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Increasing the efficacy of tumor cell vaccines by enhancing cross priming

Brian M. Andersen[a] and **John R. Ohlfest**[a,b,*]

[a]Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, 55405

[b]Department of Neurosurgery, University of Minnesota, Minneapolis, Minnesota, 55405

Abstract

Cancer immunotherapy has been attempted for more than a century, and investment has intensified in the last 20 years. The complexity of the immune system is exemplified by the myriad of immunotherapeutic approaches under investigation. While anti-tumor immunity has been achieved experimentally with multiple effector cells and molecules, particular promise is shown for harnessing the CD8 T cell response. Tumor cell-based vaccines have been employed in hundreds of clinical trials to date and offer several advantages over subunit and peptide vaccines. However, tumor cell-based vaccines, often aimed at cross priming tumor-reactive CD8 T cells, have shown modest success in clinical trials. Here we review the mechanisms of cross priming and discuss strategies to increase the efficacy of tumor cell-based vaccines. A synthesis of recent findings on tissue culture conditions, cell death, and dendritic cell activation reveals promising new avenues for clinical investigation.

1. Introduction

The less effective aseptic surgical techniques of 18th century medicine led to post-operative tumor resection infections, ultimately revealing that the immune system could eliminate tumors. William Coley, a surgeon in New York, noted many records of patients with post-operative streptococcal infections having spontaneous regression of their tumors. Attempting to recapitulate this effect, he injected live *Streptococcus* and *Serratia* bacteria into his patients' tumors. Regressions occurred in greater than 10% of cases [1, 2].

120 years later, basic knowledge of tumors, immunology, and vaccinology has enabled a number of immunotherapy approaches. Tumors express self- and neo-antigens from their aberrant genetic programs, distinguishing themselves immunologically from normal tissue. As tumors enlarge, they evade and suppress the immune system, overcoming spontaneous immune responses [reviewed in 3]. Vaccines aim to activate adaptive immune responses by providing antigens in conjunction with an immune stimulus. Although subunit and peptide vaccines have several advantages, a recent meta-analysis accounting for 3,444 patients in 173 clinical trials indicated that tumor cell-based vaccines had higher response rates (~8%)

*Corresponding Author: John R. Ohlfest, Department of Pediatrics, University of Minnesota, 420 Delaware St. SE, MMC 366, Minneapolis, MN 55455. Phone: 612-626-2491; Fax: 612-626-2490; ohlfe001@umn.edu.

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than vaccines consisting of synthesized antigens (~4%) [4]. Tumor cells provide a diverse pool of antigens that theoretically trigger concurrent CD4 and CD8 T cells responses along with tumor-reactive antibodies. A central theme of this review will be to appreciate why response rates have been so low, and provide some insight as to how the efficacy of tumor cell-based vaccines can be increased.

Thousands of cancer patients have been treated with tumor cell-based vaccines with evidence of efficacy. Melacine® is a melanoma vaccine consisting of lysates from two allogeneic melanoma lines combined with the adjuvant DETOX® Melacine® was given to 198 patients in phase II/III trials, resulting in five complete responses and seven partial responses (overall response rate 6.0%) [5]. While the overall response rate was similar to (if not lower than) that of Coley, four patients with complete responses were still alive and disease free at 7-10 years post-treatment, implying long-term disease control through memory responses. Retrospective analysis showed that the response rate was 38% in patients expressing HLA-A2 and HLA-C3 [5]. With a better safety profile and comparable complete response rate to chemotherapy or high dose IL-2, Melacine® was approved for treatment of metastatic melanoma in Canada. A tumor cell lysate vaccination known as Reniale® also improved renal cell carcinoma outcomes in a recent phase III study [6]. Nephrectomy was performed in patients with organ-confined disease, and lysate vaccines were produced from autologous tumor cells stimulated with IFNγ. Reniale® led to increased time-to-progression and survival compared to nephrectomy monotherapy (Hazard Ratio=1.58, 95% C.I. = 1.05 to 2.37 at 5 years) [6]. Recently, 10-year follow-up survival of patients with aggressive stage III disease was 58.9% in patients given Reniale® versus 36.2% in surgery monotherapy [7]. To achieve similar success in other tumor types and increase overall response rates, we must reconsider the mechanisms by which tumor cell vaccinations act.

