INTRATUMORAL ADMINISTRATION OF A MULTIGENE CONSTRUCT BY ELECTROPORATION CAN EFFECTIVELY MODULATE ANTI-TUMOR RESPONSE IN A MURINE B16.F10 MODEL

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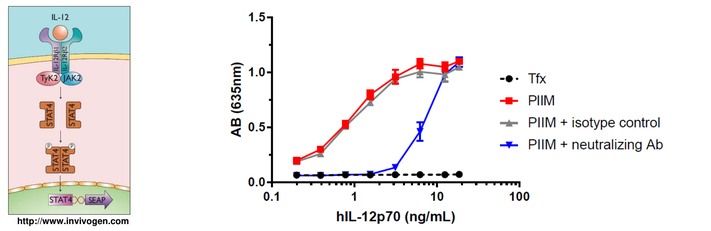
Abstract

Background: Immunomodulatory cytokines, such as IL-12, are attractive candidates for cancer immunotherapy. IL-12 is a pro-inflammatory cytokine with potent anti-tumor effects; however, systemic administration shows limited clinical efficacy and dose-associated toxicity. In preclinical and clinical studies, intratumoral (IT) delivery of IL-12 plasmid DNA by electroporation (EP) can provide a safe and effective alternative for efficacious dosing. To augment the effects of IL-12, we developed a DNA plasmid platform that allows for delivery of agents that modulate multiple immune pathways as well as tumor- or patient-specific neoantigens. Polycistronic IL-12 Immune Modulator plasmid (PIIM) is a single plasmid encoding IL-12, and a fusion of Flt3L to an antigen. Flt3L is a ligand that stimulates dendritic cell maturation and enhances antigen processing and presentation. The encoded antigen can be a viral or shared antigen, or a patient-specific neoantigen, which enables customization to patient populations, as well as providing an aid to monitoring antigen-specific immune response(s) that can be correlated to patient outcomes. Here we demonstrate the first functional characterization of PIIM. **Materials & Methods:** Two PIIM constructs were created for functional characterization: PIIM-OVA (IL12~Flt3L-OVA) for mouse experiments, and PIIM-NYESO1 (IL12~Flt3L-NYESO1) for testing in human cells. The expression and functional activity of PIIM components were determined. Treated tumors and spleens were assessed for transcriptional changes by NanoString® and phenotypic changes by flow cytometry. Systemic effects of PIIM were assessed using a syngeneic two-tumor model of B16.F10 in which only one tumor received IT-PIIM-EP while the other contralateral lesion remained untreated. **Results:** PIIM-OVA and PIIM-NYESO1 secrete functional IL-12p70, Flt3L-OVA and Flt3L-NYESO1 fusion proteins as assessed by ELISA, flow and cell-based assays. PIIM promotes DC maturation and antigen-specific T cell proliferation both *ex vivo* and *in vivo*. Hydrodynamic-based gene delivery of PIIM-OVA lead to splenomegaly and significantly increased splenic CD11c⁺ DCs. Furthermore, IT-PIIM-EP lead to generation of splenic OVA-specific CD8s and increased APM gene expression. When introduced intratumorally in a mouse two-tumor model, IT-PIIM-EP delays B16.F10 tumor growth in both treated and contralateral tumors compared to untreated controls resulting in increased overall survival. **Conclusion:** PIIM represents a novel approach to cancer immunotherapy. A combination of functional immune modulators can be expressed locally in the tumor microenvironment that increase inflammatory infiltrate, enhances antigen presentation and produces a systemic T cell response specific to the antigen encoded on the plasmid. This customizable approach has the potential to improve therapeutic outcome by enhancing adaptive-immunity and addressing patient-specific neoantigens needs.

