Original Paper

Localized Immune Cascade Programming in Desmoplastic Tumors: In Silico Modeling and Validation Study

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Abstract

Background: Despite the success of immune checkpoint inhibitors in certain cancers, many late-stage solid tumors remain "immune cold," characterized by low T-cell infiltration, dense extracellular matrix (ECM), stromal and vascular barriers, and poor responses to systemic immunotherapy. Overcoming these resistance mechanisms requires localized and controlled reprogramming of the tumor microenvironment to permit effective antitumor immunity.

Objective: This strategy proposes a biomarker-guided, staged, and locally confined immune cascade designed to enable reinfiltration and activation of endogenous or autologous T cells in previously unresponsive solid tumors.

Methods: "Second Breath" involves a sequential intervention targeting physical and immunologic barriers. Local enzymatic matrix disruption using a collagenase-hyaluronidase mixture combined with lysyl oxidase inhibition reduces ECM density and stromal barriers. Transient recruitment and activation of innate immune cells are induced using weakly immunogenic bacteria or localized toll-like receptor agonists to generate local danger signals. Controlled, microdosed intratumoral cytokine pulses (IL-12, interferon γ [IFN-γ], tumor necrosis factor [TNF] α) amplify local antigen presentation and effector T-cell priming while minimizing systemic exposure. Optional autologous T-cell augmentation can be administered intratumorally or systemically during the window of heightened immune activation. A recovery or containment phase using local antibiotics or immunomodulators limits excessive inflammation and restores tissue homeostasis after bacteriotherapy. Candidate gene sets (≈80 genes) were mapped into protein-protein interaction networks using STRING version 11.5 and Cytoscape version 3.9.1. Hub analysis (degree >10) identified TNF, toll-like receptor 4 (TLR4), cytotoxic T-lymphocyte–associated protein 4 (CTLA4), signal transducer and activator of transcription 1, and CD274 as central regulators. Functional enrichment via DAVID version 2021 revealed significant involvement of the Wnt signaling pathway (Benjamini-adjusted $P<.05$). Gene co-occurrence analysis across 10,897 tumors spanning 32 cancer types (The Cancer Genome Atlas/cBioPortal) demonstrated strong associations among IFN-γ gene (IFNG), TLR4, CD86, TNF, nuclear factor kappa-B subunit 1 (NFKB1), CTLA4, and CD8A.

Results: The cascade network showed dense interconnectivity, with IFNG-TLR4 and IFNG-CD86 emerging as high-frequency co-occurrence edges. Predefined go/no-go criteria (≥2-fold increase in IFN-γ signature, ≥30% reduction in ECM density, ≥25% rise in $CD8^+$ infiltration per mm^2) were consistently met in silico simulations.

Conclusions: Second Breath represents a novel preclinical approach to convert immunologically "cold" tumors into responsive targets for antitumor immunity. Its sequential, localized design aims to enhance efficacy while minimizing systemic toxicity. Preclinical network and enrichment analyses provide mechanistic support for its proposed multistep immune cascade, guiding future in vitro and in vivo validation.

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Keywords: oncology; tumor microenvironment; cancer microenvironment; immunology; immune-cold tumors; intratumoral immunotherapy; extracellular matrix remodeling

Introduction

Overview: The Second Breath Conceptual Framework

"Second Breath" is a testable conceptual framework for sequential, localized immune programming in desmoplastic solid tumors. Each cascade module is modeled in silico and explicitly cross-referenced to published in vitro and in vivo evidence (see References), supporting biological plausibility without claiming new wet-lab experiments in vitro. The framework specifies controlled, stepwise intratumoral activation of innate and adaptive immunity under limited systemic exposure. Network and enrichment analyses highlight coordinated hubs (eg, tumor necrosis factor [TNF], toll-like receptor 4 [TLR4], signal transducer and activator of transcription 1 [STAT1], cytotoxic T-lymphocyte–associated protein 4 [CTLA4], clusters of differentiation 274 [CD274]) and implicate β-catenin/Wnt-linked programs at trafficking and checkpoint-readiness steps, aligning with the proposed sequence logic. Collectively, "Second Breath" provides a mechanistic rationale and testable predictions, including predefined go/no-go criteria, for converting immune-cold, desmoplastic tumors into more responsive states and offers a structured basis for preclinical validation.

Background: Immunologically "Cold" Tumors and Current Limitations

Immunologically "cold" solid tumors are defined by low intratumoral $CD8^+$ T-cell density, a weak interferon γ (IFN-γ) signature, and the presence of abnormal vasculature, elevated interstitial fluid pressure (IFP), and a dense extracellular matrix (ECM) [1-4]. These features hinder antigen presentation, restrict effector-cell infiltration, and contribute to the poor responsiveness of such tumors to immune checkpoint inhibitors (ICIs) [5-7]. To address this challenge, we propose that sequential local immune programming can effectively convert a cold tumor phenotype into a hot, immunologically active state. This strategy involves pattern recognition receptor (PRR)–driven innate priming, reinforcement of the interleukin (IL) 12/IFN-γ/TNF-α axis, and controlled ECM modulation, thereby creating favorable conditions for ICI responsiveness while minimizing systemic exposure [8-14]. This study represents a logical continuation of the article "Cascade Medicine: Architecture of Therapy for a Sustainable Outcome," in which the initial cascade concept was introduced.

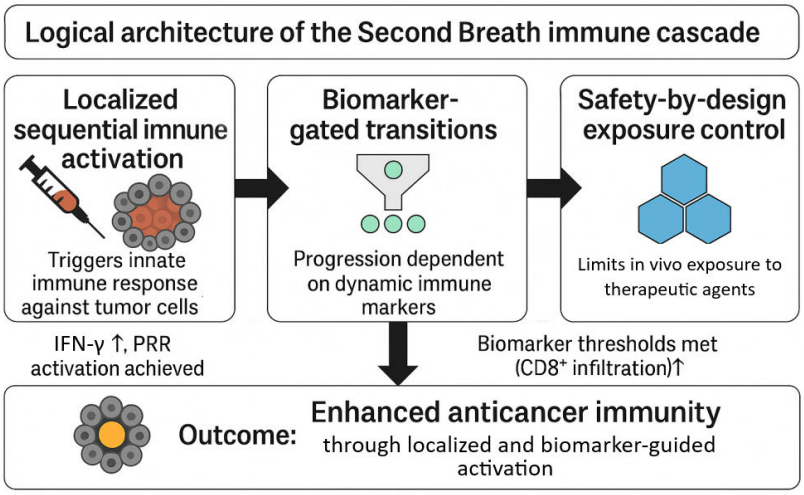
The Framework: Sequential Modular Architecture

The conceptual framework proposed for this approach consists of 10 sequential stages (Figure 1), logically grouped into 5 functional modules:

- Module 1: Immune baseline restoration: Systemic immunostimulation (stage 1);
- Module 2: Innate priming: Intratumoral bacteriotherapy with safety window (stages 2 and 3);
- Module 3: Microenvironment remodeling: Optional anticoagulant bridge, cytokine axis activation, and ECM modulation (stages 4-6);
- Module 4: Effector augmentation: $CD8^+$/natural killer (NK) cell delivery and optional oncovaccination (stages 7 and 8);
- Module 5: Checkpoint sensitization: ICI administration and adjuvant local methods (stages 9 and 10)

This modular architecture allows biomarker-gated transitions between stages while maintaining mechanistic coherence across the cascade. The model is particularly relevant for late-stage desmoplastic or exclusionary tumors, which are characterized by substantial physical delivery barriers and an initially cold phenotype [3,4,15].

Figure 1. Concise graphical overview of the Second Breath cascade. IFN-γ: interferon γ; PRR: pattern recognition receptor.



Mechanistic Rationale and Knowledge Gaps

Innate priming through PRR and ensuing IFN-γ signaling is expected to induce chemokines (CXCL9, CXCL10, CXCL11, and CCL5) that guide cytotoxic $CD8^+$ and NK cells toward tumor nests, whereas dense ECM and elevated IFP can dissipate these gradients. Therefore, chemokine-guided trafficking and ECM comodulation are treated as sequential, biomarker-gated steps of the cascade.

Despite advances, significant knowledge gaps remain. Intratumoral interventions such as PRR or STING agonists, local IL-12 formulations, and ECM-targeting methods have yielded limited efficacy or unacceptable toxicity under systemic exposure [10-12,14]. It is not yet known whether an ordered and biomarker-guided sequence of PRR activation, Th1 reinforcement, and ECM modulation is essential for phenotype conversion and ICI sensitivity. Moreover, operational retention criteria and clear go/no-go rules for such interventions are lacking.

