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## Exploiting antitumor immunity to overcome relapse and improve remission duration

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**Abstract** Cancer survivors often relapse due to evolving drug-resistant clones and repopulating tumor stem cells. Our preclinical study demonstrated that terminal cancer patient's lymphocytes can be converted from tolerant bystanders in vivo into effective cytotoxic T-lymphocytes in vitro killing patient's own tumor cells containing drug-resistant clones and tumor stem cells. We designed a clinical trial combining peginterferon  $\alpha$ -2b with imatinib for treatment of stage III/IV gastrointestinal stromal tumor (GIST) with the rationale that peginterferon  $\alpha$ -2b serves as danger signals to promote antitumor immunity while imatinib's effective tumor killing undermines tumor-induced tolerance and supply tumor-specific antigens in vivo without leukopenia, thus allowing for proper dendritic cell and cytotoxic T-lymphocyte differentiation toward

Th1 response. Interim analysis of eight patients demonstrated significant induction of IFN- $\gamma$ -producing-CD8<sup>+</sup>, -CD4<sup>+</sup>, -NK cell, and IFN- $\gamma$ -producing-tumor-infiltrating-lymphocytes, signifying significant Th1 response and NK cell activation. After a median follow-up of 3.6 years, complete response (CR) + partial response (PR) = 100%, overall survival = 100%, one patient died of unrelated illness while in remission, six of seven evaluable patients are either in continuing PR/CR (5 patients) or have progression-free survival (PFS, 1 patient) exceeding the upper limit of the 95% confidence level of the genotype-specific-PFS of the phase III imatinib-monotherapy (CALGB150105/SWOGS0033), demonstrating highly promising clinical outcomes. The current trial is closed in preparation for a larger future trial. We conclude that

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combination of targeted therapy and immunotherapy is safe and induced significant Th1 response and NK cell activation and demonstrated highly promising clinical efficacy in GIST, thus warranting development in other tumor types.

**Keywords** IFN- $\gamma$  · IFN- $\alpha$  · Peginterferon  $\alpha$ -2b · GIST · Imatinib · Immunotherapy

## Introduction

Despite effective treatments achieving remission, cancer survivors often relapse, after which interventions become largely unsuccessful. One culprit is drug-resistant clones—pre-existing and evolving continuously—and another is tumor stem cells—repopulating, resilient, and poorly understood. With their unique features, once outgrowth has occurred, both culprits can evade standard therapies and prevail. The innate and adaptive immunity have been shown to play important roles in protecting the host through tumor immunosurveillance [1–6]. Unfortunately, mechanisms of tumor-induced tolerance enable tumors to escape immunosurveillance [7]. However, the delicate balance can be restored if we can design novel treatment that can break tolerance while promote innate and adaptive antitumor immunity.

Dendritic cells (DCs) capture, process, and cross-present antigens in the context of MHC class-I and costimulatory molecules to subsets of T-lymphocytes, and play critical roles in the regulation and development of distinct immune responses [8–10] including (1) Th1 adaptive cell-mediated immunity (Th1 response) signified by interferon- $\gamma$  (IFN- $\gamma$ ) secretion and play major roles in protection against pathogens and tumors [1–6], (2) Th2, (3) Th17, and (4) T-regulatory responses (tolerance).

IFN- $\alpha$  is a type-1 IFN and a physiological danger signal [11, 12] can upregulate expression of MHC class-I molecules and costimulatory molecules on DCs, activate innate immunity, modulate DCs, promote Th1 response, help clonal expansion/survival and memory differentiation of T-lymphocytes [9–13], and has been shown to be an effective vaccine adjuvant in animal models [14] and clinical trials [15, 16]. The immunological consequences of tumor cell death induced by individual chemotherapy agents [17] and the subsequent differentiation of DCs and T-lymphocytes in the ensuing 2-week span are of pivotal importance in influencing the development toward the distinct immune responses (Th1, Th2, Th17, or tolerance). Cytotoxic chemotherapy often results in prolonged severe leukopenia depriving DCs of proper maturation (differentiation), thus often resulting in a tolerant/dysfunctional immune state.

With recognition of the IFN- $\alpha$  qualities [9–14], the pivotal role of DCs in the development of distinct immune responses [8–10] and support from our preclinical data, we hypothesize that (1) combining IFN- $\alpha$  with effective non-marrow-suppressive antitumor agent(s) could induce innate immunity and Th1 response; (2) the antitumor immunity can help eradicating tumor cells including the drug-resistant clones and tumor stem cells upfront thus improve response rate; (3) most importantly, antitumor immunity can monitor continuously and eradicate the various continuing-evolving drug-resistant clones and the resilient tumor stem cells when they first emerge at the cellular/subclinical level prior to outgrowth, and this would delay/prevent relapse, ultimately leading to the improvements in progression-free survival (PFS) and overall survival (OS).

To test the hypothesis, we designed a new strategy aiming at developing innate immunity and Th1 response concomitant with partial response (PR) or complete response (CR) achieved by effective non-marrow-

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suppressive drug therapy. Gastrointestinal stromal tumor (GIST), a sarcoma with incidence of 5000/year in US, represents an excellent model to test our hypothesis for the following reasons. First, imatinib mesylate (IM, Gleevec<sup>®</sup>, Glivec<sup>®</sup>) [18], a selective inhibitor of ABL, KIT, PDGFRA B, is highly effective, induces swift apoptosis/necrosis of GIST within 3–7 days [19], and is non-marrow-suppressive, allowing proper DC and cytotoxic T-lymphocyte differentiation toward Th1 response. Second, GIST cell alterations include cancer/testis antigens [20], tumor-antigens created by activating mutations in *KIT* (*c-kit*) or *PDGFRA* [21–24] and new mutation(s) responsible for IM resistance [25–27]. Third, IM-monotherapy trials in GIST patients have reported response rates (PR + CR) of 54% [28], 52% [29, 30], and 48% [22, 30]. The median PFS remains  $\leq 2$  years [22, 29, 30] mainly due to the development of IM resistance [25–27]. Discontinuing IM resulted in high rate of relapse due to repopulating stem cells [31]. Thus, better therapies for GIST are needed.

