consideration in vector design, vaccine development, and other biotherapeutic strategies to optimize efficacy and safety through appropriate GFP choice, dosing, and control inclusion.

INTRODUCTION

Green fluorescent protein (GFP) is a versatile reporter protein extensively used for monitoring gene expression, protein localization, and cell trafficking in both *in vitro* and *in vivo* systems. Enhanced GFP (EGFP), a commonly used variant derived from wild-type GFP, offers improved brightness, folding efficiency, and photostability compared to its wild-type counterpart [1]. Stripecke *et al*. (1999) demonstrated that GFP-derived peptides, when presented by major histocompatibility complex (MHC) class I molecules, elicited cytotoxic T lymphocyte (CTL) responses against GFP-expressing cells [2]. While some studies indicate GFP can modulate T cell activation, leading to increased expression of the activation marker CD25 and enhanced interleukin-2 (IL-2) production, others demonstrate its potential to interfere with signaling pathways, such as by reducing NF-κB activation in HEK293 and HeLa cells [3]. Consequently, the use of GFP-tagged vectors may unintentionally influence T cell responses, underscoring the need for appropriate controls to account for any GFP-specific effects [3]. However, cellular damage from GFP transfection or transduction, including direct effects (*e.g*., reactive oxygen species (ROS) generation and apoptosis induction) and indirect immune responses to the introduced vector or transgene, can also confound experimental results [4-9].

Furthermore, some GFP variants with a high net positive surface charge (supercharged GFPs) exhibit enhanced cellular uptake. This protein supercharging technique, which increases a protein's net surface charge, can also improve protein stability (*e.g*., resistance to

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temperature and proteases) and modulate protein-protein interactions [10]. Furthermore, supercharged proteins, with their high net positive surface charge, exhibit enhanced cellular uptake in both *in vitro* and *in vivo* systems [11-13]. For example, cationic supercharged GFPs (net charge +6 to +48) are efficiently endocytosed by various mammalian cell types, including chondrocytes [12, 14-16]. Consequently, supercharged GFPs have shown promise as versatile carriers for delivering various biomolecules (*e.g*., proteins, peptides, and nucleic acids) to both intracellular and extracellular compartments [14]. A supercharged GFP variant with a net charge of +36 (+36 GFP) has been shown to significantly enhance the delivery of various protein and DNA cargoes (*e.g*., antibodies, enzymes, and gene-editing tools) in both *in vitro* and *in vivo* systems [17].

Given the widespread use of GFP in gene delivery systems (*e.g*., plasmid, adenoviral, and lentiviral vectors) for tracking gene expression, and the emerging potential of +36 GFP as a delivery vehicle, we investigated the differential effects of EGFP and +36 GFP on cytokine secretion by key immune cell populations (*e.g*., dendritic cells, lymphocytes, and macrophages).

MATERIAL AND METHODS

Recombinant EGFP and +36 GFP expression and purification. The prokaryotic expression vectors pET26b-+36GFP and pET28a-EGFP, encoding supercharged GFP (+36 GFP) and enhanced GFP (EGFP), respectively, were previously constructed in our laboratory [17, 18]. Recombinant +36 GFP and EGFP were expressed in *E. coli* Rosetta (DE3) as described previously [17, 18]. Briefly, protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich) and incubating at 37°C for 3 h. His-tagged proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen) under native conditions following the manufacturer's instructions. For this study, 300 mL bacterial cultures were used for the expression and purification of both +36 GFP and EGFP. The purified proteins were dialyzed against phosphate-buffered saline (PBS) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 12% resolving gel. Protein concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The purified proteins were aliquoted and stored at -70°C.

Cytokine secretion by dendritic cells and splenocytes. The effects of EGFP and +36 GFP on TNF-α, IFN-γ, and IL-10 secretion were assessed in BMDCs, splenocytes, and BMDC-splenocyte co-cultures. BMDCs were generated from bone marrow of naïve male BALB/c mice (5–7 weeks old; Pasteur Institute of Iran) as described previously [19]. Briefly, bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco), 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech), and 10 ng/mL interleukin-4 (IL-4; PeproTech) at 37°C in a humidified 5% CO_2 atmosphere. Immature BMDCs (5×10^5 cells/well) were seeded in 6-well plates and treated with EGFP or +36 GFP (5 μg/mL) for 48 h. Splenocytes were isolated from mouse spleens by mechanical disruption followed by red blood cell lysis using ammonium-chloride-potassium (ACK) lysis buffer (Merck) as described previously [19]. Splenocytes (2×10^6 cells/well) were seeded in 6-well plates and treated with EGFP or +36 GFP (5 μg/mL) for 48 h.