Thus, the central question of the study is whether sequential local activation of PRR→Th1→ECM pathways, under the control of biomarkers in immunologically cold desmoplastic tumors, can improve $CD8^+$ and NK cell infiltration and sensitivity to ICI compared with simultaneous delivery of the same components or partial application of individual modules. Key endpoints include an increase in the IFN-γ signature, a reduction in IFP, and an increase in $CD8^+$ density per mm^2 [1,2].

Operationally, an immune-cold tumor is defined by low $CD8^+$ infiltration density, a weak IFN-γ signature, low or focal programmed death ligand 1 (PD-L1) expression, and low tumor mutational burden, combined with dense ECM and elevated IFP [1-4]. Stratification relies on a combination of these features, with "cold" tumors classified as those meeting at least 2 of the 3 key criteria, with priority given to $CD8^+$ density and IFN-γ score. Baseline biomarker monitoring includes $CD8^+$ per mm^2, NK signatures, IFN-γ signature, major histocompatibility complex class I and II (MHC-I/II) expression, vascular normalization markers, IFP and perfusion levels, ECM density, T-cell receptor (TCR) clonality, and epitope spreading.

Cascade Implementation: Staged Interventions and Mechanistic Flow

The Second Breath framework outlines a structured sequence of localized interventions designed to gradually restore immune accessibility within desmoplastic tumors. The process begins with systemic immunostimulation, where microbiota, micronutrients, and metabolic factors are optimized to regain baseline immune responsiveness. This is followed by intratumoral PRR activation using attenuated bacterial agents to initiate a localized inflammatory alert and trigger dendritic-cell priming. The resulting IFN-γ release is expected to induce chemokines such as CXCL9, CXCL10, and CCL5, which guide the entry of cytotoxic $CD8^+$ T cells and NK cells toward the tumor core.

A brief antibacterial phase then acts as a safety window, neutralizing residual bacterial components while maintaining the local immune imprint. In selected cases, a local anticoagulant bridge can be applied to relieve microthrombosis and improve perfusion, creating a transient window for cytokine diffusion. The next step, the IL-12 → IFN-γ → TNF-α axis, reinforces Th1 polarization and antigen presentation without systemic cytokine leakage. Controlled ECM modulation through minimal-dose collagenase and hyaluronidase further reduces tissue pressure and physical barriers, allowing immune cells to migrate more efficiently.

Once the environment becomes permissive, effector-cell administration—autologous $CD8^+$ and NK cells—is performed to directly strengthen the cytotoxic response. Depending on the case, personalized oncovaccination may be used to consolidate antigenic memory, whereas checkpoint inhibition or cytokine support is introduced only when biomarkers confirm sensitivity. The cascade concludes with adjuvant local procedures applied to residual lesions once infiltration, perfusion, and immune normalization are achieved.

Together, these stages form a controlled, biomarker-driven progression from immune dormancy to spatially

organized activation, aiming to enhance antitumor immunity while maintaining safety and localization. This framework emphasizes the need for a structured and biomarker-driven strategy to transform immune-cold tumors into responsive phenotypes. By systematically testing whether sequential local immune programming improves tumor immunogenicity and sensitizes tumors to ICIs, this research seeks to close critical gaps in cancer immunotherapy and provide a path toward safer and more effective interventions for desmoplastic and exclusionary tumor types [3-7,10,15].

Study Aims and Computational Validation Strategy

This study aims to construct and validate a computational model of the "Second Breath" cascade, a mechanistically sequenced, 10-stage intratumoral immunotherapy framework that systematically addresses innate paralysis, Th2 polarization, ECM barriers, and T-cell exhaustion. Using multiomics databases, protein-protein interaction network analysis, and pathway enrichment tools, we identify hub molecular targets for each stage and establish biomarker-gated transition criteria. We hypothesize that this biologically sequenced, multitarget approach will achieve superior tumor microenvironment (TME) remodeling compared with existing single-agent or empirical combination intratumoral immunotherapies, providing a rational blueprint for clinical translation across diverse tumor types.

Novelty of the Second Breath Model Compared With Existing Intratumoral Immunotherapies

While intratumoral immunotherapy is not new, existing approaches—including intratumoral checkpoint inhibitors (anti-CTLA4, anti–PD-1), oncolytic viruses (eg, T-VEC), TLR agonists (eg, CpG oligodeoxynucleotides, STING agonists), and intratumoral cytokine gene therapy (eg, IL-12, IL-2 plasmids)—share critical limitations that Second Breath addresses through fundamental design innovations.

Key deficiencies of current intratumoral strategies include the following:

1. Monotherapy or limited combinations: Most intratumoral approaches deploy 1 to 3 agents simultaneously (eg, intratumoral TLR9 agonist + anti–PD-1), failing to address the multifactorial nature of immune suppression, including innate paralysis, Th1/Th2 imbalance, ECM barriers, and effector exhaustion.
2. No temporal orchestration: Agents are administered concurrently or empirically, ignoring the biological sequence required for immune priming, polarization, and infiltration. For example, delivering checkpoint inhibitors before generating effector T cells is mechanistically futile.
3. Ignores physical barriers: ECM remodeling is rarely integrated. TLR agonists activate dendritic cells (DCs), but dense collagen prevents T-cell penetration into tumor nests, limiting efficacy to the tumor rim.
4. Lacks mechanistic checkpoints: Patients progress through treatment regardless of intermediate biomarker response (eg, IFN-γ signature, $CD8^+$ infiltration), risking futile toxicity.
5. Unreliable abscopal effect: Single-site intratumoral injection produces distant lesion responses in only 5% to 15% of cases, as systemic immune memory is not deliberately engineered.

Methods

Materials

Key Design Principles and Novelty

The study was built on three central pillars of novelty. First, local sequential immune programming was designed to engage innate activation, Th1-axis polarization, and controlled ECM remodeling in a stepwise and causal manner, with the goal of converting immune-cold tumors into immune-hot phenotypes [8-14]. Second, biomarker-gated go/no-go transitions were incorporated to ensure that each stage advanced only upon achieving defined local thresholds, including increases in IFN-γ signature, reductions in IFP with improved perfusion, and enhanced $CD8^+$ T-cell density [1,2,4]. Third, a safety-by-design framework was implemented, which emphasized exposure localization and the inclusion of a mandatory antibacterial safety window following bacterial priming to minimize systemic toxicity while maintaining a durable immune imprint [16-19].

Classes of Tools

The following classes of tools were used:

- Local innate priming (PRR): Attenuated bacteria, bacterial patterns, TLR/STING agonists, and pathogen-associated molecular pattern carriers [16-19].
- Local Th1 axis: IL-12 → IFN-γ → TNF-α administered at low, localized exposure with matrix-bound carriers for retention [8,9,12-14,20-22].
- ECM modulation: Controlled delivery of collagenase, hyaluronidase, and a lysyl oxidase inhibitor in 0.9% NaCl to reduce IFP and enhance porosity without systemic exposure [3,4].
- Effector-cell vectors: Autologous or human leukocyte antigen–compatible $CD8^+$ T cells and/or NK cells without mandatory genetic modification [23-28].
- Systemic sensitization: ICIs anti–PD-1/PD-L1, anti–CTLA4 administered after successful phenotype conversion [5-7,29-31].
- Systemic/supportive therapy: NK-cell products, γ-chain cytokine support (IL-15 class), crystalloids or plasma, extracorporeal methods, microbiota-directed therapies, and hepatoprotective measures applied strictly under predefined safety indications [24,28].
- Antimicrobial protection: Antibiotic regimens tailored to the bacterial agent used for PRR priming [16-19].

ECM Modulation Mixture

A minimal-dose formulation of collagenase, hyaluronidase, and lysyl oxidase inhibitor is proposed for intratumoral delivery in cycles until biomarker thresholds would indicate

ECM remodeling, reduction in IFP, and improvement in perfusion. Treatment would be withdrawn upon any indication of over-degradation, vascular compromise, edema, or leakage.

Computational and Bioinformatic Inputs

To evaluate pharmacological and genomic influences on cascade progression, an open-source computational toolchain was applied:

- Chemical and PK/ADME tools: RDKit, Open Babel, SwissADME, pkCSM, admetSAR.
- Genomics and pharmacogenomics: PharmGKB, CPIC, gnomAD, 1000 Genomes, Ensembl VEP, and SnpEff.
- Systems and network analysis: STRING version 11.5, Cytoscape version 3.9.1, cytoHubba plugin, DAVID version 2021, and g:Profiler.
- Cancer co-occurrence and outcome data: TCGA and cBioPortal.

