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LETTER TO THE JOURNAL

Ex vivo STAT3 phosphorylation in circulating immune cells: a novel biomarker for early cancer diagnosis and response to anti-PD-1 therapy

Basal signal transducer and activator of transcription 3 (STAT3) activation is well-documented in the tumor microenvironment (TME) due to its association with cancer prognosis [1]. However, its presence and clinical relevance in the bloodstream remain unexplored. Given that STAT3-inducing cytokines, such as interleukin-6 (IL-6), are often elevated in the bloodstream of various cancer patients [2, 3], we aimed to investigate basal STAT3 activation in blood by developing a methodology to assess *ex vivo* phosphorylation of STAT3 (pSTAT3$^{ex\ vivo}$) in circulating immune cells.

Since phosphorylation is a transient process prone to dephosphorylation, we sought to minimize the time between blood collection and the experiment. Specifically, 1) we limited the use of peripheral blood mononuclear cell (PBMC) samples to those processed within 1 hour of blood collection, and 2) immediately fixed the samples after thawing (Figure 1A). Notably, 135 non-small cell lung cancer (NSCLC) patient samples processed in this way exhibited significantly higher levels of pSTAT3$^{ex\ vivo}$-positive cells compared to healthy controls (Figure 1B and Supplementary Table S1). Prolonged handling and extended experimental steps significantly decreased pSTAT3$^{ex\ vivo}$ expression (Supplementary Figure S1), underscoring the importance of our novel approach in controlling the time between blood collection and the experiment.

We next investigated the cell types within PBMCs that express pSTAT3$^{ex\ vivo}$. CD4$^+$ T cells exhibited the highest pSTAT3$^{ex\ vivo}$ expression, followed by CD8$^+$ T cells, whereas monocytes, B cells, and natural killer (NK) cells showed minimal pSTAT3$^{ex\ vivo}$ expression (Figure 1C). Within both CD4$^+$ and CD8$^+$ T cells, pSTAT3$^{ex\ vivo}$ expression was highest in the least differentiated CD27$^+$ CD45RA$^+$ naïve subset (Figure 1C) [4]. A similar pattern was observed across multiple other cancer types (Figure 1D and Supplementary Figure S2).

Focusing on CD4$^+$ naïve T cells, pSTAT3$^{ex\ vivo}$ expression showed a stark contrast between NSCLC patients and healthy donors, even at stage I (Figure 1E). The area under the receiver operating characteristic curve for distinguishing stage I NSCLC patients from healthy donors was 0.9851, with a sensitivity of 0.92 at 95% specificity (Figure 1F). No tumor-specific or patient-specific clinical variables correlated with pSTAT3$^{ex\ vivo}$ expression in NSCLC patients (Supplementary Figure S3), while surgical removal of the tumor decreased pSTAT3$^{ex\ vivo}$ expression (Figure 1G), supporting a direct association between pSTAT3$^{ex\ vivo}$ and tumor burden. These findings underscore the potential of pSTAT3$^{ex\ vivo}$ as a blood-based diagnostic biomarker for early cancer detection, particularly useful in screening for cancer before proceeding to more invasive standard confirmation methods.

Next, we investigated the inducer of pSTAT3$^{ex\ vivo}$. Among the various cytokines tested, in vitro IL-6 stimulation of STAT3 best matched the pSTAT3$^{ex\ vivo}$ expression profiles in NSCLC patients (Supplementary Figure S4A-C). To confirm the involvement of IL-6 in pSTAT3$^{ex\ vivo}$ expression, we cultured healthy PBMCs with patient serum and assessed pSTAT3 expression. Serum-induced pSTAT3 expression tightly correlated with both the pSTAT3$^{ex\ vivo}$ levels in the serum donors and the IL-6 concentration in the corresponding serum (Figure 1H-I). Importantly, the addition of anti-IL-6Rα or anti-IL-6 antibodies to the serum completely abrogated pSTAT3 induction (Figure 1J and Supplementary Figure S4D). These findings strongly suggest that IL-6 is the predominant inducer of pSTAT3$^{ex\ vivo}$ expression.

Given the role of IL-6 in inducing pSTAT3$^{ex\ vivo}$, we further investigated their relationship. Notably, the two parameters closely followed a dose-response curve, with

Abbreviations: IL, Interleukin; NK, Natural killer; NSCLC, Non-small cell lung cancer; PBMC, Peripheral blood mononuclear cell; PR, Partial response; pSTAT3$^{ex\ vivo}$, *ex vivo* phosphorylated signal transducer and activator of transcription 3; TIL, Tumor-infiltrating lymphocyte; TME, Tumor microenvironment.