For co-culture experiments, BMDCs (5×10^5 cells/well) were seeded in 6-well plates and pulsed with EGFP or +36 GFP (5 μg/mL) for 24 h. Splenocytes (2×10^6 cells/well) were then added to the BMDC cultures at a 4:1 splenocyte:BMDC ratio, and the co-cultures were incubated for an additional 48 h.

Concentrations of TNF-α, IFN-γ, and IL-10 in the culture supernatants were measured using enzyme-linked immunosorbent assays (ELISAs) (Mabtech, Nacka Strand, Sweden). Untreated BMDCs and splenocytes served as negative controls.

Cytokine secretion by RAW 264.7 macrophages. The effects of EGFP and +36 GFP on IFN-γ, TNF-α, and IL-10 secretion were assessed in the RAW 264.7 murine macrophage cell line (National Cell Bank, Pasteur Institute of Iran). Cells (5×10^5/well) were seeded in 6-well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO_2 atmosphere. Cells were treated with EGFP or +36 GFP (5 μg/mL) for 48 h. Culture supernatants were collected, and cytokine (IFN-γ, TNF-α, and IL-10) concentrations were measured using ELISAs (Mabtech, Sweden). Untreated cells served as negative controls. The lower limit of detection for all ELISA kits was 2 pg/mL.

Ethical considerations. All animal experiments were approved by the Research Ethics Committee of the Pasteur Institute of Iran (IR.PII.REC.1400.026; approved June 7, 2021) and adhered to national guidelines for scientific research.

Statistical analysis. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). Data are presented as mean ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Characterization of purified EGFP and +36 GFP. Recombinant EGFP and +36 GFP were expressed in *E. coli* Rosetta (DE3) and purified by Ni-NTA affinity chromatography under native conditions as described previously [17, 18]. SDS–PAGE analysis confirmed the

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purity of both proteins, each appearing as a single band at approximately 27 kDa, consistent with their predicted molecular weights (Figure 1). Protein yields from 150 mL bacterial cultures were 0.6 mg/mL for EGFP and 0.4 mg/mL for +36 GFP.

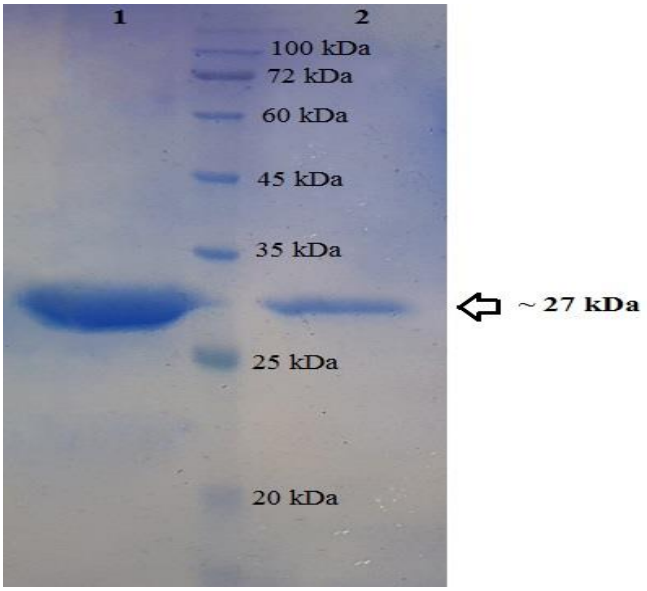


Fig. 1. SDS–PAGE analysis of purified EGFP and +36 GFP. Lane 1: Purified EGFP; Lane 2: Purified +36 GFP. The expected molecular weight of both proteins is approximately 27 kDa. MW: PageRuler™ Prestained Protein Ladder (10–180 kDa; Thermo Fisher Scientific).

Differential cytokine modulation by EGFP and +36 GFP. EGFP and +36 GFP differentially modulate cytokine secretion by DCs, splenocytes, and co-cultures: To assess the immunostimulatory effects of EGFP and +36 GFP, we measured TNF-α, IFN-γ, and IL-10 secretion in BMDCs, splenocytes, and BMDC-splenocyte co-cultures. EGFP treatment significantly enhanced TNF-α secretion by BMDCs compared to +36 GFP treatment ($P < 0.001$; Figure 2). Conversely, EGFP treatment resulted in significantly lower IL-10 secretion by BMDCs ($P < 0.05$; Figure 2). Similarly, in splenocyte cultures, EGFP stimulated significantly greater production of TNF-α and IFN-γ ($P < 0.001$ and $P < 0.05$, respectively; Figure 2) and lower production of IL-10 ($P < 0.001$; Figure 2) compared to +36 GFP. In BMDC-splenocyte co-cultures, EGFP treatment also led to increased TNF-α and IFN-γ secretion ($P < 0.05$ and $P < 0.001$, respectively; Figure 2) and decreased IL-10 secretion ($P < 0.001$; Figure 2) compared to +36 GFP.