IM was reported to induce DC-mediated natural killer (NK) cell IFN- $\gamma$  production [32, 33] and potentiate adaptive immunity through IM-off-target inhibition of KIT on DCs [34] and inhibition of Ido [35]; both IM-off-target immunological anti-GIST effects plus IM-inhibition of KIT/PDG FRA signaling contribute to the IM-monotherapy efficacy [22, 28–30] as described above and is less than satisfactory. We intend to bring out the full potential of anti-GIST immunity by a new strategy of combining peginterferon  $\alpha$ -2b (PegIFNa2b, Peg-Intron<sup>®</sup>) [36] with IM and have demonstrated significant Th1 response, innate immunity, and highly promising clinical outcome comparing to IM-monotherapy [22], strongly support all three parts of our hypothesis.

## Materials and methods

### Preclinical study

Specimens were collected under MD Anderson Institutional Review Board (IRB) protocols LAB\_00143. Primary tumor cells were isolated after digesting fresh tumor with collagenase. The chimeric *SYN-SSX* was sequenced [37]. Peripheral blood mononuclear cell (PBMC)-derived DCs were isolated by plastic adherence and culture supplemented with GM-CSF and IL-4. Cytokine cocktail consisted of TNF- $\alpha$  (R&D), IL-1 $\beta$  (R&D), IL-6 (R&D), and PGE-2 (Sigma) [38]. IL-12-p70 was analyzed using ELISA (Biosources, Camarillo, CA.) and read with UV-900 microplate reader (Bio-Tek Instruments, Winooski, VT). The plastic non-adherent cells were used to positively select CD8<sup>+</sup> T-lymphocytes using anti-CD8 monoclonal antibody (mAb) coupled to magnetic microbeads (Miltenyi Biotec, Auburn, CA).

IFN- $\gamma$ -enzyme-linked immunosorbent spot (IFN- $\gamma$ -ELISPOT) assay

CD8<sup>+</sup> T-lymphocytes were cultured in AIM-V medium supplemented with IL-2 and IL-7 and stimulated with various antigen preparations twice, total 14 days, to generate CTLs. The 96-well ELISPOT plate (Millipore, Billerica, MA) was precoated with anti-IFN- $\gamma$  antibody, incubated at 4°C overnight, plated with CD8<sup>+</sup> T-lymphocytes at  $2 \times 10^5$  cells/well, and stimulated with  $4 \times 10^4$  irradiated primary tumor cells for 40 h at 37°C. Biotinylated IFN- $\gamma$  antibody was added, followed by streptavidin peroxidase. IFN- $\gamma$  spots were counted using an ELISPOT reader.

<sup>51</sup>Cr-release assay

Cryopreserved primary tumor cells were used as targets and K562 cells as control. We labeled  $2 \times 10^6$  target cells with 100  $\mu$ Ci of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (ICN Biomedicals, Irvine, CA) and distributed 3,000 target cells in each well. Blocking experiments were performed using anti-HLA-A.B.C antibody and isotype control (Dako, Carpinteria, CA).

Clinical trial

Refer to “Results”.

Genotyping

As described previously [23].

IFN- $\gamma$ -flow cytometry

PBMCs were cultured with phorbol ester PMA (5 ng/ml) plus ionomycin (745 ng/ml) for 1 h, add brefeldin A (5 mcg/ml) and cultured for additional 4 h. After surface staining with CD4-PerCP, CD8-APC, or CD3-FITC (BD Biosciences), cells were fixed and stained with anti-human IFN- $\gamma$ -PE (BioLegend, San Diego, CA) [39]. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland).

Immunohistochemical analysis and confocal microscopy

Antigen retrieval with preheated EDTA/Tris buffer pH 9.0 for CD8, CD56, and CD4; and citrate Buffer pH 6.0 for IFN- $\gamma$ , FasL, and granzyme B. Antibodies included CD4, CD8, CD56 (Dako, Carpinteria, CA); IFN- $\gamma$  (Abcam, Cambridge, MA); and FasL and granzyme B (Novus Biologicals, Littleton, CO), goat anti-rabbit IgG antibody conjugated with Texas Red, and goat anti-mouse IgG conjugated with Alexa Fluor 488 (Novus Biologicals).

Images were acquired using Fluo View software on an Olympus FV1000 confocal laser scanning microscope.