The key effector cells and molecules involved in anti-tumor responses evoked by tumor cell-based vaccines depend on many factors. T cells, B cells, and innate cells such as NK cells and macrophages are required for tumoricidal activity of vaccines in certain experimental situations, but the CD8 T cell has received attention as a desirable tumor killer. Its unique features include detection of intracellular antigens, antigen-specific killing via cell-cell contacts and production of cytokines, and the potential for a long-lasting memory response that prevents recurrence. Understanding the mechanisms of CD8 T cell activation following vaccination (hereafter referred to as cross priming) will help to identify the key hurdles to success. Upon injection, tumor cells or cell components are taken up and processed by dendritic cells (DCs) in the skin and secondary lymphoid organs, which then bridge innate and adaptive immunity. For optimal cross priming, DCs must provide three signals: (i) antigen cross-presented in the form of a peptide—MHC I complex; (ii) co-stimulatory molecules on their surface; and (iii) secreted cytokines. Additional complexities, such as the DC subset of interest, DC trafficking, tissue of antigen challenge, and optimal CD4 T helper cell differentiation also profoundly influence CD8 T cell activation. The cellular and molecular mechanisms of cross priming, in general, have been reviewed elsewhere [8] and will only be briefly considered here to focus on tumor cell-based vaccines. Recent studies on tumor cells in culture and *in situ* shed light on potential immune signals that can be elicited for the betterment of tumor cell vaccines. This review will discuss how this data must be synthesized with knowledge of cross priming and tested in tumor cell vaccine systems to improve clinical efficacy.

2. Cross Priming in Active Immunotherapy: Activating Tumoricidal CD8 T cells

2.1 Signal One

The “altered self” hypothesis states that CD8 T cells distinguish self from non-self (such as tumor neo-antigen) by recognition of the complex of peptide antigen and the major histocompatibility complex (MHC) I [9, 10]. At first, immunologists debated how and where peptide—MHC I complex formation occurs. Does the CD8 T cell recognize the MHC I-peptide complex on the surface of the injected tumor cell or cell fragment, or do antigens transfer to another cell? While both scenarios are possible, Michael Bevan demonstrated that transfer of antigen to a host immune cell occurred during recognition of minor histocompatibility antigens. He coined the term “cross priming” to describe the process by which exogenous antigens are uptaken and loaded on MHC I rather than expressed directly [11]. Identifying and understanding the function of the cell type doing the cross priming is crucial to understanding how to best elicit a CD8 T cell response. The DC, a rare leukocyte distinct in appearance and behavior, was identified by Ralph Steinman and Zanvil Cohn in 1973 [12]. It took ten years from their discovery to confirm the DC as the most potently cross priming cell, as DC depletion resulted in the drastic reduction of killing in the mixed leukocyte reaction [13].

What features of DCs make them adept at cross priming? The most crucial is known as signal one, or cross presentation: Antigen is recognized and taken up by DCs through endocytosis, macropinocytosis, or phagocytosis, degraded by any of multiple mechanisms, and the cleaved peptide fragments 8-10 amino acids in length that are loaded onto MHC I and shuttled to the cell surface. The mechanisms of cross presentation are partially understood, but recent studies indicate that immune stimuli such as those discussed later induce changes in the routing of antigen.

2.2 Signal Two

While cross presentation of tumor antigens is necessary for CD8 T cell-mediated tumor rejection, it also requires additional DC—CD8 T cell signals. In fact, early studies that sought to reconstitute T cell priming were hampered by unresponsiveness of T cells after mono-stimulation with MHC and peptide. Jenkins and Schwartz demonstrated that antigen-loaded antigen presenting cells that were chemically fixed presented peptide to CD4 T cells, but these T cells could not proliferate without a co-stimulatory short-range signal [14, 15].

