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COUPLING INNATE AND ADAPTIVE IMMUNITY WITH YEAST-BASED CANCER IMMUNOTHERAPY

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9.1 INTRODUCTION

Many different strategies for the generation of immune responses using cancer vaccines or cancer immunotherapy are being employed for the treatment and/or prevention of human tumors. The two major elements of cancer immunotherapy are the selection of the antigens used to focus the specificity of the immune response and the approach or vehicle employed to stimulate the immune system against these antigens to eliminate target antigen-bearing tumor cells.

Recombinant nonpathogenic brewer's yeast, *Saccharomyces cerevisiae*, which we call Tarmogens™ (acronym for *tar*geted *mo*lecular immuno*gens*), are attractive vectors for cancer immunotherapy for the following reasons: (1) yeast trigger both innate and adaptive immune responses and therefore do not require additional adjuvants; (2) yeast deliver polypeptide antigens that are effectively processed into a full complement of appropriate-sized peptides competent for presentation by MHC

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class I and class II pathways, irrespective of HLA type; (3) yeast elicit potent T cell immune responses against tumor cells expressing target antigens; (4) yeast are not neutralized by the host immune system, even on repeated administration; (5) yeast-based immunotherapy is not a custom-manufactured, patient-specific vaccine, yet multiple tumor antigens may be targeted, so that treatment may be customized with a yeast "cocktail" dependent on a patient's tumor genotype profile; and (6) recombinant yeast are simple to manufacture.

This chapter discusses methods for engineering yeast to express tumor antigens and the unique properties of recombinant yeast in the activation of innate and adaptive immune responses. The broad applicability of the yeast-based immunotherapy to elicit protective T cell immune responses has been demonstrated in preclinical studies with numerous foreign, mutated, and overexpressed antigens. For instance, the therapeutic administration of yeast expressing mutated Ras protein triggered the complete ablation of carcinogen-induced mutant-Ras-bearing tumors in mice. The safety and immunogenicity of yeast Tarmogens in cancer patients are also discussed.

9.2 YEAST-BASED IMMUNOTHERAPY: MOLECULAR MECHANISMS OF ACTION

Yeast delivering tumor antigens activate innate immunity as well as the adaptive antigen-specific immune responses. The dual contributions to immune activation arise from the inherent "adjuvant-like" properties of yeast combined with direct delivery of the expressed tumor antigen for processing and presentation to the immune system. Yeast components activate the innate immune system by transmitting the "danger signals" of microbial infection through distinct surface receptors on APCs that recognize particle-associated molecular patterns (PAMPs) by pattern recognition receptors. These pattern recognition receptors include toll-like receptors (TLR1,2,4,6) and *phagocytic* receptors, such as dectin, mannose, and glucan receptors [1–3]. The inherent recognition of yeast component patterns as a "foreign" infection is not restricted to *S. cerevisiae*, but is shared with other fungi [1,4,5].

Saccharomyces cerevisiae yeast cells are avidly phagocytosed in vitro by murine bone-marrow-derived dendritic cells (DCs), human myeloid DCs and human plasmacytoid DCs, macrophages, and neutrophils, as well as human EBV-immortalized B cells [6,7] (See also Munson S, Parker J, Franzusoff A, unpublished observations). The uptake of multiple yeast per phagocytic cell has been observed within a few (0.5–6) hours after exposure, as determined by flow cytometry and fluorescence microscopy. The uptake of yeast by (DCs) triggers DC activation, maturation (including upregulation of cell surface receptors and secretion of various cytokines), and the presentation of peptides from yeast-expressed tumor antigens on MHC-I/II receptors, leading to an antigen-specific response by CD8 and CD4 T cells in the immunized hosts [8]. The elements of DC activation and antigen processing triggered by yeast exposure are elaborated in the following sections.

DC Activation and Maturation

The interaction of innate signaling receptors TLR1,2,4,6, plus phagocytic receptors on the surface of APCs, appears to be a combinatorial process that enhances and modulates the innate responses that would otherwise be achieved by binding individual innate receptors [9,10]. The consequences of TLR recognition of yeast molecular patterns by DCs results in the expression of genetic responses that are distinct from the profile elicited in response to bacterial components, viral components, or individual TLR-ligand agonists [11]. TLR2 interacts with zymosan (preparations of *S. cerevisiae* yeast cell walls) in conjunction with TLR1 and TLR6 [12,13]. TLR4 interacts with mannan on the surface of *Candida* yeast, and presumably on the surface mannan of *S. cerevisiae* [12,14]. Other receptors apparently involved in *S. cerevisiae* recognition and response are the β-glucan receptor (dectin-1 in the mouse), mannose receptor, complement receptor 3 (CR3), and the scavenger receptor CD36 [14–19]. The consequences of these multiple receptor interactions with yeast are the initiation of endocytic and phagocytic mechanisms in DCs, the activation of phagolysosomes that process the internalized yeast cells, degradation of the yeast in the endosomes and phagolysosomes, presentation of exogenous yeast-associated peptides into the MHC-II receptor pathway (within specialized endosomes), and activation of the proteasome pathway for cross-presentation of yeast-associated peptides into the MHC-I receptor pathway. As shown in Figure 9.1, the incubation of yeast also triggers upregulation of cell surface molecules, such as CD80 (B7.1) and CD86 (B7.2) and CD83 (Reference id or normal text. Kindly conform it.6,7) (see also Borges V, unpublished data). Stimulation of DCs by yeast also induces secretion of a proinflammatory panel of Th1-type cytokines, such as IL-6, IL-12, and TNFα [6,7,20,21].