## Results

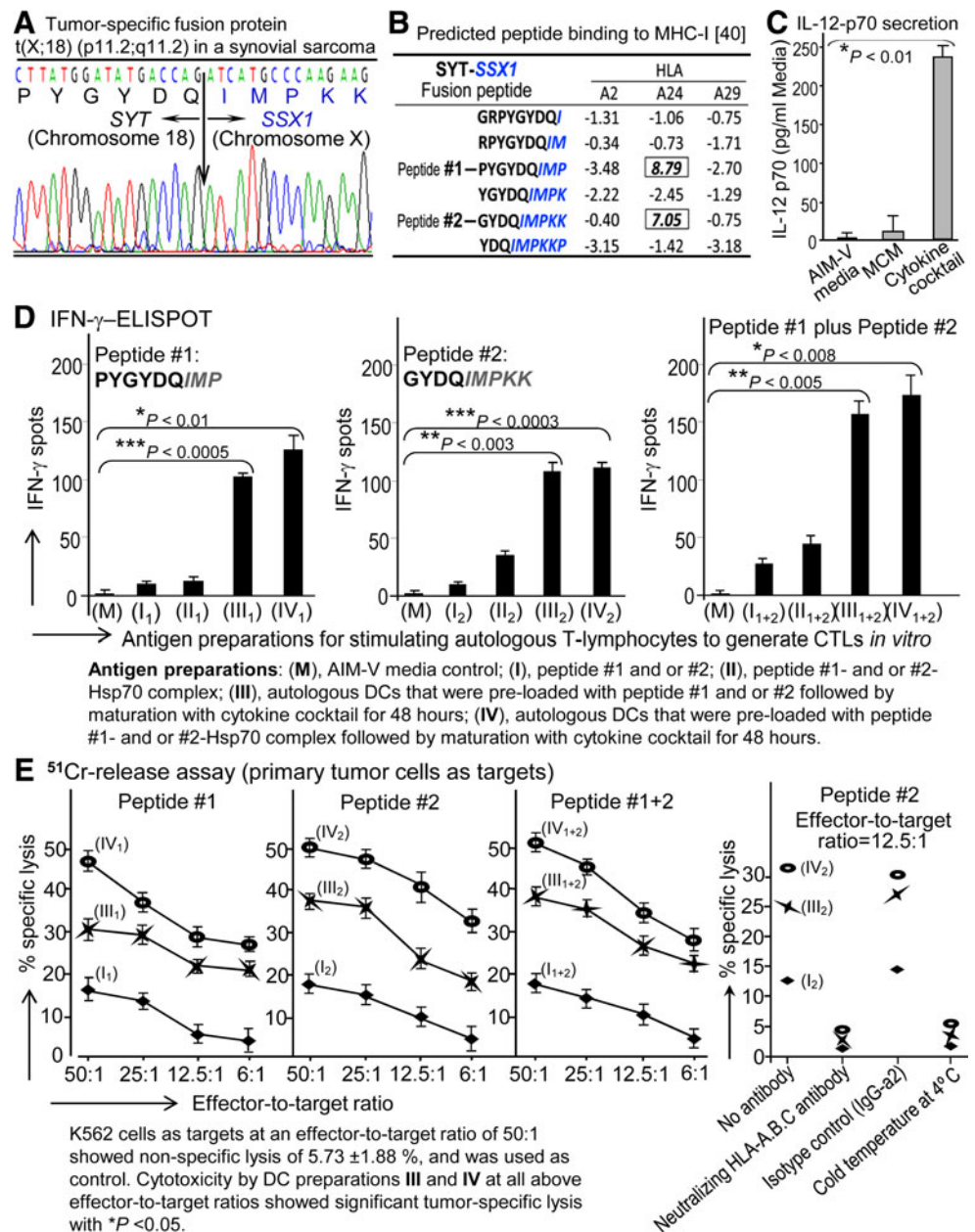
### Pre-clinical study

*Cancer patient's anergic T-lymphocytes in vivo can be converted into effective cytotoxic T-lymphocytes capable of killing patient's own primary tumor cells in vitro*

A stage III synovial sarcoma patient (HLA-A24/A29) received standard neoadjuvant chemotherapy (doxorubicin

and ifosfamide), achieved PR, and underwent surgery to achieve disease-free status. The patient later relapsed with wild spread metastasis and rapid progression failing all systematic treatment; leukapheresis was performed to collect PBMC. At the time of surgery, the post-neoadjuvant chemotherapy residual tumor consisting of resilient tumor stem cells and chemotherapy-resistant cells—as evidenced by clinical relapse and metastasis shortly after surgery along with drug resistance—were cryopreserved to serve as targets for in vitro cytotoxic T-lymphocytes (CTL) analysis as opposed to using cell lines as targets [37]. Two tumor-specific-9-mer-peptides encompassing the joining of SYT-SSX1 (Peptides #1 and 2) (Fig. 1a) exhibit high predicted binding affinity [40] with HLA-A24 class 1 molecule

**Fig. 1** CTLs targeted at patient's own tumor cells. **a** Chimeric SYT-SSX1 in a HLA-A24/A29 synovial sarcoma. **b** Predicted binding of two tumor-specific-9-mer-peptides with HLA class-I molecules. **c** IL-12-p70 secretion by mature autologous DCs. **d** IFN- $\gamma$ -ELISPOT assay. **e**  $^{51}\text{Cr}$ -release assay. CTLs that were generated by stimulation with antigen preparations III<sub>1</sub>, III<sub>2</sub>, III<sub>1+2</sub>, IV<sub>1</sub>, IV<sub>2</sub>, IV<sub>1+2</sub> can induce significant primary tumor cell lysis using IFN- $\gamma$ -ELISPOT ( $P < 0.01$ ) and  $^{51}\text{Cr}$ -release assay ( $P < 0.05$ ). The specific lysis of primary tumor cells can be abrogated by neutralizing antibody against HLA-A,B,C loci and at 4°C (e, right panel)



(Fig. 1b). DCs that were matured with a cytokine cocktail (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and PGE-2) [38] showed significant secretion of IL-12-p70 ( $P < 0.01$ , Fig. 1c). Autologous PBMC-derived CD8<sup>+</sup> T-lymphocytes were stimulated with four antigen preparations (Fig. 1d, I–IV) twice in vitro to generate CTLs. When antigen-specific peptides #1 & 2 were presented by mature DCs, we demonstrated significant cytotoxicity against primary tumor cells by both IFN- $\gamma$ –ELISPOT ( $P < 0.01$ , Fig. 1d, III & IV) and <sup>51</sup>Cr release assays ( $P < 0.05$ , Fig. 1e, III & IV). The percent tumor-specific lysis exhibited a dose-dependent relationship with effector-to-target ratio. <sup>51</sup>Cr release was completely blocked by neutralizing anti-HLA-A.B.C antibody and cold temperature at 4°C (Fig. 1e, right panel) indicating the essential role of the HLA class-I molecules and specificity of tumor lysis.

To the best of our knowledge, this is the first report using a patient's own post-chemotherapy residual drug-resistant primary tumor cells—a source of micrometastasis and recurrence—as targets for CTL assays and provided direct and convincing evidence that a terminal cancer patient's T-lymphocytes can be converted from tolerant bystanders ignoring the tumor growth in vivo into tumor-specific effective CTLs in vitro (Fig. 1d, e). Our preclinical studies served two purposes. First, these encouraging

results help to justify immunotherapy approach to overcome relapse especially when no other treatment option is in sight due to current poor understanding of the culprits causing relapse. Second, our preclinical study methods are readily applicable for testing the development of tumor-specific immunity during clinical trial by collecting PBMC and applying <sup>51</sup>Cr release or IFN- $\gamma$ –ELOSPOt assays using primary tumor cells (if available) as CTL targets. Without further dwelling on in vitro studies, we move toward our ultimate goal of restoring/stimulating antitumor immunity in vivo in cancer patients.

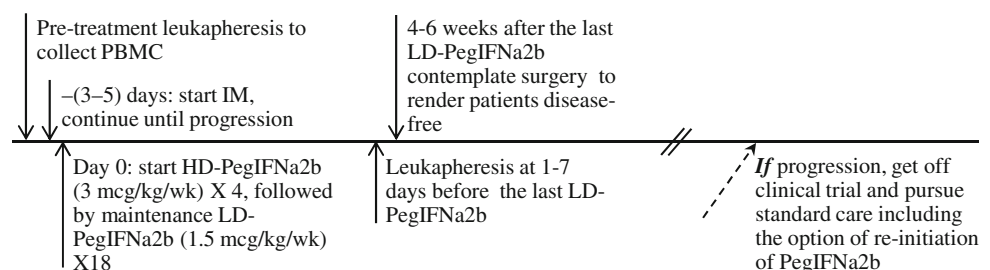
Aiming at developing antitumor immunity in parallel with achieving PR/CR by drug therapy, we initiated a new strategy of combining PegIFNa2b (3rd signal/danger signal) with IM (killing tumor cells to undermine tumor-induced tolerance and provide 1st and 2nd signals—antigen and co-stimulation) in GIST to test our hypothesis.