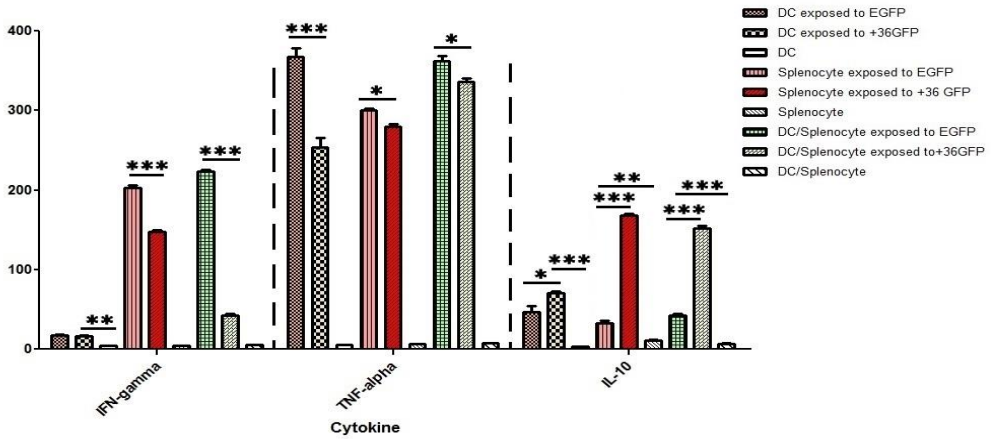


Fig. 2. EGFP and +36 GFP differentially modulate cytokine secretion by dendritic cells, splenocytes, and co-cultures. TNF-α, IFN-γ, and IL-10 concentrations in culture supernatants were measured by ELISA. Untreated cells served as negative controls. Data are presented as mean ± SD of independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

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Macrophage cytokine responses. The immunostimulatory effects of EGFP and +36 GFP on RAW 264.7 macrophages were assessed by measuring cytokine secretion. Treatment with either EGFP or +36 GFP significantly increased TNF-α and IL-10 production compared to untreated cells ($P < 0.01$ for both cytokines; Figure 3). In both treatment groups, TNF-α concentrations were consistently higher than IL-10 concentrations. Moreover, EGFP induced significantly greater TNF-α secretion than +36 GFP after 48 h ($P < 0.05$; Figure 3). IFN-γ was not detected in RAW 264.7 macrophages treated with either protein (Figure 3).

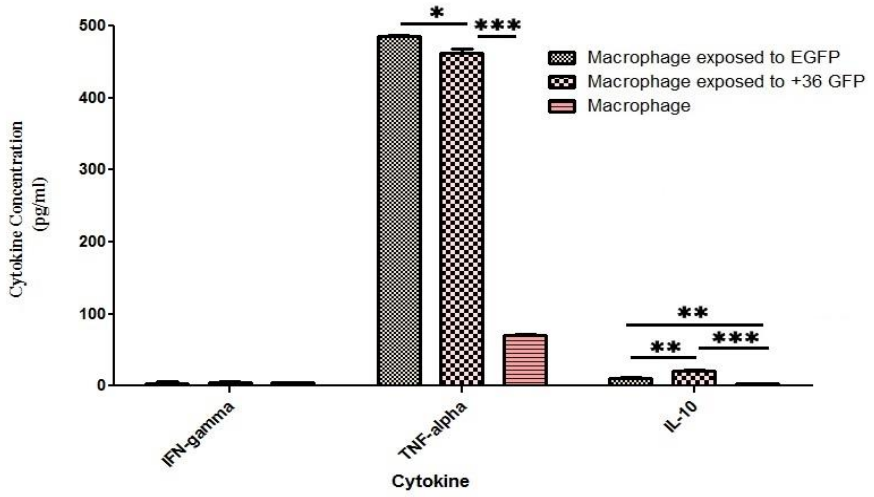


Fig. 3. EGFP and +36 GFP induce cytokine secretion by RAW 264.7 macrophages. TNF-α and IL-10 concentrations in culture supernatants were measured by ELISA. Untreated cells served as negative controls. IFN-γ was not detected. Data are presented as mean ± SD of independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