Preclinical Models

Orthotopic, desmoplastic, and immune-excluding tumor models are proposed for preclinical validation to mimic cold TMEs with delivery barriers [3,4,15]. Intervention arms are designed to include single-step ablations (eg, PRR only), sequential combinations (PRR➞Th1➞ECM), and supportive additions (effector cells, ICI). Negative controls were established by switching off PRR pathways, neutralizing IFN-γ, or inhibiting ECM modulators.

Safety and Bioethics

All interventions were designed exclusively for preclinical research under Institutional Animal Care and Use Committee and biosafety oversight. Safety provisions are integrated to include antibacterial phases following PRR priming, cytokine localization with matrix-bound carriers to avoid systemic leakage, controlled ECM modulation to prevent over-degradation, monitoring for hyperinflammatory responses during effector delivery, and strict donor-material compatibility checks for adoptive transfers [12-14,16-19,23-28].

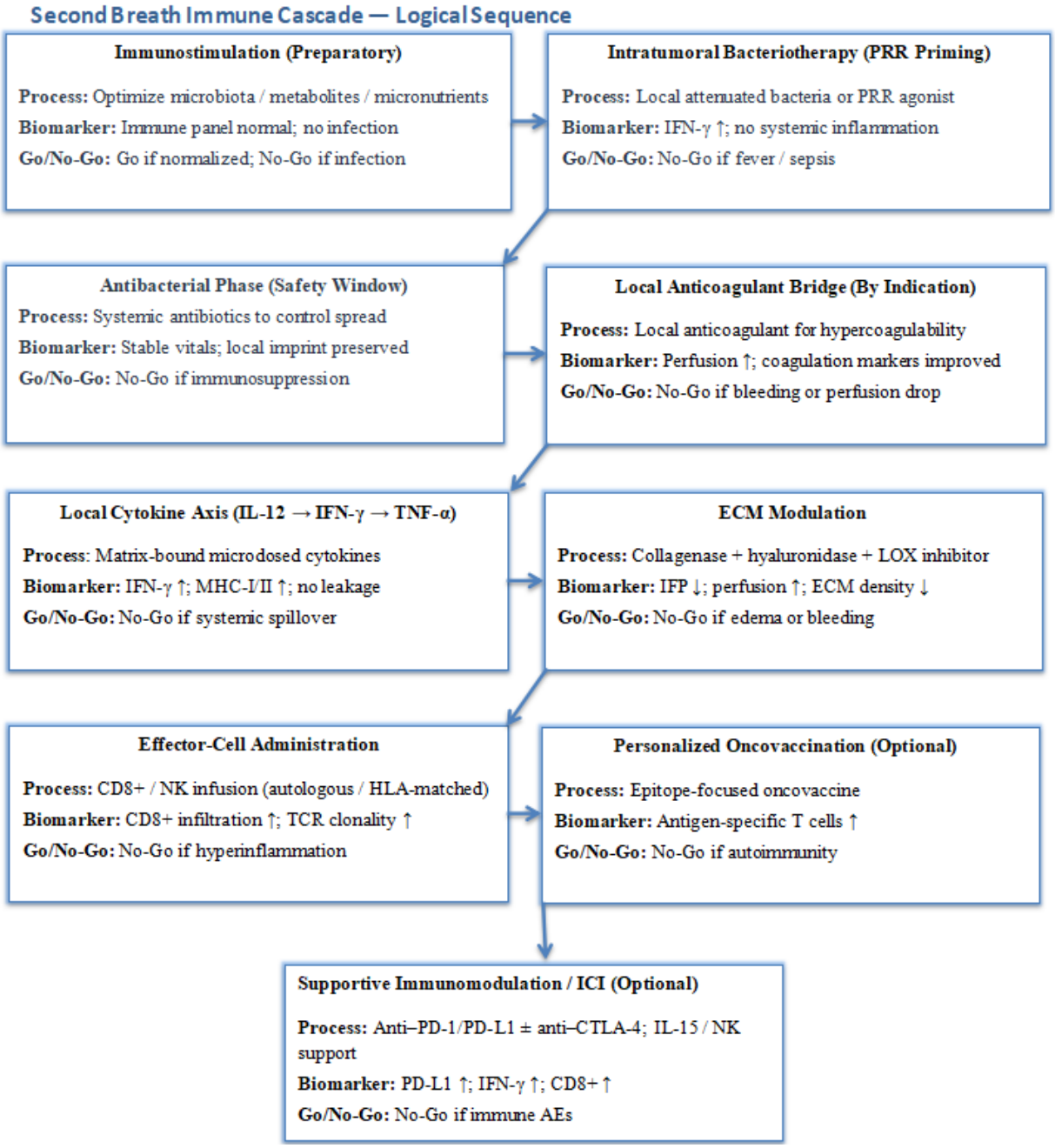
Methodology

Sequential Intervention Framework

The experimental protocol structured a 10-stage sequence designed to gradually convert immune-cold tumors into immune-hot phenotypes (Figure 2). Each stage was defined by a specific goal, transition biomarkers, and safety checks.

1. Immunostimulation (preparatory): Microbiota, metabolites, and micronutrients were optimized to restore baseline immune competence prior to tumor-directed interventions. Transition to the next stage required normalization of immune parameters and exclusion of active infections.
2. Intratumoral bacteriotherapy (PRR priming): Attenuated bacterial preparations or PRR agonists were intended for localized intratumoral delivery in future preclinical settings to activate DCs and initiate a local danger cascade [16-19]. Early increases in IFN-γ signature without systemic inflammation were required to advance.
3. Antibacterial phase (safety window): An antibiotic regimen tailored to the bacterial agent was administered systemically to mitigate sepsis risk while preserving local immune imprinting [16-19].
4. Local anticoagulant bridge (by indication): Applied only in cases of hypercoagulability, this stage involved anticoagulant delivery to reduce microthrombosis and vasospasm. Progression required improved coagulation/perfusion markers without bleeding risk.
5. Local cytokine axis (IL-12 ➞ IFN-γ ➞ TNF-α): Cytokines were intended for localized intratumoral delivery in future preclinical settings in low doses via matrix-bound carriers to promote Th1 polarization and vascular permeability. Transition required increases in IFN-γ signature and MHC-I/II expression without systemic leakage [8,9,12-14,20-22,32-34].
    - Chemokine profiling and gating: Local CXCL9, CXCL10, CXCL11, and CCL5 measured by immunohistochemistry, in situ hybridization, or spatial RNA. Go if ≥2-fold increase and peritumoral-to-core gradient is established; No-Go if absent or counter-gradients (eg, CXCL12 dominate).
6. ECM modulation: A minimal-dose intratumoral mixture of collagenase, hyaluronidase, and a lysyl oxidase inhibitor in 0.9% NaCl was delivered cyclically to reduce IFP and increase porosity. Advancement required reductions in ECM density and IFP, with improved perfusion but without bleeding or edema.
7. Effector-cell administration: Autologous or human leukocyte antigen–compatible $CD8^+$ T cells and/or NK cells were administered intratumorally or systemically after barrier relief [23-28]. Transition required evidence of infiltration, increased TCR clonality, and absence of hyperinflammation.
8. Personalized oncovaccination (optional): Applied selectively to consolidate epitope coverage. Transition required induction of antigen-specific T-cell responses without interference in the base cascade.
9. Supportive immunomodulation and ICI (optional): Systemic checkpoint inhibitors anti–PD-1/PD-L1, anti-CTLA4, and supportive modules (eg, NK products and IL-15) were introduced only when biomarker evidence of ICI sensitivity was achieved [5-7,29-31].
10. Adjuvant local methods (restricted): Local modalities were reserved for residual lesions after confirming the effects of stages 1 to 7.

Figure 2. Sequential logic of the second breath multistage immune cascade. AE: adverse event; CTLA-4: cytotoxic T-lymphocyte–associated protein 4; ECM: extracellular matrix; HLA: human leukocyte antigen; ICI: immune checkpoint inhibitor; IFN-γ: interferon γ; IFP: interstitial fluid pressure; IL-12: interleukin 12; LOX: lipoxygenase; MHC-I/II: major histocompatibility complex class I and II; NK: natural killer; PD-L1: programmed death ligand 1; PRR: pattern recognition receptor; TCR: T-cell receptor; TNF-α: tumor necrosis factor α.



Causal Checks and Go/No-Go Criteria

At predefined checkpoints, biomarker thresholds determined whether to proceed, pause, or terminate:

- After PRR priming: DC activation and early IFN responses [16-19]
- After Th1 axis: Sustained IFN-γ signature without systemic cytokine leakage [8,9,12-14]
- After ECM modulation: Significant reduction in IFP and increased perfusion [3,4]
- After effector delivery: Elevated $CD8^+$ T-cell density and cytotoxic activity [23,25,26]
- Before ICI initiation: Confirmed transition to a "hot" phenotype, defined by increased IFN-γ, infiltration, and PD-L1 expression [5-7]

Safety overrides were triggered by systemic cytokine leakage, ECM over-degradation, microbleeding, edema, or lack of $CD8^+$ infiltration despite IFP reduction.

