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Feeder Cells as Active Process Determinants in Adoptive Cell Therapy: Biological Imprinting, Critical Quality Attributes, and Feeder-Free Alternatives

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ABSTRACT

Adoptive Cell Therapy (ACT) manufacturing is commonly described as a sequence of isolation, activation, genetic modification, expansion, harvest, and formulation. Within this framework, feeder cells are frequently treated as ancillary reagents whose principal function is to increase cell number. This view is incomplete. Irradiated peripheral blood mononuclear cells, Epstein-Barr virus-transformed lymphoblastoid cells, engineered K562 derivatives, and other cellular stimulators create a dynamic signaling niche that integrates T-cell receptor or natural killer-cell receptor engagement, co-stimulation, adhesion, membrane-bound cytokines, soluble mediators, metabolic competition, and time-dependent loss of feeder viability. Consequently, feeder systems can imprint differentiation state, metabolic fitness, clonal composition, exhaustion susceptibility, trafficking competence, cytotoxicity, cytokine polyfunctionality, and in vivo persistence. In this review, feeder cells are reframed as active process determinants whose material attributes and operating parameters should be linked prospectively to product critical quality attributes. We compare feeder platforms used for tumor-infiltrating lymphocytes, conventional and engineered alpha-beta T cells, natural killer cells, gamma-delta T cells, and virus-specific T cells; examine mechanistic evidence for biological imprinting; and propose a risk-based analytical framework covering feeder identity, proliferative arrest, ligand density, residual feeder clearance, adventitious-agent control, and functional consistency. Feeder-free approaches, including anti-CD3/CD28 beads, soluble or degradable nanomaterials, ligand-presenting particles, membrane-derived stimulatory particles, cytokine-only systems, and engineered biomaterials, are then evaluated according to their ability to reproduce rather than merely replace feeder-derived signals. We argue that successful process simplification requires a defined target product profile, mechanistically anchored comparability studies, and orthogonal potency assays. Treating feeder selection as an early process-design decision may improve manufacturing robustness, facilitate comparability, and reduce the risk that expansion yield is optimized at the expense of therapeutic fitness.

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Introduction

Adoptive cell therapy encompasses therapeutic products generated from naturally occurring or genetically modified immune cells, including Tumor-Infiltrating Lymphocytes (TILs), Chimeric Antigen Receptor (CAR)-T cells, T-cell receptor-engineered T cells, virus-specific T cells, Natural Killer (NK) cells, and gamma-delta T cells. Across these platforms, ex vivo culture is not a neutral amplification step. The manufacturing environment selects among pre-existing cellular states and induces new transcriptional, metabolic, and epigenetic programs that can determine persistence, antitumor activity, and toxicity after infusion. The process therefore functions as a biological intervention rather than a simple logistics operation [1-4].

Feeder cells have historically enabled clinically relevant expansion when cytokines alone or immobilized antibodies were insufficient. Classical TIL rapid expansion protocols use a large excess of irradiated allogeneic Peripheral Blood Mononuclear

Cells (PBMCs), soluble anti-CD3 antibody, and high-dose interleukin-2 (IL-2). Virus-specific T-cell manufacturing has relied on autologous antigen-presenting cells or Epstein-Barr virus-transformed lymphoblastoid cell lines, whereas NK-cell and gamma-delta T-cell platforms frequently use irradiated K562 derivatives engineered to express co-stimulatory ligands and membrane-bound cytokines. These systems can generate large cell numbers, but their biological complexity introduces donor dependence, lot variability, residual-cell risks, and challenges for mechanistic comparability [5-12].

The central premise of this review is that feeder cells should be treated as active process determinants. Their effect is defined not only by feeder identity but also by the density and geometry of stimulatory ligands, feeder-to-effector ratio, irradiation or inactivation method, duration of cell-cell contact, cytokine environment, culture vessel, oxygen and nutrient conditions, and the kinetics of feeder death. Each of these variables can alter the magnitude, duration, and spatial organization of signaling. In turn, these process inputs can affect differentiation, clonality, metabolic state, phenotype, function, and persistence of the final ACT product [7-11,13-16].

This narrative review integrates mechanistic, translational, and regulatory evidence to address four questions: first, how different feeder platforms shape immune-cell products; second, which biological and manufacturing attributes should be considered critical; third, how feeder-free systems should be compared with cell-based stimulators; and fourth, how a risk-based control strategy can support process development and regulatory comparability. A structured literature search was conducted using PubMed/MEDLINE, regulatory guidance repositories, and citation chaining through July 2026. Priority was given to clinical manufacturing studies, direct platform comparisons, mechanistic studies linking expansion conditions to product phenotype or function, and current regulatory guidance.