IL-12p70 and Flt3L-antigen co-expressed from PIIM plasmid are functional

a) IL-12p70 Reporter Cells



b) THP-1 cell binding assay

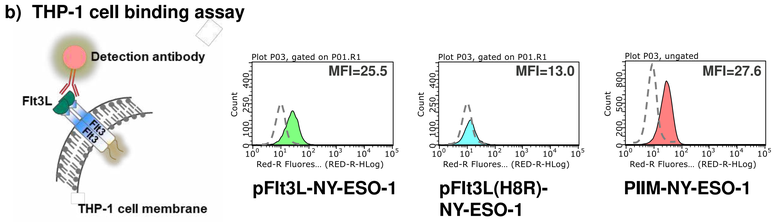
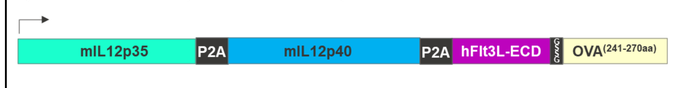


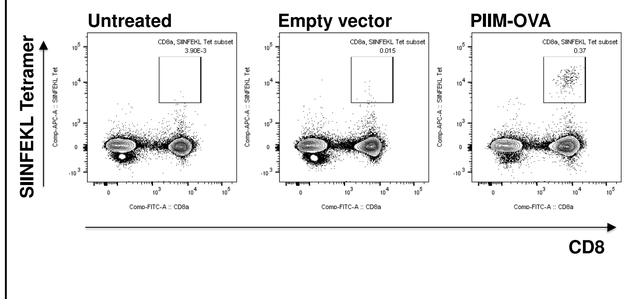
Figure 2: **a)** HEK-Blue IL-12 cells (Invivogen; hkb-il12) produce secreted embryonic alkaline phosphatase (SEAP) upon IL-12p70 signaling via a STAT4 reporter. Dose-response of hIL12-p70 derived from PIIM-NY-ESO-1 (red) on HEK-Blue cells. Signaling is blunted with use of a neutralizing IL-12 antibody (blue; R&D Systems; 125µg/mL), but not by an isotype control (gray; R&D Systems; 125µg/mL). **b)** Flt3L-NY-ESO-1, Flt3L(H8R)-NY-ESO-1 mutant, PIIM-NY-ESO-1 or transfection control (tfx) were produced by transient transfection in HEK293 cells and quantitated by ELISA. Equal amounts were incubated with Flt3⁺ human THP-1 cells to allow binding. Cell binding (shown as MFI) was detected on a Guava 12HT flow cytometer following detection with a biotinylated anti-hFlt3L antibody and streptavidin-Alexa Fluor 488 (representative flow cytometry graphs, n=3; transfection control binding is shown as gray dotted line).

Electroporation of PIIM-OVA generates SIINFEKL⁺ CD8 T cells

a) Mouse PIIM-OVA (mIL12~Flt3L-OVA) construct schematic



b) Generation of SIINFEKL⁺ CD8⁺ T cells



c) Quantitation

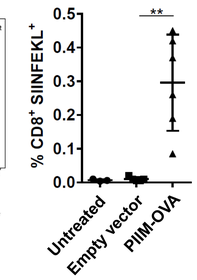


Figure 5: **a)** Schematic representation of mouse PIIM encoding a fusion protein of Flt3L and an ovalbumin fragment containing the MHC class I H-2 Kb restricted epitope, SIINFEKL (mIL12~Flt3L-OVA$^{241-270aa}$). **b)** B16.F10 cells were implanted on one flank of C57Bl/6J 6-8 week old mice. When tumors reached ~100mm^3, they were injected with plasmid DNA encoding for PIIM-OVA, empty pUMVC3 vector and electroporated or left untreated. 7 days post-treatment, lymph nodes were extracted, dissociated and stained for flow cytometry. The percentage of CD8⁺ T cells that are SIINFEKL-tetramer positive are shown in boxes on representative flow cytometry graphs (Gating: Singlet>Live>CD3⁺>CD19⁻). **c)** Consolidated data showing number of CD8⁺ SIINFEKL-tetramer⁺ T cells is shown. (n=5 animals per cohort; *Kruskal-Wallis*; ** p<0.01.)