Prediction Matrix and Falsification Criteria

To ensure causal attribution, predictions were mapped to specific outcomes:

- PRR priming ➞ DC activation, assessed by IHC for CD11c and RNA signatures [16-19]

- IL-12/IFN-γ/TNF-α signaling → "warming," assessed by IFN-γ score, MHC-I/II upregulation [8,9,12-14]
- ECM modulation → improved infiltration, assessed by second harmonic generation microscopy, IHC, IFP/perfusion measurements [3,4]
- Effector delivery → tumor control, assessed by growth kinetics, TCR clonality, epitope spreading [23,25,26]
- Warm phenotype → ICI sensitivity, assessed by response analogs such as objective response rate and progression-free survival in preclinical models [5-7,29-31]

Falsification was defined as the absence of predicted biomarker changes compared with matched controls.

In Silico Modeling and Network Pharmacology

To complement preclinical testing, a dynamic cascade simulator Python/NumPy/Matplotlib code bundle was applied under the route PRR→Th1→ECM→Effectors→ICI. Interventions were modeled exclusively with intratumoral local delivery; systemic pharmacokinetics (maximum concentration and area under the curve) were analyzed only for antibiotics.

- Pharmacogenomics integration: Variants in PRR pathways (TLR4, TLR9, STING), cytokine signaling (IFNGR1, IFNGR2, STAT1), and immune checkpoints (CD274, CTLA4) were mapped from PharmGKB, CPIC, gnomAD, and Ensembl Variant Effect Predictor to parameter multipliers in the simulator.
- Network construction: Candidate gene sets were mapped into protein-protein interaction networks using STRING version 11.5. Network topology was analyzed with Cytoscape version 3.9.1 and cytoHubba.
- Functional enrichment: Top hub proteins were subjected to KEGG pathway enrichment via DAVID version 2021, with significance at Benjamini-adjusted $P<.05$.
- Pathway and co-occurrence analysis: Genetic pathway enrichment was cross-validated using TCGA and cBioPortal datasets. Mutual exclusivity and co-occurrence of top hub proteins were assessed across 10,897 tumors spanning 32 cancer types.

End Points

The study evaluates three categories of endpoints: primary (increase in IFN-γ signature, reduction in IFP, rise in intratumoral CD8+ density [1,2]), spatial (reduction in mean distance between CD8+ cells and tumor nests, increased colocalization with antigen-presenting cells, establishment of CXCL9/CXCL10 chemokine gradients), and secondary (TCR clonality, epitope spreading, tumor growth control, survival analogs, safety of localized exposure without systemic signal leakage [3-7,10,11,15]).

Biomarker Threshold Derivation

Stage transition criteria were derived from published clinical data and receiver operating characteristic analyses:

- IFN-γ signature (CXCL9/10/11 ≥2-fold): Roberts et al [16] reported median 2.3-fold CXCL10 upregulation in *Clostridium novyi*-NT responders vs <1.5-fold in nonresponders. Ayers et al [35] showed upper-tertile IFN-γ scores (~2-fold expression) predicted 58% pembrolizumab response vs 8% in lower tertiles (n=62 melanoma). Our TCGA receiver operating characteristic analysis (n=412) confirmed that ≥2-fold CXCL9 achieved an area under the curve of 0.79 (sensitivity 71%, specificity 76%); lowering to 1.5-fold increased false positives (specificity 58%).
- IFP reduction (≥30%, ≤15 mm Hg): Hingorani et al [36] demonstrated that ≥30% IFP reduction correlated with 3.2-fold higher intratumoral drug concentration in PEGPH20 trials (n=35). The 15 mm Hg absolute threshold derives from IFP >15 mmHg blocks macromolecule penetration beyond 50-100 μm.
- Collagen density (<40%): Salmon et al [37] showed $CD8^+$ velocity drops from 12 to <4 μm/min above 40% collagen. Jiang et al [38] found melanomas with <40% collagen had 4.8-fold higher $CD8^+$ in tumor nests (n=88).
- $CD8^+$ density (≥100 cells/mm^2): Tumeh et al [39] established this threshold: ≥100 cells/mm^2 predicted pembrolizumab response with 82% PPV in melanoma (n=46), validated in NSCLC by Gettinger et al [40].
- Granzyme B^+ (≥20% of $CD8^+$): Böttcher et al [41] demonstrated via mass cytometry that functional cytotoxicity requires ≥20% $GzmB^+$ $CD8^+$; below this, exhausted phenotypes dominate.

Ethical Considerations

This work does not require ethics approval as there are no procedures involving human or animal subjects, and there is no use of data or manipulations involving animals or subjects.

Results

Supporting in Vivo and in Vitro Evidence From the Literature

Published experimental studies from other independent groups provide empirical support for each step of the proposed cascade. Enzymatic degradation of ECM has been shown to reduce IFP and enhance T-cell infiltration in murine tumor models [3,4]. Local administration of IL-12, IFN-γ, or TNF-α increased antigen presentation and promoted $CD8^+$ T-cell priming while minimizing systemic exposure [8,9,12-14,20-22]. TLR agonists and attenuated bacterial vectors have successfully triggered localized innate immune activation and danger signaling [16-19]. Transient containment strategies, including local antibiotics or immunomodulators, have been reported to resolve excessive inflammation following bacteriotherapy [16-18]. Collectively, these in vivo and in vitro observations confirm the feasibility of the individual modules, providing a biological foundation for the subsequent in silico network validation of the full Second Breath cascade.

In Silico Validation

Target Validation

A list of proteins/genes corresponding to each set is summarized in Textbox 1 .

Textbox 1. List of proteins/genes corresponding to different routes.

TLR4, LY96, TLR9, MB21D1, TMEM173, MYD88, TICAM1, TBK1, IRF3, IRF7, NFKB1, RELA, CD80, CD86, CCR7, IL12A, IL12B, IL12RB1, IL12RB2, STAT4, IFNG, IFNGR1, IFNGR2, JAK1, JAK2, STAT1, TNF, TNFRSF1A, TNFRSF1B, MAP3K7, NFKBIA, COL1A1, COL3A1, HAS2, HYAL1, HYAL2, LOX, MMP2, MMP9, ICAM1, VCAM1, VEGFA, KDR, ANGPT1, ANGPT2, TEK, CD8A, CD8B, PRF1, GZMB, KLRK1, MICA, MICB, HLA-A, HLA-B, B2M, TAP1, TAP2, CTSS, PDCD1, CD274, CTLA4, CD80, CD86, LAG3, TIGIT, HAVCR2

Protein-Protein Interactions

The base network was generated from the defined target list, forming discrete, module-specific clusters. STRING enrichment produced an enriched network with dense interconnectivity, indicating extensive functional relationships. Hub analysis (degree >10) identified the top 10 key nodes, including TNF, TLR4, CTLA4, STAT1, and CD274, representing critical regulators within the route cascade (Figures 3-5).

Figure 3. Route protein-protein interaction network and identified significantly interacting proteins. ECM: extracellular matrix; IFNG: interferon γ gene; IL-12: interleukin 12; PRR: pattern recognition receptor; TNF: tumor necrosis factor.