## Clinical study

*GIST clinical trial design is summarized in Fig. 2*

*Combination treatment of PegIFNa2b plus IM-induced significant IFN- $\gamma$ -producing-lymphocytes IFN- $\gamma$  serves*

**Fig. 2** Clinical trial design



Abbreviations: PBMC, peripheral blood monocytes; IM, imatinib; PegIFNa2b, peginterferon  $\alpha$ -2b; HD, high-dose; LD, low-dose; wk, week.

\***Eligibility:** Stages III gastrointestinal stromal tumor (GIST) patients with primary tumor  $\geq 6$  cm or stage IV (metastatic) GIST of any size.

†**End points:** Clinical endpoints include safety, response rate (complete response + partial response), time to response, progression-free survival and overall survival. Laboratory end points include NK cell activation, and Th1 immune response.

‡**Combination treatment:** For *KIT* exon 11 mutation GIST, starts IM at 400 mg/day, and for *KIT* exon 9 mutation GIST, starts IM at 800 mg/day and allow dose reduction as needed. PegIFNa2b dosage and schedule are shown in above schema. If absolute neutrophil count is  $\leq 1.5 \times 10^9/L$  or platelets is  $\leq 100 \times 10^9/L$ , PegIFNa2b dose will be on hold until bone marrow recovers.

¶**Specimens:** PBMC were collected by leukapheresis before starting IM and within a week before the last LD-PegIFNa2b. In between the two leukapheresis, PBMC were collected at specific time points using CPT Cell Preparation Tube (BD, Franklin Lakes, NJ 07417).

§**Response evaluation:** (i) RECIST, (ii) PET-CT criteria [41], and (iii) Choi criteria [42, 43].

\*\***Comparison/control:** We used IM-monotherapy CALGB150105/SWOGS0033 phase III Study results (746 patients enrolled, 382 genotyped) for comparison, it reported objective response rates and median PFS respectively for *KIT* exon 11 mutation GIST as 72% and 741 days, for *KIT* exon 9 mutation as 44% and 501 days, and for WT GIST as 45% and 384 days [22]

††**Status:** This phase II clinical trial was approved by The University of Utah IRB (IRB\_00022172) and registered with ClinicalTrials.gov (NCT00585221). Informed consent was obtained before enrollment. It was opened in May 2007, and closed for accrual by PI in July 2009 due to highly promising results and our intention to incorporate the insight gained in the interim analysis into a larger future trial.

critical function in innate, and adaptive cell-mediated immunity [2–6] is tightly regulated and produced predominantly by four cell types only, *activated* NK, NKT cells, Th1 CD4<sup>+</sup>, and Th1 CD8<sup>+</sup> CTLs [6] and is the signature of Th1 response. Five of the eight patients enrolled consented to donate PBMC. Total IFN- $\gamma$ -producing-lymphocytes and subgroups of IFN- $\gamma$ -producing -CD8<sup>+</sup>, -CD4<sup>+</sup>, and -CD4<sup>-</sup>CD8<sup>-</sup>-lymphocyte (most likely NK cells) were barely detectable before treatment (Fig. 3a, black columns), and they increased significantly to  $18 \pm 1.3\%$  ( $P < 0.0004$ ),  $32 \pm 4.8\%$  ( $P < 0.003$ ),  $15 \pm 2.5\%$  ( $P < 0.006$ ), and  $7.3 \pm 0.3\%$  ( $P < 0.0001$ ), respectively (Fig. 3a, red columns) after four HD-PegIFN $\alpha$ 2b (3 mcg/kg/week) with continuing IM. IFN- $\gamma$ -production of individual patients at single cell level by flow cytometry is shown in Fig. 3b. Subgroup analyses for Pt#7 showed that the percentage induction of IFN- $\gamma$ -producing -CD8<sup>+</sup>, -CD4<sup>+</sup>, and -NK cells was 31.57% (Fig. 3b, *u*), 11.28% (*v*), and 6.8% (*w*), respectively—signifies significant induction of Th1 response and NK cell activation; similar pattern was observed in Pt#3 (Fig. 3b, *c*, *d*, *e*). In case of Pt#7, comparing with pretreatment level (Fig. 3b, *k*), the kinetics of induction of IFN- $\gamma$ -producing-lymphocytes demonstrated 102-fold induction after 2 HD-PegIFN $\alpha$ 2b (*l*), reached a peak (861-fold) after 4 HD-PegIFN $\alpha$ 2b (*m*), notably, 4 weeks after stopping PegIFN $\alpha$ 2b (*n*) while continuing IM showed rapid decline (22-fold). After four weekly HD-PegIFN $\alpha$ 2b, the treatment was switched to 18 maintenance weekly LD-PegIFN $\alpha$ 2b (1.5 mcg/kg/week), and we observed that four of five patients' IFN- $\gamma$ -producing-lymphocytes gradually fell to non-detectable level suggesting a PegIFN $\alpha$ 2b dose-dependent effect. Our results indicate that combining IM plus 4 weekly HD-PegIFN $\alpha$ 2b is both necessary and sufficient to induce significant generation of IFN- $\gamma$ -producing-lymphocytes consisting of CD8<sup>+</sup>, CD4<sup>+</sup> T-lymphocytes, and NK cells in all patients (Fig. 3a, *b*, *b*, *g*, *i*, *m*, *o*).

*Post-combination-treatment residual tumor showed complete remission and nearly all tumor-infiltrating-lymphocytes produce IFN- $\gamma$*  All eight GIST patients enrolled in the clinical trial were diagnosed with fine needle aspiration (FNA) or biopsy, so no primary GIST cells were available to serve as targets for IFN- $\gamma$ -ELISPOT or <sup>51</sup>Cr release assay to confirm “GIST-specific” cytotoxicity. However, we studied the post-combination-treatment residual tumor from Pt#4 to compared it with the same patient's pretreatment biopsy sample and three post-IM-monotherapy residual tumors as controls (Fig. 4a, controls #1–3). The post-combination-treatment residual tumor showed pathologic CR, hyaline degeneration, necrosis, and abundant tumor-infiltrating-lymphocytes (TILs) (Fig. 4b, *m*) consisting of CD8<sup>+</sup> (*n*) and CD4<sup>+</sup> T-lymphocytes (*p*) and

CD56<sup>+</sup> NK cells (*o*). Most TILs expressed CD45RO—a memory T-lymphocyte marker (*q*) with negative isotype control (*r*). A small fraction of TILs were positive for granzyme B and FasL (*v*, *w*); on confocal microscopy, such TILs mostly co-localized with CD56 (Fig. 4c, *cc*, *gg*).