Cross-Presentation of Exogenous Yeast-Associated Antigens into the MHC-I Pathway

The classical view of MHC-restricted antigen presentation is that peptides for stimulating CD8 T cell responses via the MHC-I pathway must be derived from polypeptides endogenously expressed by the APCs. Further, this model holds that exogenous antigens are delivered exclusively by endocytosis into the MHC-II presentation pathway. More recent work has provoked revision of the classical view of MHC-I antigen presentation, since it was discovered that certain types of exogenous delivery methods were effective at loading peptides into the MHC-I pathway, and thereby stimulating activation of CD8 T cell immune responses, in a process known as *cross-priming* or *cross-presentation* [20–26].

Yeast are capable of inducing cross-priming, as shown by a variety of in vitro and in vivo studies [6,27]. In vitro, recombinant yeast expressing a model antigen, namely, ovalbumin, when incubated with DCs, were able to activate OVA-specific MHC-I restricted T cell responses. Immature murine DCs were incubated with either soluble ovalbumin alone, yeast-lacking foreign antigens (YVEC),

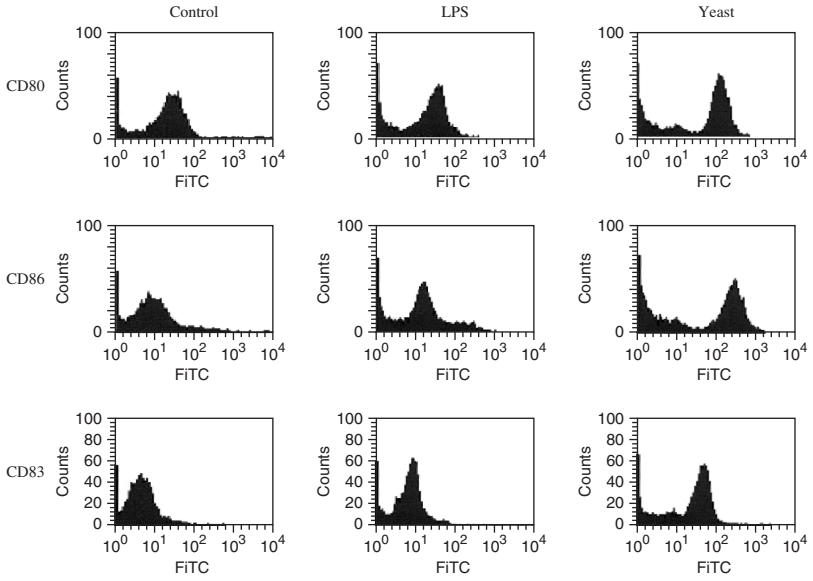


Figure 9.1. Human DC activation and maturation by bacterial LPS and yeast. Peripheral blood mononuclear cells from healthy human donors were incubated with GM-CSF and IL-4 for 5 days followed by 2 days of GM-CSF + IL-4 and incubated with saline (control), incubated with bacterial lipolysaccharide (LPS), or incubated with yeast. Flow cytometry of surface staining on DCs with CD80 (top row), CD86 (middle row), or CD83 (bottom row) antibodies is shown.

or yeast-expressing chicken ovalbumin (OVAX). The pulsed DCs were then incubated with naive OVA-specific $CD8^+$ T cells (obtained from OT-1 T cell transgenic mice). As expected, DCs pulsed with soluble ovalbumin or YVEC were unable to activate ovalbumin peptide-specific $CD8^+$ T cells. In contrast, DCs incubated with OVAX activated the ovalbumin-specific $CD8^+$ T cells and the amount of proliferation was improved in a yeast dose-dependent manner by increasing the yeast to DC ratio during pulsing [6]. Surprisingly, DCs pulsed with soluble ovalbumin mixed with YVEC also stimulated $CD8^+$ T cell proliferation [6]. However, 40,000-fold more protein is required when provided as soluble protein plus empty yeast (YVEC), compared to the amount of ovalbumin that efficiently triggers antigen-specific $CD8^+$ T cell responses when delivered inside the yeast. This result indicates that innate receptor agonists such as yeast are several orders of magnitude more effective at eliciting adaptive immune responses when the innate receptor agonists are physically associated or linked with the tumor antigens.

Tumor challenge models in immune-competent or CD8-knockout mice, or inactivating CD8 T cells with specific anti-CD8 antibodies are in vivo examples demonstrating that immunization with recombinant yeast primes tumor-targeted CD8 T cell responses [6] (Duke R, unpublished observations). Immune-competent mice immunized with OVAX were protected against challenge

with ovalbumin-expressing EL-4 tumor cells, but not to challenge with EL-4 cells that did not express ovalbumin (6) (see also Duke R, unpublished observations). The protection mediated by immunization with OVAX yeast was abolished in mice deficient in $CD8^+$ T cells (6). Thus, while not all immune responses that protect animals from tumor challenge or spontaneous carcinogen-induced tumors are CD8 T cell-restricted, this study and others have demonstrated that immunization with yeast are potent inducers of CD8 T cell responses. Furthermore, the incubation of immature DCs with yeast expressing ovalbumin, but not YVEC yeast or mock treatment, was sufficient for activating tumor protective immune responses when the OVAX-pulsed DCs were introduced back into mice challenged with ovalbumin-expressing tumors [6]. These results suggest that the mechanism of yeast-based immunotherapy requires interaction with and activation of antigen-presenting cells in immunized hosts.