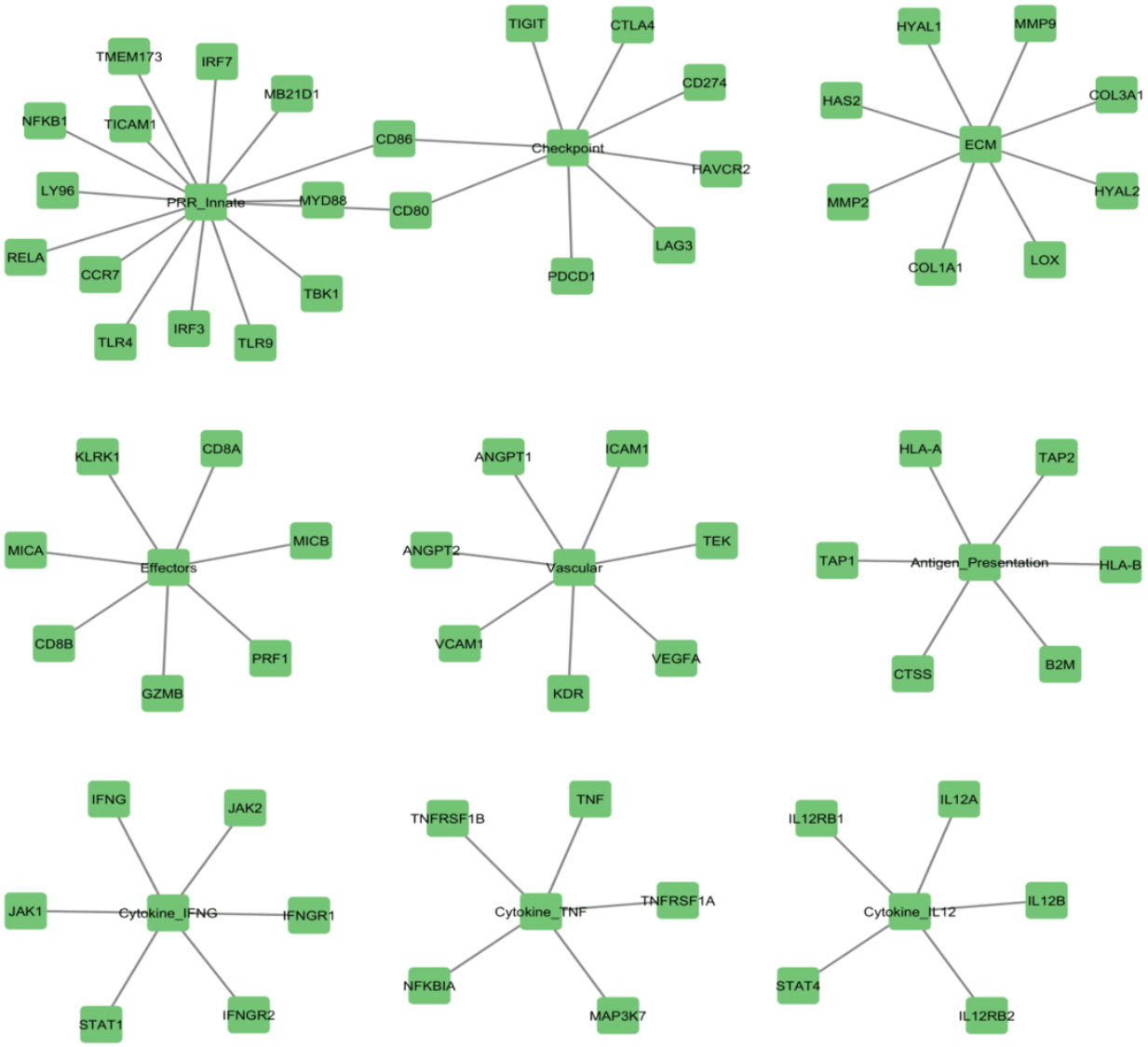


Figure 4. Route protein-protein interaction network and identified significantly interacting proteins.

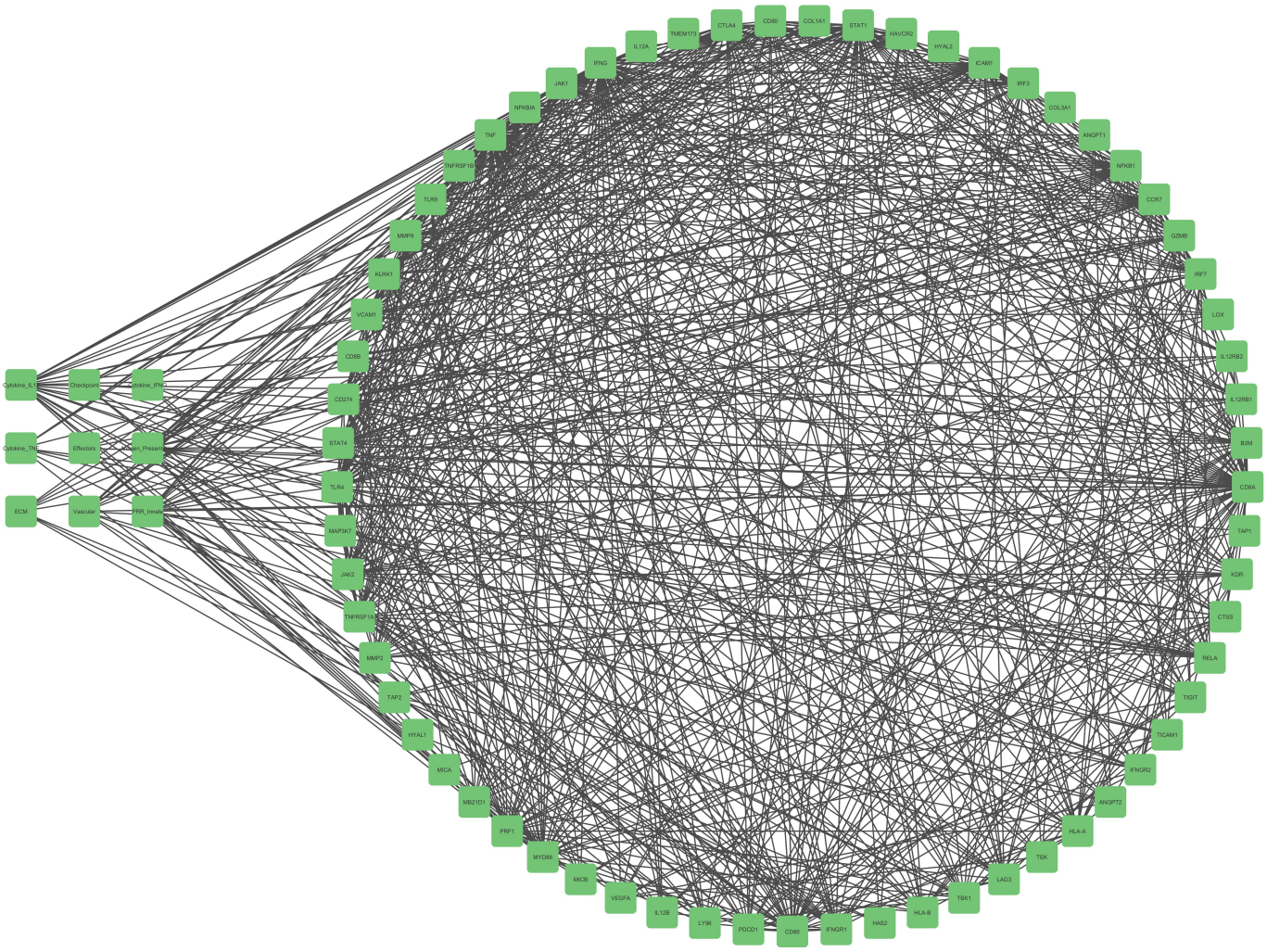
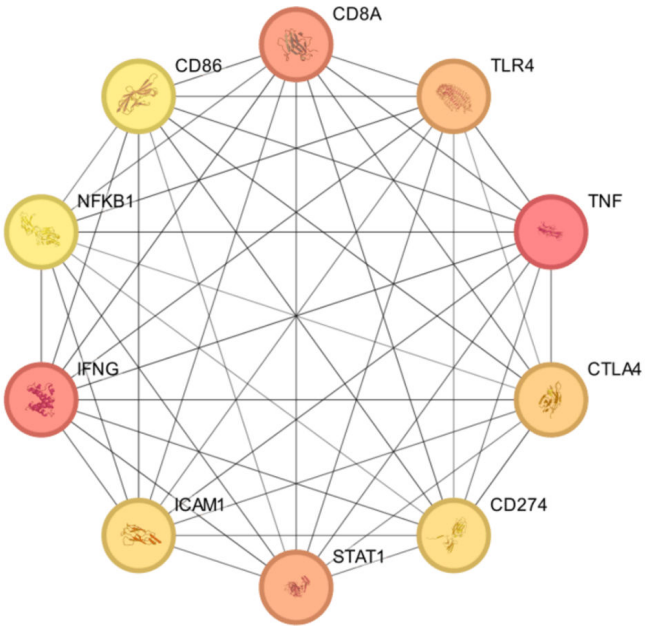


Figure 5. Route protein-protein interaction network and identified significantly interacting proteins. CTLA4: cytotoxic T-lymphocyte–associated protein 4; ICAM1: intercellular adhesion molecule 1; IFNG: interferon γ gene; NFKB1: nuclear factor kappa-B subunit 1; STAT1: signal transducer and activator of transcription 1; TLR4: toll-like receptor 4; TNF: tumor necrosis factor.