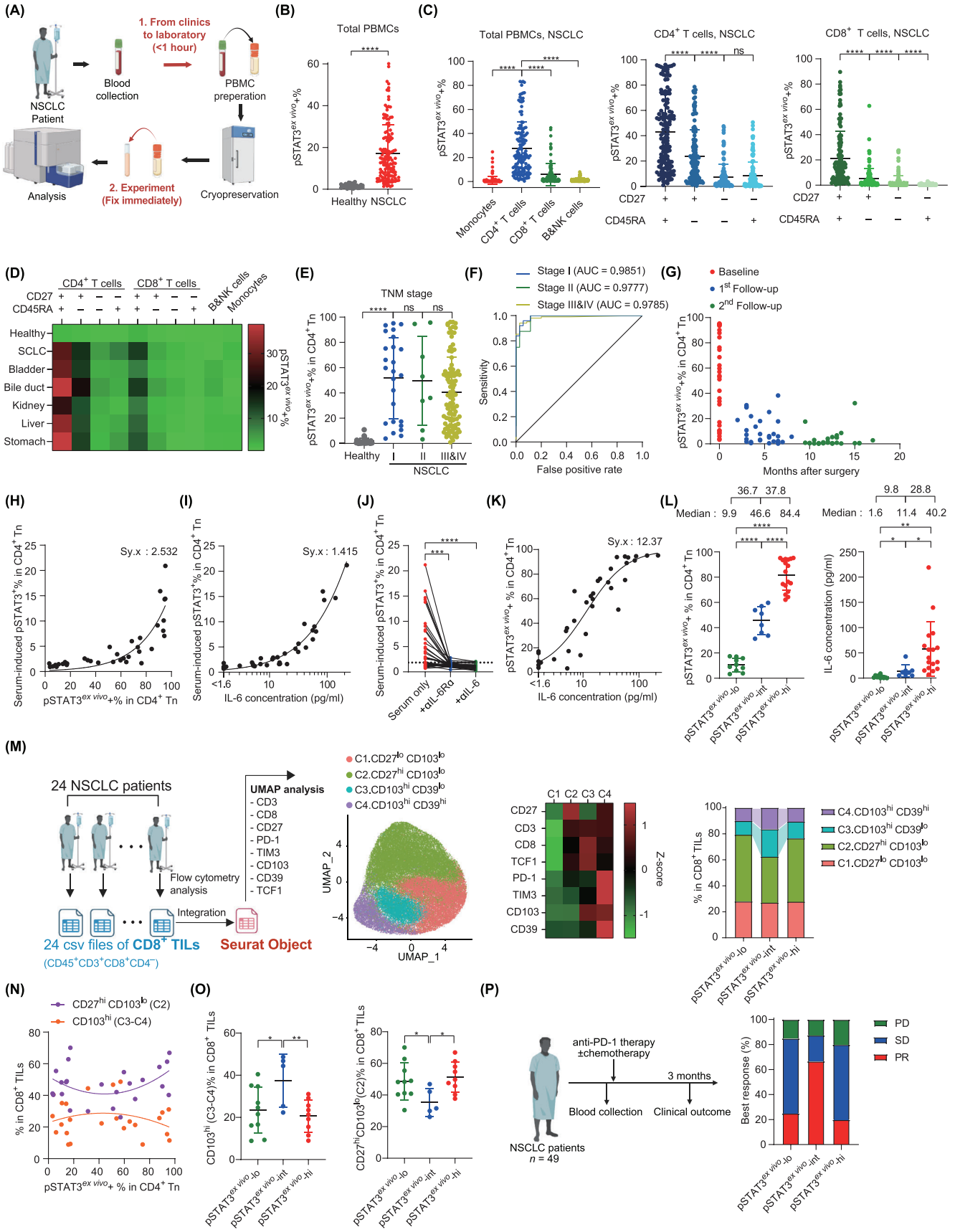

FIGURE 1 Characterization of pSTAT3$^{ex\ vivo}$ expression in circulating immune cells and its clinical relevance. (A) Methodology for assessing pSTAT3$^{ex\ vivo}$ in PBMCs. Two critical steps are highlighted: 1) minimizing the time from the clinic to the laboratory, and 2) immediately fixing the sample after thawing. (B) pSTAT3$^{ex\ vivo}$ expression in PBMCs from healthy donors ($n = 35$) and NSCLC patients ($n =$

a half-maximal effective concentration of 11.63 pg/mL (Figure 1K) [5]. Interestingly, this value is 1000-fold lower than previous in vitro estimates [6, 7] providing strong evidence that serum IL-6 can actively induce the signaling cascade. Moreover, the dose-response curve suggests that biological activity correlates with the logarithmic form of IL-6 concentration. Accordingly, while patients with low (0%-20%), intermediate (21%-60%), and high (61%-100%) pSTAT3$^{ex\ vivo}$ (pSTAT3$^{ex\ vivo}$-lo, pSTAT3$^{ex\ vivo}$-int, and pSTAT3$^{ex\ vivo}$-hi, respectively) showed a stepwise increase in pSTAT3$^{ex\ vivo}$ expression, IL-6 levels in the pSTAT3$^{ex\ vivo}$-hi group were markedly higher than in the other two groups, rendering them relatively similar (Figure 1L). These results suggest that measuring pSTAT3$^{ex\ vivo}$ expression provides a more accurate representation of systemic IL-6 activity—its capacity to trigger intracellular signaling cascades—than direct IL-6 measurements.