9.3 MUTATED Ras AND THERAPEUTIC ANIMAL TUMOR MODELS FOR YEAST-BASED IMMUNOTHERAPY

The selection of tumor antigens for targeted immunotherapy is typically based on (1) mutated proteins that arise during tumorigenesis [e.g., Ras, adenomatous polyposis coli (APC)], (2) tumor-specific protein overexpression [e.g., epidermal growth factor receptor (EGFR)], (3) differentiation, neo- and self-antigens whose expression is activated or restricted to tumor cells [e.g., melanoma antigens, mesothelin, carcinoembryonic antigen (CEA)], or (4) proteins expressed in the stroma that support tumor survival and proliferation (e.g., PSMA). However, given the multiple mechanisms by which tumor cells alter protein expression or trigger escape mutations under selective drug or immune pressure, the optimal antigens for initial therapeutic targets should be those that are critical for tumorigenesis or metastasis. Some of these targets have been identified as "antigens of addiction" because of their essential role in the cancer phenotype.

Mutated Ras protein was selected as an ideal target antigen for yeast-based immunotherapy because of its essential role as an engine driving tumorigenesis in multiple types of human cancer. The three Ras protein members, K-, H- and N-Ras, are GTPase switch proteins important for cell proliferation acting downstream of receptors that respond to external stimuli for cell division, such as EGFR. Single amino acid mutations in at least two domains in Ras (amino acids 12 or 13, and residues 59 or 61) are commonly associated with cancer as these mutations cause constitutive, signal-independent cell growth and tumorigenesis [28]. Mutations in K-Ras are present in 90% of human pancreatic adenocarcinomas, 35–40% of colorectal tumors, and 30–35% of non-small-cell lung cancer adenocarcinomas [29,30].

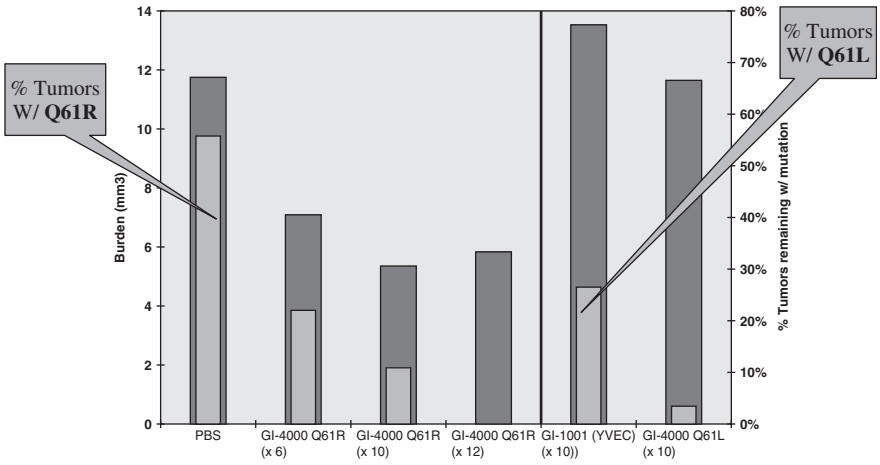
The mutated Ras proteins commonly found in cancer were expressed as tumor antigens in yeast (the GI4000 series of yeast strains). A carcinogen-induced lung tumor model was used to test the effect of the GI4000 yeast (31). In this system, one intraperitoneal injection of urethane in A/J mice spontaneously triggers 25–50

individual lung tumors per mouse, each driven by single amino acid mutations in the Ras oncoprotein. Most of the individual tumors are driven by K-Ras mutated at codon 61 (i.e., Q61R or Q61L). The genotype of some urethane-induced tumors harbor mutations at K-Ras codon 12 (i.e., G12V or G12D), while a minority of the 25–50 tumors do not appear to express mutated Ras proteins [32,33] (see also Lu Y, Franzusoff A, unpublished observations). Mice display pulmonary alveolar hyperplasia by 2 weeks posturethane exposure, adenomas by 5 weeks, and adenocarcinomas by 14 weeks postinjection. The animals expire within 12 months from tumor-associated respiratory distress.

Yeast strains were engineered to express the mouse K-Ras protein with the Q61R mutation, [strain GI4000 (Q61R)] or the K-Ras protein with the Q61L mutation [strain GI4000 (Q61L)]. After culturing the yeast and inducing expression of the mutated K-Ras proteins, the yeast were harvested, heat-killed, washed, and resuspended as intact cells in PBS. Dosing of animals is based on the number of yeast
Q3 administered (1 YU 10^7 yeast cells) with a known quantity of mutated Ras protein expressed per YU yeast at the time of administration.

The therapeutic efficacy of yeast-based immunotherapy in the urethane-induced lung tumor model was examined by initiating dosing 2–5 weeks post urethane exposure, a time at which 25–50 tumors per mouse are already present as hyperplasias or adenomas. Mice were administered 5 YU yeast per injection site (5×10^7 yeast). Animals received 6 doses every other week, 10 doses each week or 6 injections at each of two sites every other week (i.e., 12 doses) of buffered saline (PBS), control yeast that harbored no heterologous protein (YVEC/GI1001) or yeast harboring the mutated Ras proteins [e.g., GI4000 (Q61R) yeast or GI4000 (Q61L) yeast]. At 14 weeks posturethane exposure, where untreated tumors would be macroscopically visible, the tumors were excised, counted, and measured with a caliper to calculate the volume of each individual tumor, and the average tumor burden per mouse was determined. Compared with the saline-treated group, the tumor burden in mice that received 6 doses of yeast vaccine GI4000(Q61R) led to an average reduction in total tumor volume per mouse of 39%, mice that received 10 doses showed an average reduction in tumor burden of 55%, and mice that received 12 doses exhibited a 52% reduction in tumor burden (Fig. 9.1) [33].