Complementary to these findings was the discovery that at steady state, DCs induce tolerance to CD8 T cell antigens [16]. The missing signals in the case of CD4 and CD8 T cell activation were CD80 and CD86, ligands for the CD28 co-stimulatory receptor that is expressed on T cells. CD28 signaling results in the amplification of the TCR signal, inducing a proliferative response and IL-2 synthesis within the activated T cell. Following CD28 signaling, additional co-stimulatory receptor-ligand interactions are important in driving optimal T cell activation including ICOSL-ICOS, OX40L-OX40 and CD137L-CD137 among others [17]. Yet, an optimal tumor cell vaccine will induce a third class of signal in the form of secreted cytokines.

2.3 Signal Three

Further attempts were made at recapitulating cross priming *in vitro* by incubating naïve CD8 T cells with microbeads containing MHC I-peptide complexes and CD80/86. These stimuli triggered several rounds of cell division and temporary effector function, but cells were unable to reach full effector function, survive, and develop memory. When interleukin 12 (IL-12) or interferon alpha/beta (IFNα/β) were added to these cultures, however, full CD8

activation was successful [18]. Further studies of adoptive transfer of IL-12R$^{+/+}$ CD8 T cells into IL-12R$^{-/-}$ recipients demonstrated that the IL-12R on CD8 T cells was sufficient for this third signal to occur [19].

Tumor cell vaccines should therefore stimulate the production of IL-12 or IFNα/β to provide adequate signal 3 for optimal cross priming. IL-12 secretion *in vivo* can be stimulated when CD4 T cells present CD40L to CD40R expressed by DCs, a key trigger for IL-12 release [20]. Multiple methods of inducing signal three have shown promise, including providing the exogenous cytokine via genetic engineering (e.g., IL-12 producing tumor cells) or co-injection of recombinant cytokine. The use of type I interferon-promoting adjuvants as inducers of signal 3 show promise for tumor cell vaccine therapy, as they augment signals 1 and 2 through enhancing DC maturation.

2.4 Molecular Pattern Detectors: Keys to Dendritic Cell Maturation

Under steady state conditions DCs have an immature phenotype. If these cells receive antigen in isolation, tolerance ensues due to weak signal 1 and the lack of signals 2 and 3 to fully activate T cells. To mature DCs in culture and break tolerance, early DC immunologists added medium from gamma globulin-treated monocytes, which operated indirectly to trigger cytokine release [21, 22]. *In vivo*, however, DCs sense the local cytokine milieu in combination with molecular patterns of pathogens and tissue damage.

The discovery of *Toll*, a gene in *Drosophila* associated with immunity from fungal infection [23], marked the explosive appearance of the molecular pattern field. Toll-like receptors (TLRs) were found to be sensors of molecules conserved among many species of pathogens. At the same time, DC pioneers discovered maturation could occur through addition of lipopolysaccharide, a cell wall component of gram-negative bacteria [24]. TLRs thus linked the innate and adaptive immune systems through the maturation program. Upon ligation of their leucine rich repeat-containing extracellular domains with pathogen-associated molecular patterns (PAMPs), TLR cytosolic domains cluster following TLR dimerization (sometimes with a different TLR) which allows adaptor proteins to bind and signal through NFκB and MAPK. Within minutes of recognition of a microbial pattern, multiple signal transduction arms (transcriptional and post-translational) enact secretion of pro-inflammatory cytokines and increased surface expression of pre-made co-stimulatory and MHC molecules [25]. Importantly, co-administration of TLR ligands with antigens increases cross presentation (signal 1) by altering the intracellular processing route of antigen [26].

TLRs are joined by many receptor groups that spark DC maturation upon activation, as will be discussed in section 2.5. Diverse molecular structures are therefore capable of working alone or in combination to enhance maturation and cross priming.