Strikingly, almost all of the TILs actively produce IFN- $\gamma$  (Fig. 4b, *s*), in sharp contrast to the totally negative IFN- $\gamma$ -staining in the pretreatment GIST biopsy and all three post-IM-monotherapy controls (Fig. 4a, *c–f*). Uninvolved adjacent lymph nodes showed rare IFN- $\gamma$ -positive cells (*u*) suggesting that combination treatment may have induced primarily GIST-reactive TILs.

Our translational research results showed significant induction of IFN- $\gamma$ -producing -CD8<sup>+</sup>, -CD4<sup>+</sup>, -NK cell, and IFN- $\gamma$ -producing TILs—signifies induction of Th1 response and NK cell activation, thus strongly support part one of our hypothesis.

### Patients

Eight patients were enrolled (Table 1), four had stage III and four had stage IV GIST with metastasis to liver, lungs, and or peritoneum. Primary tumors (6–16 cm) originated from stomach, small intestine or rectum. The GIST natural history and response to IM treatment have been shown to correlate with histologic features, stage, anatomical site [24], and genotype [21, 22]. Three GISTs harbored *KIT* exon 11 mutations, one had *KIT* exon 9 mutation, two had wild-type (WT) *KIT* and *PDGFRA*, and two had insufficient material from FNA for genotyping.

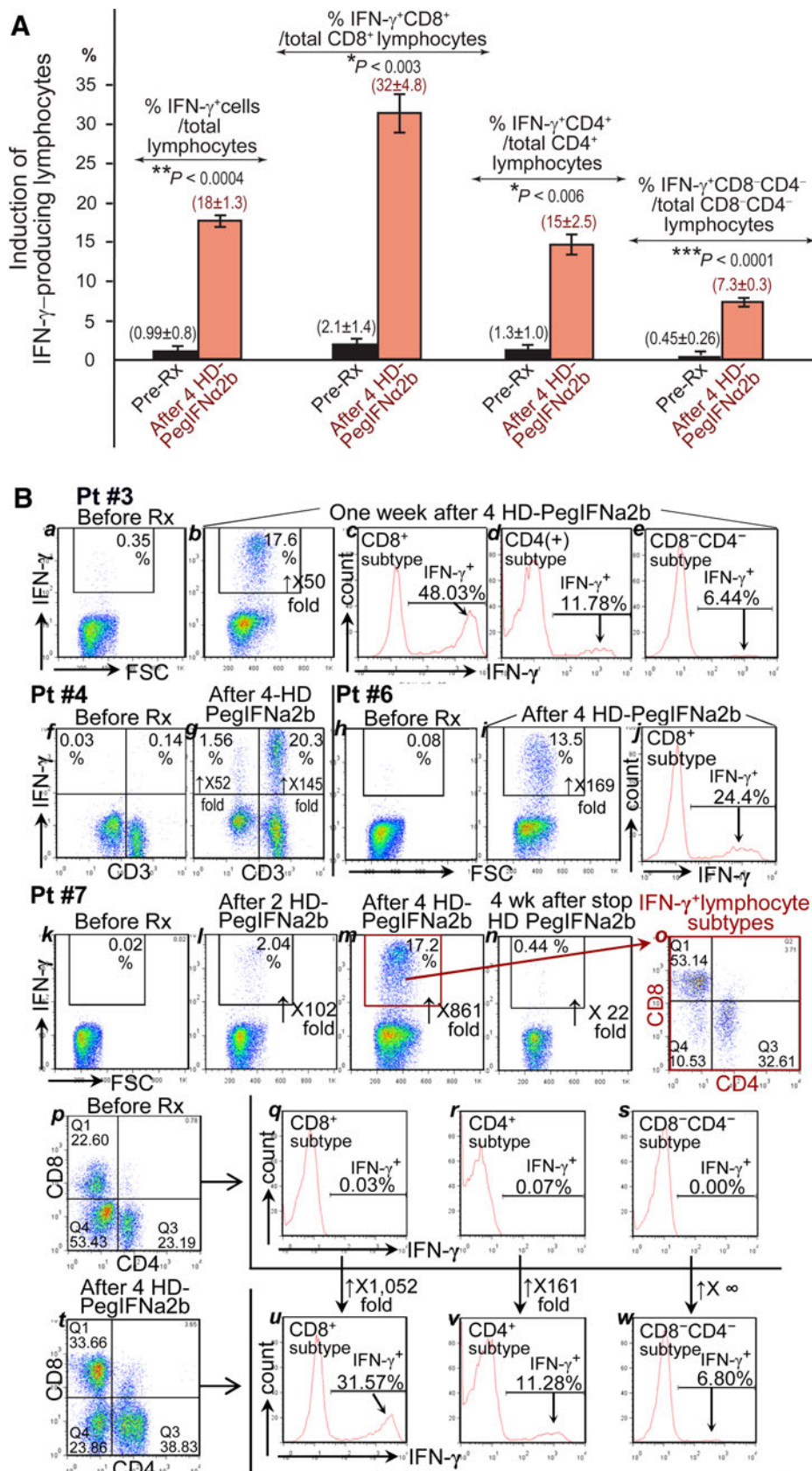
### Side effects

Pts#1–3 initially received HD-PegIFN $\alpha$ 2b at 4 mcg/kg/week, developed grade 3 neutropenia, and requiring dose reduction to 3 mcg/kg/week. Later the protocol was amended and the HD-PegIFN $\alpha$ 2b was reduced to 3 mcg/kg/week (Pts#4–8), and we observed occasional grade 1 or grade 2 neutropenia with quick recovery. Two patients (Pt#1 and 8) developed grade 3 skin rash requiring short-term steroid. All patients experienced transient low-grade fever and mild flu-like symptoms as expected.