Q4 To assess the impact of targeted yeast-based immunotherapy, the *K-ras* sequences in the residual tumors were genotyped. The tumor-sequencing results revealed
Q5 that while 56% tumors from saline or mock-yeast-treated mice harbored K-Ras Q61R mutations, the administration of increased numbers of doses of GI4000 (Q61R) yeast led to the reduction in number of Q61R-Ras-bearing tumors to the point where 12 doses resulted in complete ablation of all tumors driven by Q61R-mutated K-Ras (Fig. 9.1). Conversely, when mice were administered GI4000 (Q61L) yeast, the tumors bearing Q61L-mutated K-Ras were targeted for destruction by the yeast-mediated immune response [33]. Interestingly, when the two different yeast were injected in urethane-treated mice at different sites, or mixed in the syringe for injection at a single site, improved protection compared to individual yeast administration was observed, and some animals had eliminated all of the tumors, supporting the premise of combinatorial treatment by cocktails of



Q6 Figure 9.2. Mutation-specific tumor ablation observed when yeast vaccine administered contains the Q61R or Q61L mutation. A/J mice received a single injection of urethane, then 2 weeks later received either (1) 6 doses every 2 weeks of GI4000(Q61R) or GI4000(Q61L), (2) 10 doses of GI4000(Q61R) weekly, (3) 12 doses of GI4000(Q61R) every 2 weeks, (4) mock treatment of saline, or (5) 6 doses of GI1001(YVEC, empty yeast vector) every 2 weeks. Then 14 weeks posturethane exposure, tumors were excised, counted, and measured, and the total tumor burden was calculated. In total, 10–30 single tumors/group were used to prepare DNA and the ras sequence determined for each. Blue bars designate the total tumor burden (left axis), whereas red and orange bars designate the percentage of tumors sequenced that bear Q61R or Q61L mutations (right axis), respectively.

yeast targeting different antigens (Lu Y, Fiolkoski V, Bellgrau D, Franzusoff A, unpublished observations).

Taken together, these results showed that therapeutic immunization with yeast bearing mutated Ras proteins was able to activate the immune system to eliminate all tumor cells driven by a single amino acid Ras mutation with yeast-based targeted immunotherapy. Tumor-targeted immune responses were enhanced with repeated yeast immunizations, even when administered weekly. Furthermore, although humans typically harbor cancers arising from a single tumor source, these results underscore the importance of genotyping human tumors for the specific Ras mutation driving the cancer, so that the appropriate mutated Ras-bearing yeast will be administered.

9.4 EXPANDING THE CATALOG OF YEAST-EXPRESSED TUMOR ANTIGENS

Besides the GI4000 yeast targeting individual Ras mutations, several GI4000 yeast were engineered to express multiple Ras mutations as fusion proteins. All three

different yeast strains currently being tested in the clinic harbor one of the three most common amino acid mutations at position 12 (G12V, G12 C or G12D), in addition to both Q61R and Q61L mutations described above. Other yeast strains have been engineered to express all of the most common Ras mutations found in human cancer. These strains, namely, RastaFAR GI4000 yeast, express fusion proteins with multiple cassettes of Ras polypeptide harboring different Ras mutations. Each of these polypeptide cassettes is sufficiently long so that epitopes for MHC-I and MHC-II presentation may be randomly generated in all different frames and lengths by antigen-presenting cells to support the diversity of HLA binding specificities found in humans.

Table 9.1 lists additional yeast strains that have been engineered to express different tumor antigens. GI3000 yeast express EGFR as an intracellular protein to target tumors overexpressing EGFR. The administration of GI3000 yeast expressing EGFR has resulted in the breaking of immune tolerance to gliomas overexpressing EGFR (pareas Lu Y, et al, manuscript in preparation). The MUC1 and CEA proteins expressed in GI6000 yeast have been characterized as overexpressed differentiation or self-antigens in several ductal cancers, including breast, pancreatic, and colorectal carcinomas. The apparent role of these tumor proteins in metastasis combined with the availability of well-defined transgenic systems featuring them makes these antigens especially attractive targets for yeast-based immunotherapy. Because of the apparent role of these tumor-associated proteins in metastasis, and because of the availability of well-defined tumor models, including transgenic animal tumor models, these proteins are attractive target tumor antigens for yeast-based immunotherapy (Borges V, Bellgrau D, Lu Y, Franzusoff A, unpublished observations and Hodge JW, personal communication). The melanoma-associated MART1 protein expressed in GI7000 yeast has elicited antigen-specific immune responses

Q7

TABLE 9.1. Tumor Antigens Expressed in Yeast for Cancer Immunotherapy

Yeast Product Name	Tumor Antigen	Immunogenicity	Tested in Tumor Protection Model	Source[a]
GI-3000	EGFR (human or rat)	Yes	Yes	1
GI-4000	Common mutations in human, mouse or rat K-, H-, or N-Ras	Yes	Yes	Refs. 34,35
GI-6000	Muc1 (human)	No	Yes	2
	CEA	Yes	NT[b]	3
GI-7000	Mart-1 (human)	Yes	Yes	4

[a] *Sources:* (1) Lu Y et al, manuscript in preparation; (2) Borges V, Bellgrau D, Lu Y, Franzusoff A, unpublished observations; (3) Hodge JW, personal communication; (4) Lu Y, Franzusoff A, unpublished observations.