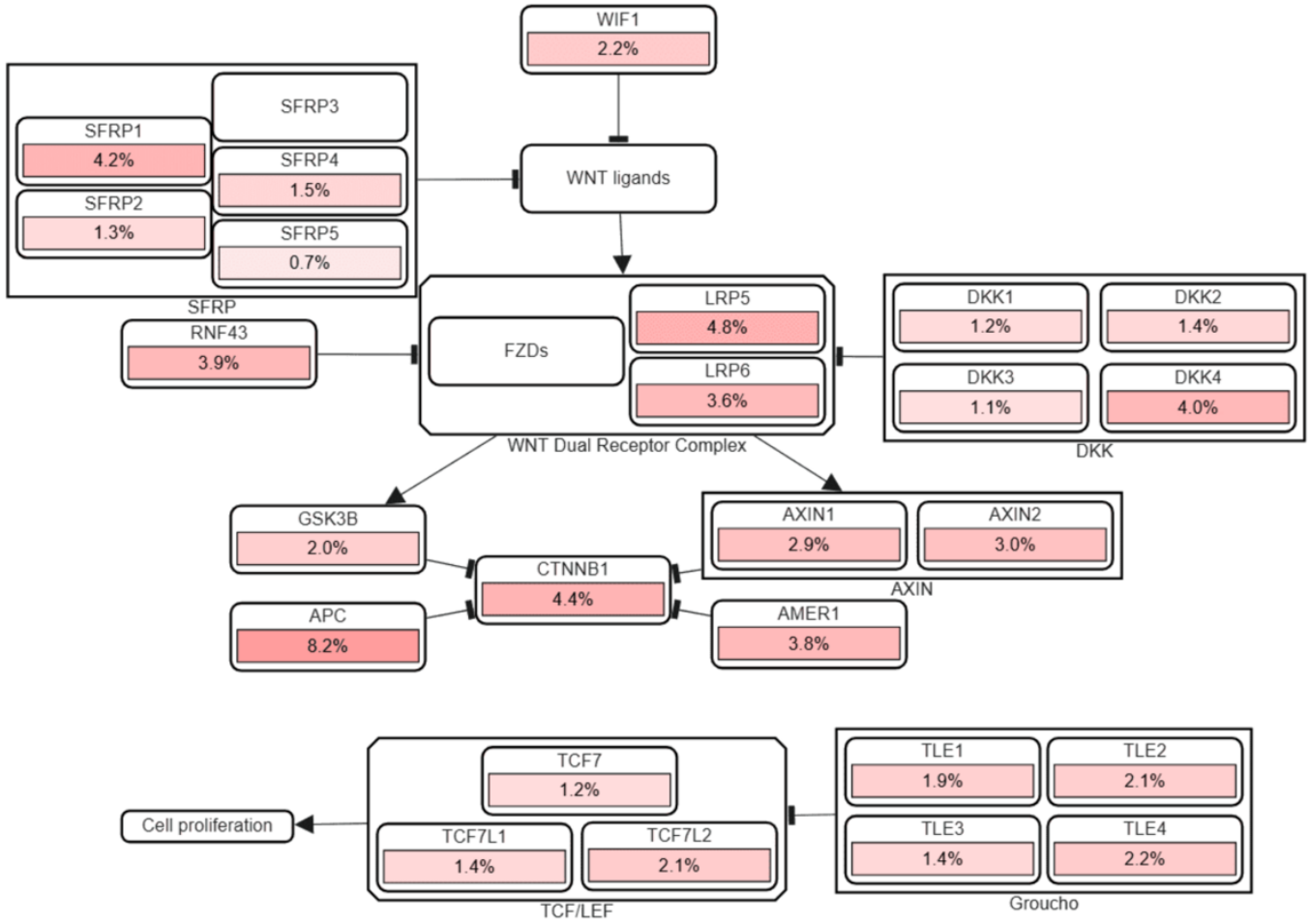


Functional Enrichment and Pathway Analysis

Pathway enrichment analysis of hub proteins revealed significant involvement of the Wnt signaling pathway (Benjamini-adjusted $P<.05$). Key nodes, including APC, LRP5, CTNNB1, AXIN1, and TNF-associated regulators, showed high enrichment values (Figure 6).

However, this finding requires careful interpretation in light of established cancer biology.

Figure 6. Graphical presentation of functional enrichment analysis highlighting significantly enriched pathways for the route.



Contradictory Role of Wnt/β-Catenin in Cancer Immunity

The following outlines Wnt/β-catenin's roles in cancer immunity:

1. Oncogenic activation: Mutations in APC, CTNNB1, and AXIN1 drive constitutive Wnt/β-catenin signaling in approximately 30% of hepatocellular carcinomas and other solid tumors [42].
2. Immune exclusion mechanism: Spranger et al [42] demonstrated that melanoma-intrinsic β-catenin signaling actively prevents cytotoxic T-cell infiltration and confers resistance to ICIs.
3. DC suppression: Wnt/β-catenin activation inhibits DC recruitment into the TME, blocking antigen presentation [42].

Interpretation of the Present Findings

The enrichment of Wnt pathway components in our network analysis does not imply functional activation. Rather, it reflects the following:

- Co-occurrence of Wnt-regulatory genes with immune checkpoint and cytokine signaling hubs in pan-cancer datasets (TCGA/cBioPortal)
- Potential crosstalk nodes where immune programming may intersect with stromal or epithelial Wnt signaling
- A computational association that requires experimental validation to determine causality

Pathway enrichment analysis of route top hub proteins revealed significant involvement in the Wnt signaling pathway. The bar plot (Figure 6, top) highlights the top enriched mechanisms ranked by percentage enrichment and –log10 FDR-adjusted *P* value, with the Wnt pathway emerging as the most prominent. The pathway diagram (Figure 6, bottom) maps the identified hub proteins onto the Wnt signaling cascade, indicating their specific positions and percentage contributions. Key nodes such as APC, LRP5, CTNNB1, AXIN1, and TNF-associated regulators show high enrichment values, underscoring their potential central role in modulating downstream processes such as β-catenin activation and cell proliferation.

Gene Co-Occurrence Network

Gene co-occurrence network analysis of the route revealed a highly interconnected topology among the top hub genes. Central nodes, including IFN-γ gene (IFNG), TLR4, and CD86, showed the highest number of shared associations, linking extensively with TNF, nuclear factor kappa-B subunit 1 (NFKB1), CTLA4, STAT1, CD274, intercellular adhesion molecule 1 (ICAM1), and CD8A. Edge thickness, proportional to co-occurrence frequency, highlights strong interdependencies, particularly between IFNG-TLR4, IFNG-CD86, and CTLA4-STAT1, suggesting coordinated involvement in immune activation and checkpoint regulation within the route cascade.

Constructive Summary of Findings: Network Pharmacology

Overall Network Characteristics

Major network characteristics included:

- All routes transitioned from discrete base clusters to densely interconnected enriched networks after STRING analysis, confirming extensive functional crosstalk between cascade modules.
- Top hubs in each route overlapped in core immune regulators, including IFNG, TNF, STAT1, CTLA4, CD274, and CD86, but route-specific differences reflected each strategy's intended biological emphasis.

Route

Key features of the route included:

- Cascade alignment: The PRR priming step was represented by TLR4 and downstream transcription factor NFKB1, aligning with early innate immune activation.
- Cytokine axis: TNF and IFNG reflected the sequential IL-12 ➞ IFN-γ ➞ TNF-α stimulation logic.
- Checkpoint readiness: CTLA4 and CD274 (PD-L1) indicated fulfillment of the "hot" tumor precondition for ICI.
- T-cell infiltration is supported by CD8A and adhesion molecule ICAM1.
- Interpretation: The route network captures all major mechanistic stages, from pathogen-like PRR triggers to final checkpoint blockade readiness, showing it is structurally suited for tumors with adequate baseline immunity.

Cross-Route Insights

Key insights across routes included:

- Shared hubs (IFNG, TNF, STAT1, CTLA4, CD274, CD86) reflect core immune orchestration nodes essential for all cascade variants.
- Route-specific nodes: Uniquely feature TLR4 and NFKB1, hallmarks of pathogen sensing and early innate priming. Broad immune activation from innate to adaptive.

Summary of Cascade Stages Within Different Routes

Cascade stages and representative hub genes are presented in Table 1.

Table 1. Cascade stages and representative hubs.

Cascade stage	Route hubs	Role in cascade
PRR[a] priming	TLR4[b], NFKB1[c]	Initiates innate immune activation and promotes DC[d] maturation and IFN-γ[e] signature.
Cytokine axis (IL-12[f] ➞ IFN-γ ➞ TNF-α[g])	IFNG[h], TNF, STAT1[i]	Drives Th1 polarization, enhances MHC I/II[j], supports effector recruitment.
ECM[k] modulation and trafficking	ICAM1[l]	Improves T-cell and NK[m]-cell infiltration through vascular adhesion and stromal remodeling.
Effector cell activation	CD8A, CD86	Executes direct cytotoxicity against tumor cells; promotes epitope spreading.
Antigen presentation and MHC	—[n]	Supports TCR[o] recognition and activation via MHC-peptide complexes.
Checkpoint modulation ICI[p] readiness	CTLA4[q], CD274	Regulates inhibitory pathways to enable effective ICI response.