### Response rate

Despite multiple poor prognostic factors, combination treatment of IM plus PegIFN $\alpha$ 2b achieved a response rate (PR + CR) of 100% by all three evaluation criteria, RECIST, PET-CT scan criteria [41], and Choi criteria [42, 43], contrasting to the reported GIST IM-monotherapy response rates of 54% [28], 52% [29, 30], and 48% [22, 30]. Pt#2 achieved PR by Choi criteria at week 8 and was not assessable (NA) by PET-CT criteria because the tumor

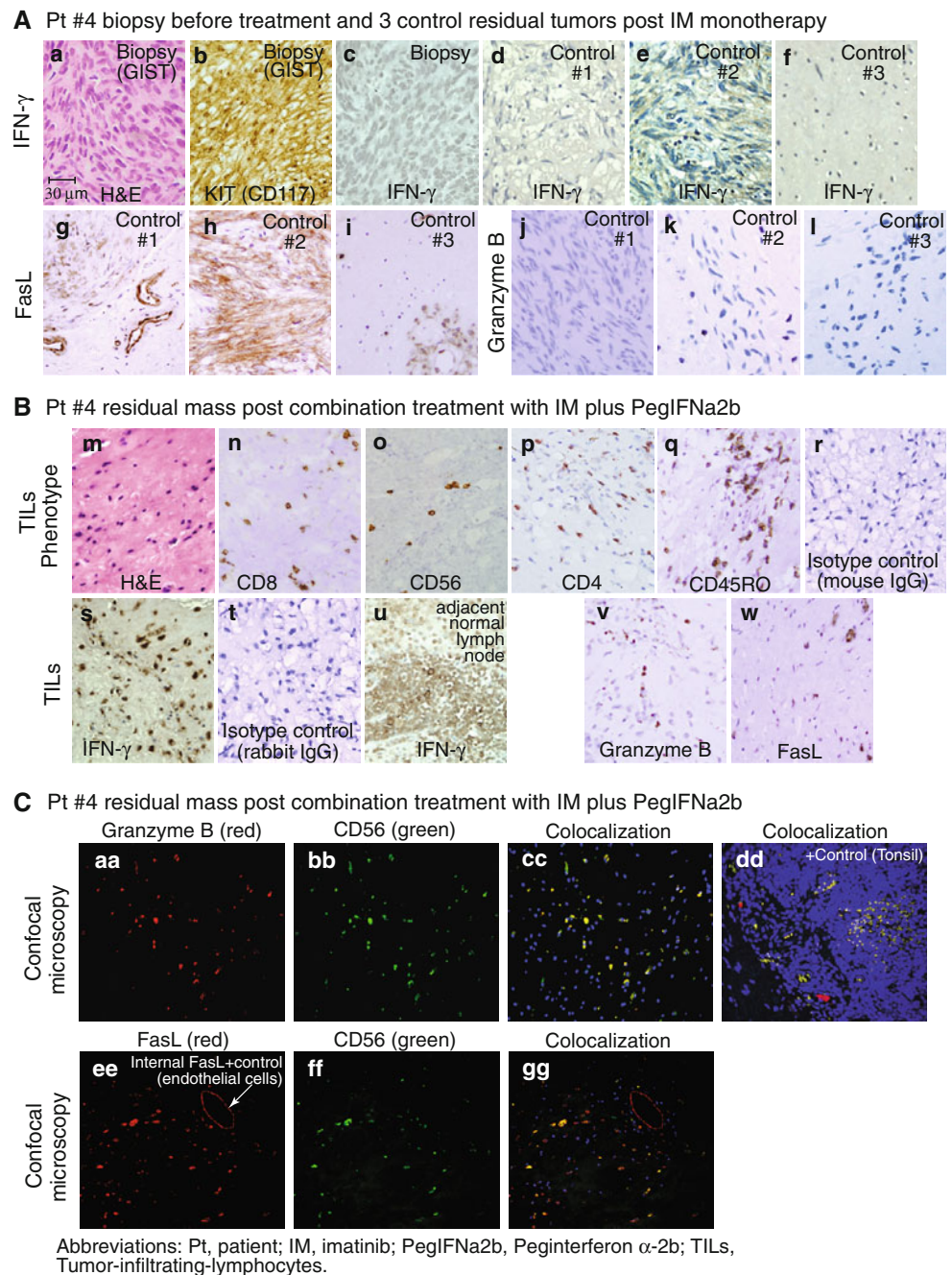
**Fig. 3** Flow cytometry analysis of IFN- $\gamma$ -producing-lymphocytes. **a** Bar graph demonstrating induction of IFN- $\gamma$ -producing-lymphocytes before (black bars) and after (red bars) combination treatment with IM plus PegIFNa2b. **b** Flow cytometry at single cell level. IFN- $\gamma$ -producing-lymphocytes were barely detectable before treatment (a, f, h, k) and were induced significantly ( $P < 0.003$ ) in total lymphocytes (b, g, i, m), subtype of CD8 $^+$  lymphocytes (c, j, u), CD4 $^+$  lymphocytes (d, v), and CD8 $^-$ CD4 $^-$  cells (most likely NK) (e, w) after IM plus 4HD-PegIFNa2b



Abbreviations: Rx, treatment; HD-PegIFNa2b, high-dose Peginterferon  $\alpha$ -2b.



**Fig. 4** Immunohistochemical studies. **a** TILs of pretreatment biopsy of Pt#4 and three post-IM-monotherapy GISTs (Controls #1–3) showed no IFN- $\gamma$  production. **b** Pt#4 post-combination-treatment residual tumor. It showed pathologic CR with extensive hyaline degeneration, necrosis, and numerous TILs (*m*), consisting of CD8<sup>+</sup> (*n*), CD56<sup>+</sup> (*o*), and CD4<sup>+</sup> (*p*) lymphocytes. Strikingly, nearly all TILs produced IFN- $\gamma$  in situ (*s*), and most TILs expressed the memory T-cell marker CD45RO (*q*). **c** Confocal microscopy on Pt#4 post-combination-treatment residual tumor



was not FDG-avid or by RECIST, because the tumor was surgically removed 6 weeks after completion of HD-Peg-IFNa2b when tumor size had reduced by 28% and became amenable to surgery, resulting in insufficient time for RECIST evaluation [44]. In comparison, 13% (49/382) of genotyped GIST patients were categorized as NA by RECIST in the S0033 trial [22]. Pt#4 had a large rectal primary (9.3 cm; Fig. 5, top row), homozygous mutation in *KIT* exon 11 [23], and high mitotic count (20/50 high power field), nonetheless achieved pathologic CR with abundant IFN- $\gamma$ -producing TILs (Fig. 4b, *s*). Pt#5 (9.8 cm primary gastric GIST and extensive liver lesions) and Pt#7