[b] Not tested to date.

and tumor protection in transplant and transgenic animal tumor models (Fujita et al., manuscript submitted). Interestingly, since MART1 is not fundamental to the malignant phenotype (unlike EGFR or mutated Ras), eventually cells from treated animals shed the MART1 antigen under immune pressure (Fujita et al., manuscript submitted), as would be predicted from the discussion above. The breadth of results demonstrating the immunogenicity of multiple antigens and tumor protection against these different targets illustrates the potential of yeast as a vector for therapeutic cancer vaccines. All of the tumor antigens expressed in yeast tested to date have been immunogenic and have promoted antitumor activity, even in transgenic or homologous model systems.

Yeast-based immunotherapy has also been used to target immune responses against foreign antigens for infectious disease targets such as HIV, HCV, influenza, and fungi. A yeast vaccine expressing an HCV NS3 and core fusion protein is currently being tested in a phase I clinical trial. Further discussion of yeast-based immunotherapy for infectious disease indications is, however, beyond the scope of the current review.

9.5 YEAST-BASED VACCINE VECTOR DESIGN AND STRATEGIES

Results with the urethane-induced tumor model showed that increasing the number of doses of yeast-based immunotherapy improves efficacy in animal tumor models. Studies performed with a variety of target antigens have revealed that increasing the amount of antigen expressed per yeast cell is also important for enhancing antigen-specific immune responses. Thus, the number of yeast administered and the amount of antigen per yeast are two critical elements of yeast-mediated immune responses. In this section, the methods employed to increase the amount of heterologous antigen expressed while culturing yeast will be described. There are many parameters within vector design that likely impact heterologous protein expression. For this discussion, the focus will be on two key elements of yeast expression vector engineering: plasmid copy number and the yeast promoter used to express the heterologous protein.

Controlling Plasmid Copy Number

Gene expression cassettes may be integrated into the yeast genome as single or several units, or may be maintained as extrachromosomal, or episomal, plasmid elements at low, intermediate, or high copy number. Episomal plasmids are inherently unstable because of the extra metabolic cost required by the cell to replicate the episomal plasmid for cell division. Thus, yeast that harbor mutations in one or more biosynthetic gene products are considered auxotrophic for a particular biochemical pathway. The standard auxotrophies used for yeast engineering include mutations in the synthesis of individual amino acids, such as histidine (*his3* mutations) or leucine (*leu2* mutations), or pathways for nucleic acid precursors

such as uridine (*ura3* mutations) or adenine (*ade2* mutations). A parent yeast that harbors these mutations can be "rescued" by addition of the end product to the media, or by introduction of the wild-type gene for that pathway on an episomal plasmid element to convert the cell from auxotrophy to prototrophy. Therefore, a plasmid engineered to express a heterologous protein also encodes the protrophic gene so that the plasmid will be retained by the cell under selective growth conditions. When the selection is no longer applied, then the yeast will no longer need to replicate the plasmid for survival and the episomal plasmid will be diluted by cell division until new daughter cells will be produced that no longer possess any episomal plasmids.

The advantage of integrating the heterologous expression cassette into the yeast genome is the inherent stability of replicating chromosomes with each cell division. This means that yeast can be cultured in rich, nonselective media that allow for shorter generation times and the density of yeast number per liter of culture is typically an order of magnitude higher than that of yeast grown in minimal selective media. However, the number of copies of the desired gene product is then limited to the number of copies integrated into the yeast genome.

In contrast, episomal plasmids may be engineered to encode replication regulatory sequences that dictate how many plasmid copies will be maintained in yeast cells with each cell division. One category of vectors encodes an yeast origin of DNA replication, the autonomously replicating sequence (ARS) plus a centromeric DNA element (CEN) that binds the yeast mitotic spindle, enabling accurate plasmid segregation. This class of plasmids is commonly referred to as "low copy" because accumulation is limited to approximately two to three copies per cell. The other major regulatory element, known as the *origin of replication*, is derived from a naturally occurring circular DNA found in yeast strains, and is also called the *2-μm circle*. This 2-μm origin or replication drives plasmid high-copy accumulation to ~40–60 molecules per cell. These vectors maintain chromosome-like stability by signaling the recruitment of specialized plasmid-partitioning machinery [35,36]. Thus, the highest possible heterologous protein expression is typically achieved by employing 2-μm-based vectors for expression cassette gene copy numbers higher than those that could be obtained with either chromosomal integration or expression from the low-copy *ARS-CEN*-based vectors.