2.5 Death and Danger Sensors in Dendritic Cells and Their Roles in Cross-Priming

All cells undergo stress responses in situations of environmental hostility, including mechanical trauma, nutrient starvation, temperature extremes, DNA damage, infection and hypoxia. These insults result in the release of pre-made molecules or the production of stress-related molecules that are subsequently released. Active or passive death can lead to the release of different “danger” molecules, and growth conditions before death directly impact the expression of molecules to be released by these processes. Numerous studies document the production and biologic function of stress-associated molecules in malignant and non-malignant cells. Such studies have laid the foundation for identifying the internal arsenal of molecules within tumor cells that may enhance cross priming under the proper conditions. Shortly after the discovery of TLRs, Matzinger and Fuchs proposed that the concept of the immune system’s discrimination between self and non-self was incorrect.

After all, this dichotomy failed to explain how autoimmunity occurs. They argued, rather, that the immune system's "switch" was sensitive to danger signals, be they from pathogens or from pathologically dying self-cells [27, 28]. Endogenous danger signals from stressed, dying, or dead cells were termed the "alarmins," and these molecules are largely detected by the same pattern recognition receptors as PAMPs. Since their inception, their identification has been provocative, arduous and controversial.

In 2000, Shi and colleagues reported the intrinsic adjuvant activity of cytosolic proteins within multiple syngeneic cell lysates. The lysates enhanced T cell killing following co-injection with model antigens such as chicken ovalbumin [29]. These findings reveal many key properties of alarmins:

1. Cells passively release pre-made alarmins upon instantaneous death (necrosis) or apoptosis;
2. UV irradiation, chemotherapy, or heat shock can boost adjuvanticity; and
3. Malignant and non-malignant cells can release alarmins.

In addition, this study set the stage for the molecular identification of alarmins themselves. We now know that alarmins constitute a diverse group of proteins and non-proteins that activate TLRs, nod-like receptors, purinergic receptors, and scavenger receptors. Excellent reviews on the many effects on sites of inflammation can be found elsewhere [30, 31]. Relevant to the use of tumor cell vaccines are the effects of these molecules on cross priming, and how tissue culture can enrich for these molecules.

The currently characterized alarmins make up diverse groups of molecules from all cellular compartments (Table 1). Most important to cross priming are their target receptors, known for their pattern recognition and connections with signals 1,2, and 3. Lesser studied alarmin sensors are the C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and nod-like receptors (NLRs). They can synergize with TLRs, and their effects on cross priming are less well understood and undoubtedly complex. These receptors could exert powerful biologic effects and demand as much attention as is given to TLRs for their potential as tumor vaccine targets.

CLRs are scavenger receptors that serve as a recognition and entry point for specific glycosylated antigens [32]. Closely linked to entry is the endocytic routing of an antigen taken up after binding CLRs; therefore, recognition by CLR significantly influences in the intracellular route of the antigen within the cell. As tumor antigens are frequently alternatively glycosylated [33], the role of CLRs in tumor antigen processing is a potential mechanism for selective priming against tumor antigens. For example, some CLRs expressed by DCs such as DC-SIGN recognize malignancy-associated glycosylation patterns selectively [34]. As discussed later, CLR binding by itself often leads to tolerance [35], but its potential to contribute to cross priming via antigen uptake and routing in combination with other maturation stimuli is relatively unexplored.

RLRs and NLRs are cytosolic receptors that bind diverse ligands, including single- and double-stranded RNA, double-stranded DNA, and cathepsin enzymes that are released as a result of phagosome rupture. These receptors play an integral part in vaccines empirically established in the past, from live attenuated viral vaccines to aluminum-containing vaccines [36, 37]. Providing ligands for these receptors in purified form and in combination with tumor antigens in various forms has yielded inconsistent results. An alternative to this exogenous source is to make use of tumor cells in culture that produce their own ligands for pattern recognition receptors. A wealth of literature indicates there are many avenues one might use to boost cross priming through induction of these endogenous innate ligands.