Feeder Cells as Integrated Signaling Niches Beyond Numerical Expansion

A feeder cell is often operationally defined as a replication-incompetent cell that supports the growth of another cell population. In ACT manufacturing, however, the feeder is better understood as a transient cellular microenvironment. It presents membrane-bound ligands, captures or displays antibodies through Fc receptors, provides adhesion surfaces, releases cytokines and metabolites, consumes nutrients, and undergoes stress and cell death after irradiation. These features create a time-varying niche that cannot be described adequately by a single input such as feeder-to-effector ratio [7-9,13,17].

Three categories of signals are particularly relevant. Signal 1 engages the T-cell receptor, CAR-associated signaling machinery, or activating NK receptors. Signal 2 is delivered through co-stimulatory ligands such as CD80, CD86, CD70, 4-1BB ligand, OX40 ligand, ICAM-1, and related molecules. Signal 3 comprises soluble or membrane-bound cytokines, including IL-2, IL-7, IL-15, and IL-21. Feeder systems can also contribute a fourth layer: physical and temporal organization. Cell-cell synapses constrain receptor spacing, local ligand concentration, contact duration, and serial engagement in ways that differ from persistent antibody-coated beads or soluble agonists [8-11,18-20].

The biological consequence is that equal fold expansion does not imply equivalent products. Two cultures may achieve the same cell dose while differing in CD4-to-CD8 composition, memory-state distribution, activation thresholds, cytokine polyfunctionality, cytotoxicity, mitochondrial reserve, telomere dynamics, repertoire diversity, and susceptibility to activation-induced cell death. Process development that uses total viable cell number as the dominant endpoint may therefore optimize productivity while unintentionally reducing therapeutic fitness [7,9,11,15,21].

Temporal Signaling and the Kinetics of Feeder Disappearance

Irradiation is intended to prevent feeder proliferation, but it does not instantaneously eliminate feeder function. Irradiated cells can remain metabolically active, retain surface ligands, bind activating antibodies, and release soluble factors for variable periods. Their progressive apoptosis changes the stimulatory environment over time. Thus, the effective stimulation profile is shaped by the irradiation dose, cell type, pre-irradiation state, cryopreservation history, handling interval, and culture conditions. UVC-mediated inactivation, for example, can suppress proliferation while altering cellular energy metabolism and increasing surface retention of bound anti-CD3 antibody, illustrating that the inactivation method itself can change the quality of stimulation [22].

Temporal control is also important because persistent stimulation can drive differentiation and dysfunction. In a direct comparison of cell-based artificial antigen-presenting cells and anti-CD3/CD28 beads, transient cell-based stimulation favored CD8-positive T-cell expansion, preservation of stem-like memory features, cytokine polyfunctionality, persistence, and antitumor activity, whereas prolonged bead contact promoted more differentiated products. The general implication is not that cellular feeders are intrinsically superior, but that signal duration and reversibility are critical process parameters that must be measured and controlled [15].

Feeder Platforms Across Act Modalities

Irradiated PBMC Feeders in TIL Rapid Expansion

The classical TIL manufacturing paradigm consists of an initial outgrowth or pre-rapid expansion phase followed by a Rapid Expansion Protocol (REP). In widely used REP formats, TILs are stimulated with soluble anti-CD3 antibody, high-dose IL-2, and approximately a 200-fold excess of irradiated allogeneic PBMC feeders for about 14 days. The system can produce clinically relevant cell numbers and has supported the development of TIL therapy from early melanoma studies to contemporary commercial manufacturing [3-6].

PBMC feeders provide heterogeneous support because they contain monocytes, B cells, T cells, NK cells, and other subsets whose abundance and activation state vary by donor and collection method. Fc-receptor-bearing cells can present anti-CD3 antibodies, while accessory cells contribute adhesion molecules, cytokines, and co-stimulatory signals. The use of pooled allogeneic donors may improve consistency relative to a single donor and increase allostimulatory support, but it also expands the material-control burden. Donor eligibility, collection conditions, storage, pooling strategy, irradiation, and infectious-disease testing become part of the manufacturing control space [5,6,22,23].

PBMC feeders can influence phenotype beyond total expansion. Conventional REP conditions often produce highly activated effector-memory cells, and the balance between rapid proliferation and retention of less differentiated cells remains a central optimization problem. Addition of 4-1BB agonism during REP increased CD8-positive T-cell yield, preserved CD28 expression, enhanced perforin and granzyme expression, and improved cytokine secretion, demonstrating that an incompletely supplied co-stimulatory axis can materially alter the product even when the core feeder system is unchanged [9].

The culture device also interacts with feeder biology. Compartmentalized bags and gas-permeable systems can improve closure, reduce handling, and alter cell density, oxygen transfer, and the probability of feeder-effector contact. Side-by-side studies have shown that changes in vessel configuration can maintain viability and expansion while shifting CD4/CD8 composition or enriching tumor-reactive subsets. The feeder system should therefore be evaluated as part of a process unit operation rather than as an isolated reagent [24,25].