One additional strategy for amplifying plasmid copy number is that a particular mutation in the *LEU2* gene product, known as the *leu2d* mutation, may also be recruited as a selectable marker for replicating plasmids [37]. The defective leu2d protein is unable to support leucine biosynthesis unless the protein is present in high numbers in the cell (i.e., mass action). Thus, yeast prototrophy achieved with the *leu2d* gene encoded on the vector results in very high copy numbers—50–200 copies per cell—when cells are grown in absence of added leucine to the culture media. In practice, though, selection using *leu2d* alone is more complex for initial clone isolation, presumably because of the requirement to achieve immediate high copy number of the episomal plasmids to support auxotrophic growth in the absence of added leucine. Hence, most vectors that utilize *leu2d* for selection also include another prototrophic selection, such as *URA3*, so that the clones harboring

the desired expression vector may be selected by growth in the absence of added uridine and once established, the copy number may be enhanced by eliminating added leucine to the culture media. Our studies have indicated that target genes expressed from *URA*3 + *leu*2*d* based vectors generate two- to fivefold more heterologous antigen in growth medium lacking both leucine and uracil than in medium lacking uracil alone (King T, Lu Y, Guo Z, Kelley V, Franzusoff A, unpublished observations).

Promoter Choice: Inducible, Constitutive, or Repressible

The second key element for engineering high-level heterologous protein expression is the choice of promoter for regulating transcription of the heterologous gene (factors that regulate the efficiency of translation initiation or transcription termination are not reviewed here). The type of promoter employed will be influenced by several interrelated factors, such as the need to control the timing of antigen expression, the impact of foreign protein expression on yeast cell health, and whether multiple antigens are produced from one or multiple plasmids within the yeast. The ability to control the rate of mRNA (and protein) accumulation is useful in cases where the antigen is toxic to the yeast cell and/or prone to aggregation. For such proteins, the investigator may need to have control over the rate and timing of mRNA synthesis. This may be achieved through the use of so-called rheostatic promoters—those that can be regulated by the addition of chemical compounds to the cell growth medium. By lowering the transcription rate (e.g., by adding a repressor compound) or initiating transcription late in the yeast culturing process, a heterologous protein that interferes with normal yeast growth may be better produced to higher levels, in addition to yielding higher-density cell harvest. For aggregation-prone proteins, slow or delayed synthesis is sometimes the key to avoiding these unproductive complexes because the protein molecules of interest are allowed to completely fold before encountering other partially folded proteins. From the process development standpoint, some of these controls are achieved by growing yeast at lower- or higher-than-optimal temperatures. Wild-type yeast strains grow most rapidly at 30°C, but can be cultured at 4–37°C. A second advantage of rheostatic promoters is the ability to evaluate the consequences of antigen content independent of the number of yeast administered. Some useful rheostatic promoters for use in *S. cerevisiae* include those that can be induced (e.g., *CUP1*), repressed (*MET25*) or tuned in either direction (e.g., *GAL1–10*, tetracycline-inducible or -repressible variants) [38–42].

The use of constitutive promoters may circumvent limitations of rheostatic promoters and can be useful for simpler proteins that are not prone to aggregation or that cause toxicity to the yeast. The compounds used to regulate rheostatic promoters are in some cases toxic and need to be removed from bulk yeast drug substance, which may complicate manufacturing and testing for clinical applications. These substances must be added at a defined point in the cell growth cycle, which imposes the need to closely monitor cell growth rate during the production run. The use of a constitutive promoter can mitigate or even bypass these problems because

no promoter-activating compounds need to be added. Furthermore, transcription rates from constitutive promoters are frequently higher than those for rheostatic promoters, resulting in higher total levels of heterologous protein. Examples of strong constitutive promoters in *S. cerevisiae* include *ADH1* (alcohol dehydrogenase), *ENO2* (enolase), *TEF1*, *TEF2* (translation elongation factors 1 and 2), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). The endogenous products of these promoters are among the most abundant in the yeast cell under routine culture conditions (e.g., glucose plus nitrogen source). Promoters normally used to make components of the protein synthesis machinery are excellent choices for the present application because they generally drive transcription of essential, single-copy genes that must be activated to achieve protein synthesis. Thus, heterologous protein expression driven by a constitutive promoter that is normally used in ribosomal biogenesis will correlate directly with bulk cellular protein synthesis.

Finally, promoter choice may be influenced by the need to create multiantigen products. Yeast have been engineered for immunotherapy to express two different antigens at the same or different levels, raising the question as to whether both genes should be expressed from the same or different promoters. The *CUP1* promoter recruits a transcription factor (Ace1p) whose amount is limiting for maximal transcription of one mutated Ras protein, like that engineered for the GI4000 strains. Overexpression of the *ACE1* gene on a 2-μm-based plasmid was observed to result in doubling the quantity of *CUP*-driven expression of mutated Ras protein produced on copper addition (King T, Lu Y, Franzusoff A, unpublished observations). This example illustrates that a promoter can have limited capacity to drive transcription because of a limited supply of a promoter-specific transcription factor (or Factors). If two different antigens should be highly expressed, the best expression strategy may therefore be to utilize two strong promoters, such as *TEF2* and *GAPDH*, that use independent regulatory factors.

This discussion illustrates that there are numerous ways to express tumor antigens in yeast. It is also important to point out that antigens may be localized to different compartments in yeast and still be immunogenic, specifically, as cytosolic proteins or membrane proteins, and even secreted into the periplasm, as long as the protein remains behind the cell wall or is somehow retained by yeast after administration [6]. Success can be achieved with various strategies, and the optimal system will be empirical, depending on the number of tumor antigens being expressed and how their biochemical properties affect protein accumulation and yeast cell metabolism. In the end, the optimal yeast expression system will result in high-level heterologous protein levels with a simple manufacturing protocol that does not involve many postculturing steps to reduce the potential for toxic byproducts of manufacturing.