(11.4 cm primary gastric GIST) achieved radiographic near-CR of primary GIST (Fig. 5, 2nd and 3rd row), such that surgeries were no longer indicated—great clinical benefit. Pt#6 harboring WT GIST with extensive liver metastasis, bilateral lung metastasis, and peritoneal implants achieved PR by all three criteria at week 8 (Table 1; Fig. 5, 4th row); however, on day 369 of PR—7 months after completion of PegIFNa2b—while continuing IM, four existing lesions showed increased SUV. HD-PegIFNa2b was then re-initiated with continuation of IM 400 mg/day and resulted in a second PR (Fig. 5, last 2 rows), which lasted 430 days while off both IM and

**Table 1** GIST patient characteristics, stage, genotyping, response rate, and remission duration

Pt ID (age; PS <sup>a</sup> )	Stage; mitosis/50HPF; primary site (size); metastatic sites	KIT genotyping (mutational analysis)	Response Evaluation		Continuing PR/CR or PFS <sup>c</sup> ; [S0033 Study genotype-specific median PFS; 0.95UCL] <sup>f</sup> Days
			PET-CT <sup>b</sup>	Choi <sup>c</sup>	
#1 (82;0)	III; < 5; gastric (15.7 cm)	FNA, insufficient for genotyping	PR at wk 8	PR at wk 17	PR at mo 46 >1,572 (4.3 yr);
#2 (82;0)	III; < 5; gastric (6 cm)	KIT exon 11, GTT → GAT,V559D	Not FDG avid	PR at wk 8	NA <sup>e</sup> (28%↓ at wk 8) >1,488 (4.1 yr); [741; 1,035]
#3 (53;0)	IV; 40; SM (5.5 cm); liver met	KIT exon 11, 558_560del	PR at wk 9	PR at wk 9	PFS = 765 (2.1 yr); [741; 1,035]
#4 (46;0)	III; 20; rectal (9.3 cm)	KIT exon 11, homozygous GTT → GAT,V560D	PR at wk 12	PR at wk 12	>1,319 (3.6 yr); [741; 1,035] (pathologic CR)
#5 (42;1)	IV; gastric (9.8 cm); liver	Wild-type KIT & PDGFRA	PR at wk 9	PR at wk 9	>1,298 (3.6 yr); [384; 784]
#6 (52;0)	IV; gastric (4.5 cm, resected); liver, lung, and peritoneal implants	Wild-type KIT & PDGFRA	PR at wk 8	PR at wk 8	PFS = 799 (2.2 yr) <sup>h</sup> ; [384; 784]
#7 (84;1)	III; gastric (11.4 cm)	Insufficient material from FNA for genotyping	PR at wk 13; near-CR at mo 6	PR at wk 13; near-CR at mo 6	NA, died of unrelated cause in remission with radiographic near-CR
#8 (77;0)	IV; 17; SM (16 cm); liver	KIT exon 9, A502_Y503 dup	PR at wk 10	PR at wk 10	>1,179 (3.2 yr); [501; 881]

Pt patient, HPF high power field, PET-CT positron emission tomography-computed tomography, PR partial response, CR complete response, yr year, wk week, mo month, PFS progression-free survival, 0.95UCL 95% upper confident level, IM imatinib, SM small bowel, FNA fine needle aspiration, FDG fluorine-18 fluorodeoxyglucose

<sup>a</sup> PS, ECOG performance status at the time of diagnosis. After treatment as of November 7, 2011, all PS = 0

<sup>b</sup> PET-CT criteria of PR: A decrease in the standardized uptake value by 25% [41]

<sup>c</sup> Choi criteria of PR: Decrease in tumor size more than 10% OR decrease in tumor density more than 15% [42, 43]

<sup>d</sup> RECIST criteria of PR: 30% decrease in the sum of the longest dimension or target lesions

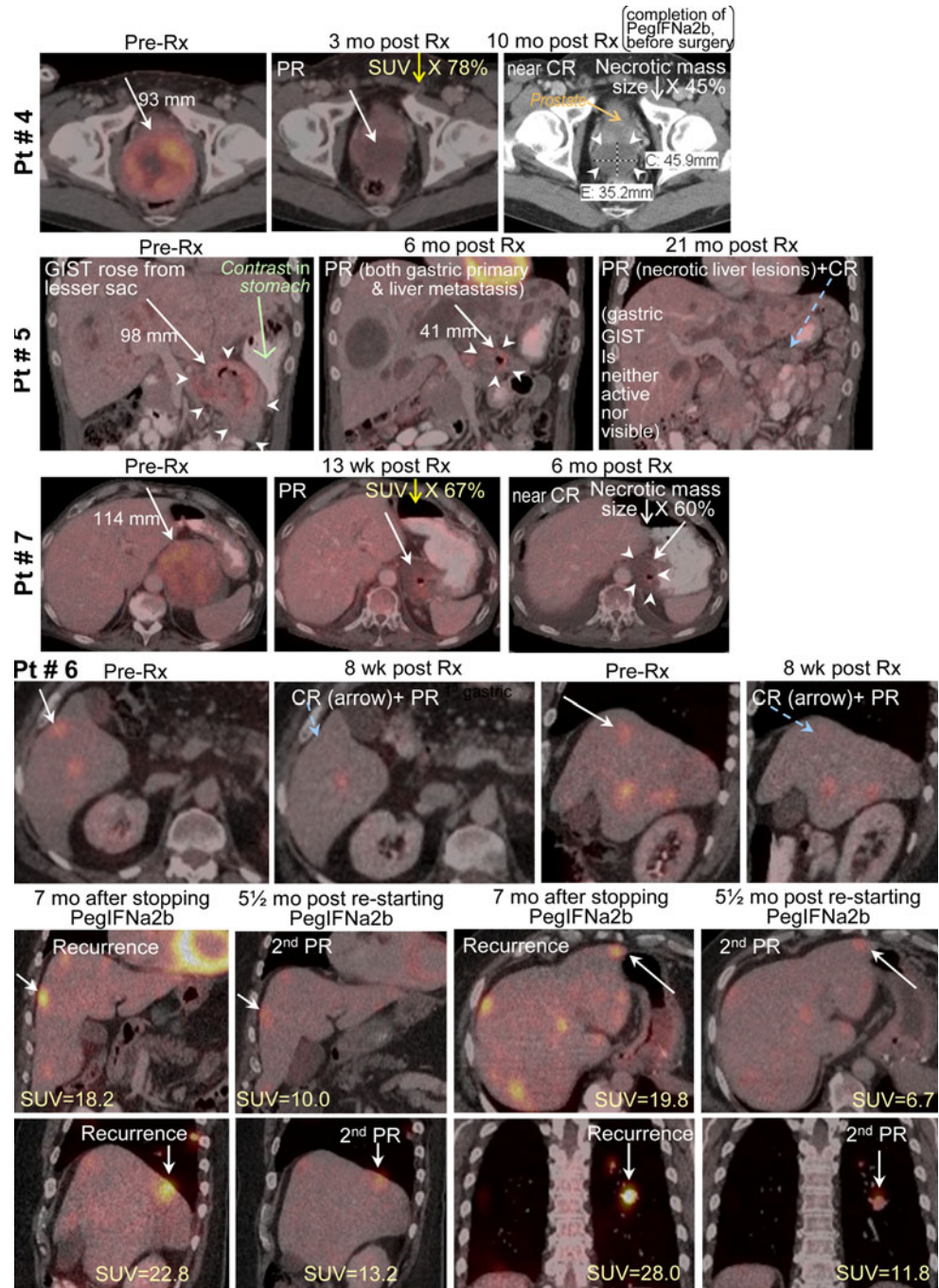
<sup>e</sup> Continuing PR/CR and PFS was calculated as of November 7, 2011