Polycistronic IL-12 Immune Modulator (PIIM)

1) Multicistronic DNA construct encoding the following proteins:

pIL12-P2A
- Increases TILs & NK cells
- Increases IFNγ signature
- Increases APM
- Increases anti-angiogenic factors

+

Flt3L-ECD
- Stimulates DC maturation
- Enhances antigen processing & presentation

+

Neo/patient-specific antigen
- Monitor antigen-specific immune responses correlated to patient outcomes
- Potential therapeutic for antigen-expressing tumors

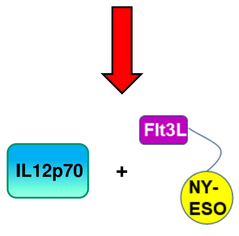
Fusion protein

2) Delivered via electroporation using Oncosec's GENESIS Electroporation Technology

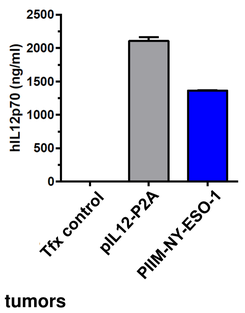
[In vivo EP conditions: 400V/cm, 8 x 10ms pulses]

Expression of IL-12p70 and Flt3L-NY-ESO-1 in vitro and in B16.F10 tumors

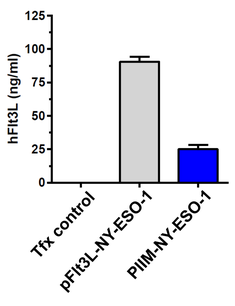
a) Schematic representation of PIIM with Flt3L fused to the shared antigen NY-ESO-1



b) Expression of IL-12p70



c) Expression of Flt3L-Ag



d) In vivo expression in B16.F10 tumors

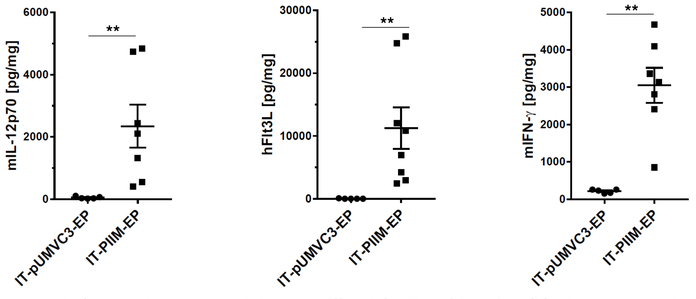
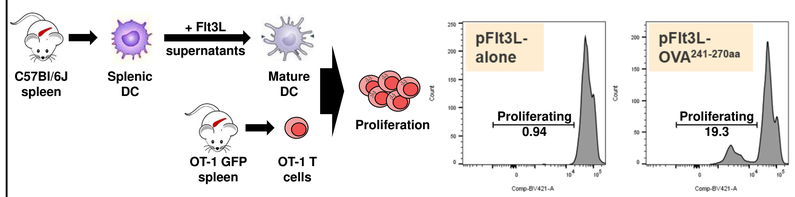


Figure 1: **a)** Schematic representation of PIIM (hIL12~hFlt3L-NY-ESO-1). **b)** Amount of hIL-12p70 secreted by HEK293 cells following transient transfection with pIL12-P2A, PIIM-NY-ESO-1 or transfection (tfx) control (72hr harvest; n=3; hIL-12p70 DuoSet ELISA DY1270). **c)** Amount of hFlt3L expressed and secreted by HEK293 cells following transient transfection with pFlt3L-NY-ESO-1, PIIM-NY-ESO-1 or transfection control (n=3; hFlt3L DuoSet ELISA DY308). **d)** The murine PIIM-NYESO analogue (mIL12~hFlt3L-NY-ESO-1) was electroporated into established B16-F10 lesions. After 48hr, the expression of mIL-12p70 (left panel), hFlt3L-NY-ESO-1 (middle panel) and the IL-12-induced cytokine IFN-γ (right panel) was measured in the tumor lysate (n=6-8; *Mann-Whitney*; ** p<0.01; R&D Systems DuoSet ELISAs DY419, DY308 and DY485).