Understanding how to optimize the production of the molecule or molecules that best enhances cross priming will not be a trivial task; however, such knowledge is required to move the field forward and enhance the efficacy of tumor cell vaccines.

2.6 Considerations of DC Subsets, Timing, and Anatomy

Diverse vaccination responses are elicited from injection of the same agent depending on the DC subset activated, timing of vaccination, route, and location of injection. DCs are a heterogeneous population that can be divided functionally and phenotypically into many subsets. The main division exists between conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs and not pDCs have been established as the primary cross priming subset [38], which are then divided into migratory and lymphoid organ-resident DCs [Table 2, 39-44]. Migratory DCs sample antigen in their tissues of residence in an immature state. Once receiving a maturation stimulus they undergo a program that increases surface expression of MHC and costimulatory molecules, decreases their uptake of extracellular material, and migrate to lymph nodes for presentation of antigen to CD4 and CD8 T cells. Lymphoid organ-resident DCs, in contrast, receive antigens that enter the lymph tissues as part of lymphatic drainage. Antigen presentation in a lymph node draining a vaccination site can occur in the first 12 hours by lymphoid resident DCs, followed, roughly 24 hours later, by migratory DCs. These two waves of antigen presentation are required for optimal priming of CD4 T cells [45, 46], but presentation waves have not been as well studied in cross priming. T cell-DC contacts are believed to remain stable for 2-24 hours, leaving open the possibility that TCR signaling must be prolonged for up to 48 hours for optimal cross priming to occur [47, 48].

The two-wave model of antigen presentation raises two important variables into the rational design of vaccines: location and timing. One must first match the route of injection with the anatomical compartments of the most potently cross-priming DCs. Intradermal vaccination was superior to intravenous and intraperitoneal injection in controlling tumor and inducing IFNγ-secreting antigen-specific CD8 T cells in a murine model of melanoma [49]. In clinical practice, however, subcutaneous and intradermal injection have not undergone direct cross priming comparison, which must be tested with regard to tumor type and site. Due to recent findings that tissue-specialized DCs impart tissue-specific homing properties to CD8 T cells [50], and the lymph nodes nearest to a tumor are often dysfunctional [51], the location of the tumor almost certainly affects the optimal location of vaccination. Rationally changing the injection site is rarely considered in clinical trial design (e.g., one could select vaccination sites further from the tumor draining lymph nodes, which is likely variable depending on where metastases are present). Careful attention to the route of administration and injection site in relation to the tumor is warranted, particularly in clinical trials.

Tumor vaccinologists have learned from the more experienced infectious disease field that prime—boost strategies are capable of powerful CD8 T cell expansion and cytotoxic function. The majority of anti-pathogen prime-boost strategies involved the use of live virus and DNA vaccines, but could subunit or tumor cell vaccines also benefit from prime—boost optimization? A recent study by Wick and colleagues attempted to answer this question by mimicking viral infection in a four-consecutive day “cluster” homologous prime—boost regimen [52]. In contrast to tumor cell injection, antigen in a replicative viral infection persists and induces direct presentation in many host cells for days. This persistence of antigen might be recapitulated in re-delivery of tumor cell vaccine on consecutive days. By injecting mice with whole protein or peptide plus the TLR3/MDA5/inflammasome agonist poly (I:C), four days in a row, followed by a homologous boost, the CD8 T cells specific for a single epitope expanded to nearly 50% of the CD8 T cell compartment, comparable to infection models [53]. These primed CD8 T cells were capable of producing large amounts of IFNγ and eliminating established tumors [52]. While these studies indicate promise,

studies must be done to test the benefit of tumor lysate "cluster" vaccination. Unfortunately, little is known regarding how the timing of cancer vaccines may change clinical efficacy, and most clinical protocols have used intervals weeks to months apart. Consecutive immunizations may be counter-indicated with some adjuvants. For example, week-long daily vaccination with CpG ODN 1826 (type B) damages secondary lymphoid organs and leads to diminished CD8 T cell responses in mice, while the same timing and duration of poly (I:C) vaccination leaves immune organs intact [54]. However, as TLR expression in distinct leukocyte subsets in humans differs significantly from that of mice [55], it is important to consider the cells targeted by the adjuvant used in each species. Testing variations in vaccine adjuvant, site of injection, and frequency must occur in humans to move the field forward. We advocate strongly that issues of optimal timing and injection site should be prospectively compared in Phase I and II clinical trials.