Given the potential of pSTAT3$^{ex\ vivo}$ as a marker of systemic IL-6 activity, we investigated the relationship between pSTAT3$^{ex\ vivo}$ and immune cell populations. Initially, we compared pSTAT3$^{ex\ vivo}$ expression with the distribution of immune cell populations in peripheral blood. However, we did not observe any gross alterations (Supplementary Figure S5). We then explored immune cell populations within the TME. Notably, the frequency of M2-like CD206^{hi} tumor-associated macrophages and CD39^{hi} regulatory T cells correlated with pSTAT3$^{ex\ vivo}$ and were most abundant in the pSTAT3$^{ex\ vivo}$-hi group (Supplementary Figure S6A-G), suggesting a link between systemic IL-6 activity and the formation of immunosuppressive TMEs.

Interestingly, the distribution of CD8^{+} tumor-infiltrating lymphocytes (TILs) showed a unique relationship with pSTAT3$^{ex\ vivo}$ expression. We performed uniform manifold approximation and projection analysis on CD8^{+} TILs by integrating flow cytometry data from 24 NSCLC patients (Figure 1M). Among the four CD8^{+} TIL clusters, those with high CD103 expression (C3.CD103^{hi}CD39^{lo} and C4.CD103^{hi}CD39^{hi}) were specifically elevated in the pSTAT3$^{ex\ vivo}$-int group, whereas the C2.CD27^{hi}CD103^{lo} cluster was reduced (Figure 1N-O and Supplementary Figure S6H). Given that CD103^{+}CD8^{+} TILs include tumor-reactive cells (Supplementary Figure S7) [8, 9], these results suggest that the accumulation of tumor-reactive CD8^{+} T cells within tumors increases at a specific range of systemic IL-6 activity but diminishes at higher levels.

Given the strong correlation between pSTAT3$^{ex\ vivo}$ expression and key elements of anti-tumor immunity, we hypothesized that pSTAT3$^{ex\ vivo}$ expression could serve as a prognostic marker for cancer immunotherapy. We analyzed pSTAT3$^{ex\ vivo}$ expression in pre-therapy PBMCs