Parameters of Dose Responses with Yeast-Based Immunotherapy

Four parameters of dosing with recombinant yeast have been investigated: (1) the amount of antigen expressed per yeast cell, (2) the number of yeast administered per dose, (3) the number of doses administered over time, and (4) the

route of administration. The importance of these four parameters to efficacy of yeast-based immunotherapy have been elucidated with in vitro and in vivo studies of T-cell-dependent immune responses and tumor protection, respectively. These studies were performed with yeast expressing several different tumor or infectious disease antigens, so the identity of the antigen does not seem to influence the reproducibility of observations related to the parameters of dosing efficacy.

Increasing the number of yeast and the amount of antigen expressed per yeast appears to improve the relative abundance of CTLs in immunized animals, particularly for foreign antigens and mutated self-antigens. As described in Section 9.4, the amount of antigen per yeast may be modulated by using different promoters and plasmids to express the same antigen at different levels in different yeast strains, or to modulate the activity of an inducible promoter by adjusting the amount of inducer (e.g., copper) added to the cultures for activating antigen expression in yeast. Thus, for foreign antigens (HIV-Gag, HCV NS3 and core proteins, influenza HA and M1 antigens) and mutated self-antigens (mutated Ras), a "more is better" paradigm appears to be operative, namely, more yeast administered plus more antigen expressed per yeast. Immune efficacy with recombinant yeast expressing lower amounts of antigen may still be achieved by increasing the number of yeast administered per dose. Similarly, fewer yeast are needed to exceed the threshold of immune efficacy if the heterologous antigen is expressed at very high levels in yeast, specifically, when the antigen is expressed as high as 5–10% of total yeast cell protein [21] (see also Franzusoff A, Duke R, unpublished observations). Surprisingly, for targeting self-antigens such as wild-type EGFR and CEA, the results from preliminary studies suggest that the administration of fewer yeast, albeit with high levels of heterologous antigen expressed per yeast, may be more efficacious than dosing with higher numbers of yeast (Hodge JW, personal communication; Lu Y, Franzusoff A, manuscript in preparation). The reasons for this alternate paradigm are currently being investigated.

Activating T cell responses by immunization via multiple different routes of administration has been studied with in vitro and in vivo models and with different yeast-expressed antigens. Immune responses have been assessed by multiple parameters, namely, by lymphocyte proliferation, CTL, ELISpot (enzyme-linked immunospot assay) and intracellular cytokine secretion assays, to compare the impact of administering yeast by subcutaneous, intranasal (IN), oral, intraperitoneal (IP), and intravenous (IV) routes. Each of these routes of administration were effective for activating antigen-specific immune responses with recombinant yeast. Interestingly, more profound differences were observed when animals were challenged with tumors implanted in different locations. Subcutaneous and intranasal administration of yeast-based immunotherapy were equally effective against challenge with tumors implanted subcutaneously. However, when tumors were implanted intracranially, administering recombinant yeast by the intranasal and oral routes achieved significantly more protection against tumor challenge than did subcutaneous dosing (Lu Y et al, manuscript in preparation).

Thus, the choice of administration route may be important for specific disease indications, since T cells that become activated by one route of administration are

likely to recirculate in the related lymphoid tissues for surveying the appearance of target antigens presented by diseased cells. For instance, the intranasal and oral routes are effective for activating mucosal-tissue-associated lymphoid surveillance. Hence, some diseases that are associated with mucosal tissues, such as lungs, intestines as well as head and neck cancers, may receive more attention of T cells activated by the mucosal route. The relative benefit of route of administration for specific indications is admittedly speculative.

In summary, yeast-based immunotherapy surprisingly exhibits dose–response parameters commonly associated with dosing small-molecule drugs. In this regard, the amount of antigen in the yeast, the number of yeast given, the number and schedule of immunizations, and the route of administration are all factors contributing to the extent of the immune response and its effectiveness in a given model system. These factors may contribute different levels of importance depending on the antigen and model used, and thus represent an empirical guide for testing. These preclinical findings continue to be evaluated with more antigens engineered into the yeast and with cancer models of spontaneously induced tumors or infectious diseases that involve different organs. The more conclusive tests of these hypotheses will require randomized controlled trials with patients harboring these different diseases.

9.6 PRECLINICAL SAFETY STUDIES AND CLINICAL TRIALS IN CANCER WITH YEAST-BASED IMMUNOTHERAPY

Preclinical Safety Studies with Yeast-Based Immunotherapeutics

Yeast-based vaccines expressing three different antigens have been tested in five separate toxicity studies in rabbits conducted under good laboratory practices (GLP). Rabbits were injected subcutaneously with 0.5–100 YU per injection site, and up to 13 weekly injections before histopathological analysis. Aside from occasional minor injection site reactions that resolved after 2 weeks' recovery, none of the rabbits showed signs of abnormality, aside from increased levels of circulating neutrophils. The equivalence of the safety profile from the five GLP toxicity studies implies that the yeast vector, not the expressed antigen, has the greatest influence on the reactions to immunization. Indeed, the investigational new drug (IND) application for the three GI4000 series of products that are being tested in the clinic in patients with cancers harboring mutated Ras was filed with a single chemistry manufacturing and controls (CMC) section, by agreement with the FDA. However, because of the mechanism of action elucidated by the study with urethane-induced tumors in mice (described in Section 9.2), only one of the three GI4000 yeast is administered to cancer patients. The selection of which GI4000 yeast to administer is governed by the identification of the Ras mutation genotype in the patient's tumor to match the yeast expressing the relevant mutated Ras polypeptide.