DISCUSSION

GFP, a ~27 kDa protein comprising 238 amino acids, is widely used as a reporter in various biological applications, including detecting transgene expression, monitoring gene expression, visualizing or tracking proteins via fusion constructs, and tracking cell lineage and migration [3, 20]. While traditionally considered biologically inert, GFP has been shown to exert various biological effects, particularly on immune cell function, including cytokine production and inflammatory signaling pathways. For example, Koelsch *et al*. (2013) demonstrated a significant decrease in CD25 (IL-2Rα) expression in both $CD4^+$ and $CD8^+$ T cells isolated from mice transfected with a GFP-encoding vector [3]. Furthermore, GFP has also been shown to modulate the nuclear factor-κB (NF-κB) pathway, potentially reducing IL-2 secretion and CD25 expression in activated T cells. However, one study using a NF-κB-luciferase reporter system in HEK-293T cells found that EGFP expression interfered with polyubiquitination processes, decreasing NF-κB activation [21]. However, conflicting reports exist regarding the *in vitro* effects of GFP and its derivatives on human and murine T cell activation, with some studies showing immunostimulatory effects while others report immunosuppressive or no effects [3, 21, 22]. These varied findings suggest that different GFP variants, such as EGFP and +36 GFP, may exert distinct effects on T cell activation, potentially due to differences in their structure or intracellular localization. Indeed, the biological effects of GFP can vary depending on the GFP variant (*e.g*., EGFP vs. +36 GFP), the cell type (*e.g*., T cells vs. macrophages), and the experimental conditions (*e.g*., transfection method, duration of expression) [23]. Our study reinforces this point, demonstrating differential effects of EGFP and +36 GFP on immune cells, specifically highlighting differential modulation of TNF-α and IL-10 secretion in DCs and macrophages.

Our data demonstrate that EGFP stimulates cytokine secretion by DCs, splenocytes, and DC-splenocyte co-cultures. Specifically, EGFP induced significantly greater TNF-α and IFN-γ secretion compared to IL-10. These elevated TNF-α and IFN-γ concentrations suggest a Th1-biased immune response. EGFP also significantly increased TNF-α secretion by macrophages. This observation aligns with a previous study demonstrating robust CTL responses against EGFP-expressing leukemia cells in mice challenged with BM185/EGFP leukemia [2]. This highlights the potential role of CTLs in mediating anti-tumor immunity against EGFP-expressing cells. Another study evaluated GFP's potential as an adjuvant to enhance T-cell immunity against the melanoma tumor antigen MART1. Transducing DCs with a GFP-expressing adenovirus, followed by pulsing with MART1, significantly increased the percentage of MART1-specific $CD8^+$ T cells (28%) compared to DCs pulsed with MART1 alone (11%; $P < 0.01$), DCs transduced with a null adenovirus and pulsed with MART1 (11.7%; $P < 0.02$), or DCs pulsed with MART1 and loaded with GFP

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protein (12.2%) [24]. Previous studies have demonstrated that certain GFP variants, including those similar to the ones used in this study, can elicit CTL responses [25-27]. This CTL response is likely initiated by the recognition of GFP peptides presented on MHC class I molecules by CTLs [2]. However, EGFP immunogenicity can be significantly influenced by the animal model (with varying responses between strains) and the route of administration (*e.g*., subcutaneous or intravenous), which can alter both the magnitude and type of immune response elicited [2, 20]. For example, EGFP elicited a robust antibody response in BALB/c mice, but only a weak response in C57BL/6 mice [20]. Computational epitope prediction and *in vitro* MHC binding assays have demonstrated that mouse MHC Class I (H2-Kd) molecules present a naturally occurring EGFP epitope. This presentation initiates a T-cell activation cascade, triggering intracellular signaling events that lead to T-cell proliferation and effector functions [4].

Conversely, some studies suggest that GFP can exhibit cytotoxicity under specific conditions (*e.g*., high expression levels, specific cell types) by inducing apoptosis [5]. Supporting this, caspase-3 expression was significantly increased in cells transfected with a GFP-expressing vector compared to control vector-transfected cells [5]. Increased ROS production in GFP-expressing cells has also been linked to cytotoxicity [4], likely through oxidative damage to cellular components such as DNA, proteins, and lipids. The relationship between GFP immunogenicity (MHC-I presentation and CTL activation) and increased ROS production remains unclear. One potential link could be granzyme B released by activated CTLs, which can trigger mitochondrial ROS release by cleaving caspase-3 and nuclear lamin, thereby disrupting mitochondrial membrane integrity [28, 29].