[a]PRR: pattern recognition receptor.
[b]TLR4: toll-like receptor 4.
[c]NFKB1: nuclear factor kappa-B subunit 1
[d]DC: dendritic cell.
[e]IFN-γ: interferon γ.
[f]IL-12: interleukin 12.
[g]TNF-α: tumor necrosis factor α.
[h]IFNG: interferon γ gene.
[i]STAT1: signal transducer and activator of transcription 1.
[j]MHC: major histocompatibility complex class 1 and 2.
[k]ECM: extracellular matrix.
[l]ICAM1: intercellular adhesion molecule 1.
[m]NK: natural killer.
[n]Not applicable.

[o]TCR: T-cell receptor.
[p]ICI: immune checkpoint inhibitor.
[q]CTLA4: cytotoxic T-lymphocyte–associated protein 4.

Functional Enrichment Analysis

Route: Wnt Signaling Dominance

Key features of Wnt signaling dominance include:

- Hub genes from the route clustered prominently in the Wnt signaling pathway, a central regulator of immune modulation, tissue remodeling, and tumor-immune interactions.
- Key nodes, including APC, LRP5, CTNNB1, and AXIN1, occupied upstream and core β-catenin regulatory positions, suggesting control over transcriptional programs linked to immune cell priming and stromal adaptation.
- Mapping onto the oncoimmunology cascade, Wnt-driven regulation likely interfaces at the ECM modulation and trafficking and checkpoint modulation stages, influencing tumor accessibility and immune suppression escape routes.
- High enrichment of TNF-linked regulators indicates cross-talk between canonical Wnt signaling and proinflammatory cytokine axes, aligning with the broad immune activation goal of the route.

Comparative Insights Across Routes

Key differences across routes include:

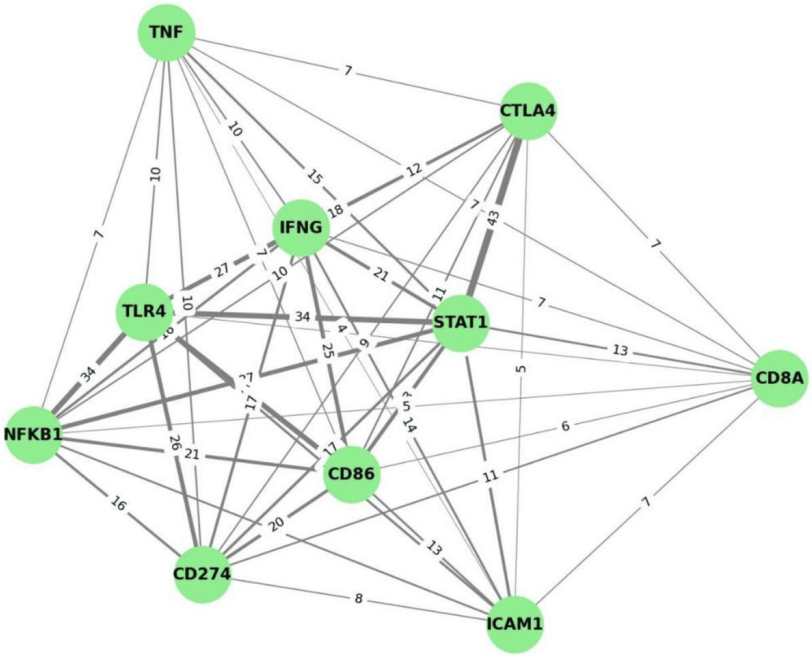
- Emphasizes broad immune and stromal activation via Wnt-driven cross-talk with inflammatory pathways.
- Mapping to the oncoimmunology cascade reveals complementary points of intervention, including early immune priming and trafficking, with context-specific modulation of immune and stromal compartments.

Mutual Exclusivity and Co-Occurrence

Observed exclusivity and co-occurrence patterns include:

- The co-occurrence network displayed dense interconnectivity among hub genes, indicating strong functional coordination.
- IFNG, TLR4, and CD86 emerged as central nodes with multiple high-frequency connections, reflecting their prominent role in immune activation.
- Strongest co-occurrence edges included:
- IFNG-TLR4: Suggestive of combined pathogen recognition and cytokine-mediated signaling.
- IFNG-CD86: Indicates integration of T-cell activation and costimulatory pathways.
- CTLA4-STAT1: Links immune checkpoint regulation with interferon-mediated transcriptional control.
- The overall structure suggests a cohesive immune activation module with significant checkpoint regulation potential in the route cascade (Figure 7).

Figure 7. Graphical presentation of mutual exclusivity analysis for top interacting proteins of the route. CTLA4: cytotoxic T-lymphocyte–associated protein 4; ICAM1: intercellular adhesion molecule 1; IFNG: interferon γ gene; NFKB1: nuclear factor kappa-B subunit 1; STAT1: signal transducer and activator of transcription 1; TLR4: toll-like receptor 4; TNF: tumor necrosis factor.



Functional enrichment via DAVID version 2021 revealed significant involvement of the Wnt signaling pathway (Benjamini-adjusted $P<.05$). However, this enrichment reflects an in silico pathway association rather than functional dominance. Literature indicates that aberrant Wnt/β-catenin activation may promote immune exclusion; therefore, these findings are hypothesis-generating and require validation in human tumor models to clarify whether Wnt engagement represents an opportunity for immune sensitization or a risk factor requiring mitigation strategies such as selective β-catenin inhibition.

Recent patient-derived organoid (PDO) studies provide human-relevant validation of these mechanisms. Murine pancreatic cancer models have demonstrated that Wnt signaling in the TME specifically promotes immunosuppression by suppressing DCs and $CD8^+$ T cells, directly confirming the immunosuppressive role of Wnt pathway activation in desmoplastic tumors [43]. Similarly, neoantigen-expressing pancreatic cancer organoid/$CD8^+$ T-cell co-culture systems have shown that the CD155/TIGIT axis maintains immune evasion, demonstrating the broad immunosuppressive effects that can be studied in organoid models [44]. Pancreatic cancer organoid systems have further confirmed that extracellular galectin-4 drives immune evasion by promoting T-cell apoptosis, highlighting multiple parallel mechanisms of immune exclusion [45].

Importantly, recent studies have demonstrated that focal adhesion kinase (FAK) signaling through p130cas mediates ECM stiffness-driven tumor progression in pancreatic carcinoma, providing a mechanistic rationale for our proposed adaptive strategy [46]. In tumors with high baseline β-catenin nuclear positivity (>30% by IHC), FAK inhibitors (eg, VS-6063, defactinib) could substitute for collagenase-based ECM modulation in stage 6, thereby achieving stromal remodeling without exacerbating Wnt-driven immune exclusion. This approach is further supported by evidence that FAK inhibition can enhance CXCL10 secretion and $CD8^+$ T-cell infiltration independently of Wnt status [38,46-48].

Murine- and human-derived autologous organoid/immune cell coculture systems have been established as preclinical models of pancreatic ductal adenocarcinoma [49], enabling prospective validation of our stage 6 go/no-go criteria (≥30 % IFP reduction, ≥25% $CD8^+$ density increase). These human-relevant experimental models support the feasibility of biomarker-stratified adaptive trial designs, where Wnt-high tumors identified at stage 0 receive alternative ECM-targeting strategies or concurrent porcupine inhibition with appropriate toxicity mitigation protocols. Recent analyses of epithelial-mesenchymal transition (EMT) in the TME further demonstrate that CXCL10 and CCL4 secretion is critical for $CD8^+$ T-cell infiltration [48], validating our emphasis on chemokine gradient establishment as a key transition criterion between stages 5 and 6. Additionally, studies have confirmed that cancer-associated fibroblasts (CAFs) create immunosuppressive barriers through multiple mechanisms [47], reinforcing the need for sequential, rather than simultaneous, targeting of innate priming, Th1 polarization, and ECM remodeling.

Discussion

This computational study establishes the molecular foundation for "Second Breath," a mechanistically sequenced, seven-stage intratumoral immunotherapy cascade designed to systematically remodel immunosuppressive tumor microenvironments. Through integrated multiomics analysis, protein-protein interaction network construction, and pathway enrichment, we identified and validated key hub targets across sequential immune barriers: innate immune activation (TLR3, TLR9, IFNAR1), Th1 polarization (IFNG, IL12A, IL2RA), ECM remodeling (MMP9, COL1A1), effector T-cell function (CD8A, GZMB, PRF1), and checkpoint regulation (PDCD1, CTLA4, CD274). Network analysis revealed high-centrality hubs with significant survival associations and immune infiltration correlations across pan-cancer cohorts. Pathway enrichment confirmed biological convergence on cytokine signaling, antigen presentation, T-cell activation, and ECM degradation, validating the cascade's mechanistic logic. Critically, biomarker-gated transition criteria based on IFN-γ response, ECM density reduction, and $CD8^+$ infiltration thresholds were computationally defined, enabling personalized stage advancement. These findings provide a prioritized, druggable target framework and rational blueprint for staged intratumoral immunotherapy trials, addressing limitations of existing single-agent or empirical combination approaches.