Expressed Flt3L-antigen matures DCs and presents antigen to CD8 T cells

a) Mouse DCs matured with pFlt3L-OVA present antigen to co-cultured OT-1 T cells



b) Maturation of human iDCs with Flt3L-NY-ESO-1

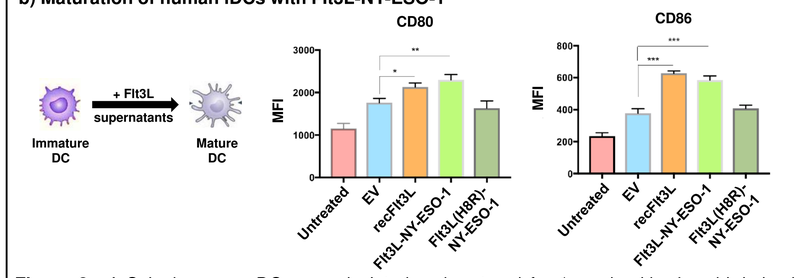
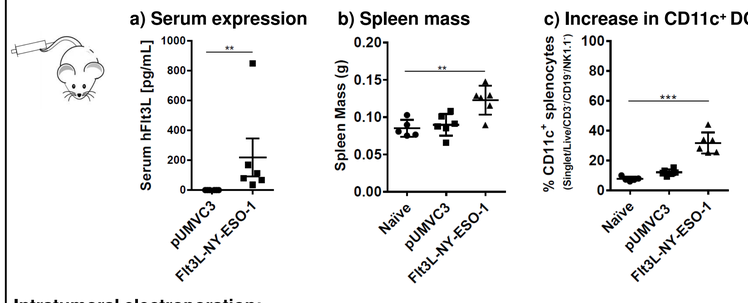


Figure 3: **a)** Splenic mouse DCs were isolated and matured for 1 week with plasmid-derived Flt3L supernatants. Mature DCs were then co-cultured with magnetically sorted OT-1 CD8 T cells loaded with proliferation dye (BD, Violet 450 #562158) . After 4 days, the percentage of OT-1 proliferation was determined (Representative dilution series shown). **b)** Human iDC's cultured ex-vivo were either untreated (pink), treated using 200ng/mL recombinant hFlt3L (orange), plasmid-derived hFlt3L-NY-ESO-1 (light green), mutated Flt3L(H8R)-NY-ESO-1 (dark green) or empty vector (EV, blue). (*Kruskal-Wallis;* *p<0.05, **p<0.01, ***p<0.001).

Flt3L-NYESO-1 is functional in vivo when delivered IV or by intratumoral EP

Hydrodynamic delivery:



Intratumoral electroporation:

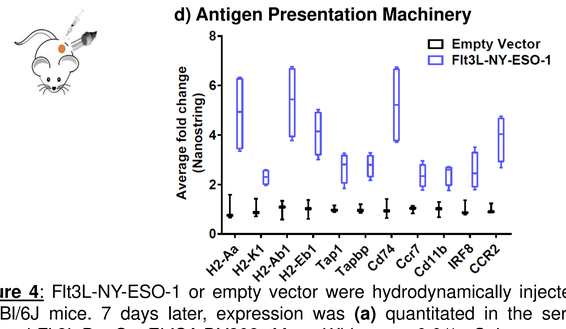
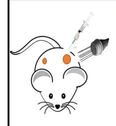


Figure 4: Flt3L-NY-ESO-1 or empty vector were hydrodynamically injected into the tail vein of C57Bl/6J mice. 7 days later, expression was **(a)** quantitated in the serum (n=6 animals per cohort; hFlt3L DuoSet ELISA DY308; *Mann Whitney*, p<0.01). Spleens were **(b)** massed and **(c)** dissociated for analysis by flow cytometry to determine percent CD11c⁺ splenocytes (*Kruskal-Wallis;* **p<0.01, ***p<0.001). For IT EP expression, established B16.F10 tumors were injected with plasmid DNA encoding for Flt3L-NY-ESO-1 or empty vector (and electroporated. Tumors were collected 7 days post-treatment. RNA was extracted using Trizol® and 50ng of total RNA was used for NanoString®. **(d)** Induction in expression of antigen presentation machinery genes (APM) over no treatment levels was assessed by NanoString® nCounter technology (fold-change; *Holm-Sidak method;* p<0.05).