<sup>f</sup> S0033 IM-monotherapy genotype-specific median PFS(PR + CR) of KIT exon 11 mutation, exon 9 mutation, and wild-type GIST were reported as 741 days/(72%), 501 days/(44%), and 384 days/(45%) respectively [22]; the 0.95 UCL of KIT exon 11 mutation taking IM 400 mg, exon 9 mutation taking IM 800, and wild-type GIST taking IM 400 mg are 1035, 881, and 784 days, respectively [personal communication with Dr. Michael C. Heinrich, the PI of IM-monotherapy phase III S0033 Study]

<sup>g</sup> NA: Not assessable by RECIST. RECIST requires prolonged “Time-to-Response” [44], and when the GIST reduced by 28% becoming surgically resectable at week 8, Pt #2 opted for surgery prematurely and left with insufficient time for RECIST evaluation

<sup>h</sup> Pt#6 responded with a 2nd PR after re-initiation of HD-PegIFNα2b, Pt #6 decided to stop both imatinib and PegIFNα2b, eventually recurred while off all treatment. 1st PR = 369; 2nd PR = 430 days; total PFS = 799 days

**Fig. 5** PET-CT and CT scans. Top three rows show that the combination treatment resulted in swift radiographic CR or near-CR of large primary GIST of Pts#4, 5, and 7 (9.3, 9.8, and 11.4 cm). Pt#6 showed mixed PR and CR (fourth row). Last two rows illustrate the second PR (after emergence of IM resistance) induced by re-initiation of PegIFNa2b



Abbreviations: Pt, patient; PegIFNa2b, peginterferon  $\alpha$ -2b; Rx, combination treatment of IM plus PegIFNa2b; mo, month; wk, week; PR, partial response; CR, complete response; SUV, standardized uptake value of PE-CT scan.

PegIFNa2b. Induction of a second PR after tumor progression (due to IM resistance) is unprecedented.

*Progression-free survival and overall survival*

After a median follow-up of 3.6 years (3.2–4.3 years), the OS is 100% with PS = ECOG 0 for all seven evaluable patients. Pt#1 had unsuccessful genotyping and was

grouped with *KIT* exon 11—the most favorable group—for the evaluation. Pt#7 died at age 85 of unrelated illness while in remission with a radiographic near-CR (Table 1; Fig. 5, 3rd row). The PFS of Pt#6 and the continuing PR/CR of Pts#1, 2, 4, 5, and 8—a total of 6 out of 7 evaluable patients—exceed not only the genotype-specific median PFS [22] but also the upper limit of the 95% confidence level of the genotype-specific PFS of S0033

IM-monotherapy trial (Table 1, last column; S0033 Study data are in brackets) [Personal communication with Dr. Michael C. Heinrich, PI of IM-monotherapy phase III S0033 Study]. Pt#3 harbored aggressive GIST with extensive liver metastasis and achieved PR at week 8 by all three criteria, but three out of the numerous liver lesions showed evidence of increased SUV on PET-CT scan without new lesions on day 765, and the genotype-specific PFS was slightly longer than that of S003 IM-monotherapy trial [22].

Taken together, combination treatment with IM plus PegIFN $\alpha$ 2b was well tolerated, safe, demonstrated a 100% response rate (PR + CR), 100% OS rate, and substantially prolonged continuing PR/CR (5 patients) and PFS (2 patients) after a median follow-up of 3.6 years (3.2–4.3 years), and strongly supports part two and three of our hypothesis.

## Discussion

In CML, combining two active agents, peginterferon- $\alpha$ 2a (Pegasys<sup>®</sup>) and IM, demonstrated tolerability and improved efficacy over IM-monotherapy [45], but the immunological implications of IFN- $\alpha$  was not addressed. Our GIST study of adding PegIFN $\alpha$ 2b (immunotherapy) to the current standard IM (targeted therapy) demonstrated significant induction of innate immunity and Th1 response and highly promising clinical outcome comparing to IM-monotherapy albeit small group study, and strongly supported our hypothesis. Although IM has off-target effect of activating innate [32, 33] and potentiating adaptive immunity [34, 35], the significant induction of IFN- $\gamma$ -producing-lymphocytes (Fig. 3a) in this study is mainly attributed to PegIFN $\alpha$ 2b rather than IM alone because stopping HD-PegIFN $\alpha$ 2b (Fig. 3b, n) or switching to maintenance LD-PegIFN $\alpha$ 2b while continuing IM resulted in a sharp decline of IFN- $\gamma$ -producing-lymphocytes to barely detectable level. This new concept/strategy of combining immunotherapy with effective non-marrow-suppressive treatments might be beneficial to other cancer types as well. Combining IFN- $\alpha$  or peginterferon  $\alpha$  with radiation, hormone/hormone antagonist, small molecule targeted therapies, or monoclonal antibodies in radiosensitive tumors, prostate, breast, pancreatic, melanoma, hepatocellular, colorectal, and sarcoma may help delay/prevent relapse and warrant further investigations.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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