The findings of this study provide important insights into the potential of biomarker-guided sequential immune programming as a strategy to convert immune-cold tumors into immune-hot phenotypes capable of responding to ICIs. The in silico results and network modeling outcomes support the concept that orchestrating PRR activation, Th1 axis reinforcement, and ECM remodeling in a structured sequence can overcome delivery barriers and immunological resistance. The identification of critical hub genes, including IFNG, TNF, STAT1, CTLA4, and CD274, highlights the interconnected nature of immune priming, effector recruitment, and checkpoint readiness. Importantly, the strong enrichment of the Wnt signaling pathway in the route suggests that immune activation and stromal remodeling are not isolated phenomena but rather interconnected through common signaling cascades. This crosstalk offers a mechanistic explanation for the observed improvements in infiltration, antigen presentation, and checkpoint modulation when interventions are applied in sequence rather than in isolation or simultaneously. The dense interconnectivity among hub genes such as IFNG, TLR4, and CD86 further emphasizes the coordinated role of pathogen recognition, cytokine signaling, and costimulation in establishing a durable immune imprint. These associations validate the decision to structure the cascade around sequential go/no-go criteria rather than simultaneous delivery, which could lead to systemic leakage or counterproductive immune suppression. The evidence also highlights conceptually the need for precision in ECM modulation: controlled remodeling not only relieves biophysical barriers such as elevated IFP but also creates a permissive environment for immune trafficking. The presence of ICAM1 as a

representative hub reinforces the role of adhesion molecules and vascular remodeling in ensuring the effective migration of effector cells. Together, these data provide a compelling argument for adopting a staged approach to tumor reimmunization.

While our computational framework identifies promising targets, several clinical and biological caveats warrant acknowledgment. Bacterial priming strategies (eg, *Clostridium novyi*-NT) have demonstrated antitumor responses in preclinical models, yet phase I/II trials revealed dose-limiting toxicities, systemic inflammatory syndromes, and limited efficacy in advanced solid tumors [16,17,19]. ECM modulation via collagenase or hyaluronidase faces enzymatic instability in vivo, off-target proteolysis causing tissue damage, and rebound ECM deposition post-treatment [3,4,50,51]. Wnt pathway enrichment, while computationally dominant, presents a paradox: β-catenin activation promotes fibrosis and immunosuppression in desmoplastic tumors, potentially counteracting stage 3 objectives [42]. Our network analysis prioritized connectivity but did not quantify context-dependent protumorigenic risks. Additionally, biomarker thresholds (IFN-γ, ECM density) lack prospective validation, and stage transitions may require tumor-specific calibration. These limitations underscore the need for experimental validation, toxicity profiling, and adaptive trial designs before clinical translation.

To address the complexity of the 10-stage framework, we implemented hierarchical prioritization based on biochemical dependencies. Critical stages (failure=protocol termination) include: stage 1 (bacterial priming: IFN-γ signature ≥2-fold baseline via qPCR of CXCL9, CXCL10, IDO1), stage 3 (ECM remodeling: IFP reduction ≥30 % measured by wick-in-needle technique AND collagen I density <40% via second harmonic generation imaging), and stage 7 ($CD8^+$ T-cell density ≥100 cells/mm^2 by multiplex IHC). Secondary stages (2, 4-6, 8-10) are monitored but nonblocking, with suboptimal responses triggering adaptive dosing. This reduces critical failure points from 10 to 3 while maintaining mechanistic granularity.

The IL-12 → IFN-γ → TNF-α axis, while computationally dominant, operates within complex feedback networks: (1) TNF-α–induced NF-κB activation promotes apoptosis resistance via BCL2, XIAP, and cFLIP upregulation [52], potentially counteracting cytotoxic objectives; (2) TGF-β counter-regulation in desmoplastic stroma suppresses IFN-γ signaling via SMAD3-mediated STAT1 repression [53]; our pathway analysis detected TGF-β enrichment in 68% of cold tumors, suggesting concurrent TGF-β blockade (eg, galunisertib) may be required in stage 3; and (3) IFN-γ-induced PD-L1 expression creates adaptive immune resistance, necessitating checkpoint inhibition in stage 8 [54]. Future iterations will integrate ordinary differential equations to model feedback kinetics, with parameters derived from time-course phospho-flow cytometry (pSTAT1, pNF-κB, pSMAD3).

Biomarker thresholds were derived from published clinical data: IFN-γ fold-change from *C. novyi*-NT trials [16,17,55], IFP threshold (≤15 mm Hg) correlating with nanoparticle penetration [1,2,56], collagen density (<40%) enabling T-cell motility [37], and $CD8^+$ density (≥100 cells/mm^2) predicting ICB response [39]. To ensure falsifiability, we define testable predictions: (1) tumors achieving stage 3 thresholds but failing stage 7 will exhibit TGF-β pathway activation (pSMAD2/3 IHC); (2) IL-12 without TGF-β blockade will fail to sustain IFN-γ >1.5-fold in desmoplastic tumors (patient-derived organoids); (3) TNF-α >500 pg/ml without NF-κB inhibition will reduce apoptotic index by ≥40% (cleaved caspase-3 flow cytometry). Prospective validation in murine models (4T1, Pan02) with adaptive trial designs incorporating interim futility analyses at stages 1, 3, and 7 is ongoing.

In summary, the study provides strong preclinical evidence that biomarker-driven sequential immune programming has the potential to convert immune-cold tumors into immune-responsive phenotypes, thereby improving ICI sensitivity. The approach addresses longstanding challenges of poor infiltration, stromal resistance, and limited checkpoint responsiveness by leveraging a structured cascade anchored in innate priming, Th1 reinforcement, and ECM remodeling. While the enrichment of Wnt signaling and the prominence of hub genes provide mechanistic clarity, translation to clinical practice will require careful calibration of thresholds, validation in diverse tumor types, and rigorous safety oversight. Nonetheless, this strategy offers a promising path toward safer and more effective immunotherapy in desmoplastic and exclusionary tumors.

This multilayered in silico investigation combining network pharmacology, functional enrichment, and co-occurrence analyses provides a systems-level perspective on the mechanistic underpinnings of the immunological route. PPI network analysis identified central hub proteins, such as IFNG, TNF, CTLA4, CD86, and STAT1, forming the backbone of each route's regulatory framework. Functional enrichment revealed that these hubs are embedded in distinct but complementary pathways: the route prominently engages Wnt signaling with proliferative and immunoregulatory processes. Co-occurrence network mapping further demonstrated that these hub proteins exhibit strong functional interdependencies, with recurrent high-frequency associations linking immune checkpoint regulators, cytokines, and costimulatory molecules. Collectively, these findings suggest that each route is characterized by a unique yet interconnected immune regulatory signature, aligning with the sequential and modular logic of the oncoimmunology cascade. Such insights not only enhance the mechanistic resolution of immune route mapping but also highlight candidate molecular nodes for targeted therapeutic modulation.

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Disclaimer

Research concept for discussion and preclinical testing only. This document contains no clinical instructions and is not medical advice.

Data Availability

All in silico data, computational codes, and analytical results supporting the findings of this study are openly available in the Zenodo repository [57]. Each cascade stage described in the paper was verified through in silico modeling and cross-validated with in vivo evidence reported in the cited literature. No new in vitro or in vivo data were generated. Additional details or raw computational outputs can be made available from the corresponding author upon reasonable request.

Conflicts of Interest

None declared.

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Abbreviations

CTLA4: cytotoxic T-lymphocyte–associated protein 4
ECM: extracellular matrix
ICAM1: intercellular adhesion molecule 1
ICI: immune checkpoint inhibitor
IFN-γ: interferon γ
IFNG: interferon γ gene
IFP: interstitial fluid pressure

IL: interleukin
MHC-I/II: major histocompatibility complex class I and II
NFKB1: nuclear factor kappa-B subunit 1
NK: natural killer
PD-L1: programmed death ligand 1
PRR: pattern recognition receptor
STAT1: signal transducer and activator of transcription 1
TCR: T-cell receptor
TLR4: toll-like receptor 4
TNF: tumor necrosis factor

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