K562-Based Artificial Antigen-Presenting Cells for Alpha-Beta T Cells

K562 cells are attractive scaffolds for Artificial Antigen-Presenting Cells (aAPCs) because they are readily expanded, amenable to stable genetic modification, and have limited endogenous HLA class I and class II expression. Engineered derivatives can express

Fc receptors for antibody loading, co-stimulatory ligands, adhesion molecules, cytokines, or introduced HLA molecules. Master and working cell banks can be established, offering a potentially more standardized alternative to freshly sourced PBMC feeders [7,8,26].

In TIL manufacturing, K562-derived aAPCs have achieved expansion comparable to PBMC feeders at substantially lower feeder-to-TIL ratios. Direct-expansion studies reported enrichment of CD8-positive cells, maintenance of CD27 and CD28, low frequencies of FOXP3-positive CD4 cells, preserved tumor reactivity, and the ability to undergo secondary expansion. Clinical-grade aAPCs expressing CD86, 4-1BB ligand, and membrane-bound IL-15 also generated TILs with a broadly comparable effector-memory profile, while transcriptomic analysis showed enrichment of Wnt and stem-cell-associated pathways. These observations directly support the concept of biological imprinting by the feeder platform [7,8].

The modularity of K562 aAPCs permits deliberate control of co-stimulatory combinations. K562 cells expressing CD80, CD70, and 4-1BB ligand expanded antigen-specific T cells more efficiently than cells expressing partial ligand combinations, consistent with non-redundant signaling through CD28, CD27, and 4-1BB. Nevertheless, greater stimulation is not necessarily better: excessive ligand density, repeated exposure, or prolonged contact may accelerate differentiation or activation-induced cell death. Platform design should therefore define an intended signaling profile rather than maximize every activating input [18,19].

NK-Cell Feeder Systems

NK-cell manufacturing provides the clearest example of feeder-dependent signal engineering. Wild-type K562 cells are susceptible to NK-cell recognition, but dramatic expansion generally requires engineered ligands. Co-expression of membrane-bound IL-15 and 4-1BB ligand produced robust and selective NK-cell expansion without parallel T-cell outgrowth. Subsequent platforms using membrane-bound IL-21 with 4-1BB ligand enabled sustained proliferation and large cumulative expansion, supporting repeated stimulation and clinical-scale production [10-13].

Membrane-bound cytokines do more than substitute for soluble cytokine supplementation. Their spatial restriction and receptor-proximal delivery create high local signal intensity during cell-cell contact. IL-15 and IL-21 also have distinct biological consequences. Comparative studies indicate that the sequence and duration of exposure to these cytokines can modify proliferation, differentiation, metabolism, polyfunctionality, receptor expression, and *in vivo* activity. The feeder genotype and stimulation schedule should therefore be regarded as product-defining variables, particularly for multi-dose or off-the-shelf NK-cell strategies [11,16,29].

The use of feeder-derived membrane particles illustrates a transition between cellular and non-cellular stimulation. Plasma-membrane particles generated from K562 cells expressing membrane-bound IL-21 and 4-1BB ligand can expand NK cells without introducing intact proliferative feeder cells into the culture. This approach retains native membrane organization and multivalent signaling while simplifying residual-cell control. It also demonstrates that the critical element may be the organized ligand-bearing membrane rather than the viability of the feeder itself [19].

Autologous or allogeneic PBMC feeders have also been used for NK-cell expansion in combination with anti-CD3 and IL-2. These systems can be accessible and GMP compatible, but they inherit

donor variability and may expand contaminating T cells unless depletion and release controls are robust. The relative importance of feeder source, starting-cell composition, and cytokine exposure must be resolved for each intended NK-cell product [29-31].

Gamma-Delta T Cells and Other Unconventional Lymphocytes

Gamma-delta T cells are present at low frequency in peripheral blood and frequently require strong proliferative support to achieve therapeutic doses. Engineered K562 aAPCs expressing combinations of CD64, CD86, 4-1BB ligand, anti-CD3 single-chain fragments, and cytokine receptors have supported large-scale expansion in conventional vessels and gas-permeable bioreactors. Compared with zoledronic acid and IL-2 alone, repeated aAPC stimulation can extend proliferation and enhance effector function [20,21,26].

The same principles apply to other lymphocyte populations, including invariant natural killer T cells and selected antigen-specific alpha-beta T-cell clones. Feeder composition can determine which subsets dominate, how strongly antigen specificity is retained, and whether repeated stimulation preserves or erodes proliferative capacity. This is particularly relevant when the starting population is rare and the manufacturing process may amplify only a small number of founder clones.

Virus-Specific T Cells and Lymphoblastoid Feeder Systems

Virus-specific T-cell manufacturing historically used autologous antigen-presenting cells or Epstein-Barr virus-transformed lymphoblastoid cell lines to provide physiologic antigen processing and co-stimulation. Such systems enabled effective clinical products for post-transplant viral disease and established the feasibility of repeated antigen-specific stimulation. However, they require individualized or banked antigen-presenting cells, extended manufacturing timelines, viral and adventitious-agent controls, and careful assessment of non-target antigen responses [27,28].