135). (C) pSTAT3$^{ex\ vivo}$ expression across different immune cell types within the PBMCs of NSCLC patients ($n = 135$). (D) Heatmap showing cellular expression profiles of pSTAT3$^{ex\ vivo}$ in various cancer patients, color-coded from green (low) to red (high). (E) pSTAT3$^{ex\ vivo}$+ frequency in CD4^{+} naïve T cells (Tn) from healthy donors ($n = 35$) and NSCLC patients at different TNM stages ($n = 25$, 8, and 102 for stages I, II, and III&IV, respectively). (F) Receiver operating characteristic curves of patients at different TNM stages and their area under the curve (AUC) values. (G) pSTAT3$^{ex\ vivo}$ expression dynamics following surgical removal of tumor. Blood samples were collected at the time of surgery, and at two intervals: 2.5-8.0 months (first follow-up) and 9-15 months (second follow-up) post-surgery ($n = 32$ for each interval). (H-I) Correlation between serum-induced pSTAT3^{+} frequency in CD4^{+} Tn cells and (H) pSTAT3$^{ex\ vivo}$+ frequency in CD4^{+} Tn cells of serum donors or (I) IL-6 concentration in the serum ($n = 35$). The lines represent non-linear regression using the exponential growth curve (H) or Hill equation (I). The Sy.x values calculated with the fitting models are shown. (J) Effect of anti-IL-6Rα or anti-IL-6 antibodies on serum-induced pSTAT3 expression. The dashed line represents the value from samples cultured in media instead of serum. (K) Relationship between serum IL-6 concentration and pSTAT3$^{ex\ vivo}$+ frequency in CD4^{+} Tn cells. The line represents non-linear regression using the Hill equation, with the Sy.x calculated from the fitting model shown. (L) pSTAT3$^{ex\ vivo}$+ frequency in CD4^{+} Tn cells (left) and serum IL-6 concentration (right) in three NSCLC patient groups ($n = 10$, 8, and 17 in pSTAT3$^{ex\ vivo}$-lo, pSTAT3$^{ex\ vivo}$-int, and pSTAT3$^{ex\ vivo}$-hi, respectively). The median for each group and the difference between medians are indicated above. (M) Uniform manifold approximation and projection (UMAP) analysis of CD8^{+} TILs. Tumor-infiltrating immune cells from 24 NSCLC patients were analyzed by flow cytometry. Molecular expression levels of CD8^{+} TILs were exported into CSV files and integrated into a Seurat object. UMAP was analyzed based on the expression levels of the indicated molecules. A heatmap shows molecular expressions in each cluster, and a bar graph shows the distribution of each cluster across 3 patient groups. (N) Correlation between pSTAT3$^{ex\ vivo}$+ frequency in CD4^{+} Tn cells and the frequency of CD103^{hi} (C3–C4) or CD27^{hi}CD103^{lo} (C2) clusters in CD8^{+} TILs ($n = 24$). (O) Frequency of CD103^{hi} (C3–C4) (left) or CD27^{hi}CD103^{lo} (C2) clusters (right) in CD8^{+} TILs for each patient group ($n = 10$, 5, and 9 for pSTAT3$^{ex\ vivo}$-lo, pSTAT3$^{ex\ vivo}$-int, and pSTAT3$^{ex\ vivo}$-hi, respectively). (P) Frequency of patients with PR, SD, or PD at 3 months post-therapy in each patient group ($n = 20$, 24, and 5 in pSTAT3$^{ex\ vivo}$-lo, pSTAT3$^{ex\ vivo}$-int, and pSTAT3$^{ex\ vivo}$-hi, respectively). All bar graphs represent mean values with error bars. Statistical significance was determined using either an unpaired or a paired Student's *t*-test. Values of $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ were considered significant. Some of the images are generated by *Biorender.com*. Abbreviations: STAT3, phosphorylated signal transducer and activator of transcription 3; pSTAT3$^{ex\ vivo}$, *ex vivo* phosphorylated STAT3; PBMC, peripheral blood mononuclear cell; NK, natural killer; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; Tn, naïve T cells; AUC, area under the curve; IL-6, interleukin-6; Sy.x, standard error of estimates; UMAP, uniform manifold approximation and projection; TILs, tumor-infiltrating lymphocytes; PD-1, programmed death-1; PR, partial response; SD, stable disease; PD, progressive disease; TCF1, T cell factor 1, TIM3; T-cell immunoglobulin and mucin domain 3.

from 49 NSCLC patients who received anti-PD-1 therapy (± chemotherapy) (Supplementary Table S2). The pSTAT3$^{ex\ vivo}$-hi group showed the worst response (partial response (PR) 20%; Figure 1P), consistent with their highly immunosuppressive TMEs. Notably, the pSTAT3$^{ex\ vivo}$-int group exhibited significantly better outcomes than pSTAT3$^{ex\ vivo}$-lo (PR 66.7% versus 25%; Figure 1P), suggesting a potential connection with CD103^{+}CD8^{+} T cells. Moreover, the biomarker performance of pSTAT3$^{ex\ vivo}$ was significantly greater than tumor PD-L1 expression (Supplementary Figure S8). These findings suggest an unappreciated non-linear relationship between systemic IL-6 activity and anti-PD-1 responsiveness.

Collectively, these findings suggest that systemic IL-6, despite its extremely low concentration (median ~10 pg/mL) [3], can actively induce the STAT3 signaling cascade in vivo and modulate anti-tumor immunity. Our refined methodology enabled quantification of systemic IL-6 activity as pSTAT3$^{ex\ vivo}$, which could serve as a biomarker for cancer diagnosis and a predictor of responsiveness to anti-PD-1 therapy. Overall, our study offers new avenues for exploring systemic cytokines in various disease models, as demonstrated in our cancer patient cohort.

AUTHOR CONTRIBUTIONS

Sung-Woo Lee and Jae-Ho Cho administered this project; Sung-Woo Lee, Young Ju Kim, and Jae-Ho Cho conceptualized, designed methodologies, performed formal analysis, curated data, and wrote the original draft; Sung-Woo Lee, In-Jae Oh, and Jae-Ho Cho supervised, acquired funding, reviewed and edited the manuscript; Saei Jeong, Kyung Na Rho, Jeong Eun Noh, Hee-Ok Kim, Hyun-Ju Cho, Yoo Duk Choi, Deok Hwan Yang, Eu Chang Hwang, Woo Kyun Bae, Sook Jung Yun, Ju Sik Yun, Cheol-Kyu Park, and In-Jae Oh curated resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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DATA AVAILABILITY STATEMENT

All data are included within the article and its supplemental information.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Boards of Chonnam National University Medical School and Hwasun Hospital (CNUHH-2022-021 and CNUHH-2024-034). All cancer patients provided written informed consent. Written informed consent from healthy donors from the Korean Red Cross was formally waived in accordance with the Korean Bioethics and Safety Act.

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