GFP has been shown to elicit both humoral and cellular immune responses, as well as cytotoxic effects (including apoptosis and reduced cell viability) in various animal models, including mice, rats, dogs, and monkeys. Given that GFP can induce ROS production and oxidative stress in certain cell types, GFP analogs like CFP and YFP, which reportedly induce lower levels of oxidative stress, may be preferable alternatives for cell lines sensitive to oxidative stress [4-8].

Given EGFP's utility as a reporter gene and its inherent immunological properties, researchers have investigated supercharged proteins—proteins with a high net positive charge that enhance cellular uptake—including modified GFPs, as potent vehicles for delivering macromolecules into mammalian cells [17]. Our group has demonstrated effective delivery of the full-length human papillomavirus (HPV)-16 E7 antigen, a key oncoprotein in HPV-related cancers, using +36 GFP as a delivery vehicle. Both *in vitro* and *in vivo* experiments showed increased E7 expression in target cells and a robust E7-specific immune response, indicating successful antigen delivery and presentation. This approach holds promise for E7-targeted immunotherapies against HPV-associated malignancies. The supercharged nature of +36 GFP likely facilitates its efficient cellular entry and enhances E7 transfection. Moreover, immunization of mice with +36 GFP complexed (+36 GFP + E7) or fused (+36 GFP-E7) with the E7 antigen significantly elicited a Th1 cellular immune response and granzyme B secretion. Importantly, mice immunized with +36 GFP-E7 constructs exhibited significantly greater protection against TC-1 tumor challenge (approximately 67%) compared to E7 alone (approximately 33%). These findings highlight the potential of +36 GFP as an effective antigen delivery system for eliciting potent anti-tumor immune responses [17]. Krishnan *et al*. (2018) investigated the effects of cationic GFPs on chondrocytes and found no adverse effects on cartilage matrix turnover, cell viability, or metabolic rate, indicating biocompatibility with these cells [14]. While our previous work demonstrated the antiviral activity of +36 GFP, showing up to 75% reduction in hepatitis C virus (HCV) replication in Huh7.5 cells, 49% reduction in HIV replication in HeLa cells, and 43% reduction in HSV replication in Vero cells [30], the present study focuses on its immunostimulatory effects. Specifically, we investigated the differential effects of +36 GFP and EGFP on cytokine secretion from immune cells *in vitro*. We found that +36 GFP induced a distinct cytokine profile compared to EGFP. Although +36 GFP induced lower levels of IFN-γ and TNF-α, it stimulated significantly higher IL-10 secretion. These observed differences in cytokine induction may be attributed to the distinct structural and physicochemical properties of EGFP and +36 GFP, such as the increased positive charge and enhanced cellular uptake of +36 GFP, which could influence its interactions with immune cells and subsequent signaling pathways. The increased positive charge of +36 GFP might lead to stronger interactions with negatively charged cell surface molecules, potentially altering receptor clustering and downstream signaling cascades. The enhanced cellular uptake of +36 GFP could also affect its intracellular localization and interactions with various cellular components, including those involved in cytokine regulation.

This study demonstrates the differential immunomodulatory effects of EGFP and its supercharged variant, +36 GFP. EGFP induced a more pro-inflammatory cytokine profile, while +36 GFP promoted a potentially more regulatory response, characterized by increased IL-10 secretion and decreased IFN-γ and TNF-α. These findings underscore the importance of considering the immunological properties of GFP variants when designing experiments, particularly those involving immune cells or *in vivo* models. In the context of vaccine development using GFP-linked antigens, careful assessment of immunogenicity and toxicity in relevant animal models is crucial to avoid non-specific immune responses and optimize vaccine efficacy. Dose optimization of supercharged GFP variants like +36 GFP

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is particularly important to balance immunogenicity with potential adverse effects. The immunomodulatory properties of GFP variants, including their demonstrated ability to enhance antitumor immunity and inhibit viral replication, hold promise for therapeutic applications. However, careful consideration of factors such as GFP variant selection, dose, route of administration, animal model, and target cell type is essential to mitigate potential negative effects, including unwanted immune activation, cytotoxicity, and interference with normal cellular functions. Ultimately, a thorough understanding of the immunological and cellular effects of GFP variants is critical for their safe and effective application in research and therapeutic settings.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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