Feeder-free peptide pools, viral vectors, nucleic-acid-loaded APC substitutes, and cytokine-based enrichment strategies can shorten production and reduce dependence on living antigen-presenting cells. Yet the transition from lymphoblastoid feeders to defined reagents must preserve epitope breadth, HLA restriction, CD4/CD8 balance, and functional avidity. A higher numerical yield with a narrower or less persistent repertoire may not constitute a clinically meaningful improvement [27,28,40].

Biological Imprinting by Feeder Systems Differentiation and Memory State

The differentiation state of an ACT product is associated with proliferative reserve, persistence, and antitumor efficacy. Feeder-mediated T-cell receptor signaling, co-stimulation, and cytokine exposure regulate the balance among stem-cell memory, central memory, effector memory, and terminal effector states. High-intensity or persistent stimulation may increase immediate cytotoxicity while reducing long-term proliferative capacity. Conversely, transient or appropriately tuned stimulation can support expansion while preserving CD27, CD28, CCR7, CD62L, and other markers associated with less differentiated states [9,15,32].

Feeder cells can also create subset-specific selection. 4-1BB signaling preferentially supports activated CD8-positive cells, CD28 signaling can favor cells retaining CD28 competence, and membrane-bound cytokines can differentially support NK-cell maturation states. As a result, the observed phenotype at

harvest reflects both induction and selection. Distinguishing these mechanisms requires longitudinal sampling rather than end-point analysis alone [9,18,29].

Metabolic Programming and Stress Adaptation

Expansion requires rapid biomass generation and imposes substantial metabolic stress. Feeder cells influence this environment by consuming glucose, amino acids, and oxygen; releasing metabolites; modifying pH; and altering the effective cytokine concentration. Their death can release nucleotides, lipids, damage-associated molecules, and intracellular components. These effects may change glycolytic dependence, mitochondrial biogenesis, oxidative phosphorylation, reactive-oxygen stress, and resistance to nutrient deprivation. Although metabolic assays are not routinely included in product release, they can be highly informative during process development and comparability studies [16,22,32].

For NK cells, exposure to membrane-bound IL-15 versus IL-21 can produce distinct proliferative and functional trajectories, including differences in expansion durability, receptor expression, metabolic fitness, and senescence. For T cells, persistent stimulation and high-dose IL-2 can favor rapid effector differentiation, whereas cytokine combinations containing IL-7 or IL-15 may better preserve memory-associated states. These relationships are context dependent and should be evaluated using the final intended stimulation platform rather than inferred from cytokine identity alone [11,13,16,29].

Transcriptional and Epigenetic State

Feeder-induced signaling activates transcription factors including NFAT, NF-kappaB, AP-1, STAT proteins, T-bet, Eomes, and MYC. The duration and combination of these signals can stabilize chromatin states associated with effector differentiation or exhaustion. Direct evidence from aAPC-expanded TILs showed altered transcriptional programs, including enrichment of Wnt and stem-cell-associated pathways, without a major loss of clonal diversity. These findings indicate that feeder choice can modify cell state even when conventional phenotypic and cytotoxicity assays appear similar [7].

Single-cell RNA sequencing, chromatin-accessibility profiling, DNA-methylation analysis, and multi-omic integration can therefore serve as development tools for identifying hidden process effects. These assays are not expected to become routine lot-release tests in most programs, but they can define a mechanistic fingerprint that supports platform selection, comparability, and identification of simpler surrogate assays. A rational strategy is to use high-dimensional assays during process characterization and then translate the most informative signals into robust flow-cytometric, metabolic, or functional readouts.

Clonal Repertoire and Antigen Specificity

Feeder systems can preserve, broaden, or distort the clonal repertoire. Polyclonal anti-CD3 stimulation may expand both tumor-reactive and bystander T cells, while antigen-specific APCs favor selected clonotypes. Strong allogeneic stimulation can introduce expansion pressure unrelated to the intended antigen. Conversely, poorly stimulatory feeder lots may permit only highly proliferative clones to dominate. Repertoire analysis should therefore assess diversity, dominance, and retention of known tumor- or virus-reactive clonotypes rather than relying solely on total T-cell purity [7,8,27].

The acceptable degree of repertoire change depends on the product concept. For a polyclonal TIL product, broad repertoire preservation may be desirable; for a defined TCR-engineered product, uniform transgene expression and functional avidity may be more important; for virus-specific T cells, epitope breadth and HLA coverage may be critical. Feeder-related CQAs should consequently be linked to a product-specific target profile rather than applied identically across ACT classes.

Functional Potency, Persistence, and Safety

Immediate cytotoxicity is only one component of potency. Feeder-conditioned products may differ in serial killing, cytokine polyfunctionality, proliferative response after antigen encounter, resistance to suppressive cytokines, trafficking receptor expression, and survival after cytokine withdrawal. Transiently stimulated CAR-T cells, for example, demonstrated superior persistence and antitumor activity in vivo despite the use of similar receptor specificity. For TILs, 4-1BB co-stimulation enhanced survival-associated proteins and functional output [9,15].