GI4000-01 Phase I Safety and Immunogenicity in Metastatic Cancer

In a phase I clinical trial of GI4000 series yeast, 33 subjects with advanced pancreatic or colorectal cancer were enrolled. Greater than 90% of the patients enrolled in the trial had metastatic disease at the time of enrollment, and subjects had received an average of three previous therapy regimens prior to participation in the GI4000-01 trial.

The design for this "first time in human" study was a dose escalation trial, in which cohorts received either 0.1, 1, 5, 10, 20, or 40 YU of the mutation-matched GI4000 yeast administered subcutaneously for 5 weekly doses. Subjects were monitored for safety, local injection site reactions, complement cascade activation postinjection, and antigen-specific immunologic responses. No dose-limiting local or systemic toxicities were observed at any of the doses tested. The majority of subjects exhibited antigen-specific responses as demonstrated by lymphocyte proliferation and/or intracellular cytokine staining assays. Several subjects continue to be followed for survival. A manuscript describing the results of this study is in preparation.

GI4000-02 Phase II Testing in Resected Pancreas Cancer

GI4000-02 is a multicenter, double-blind, placebo-controlled, adjuvant trial comparing GI4000 in combination with a gemcitabine regimen versus a gemcitabine regimen alone in patients with successfully resected *ras*-mutation-positive pancreas cancer. Another enrollment criterion is that the surgery have a tumor resection status of R0 or R1, indicating that postresection, the pathology shows a resection margin that is free of even microscopic disease (R0), or that there is evidence of microscopic disease at the resection margin, but no macroscopic disease (R1).

Subjects will receive three doses of GI4000 yeast after resection, but prior to initiation of gemcitabine. Monthly doses of GI4000 or saline placebo are administered after the start of gemcitabine during the drug holiday between gemcitabine cycles. The primary endpoint of the trial is recurrence-free survival at 15 months after randomization.

9.7 CONCLUSIONS

This chapter summarizes key aspects of using the recombinant yeast platform technology for cancer immunotherapy. The danger signals from recombinant yeast immunization trigger a multifactorial innate immune response. This innate immune response profoundly impacts and activates the antigen-presenting cells, which promotes antigen-specific T cell responses. The yeast-based immunotherapy platform has been used for immune responses against a variety of foreign, mutated, or self-antigens.

The expression of polypeptide tumor antigens in yeast promotes the processing of a comprehensive assortment of peptides in these yeast-activated APCs.

The delivery of this broad catalog of peptides into antigen presentation pathways increases the probability that MHC receptors from diverse HLA genotypes will be able to bind peptides derived from yeast-expressed antigens. As a result, even tumors bearing single amino acid mutations in Ras, or overexpressing self-antigens, are capable of being targeted for destruction by yeast-mediated immune responses in a broad range of immunized hosts. The specific targeting and ablation of tumor cells that is highly desirable for the immune protection against cancer may be achieved without off-target side effects.

Through the many studies with yeast-based immunotherapy in animals and the phase I clinical trial in cancer and the phase Ib trial in chronic hepatitis C infection, numerous significant (including some surprising) observations about the use of recombinant yeast for activating innate and antigen-specific immune responses have emerged:

Antigen delivery and yeast-mediated immune responses

- Dosing with recombinant yeast drives a Th1-type cytokine response profile, including TNFα, GM-CSF, and IFNγ.
- Recruitment and activation of APCs does not require the addition of artificial adjuvants.
- Yeast-expressed antigens are delivered into the MHC-I pathway by nonclassical "cross-presentation", in addition to classical MHC-II presentation of yeast-delivered antigens.
- Orders-of-magnitude improvement in antigen-specific $CD8^+$ T cell responses when target antigen is expressed in yeast compared to mixing soluble protein with empty yeast.

Vaccine efficacy

- Recombinant yeast are immunogenic by multiple routes of administration.
- Yeast-based immunotherapy is able to break immune tolerance to self-antigens.
- The potential for administering "cocktails" of yeast expressing different antigens may be adopted for customizing the immunotherapy according to the target tumor genotype.
- The yeast may be immunized repeatedly without eliciting neutralizing antibodies that interfere with improvements in antigen-specific immune response.
- The clean safety profile observed with dosing recombinant yeast in animals and humans.

Vaccine manufacturing

- Heat-killing yeast does not destroy the immunogenicity of yeast-expressed antigens (although this must be confirmed for each new antigen tested).
- The simplicity and scaleability of the manufacturing process for yeast-based vaccines

In closing, immunotherapy represents an attractive strategy for the treatment of cancer. Nevertheless, the challenge of dealing with high-burden tumors in late-stage cancer patients should not be underestimated. The phase II trial design that we have adopted for testing yeast-based immunotherapy in pancreas cancer specifically avoids the complications of high-burden cancer (see Section 9.6, paragraph on GI4000-02 phase II testing in resected pancreas cancer). The range of potential obstacles that may emerge with treating high-burden tumors include immunosuppressive factors such as TGFβ, amplification of regulatory T cells, as well as the potential for reduced numbers of nascent immune cells to promote antigen-specific immune responses in patients with advanced cancer. Fortunately, cancer immunotherapy may be combined with other strategies that target these hurdles contributed by high tumor burden, such as targeted therapy with monoclonal antibodies or small-molecule drugs and chemotherapeutic regimens. We are currently exploring the impact of combining yeast-based immunotherapy with approaches that are predicted to reduce or overcome these hurdles.

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