Electroporation of PIIM-NY-ESO-1 results in B16.10 tumor growth delay

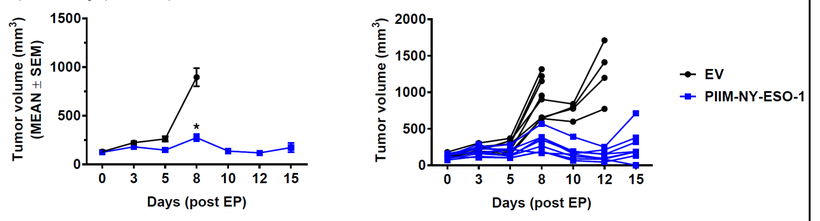


Groups:
Untreated (not shown)
PIIM-NY-ESO-1 (50µg)
pUMVC3 (EV) (50µg)

At time of treatment:
Primary lesion: 125-160mm^3
Contralateral lesion: 30-35mm^3

EP conditions:
400V/cm
8 x 10ms pulses
Treatment on Day 0 and 6

a) Primary (treated) lesion



b) Contralateral (untreated) lesion

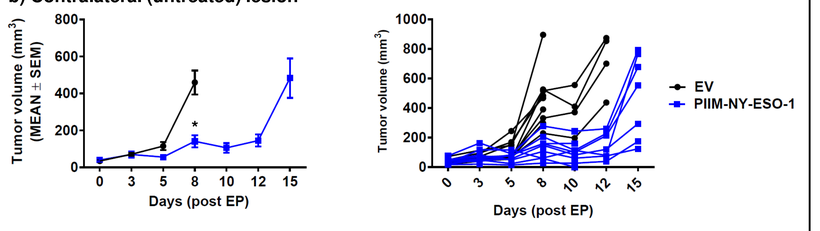
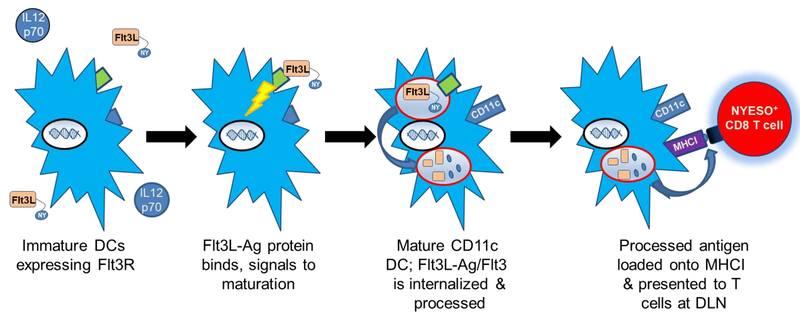


Figure 6: B16.F10 cells were implanted on both flanks of C57Bl/6J 6-8 week old female mice at different densities (1x10^6 primary, 0.25x10^6 contralateral). Tumor on one flank **(a)** was treated by electroporation while **(b)** the tumor on the other flank was left untreated. Both tumors were measured 3 times a week and mice were sacrificed when they reached max allowed tumor burden (1500 mm^3; calculated using the formula $V_T = a^2 \times b / 2$, where a = smallest diameter and b = perpendicular diameter). Left plots represent consolidated data; spaghetti plots for individual animals are shown on the right. (n=9 per group; *Bonferroni-Dunn*; *p<0.05)

Summary and Conclusions

Hypothetical Model:



- PIIM-NY-ESO-1 co-expresses IL-12p70 and Flt3L-NY-ESO-1 both *in vitro* and in B16.F10 tumors; moreover, both proteins appear to be fully functional.
- Mouse DCs matured with pFlt3L-OVA can present SIINFEKL to OT-1 T cells
- NanoString® analysis of B16.F10 tumors electroporated with hFlt3L-NY-ESO-1 reveals an increase in APM and TIL genes.
- Electroporation of PIIM-OVA into B16.F10 tumors generates CD8⁺ SIINFEKL-tetramer⁺ T cells in the draining lymph node.
- IT-EP of PIIM-NY-ESO-1 controls tumor growth in a contralateral B16.F10 tumor model, similar to results seen with EP of IL12-P2A alone.
- **Summary:** PIIM represents a molecular platform using the DC maturation factor Flt3L fused to a antigen (viral, shared, or a patient-specific neoantigen) co-expressed off a single DNA plasmid along with the potent immunomodulatory cytokine IL-12p70.