Feeder-related safety concerns include residual viable feeder cells, unintended alloreactive or autoreactive expansion, transfer of adventitious agents, residual transforming viruses in lymphoblastoid systems, and unwanted cytokine or activation profiles. For K562-derived feeders, residual tumor-cell contamination is a specific concern even when the feeder is irradiated. Product release must therefore include validated residual-feeder detection with sensitivity justified by risk, together with evidence that inactivation consistently prevents replication [34-39].

Critical Material Attributes and Critical Process Parameters

A quality-by-design approach begins with a target product profile and a mechanistic understanding of how materials and process parameters affect Critical Quality Attributes (CQAs). For feeder-supported ACT, the feeder should be controlled as a complex biologic input. Its critical material attributes include identity, purity, cell-bank history, genetic stability, ligand expression, cytokine expression, viability at use, sterility, mycoplasma status, adventitious-agent status, and functional stimulatory capacity. The relevance of each attribute depends on the intended mechanism and on whether the feeder is autologous, donor-derived, immortalized, or genetically engineered [34-39].

Critical process parameters include feeder-to-effector ratio, timing of feeder addition, irradiation or inactivation dose, interval between inactivation and use, number of restimulations, antibody loading, cytokine concentration, culture density, vessel geometry, agitation, oxygen transfer, media exchange, and duration of contact. Many of these parameters interact. For example, a lower feeder ratio may be compensated by higher ligand density or prolonged exposure, but the resulting cell state may not be equivalent. Multivariate process characterization is therefore preferable to one-factor-at-a-time optimization [7-9,15,22].

The most useful control strategy separates three analytical layers. First, feeder release tests establish that the feeder lot is suitable for use. Second, in-process tests confirm that the co-culture is behaving as intended. Third, drug-product release tests demonstrate identity, purity, safety, and potency of the therapeutic cells. A feeder lot that passes identity and sterility testing may still be unsuitable if its functional ligand density or stimulatory capacity has drifted. Conversely, a modest change in feeder phenotype may be acceptable if a validated functional assay and final-product comparability data demonstrate preserved performance [34-39].

Table 1: Representative Feeder Platforms and Their Process-Defining Features

Platform	Typical ACT use	Principal signals	Advantages	Key risks/limitations
Irradiated allogeneic PBMCs	TIL REP; polyclonal T-cell expansion	Fc-mediated anti-CD3 presentation; heterogeneous co-stimulation; soluble mediators; allostimulation	Long clinical history; strong expansion; familiar workflow	Donor and subset variability; infectious-disease testing; high feeder ratio; residual-cell and sourcing burden
Autologous PBMCs	NK cells; selected T-cell workflows	Accessory-cell support; anti-CD3 presentation; autologous cytokine milieu	Reduced allogeneic input; accessible in some settings	Consumes patient material; variable composition; may co-expand T cells
EBV-transformed lymphoblastoid cells	EBV-specific and other virus-specific T cells	Physiologic antigen processing and presentation; strong B-cell co-stimulation	Broad antigen presentation; established clinical precedent	Viral and adventitious-agent control; long lead time; individualized or banked material
K562-based aAPCs	TILs; CAR/TCR T cells; NK cells; gamma-delta T cells	Engineered Fc receptors, co-stimulatory ligands, adhesion molecules, membrane cytokines	Bankable; modular; lower lot variability; high expansion efficiency	Tumor-derived cell line; genetic stability; residual feeder detection; inactivation validation
Feeder-derived membrane particles	NK cells and emerging platforms	Native membrane organization of cytokines and co-stimulatory ligands without intact cells	Reduced residual viable-cell risk; multivalent signaling	Particle characterization, dose definition, manufacturing consistency
Engineered stromal or APC-like cells	Stem-cell-derived immune cells; specialized differentiation systems	Developmental niche, cell-cell adhesion, cytokines and extracellular matrix	Can reproduce lineage-specific biology	High complexity; xenogeneic risk in some systems; difficult comparability

Proposed Feeder-Specific Critical Quality Attributes

Feeder CQAs should be justified by a causal chain linking feeder attributes to process performance and drug-product quality. A practical framework includes identity and authenticity; genetic and phenotypic stability; proliferation arrest; functional ligand density; cytokine display or secretion; viability and apoptosis kinetics; sterility and adventitious-agent control; and residual feeder clearance. For donor-derived feeders, donor eligibility, pooling, collection, transport, cryopreservation, and thaw recovery are additional material attributes [34-39].

Functional potency of the feeder should not be defined solely by its ability to induce fold expansion. A composite feeder assay may include expansion kinetics, viability, subset composition, activation-marker induction, and a functional readout in a standardized responder-cell system. Reference responder cells, control charts, and system-suitability criteria can help separate feeder-lot effects from patient starting-material variability. Where a single assay cannot capture the mechanism, an assay matrix is preferable [35,38].

Residual feeder-cell testing requires special attention. Methods may include flow cytometry using feeder-specific markers, quantitative PCR for feeder-specific genomic sequences or transgenes, digital PCR, short tandem repeat analysis, or assays targeting lineage markers absent from the therapeutic product. The assay sensitivity should be linked to the maximum administered dose and the biological risk of the feeder. Validation should address specificity in the presence of large numbers of therapeutic cells, sample representativeness, and the possibility of damaged but metabolically active feeder cells [36-39].

Table 2: Risk-Based Analytical Framework for Feeder Cells

Attribute	Primary risk	Potential analytical approach	Control point
Identity/authenticity	Cell-line misidentification or donor mix-up	STR profile; lineage markers; donor traceability; transgene identity	At cell-bank qualification and lot release
Genetic stability	Loss or drift of stimulatory transgenes; emerging subclones	Copy number; sequence confirmation; karyotype/genomic stability; expression trend	Bank qualification and periodic monitoring
Functional ligand density	Under- or over-stimulation and altered imprinting	Quantitative flow cytometry; calibrated binding sites/cell; ligand occupancy	Each feeder lot or qualified campaign
Proliferation arrest	Feeder outgrowth in culture or patient exposure	Post-inactivation growth assay; extended culture; replication marker assessment	Validated inactivation process plus lot verification
Viability/apoptosis kinetics	Uncontrolled signal duration and debris burden	Viability over time; annexin V/7-AAD; metabolic activity; ligand retention	Process characterization; targeted in-process monitoring
Functional stimulatory capacity	Lot passes identity but fails to support intended product	Standardized responder-cell expansion, phenotype and functional assay	Lot release with reference control
Microbiological safety	Transmission of bacteria, fungi, mycoplasma or viruses	Sterility, mycoplasma, endotoxin, adventitious-agent testing	Per applicable GMP and cell-bank strategy
Residual feeder clearance	Infusion of tumor-derived or allogeneic feeder cells	Flow cytometry, qPCR/dPCR, STR or transgene-specific assay	Final product release or validated clearance strategy
Secretome/cytokine activity	Unintended differentiation or toxicity-related imprinting	Targeted cytokine panel; functional receptor-reporter assay	Characterization and change assessment
Process consistency	Drift in feeder-to-effector interaction	Feeder ratio, contact duration, mixing, culture density, vessel and medium controls	In-process and continued process verification

Feeder-Free Alternatives

Anti-CD3/CD28 Beads and Polymeric Nanomatrices

Immobilized anti-CD3 and anti-CD28 antibodies enabled prolonged T-cell proliferation in the absence of exogenous feeder cells and became foundational to modern CAR-T manufacturing. Magnetic beads provide standardized activation, are compatible with closed systems, and can combine selection and stimulation. Polymeric nanomatrices and related reagents can be removed or diluted without magnetic separation. These systems substantially reduce biological raw-material complexity [14,15,32,42,45].

The principal limitation is that immobilized reagents may deliver prolonged or spatially uniform stimulation that differs from dynamic cell-cell interactions. Bead-to-cell ratio, bead size, antibody density, removal time, and mixing influence signaling intensity and duration. Persistent bead exposure can drive differentiation and reduce functional quality, whereas early bead removal or degradable matrices may preserve less differentiated states. Thus, feeder-free does not mean parameter-free [15,32,42].

Soluble, Plate-Bound, and Multimeric Agonists

Soluble anti-CD3, plate-bound antibodies, tetrameric antibody complexes, and Fc-based multimers can activate T cells without living feeders. These approaches are operationally simple and may be useful at small scale, but they can create gradients, uneven surface presentation, or vessel-dependent activation. Translation to closed, large-scale manufacturing requires control of surface chemistry, mixing, adsorption, and reagent clearance. The absence of a living feeder reduces adventitious-agent risk but may increase dependence on precise physical presentation.

Ligand-Presenting Particles and Membrane-Derived Systems

Synthetic particles can present anti-CD3, anti-CD28, 4-1BB

ligand, cytokines, or adhesion molecules at defined density and spacing. Nanoparticles and microcarriers offer tunable geometry and may be engineered to degrade after a programmed interval. Membrane-derived particles occupy an intermediate position: they preserve native membrane orientation and multivalency while eliminating intact feeder cells. PM21 particles are a clinically relevant example for NK-cell expansion [19].

The development challenge is analytical. Particle size distribution, ligand density, orientation, potency, aggregation, storage stability, and dose must be controlled. A mass-based particle dose may not correlate with functional surface area or accessible ligand number. As these systems become more complex, they may reproduce many of the characterization burdens of cell-based feeders, although with improved standardization and residual-cell safety.

Cytokine-only and Small-Molecule-Supported Expansion

Cytokine-only systems are attractive because they minimize cellular and particulate raw materials. IL-2, IL-7, IL-15, and IL-21 can be used alone or in combination, and pathway-modulating small molecules may alter differentiation, metabolism, or exhaustion. Recent preclinical work has demonstrated feeder-free TIL expansion using anti-CD3/CD28 stimulation with lower IL-2 exposure and additional cytokines. Nevertheless, cytokine-only or antibody-plus-cytokine systems may not fully reproduce adhesion, synapse geometry, or membrane-restricted signaling [40,46].

Such platforms should be evaluated against a predefined quality target rather than against historical fold expansion alone. Important endpoints include expansion success across poor-quality starting materials, preservation of tumor-reactive clones, serial killing, metabolic reserve, cytokine withdrawal survival, in vivo persistence, and resistance to suppressive conditions.

Engineered Biomaterials and Microphysiologic Systems

Emerging biomaterials can control ligand spacing, stiffness, degradability, and temporal release. Hydrogels, supported lipid bilayers, microfluidic contact systems, and three-dimensional scaffolds may reproduce selected aspects of an immunological synapse while avoiding living feeders. These approaches also create opportunities for process analytical technologies, including real-time imaging, metabolic monitoring, and controlled stimulus withdrawal. Their translational value will depend on scalability, GMP-grade component availability, closed-system compatibility, and a clear demonstration that added engineering complexity produces clinically relevant quality gains.

Table 3: Comparison of Feeder-Free Expansion Strategies

Approach	Signal architecture	Strengths	Limitations	Best-aligned use
Anti-CD3/CD28 magnetic beads	Defined and widely used; supports selection plus activation	Closed-system compatibility; extensive CAR-T experience	Signal persistence; bead removal; bead-to-cell ratio dependence	CAR-T and TCR-T manufacturing
Polymeric or soluble nanomatrix	Multivalent activation without permanent beads	Simplified removal; tunable antibody density	Matrix consistency; dose metric; scale-up mixing	T-cell activation and gene-modified products
Plate-bound or soluble agonists	Antibody-mediated receptor engagement	Low complexity and cost in development settings	Open handling; vessel effects; difficult large-scale uniformity	Early development and small-scale workflows
Synthetic ligand-presenting particles	Defined co-stimulation/ cytokine presentation	Tunable geometry, density and degradability	Complex characterization; aggregation and stability	Next-generation T- and NK-cell platforms
Feeder-derived membrane particles	Native membrane multivalency without intact cells	Strong NK expansion; lower residual viable-cell risk	Biological source remains; particle potency and consistency	NK-cell expansion
Cytokine-only or cytokine plus soluble activator	No cell-based stimulant	Simple raw-material chain; easier residual control	May not reproduce synapse or adhesion signals; variable robustness	Selected TIL, NK and virus-specific workflows
Engineered hydrogels/ microcarriers	Spatially and temporally controlled signaling	Programmable stimulation and scale-out potential	Early translational maturity; device and material burden	Future automated and continuous platforms

Comparability When Replacing Feeder Cells

Replacing a feeder system is a major manufacturing change because it can alter multiple product attributes simultaneously. A successful comparability exercise should begin with a mechanistic map of the signals removed, retained, or newly introduced. For example, replacing PBMC feeders with anti-CD3/CD28 beads changes not only raw-material sourcing but also Fc-mediated presentation, adhesion, cytokine milieu, allostimulation, contact kinetics, and stimulus persistence. A comparison limited to total expansion, viability, CD3 purity, and short-term cytotoxicity is unlikely to be sufficient [35,38,39].

A tiered comparability strategy can reduce risk. Tier 1 should evaluate manufacturability: success rate, kinetics, yield, viability, process duration, and closure. Tier 2 should evaluate identity and phenotype: subset composition, memory state, activation, exhaustion, homing receptors, and transgene expression where relevant. Tier 3 should evaluate function: antigen-specific cytotoxicity, cytokine polyfunctionality, proliferation after re-challenge, serial killing, and resistance to suppressive conditions. Tier 4 should assess higher-order attributes such as repertoire, transcriptomic state, metabolism, and in vivo persistence in representative models. The depth required depends on development stage and the magnitude of the process change [35,38].

Acceptance criteria should be prospectively justified and tied to clinical knowledge where available. Statistical equivalence may be unrealistic for highly variable autologous products; a totality-of-evidence approach using predefined quality ranges, multivariate similarity, and demonstration of preserved mechanism of action may be more appropriate. Bridging lots should include difficult starting materials because average healthy-donor performance

can conceal clinically important differences in process robustness.

Regulatory and GMP Considerations

Regulatory guidance for cell and gene therapy emphasizes control of source materials, process design, in-process testing, potency, and comparability. Human- and animal-derived materials raise specific concerns regarding identity, consistency, and transmission of adventitious agents. These principles apply directly to donor-derived feeder cells and indirectly to immortalized or genetically engineered feeder-cell banks. Sponsors should define the feeder’s role, qualification strategy, testing program, traceability, and clearance or inactivation controls in the chemistry, manufacturing, and controls documentation [34-39].

Potency assurance is broader than a final release assay. Current FDA thinking describes potency assurance as an integrated strategy involving manufacturing process design, material control, process control, in-process testing, and lot-release assays. For feeder-supported ACT, this means that feeder qualification and functional consistency are part of potency assurance, not merely upstream raw-material management. A final cytotoxicity assay cannot fully compensate for an uncontrolled feeder system if the process can generate products with divergent persistence or differentiation states [34,35].

Cell-bank strategy is central for K562-derived and lymphoblastoid feeders. Master and working banks should be established under controlled conditions with identity, sterility, mycoplasma, adventitious-agent, and genetic-stability testing appropriate to the source and engineering history. Transgene expression should be trended over passages, and maximum population-doubling or passage limits should be justified. Changes in vector, clone, bank,

medium, cryopreservation, or irradiation process may require formal comparability assessment [36-39].

Residual-feeder specifications should be based on risk. For tumor-derived K562 feeders, a highly sensitive and specific residual assay is generally warranted. For allogeneic PBMC feeders, the risk profile differs but still includes residual non-product cells and immunologic effects. Manufacturing controls should also address segregation, irradiation equipment qualification, chain of identity, and prevention of cross-contamination between feeder and therapeutic-cell operations.

A Proposed Development Roadmap

First, define the intended biological state of the therapeutic product. The target should include not only cell number and viability but also subset composition, differentiation state, functional profile, persistence-related attributes, and clinically relevant potency. Without this definition, feeder selection becomes a trial-and-error exercise dominated by fold expansion.

Second, map the feeder's signal architecture. Document the relevant receptor-ligand interactions, membrane-bound cytokines, soluble factors, adhesion pathways, and temporal profile. This map should identify which signals are essential, which are redundant, and which may create undesired differentiation or safety risks.

Third, establish feeder CQAs and process parameters using designed experiments. Evaluate feeder ratio, ligand density, inactivation method, contact duration, restimulation frequency, cytokines, and vessel conditions in combination. Use both conventional assays and selected high-dimensional analyses to identify latent process effects.

Fourth, build an orthogonal potency strategy. A suitable matrix may combine an immediate mechanism-based assay, such as antigen-specific killing or cytokine release, with a proliferative or persistence-related assay and a phenotypic surrogate linked to clinical performance.

Fifth, evaluate feeder-free alternatives by signal equivalence rather than by nomenclature. The question is not whether a platform contains living cells, but whether it delivers the intended signals with improved control, scalability, safety, and product quality.

Finally, lock the process only after testing clinically representative starting material. Autologous ACT processes must tolerate lymphopenia, prior therapy, low T-cell fitness, tumor-derived suppressive cells, and variable starting composition. A feeder system that performs well only with healthy-donor cells is not necessarily robust for clinical manufacturing.

Future Directions

Future feeder platforms are likely to become increasingly programmable. Cell-based aAPCs may incorporate inducible ligand expression, suicide switches, standardized genomic landing pads, or tunable membrane cytokines. Non-cellular systems may use degradable particles, supported membranes, or biomaterials that deliver defined sequences of stimulation. The objective will shift from maximal expansion toward controlled generation of a desired cellular state.

Single-cell and multi-omic technologies will improve the resolution of feeder-product relationships. Integrating longitudinal process data with final-product phenotype and clinical outcomes may identify compact signatures that predict persistence or response.

Machine-learning models could then support feeder-lot release, process parameter selection, and early detection of manufacturing drift, provided that models remain interpretable and are trained on sufficiently diverse clinical material.

A major unresolved issue is whether feeder-induced states remain stable after infusion. Some *ex vivo* signatures may rapidly normalize *in vivo*, whereas others may reflect durable epigenetic programming. Paired analyses of manufacturing samples, infusion products, and post-infusion cells are needed to separate transient activation markers from clinically meaningful imprinting.

The field should also standardize reporting. Publications should describe feeder source, cell-bank status, genetic modifications, irradiation method and dose, feeder-to-effector ratio, antibody loading, cytokine schedule, vessel, restimulation timing, residual-feeder assay, and final-product phenotype. Incomplete reporting currently limits reproducibility and cross-platform comparison.

Conclusions

Feeder cells are not passive accessories in ACT manufacturing. They are dynamic, multicomponent signaling systems that can determine which immune-cell states are selected, induced, and preserved during expansion. Their influence extends from immediate yield to differentiation, metabolism, repertoire, potency, persistence, and safety. Accordingly, feeder identity, ligand architecture, inactivation, contact kinetics, and functional consistency should be incorporated into the process control strategy and linked to product CQAs. Feeder-free systems offer important advantages in standardization, biosafety, and operational simplicity, but they should be judged by their ability to reproduce the desired biological state rather than by their removal of living cells alone. A mechanistically anchored, risk-based approach to feeder selection and replacement can help ensure that manufacturing simplification does not trade away therapeutic fitness.

Declarations

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