3. Rational Design of Tumor Cell Vaccines Aimed at Cross Priming Signals

Recent technology has enabled the identification of many tumor-associated antigens and the production of synthetic antigen (e.g., peptide, subunit) vaccines [56, 57]. Despite these efforts, to date tumor cell-based vaccines have triggered double the response rate (~8%) relative to vaccines consisting of synthesized antigens (~4%) [4]. At least part of this difference in success lies in the challenge of variable antigen expression among cells in a given tumor and between tumors of different individuals. Vaccinating with too few antigens will result in selection of antigen loss variants and tumor progression. This has been elegantly demonstrated in animal models by Schreiber and colleagues, indicating loss of single immunodominant CD8 T cell epitope can be adequate for immune escape [58]. In humans, a notable example includes glioblastoma patients vaccinated with a peptide derived from the EGFRvIII mutation, in which recurrent tumors were negative for EGFRvIII despite being positive at enrollment [59]. To induce a broad response that targets a large variety of peptide—MHC I complexes on the tumor cell, vaccines must provide adequate quantities of multiple antigens.

At the time of publishing this article, clinicaltrials.gov contained over 900 search hits for "tumor cell vaccines," the majority of which place great faith in *ex vivo* tumor cell culture to provide such a diverse pool of antigens. As in the past, the success or failure of these trials will likely depend on all-too-often neglected aspects of tissue culture and vaccine production that affect the three required cross priming signals. With new knowledge of endogenous adjuvants, starting new and working stepwise is the most rational approach for moving beyond the current modest success. For example, we have yet to make basic head-to-head comparisons of the cross priming abilities of vaccines in their crudest forms—that is, surgically resected tumor specimen versus the same tumor cells expanded *ex vivo* in tissue culture. While we know that tumors produce and secrete many immunosuppressive cytokines, new evidence of tumor heterogeneity emphasizes the importance of greater diversity of antigen pools.

Tissue culture differs from *in vivo* tissues in many aspects, including nutrient supply, dimensionality of substrate, and oxygen tension. These differences result in many changes, including divergence in antigen expression, decrease in heterogeneity, and loss of stromal cells to which immunization can target. Studies such as that by Lee and colleagues have made us optimistic that tissue culture will be more desirable, even if initially inferior to fresh tumor specimen, once we identify crucial factors that govern cross priming. They observed dramatic and irreversible gene expression changes in glioma cells grown in serum-containing media [60]. Culturing cells in neural stem cell (NSC) media with basal fibroblast growth factor and epidermal growth factor resulted in a relatively preserved phenotypic and genotypic profile [60]. When glioma cells from NSC media were used for vaccine

immunotherapy of glioma-bearing mice, they resulted in superior tumoricidal CD8 T cell responses and increased survival compared to vaccines from serum-containing media [61].

Tissue culture is therefore a double-edged sword in that it risks inducing an artificial phenotype, yet proper manipulation may result in a vaccine superior to the phenotype of the tumor itself. Not only are culture conditions important for enrichment of antigen and adjuvant expression during tumor culture, but methods of cell killing are also crucial for vaccine activity [62]. In section 3 we discuss the culture and harvest stages of tumor cell vaccine production and how they impact cross priming.

3.1 Oxygen

The effect of oxygen on tumor cell immunogenicity in the context of vaccines is poorly understood. It has long been known that oxygen level varies dramatically within tumors, depending on proximity to capillaries, and that radiation resistance is linked to tumor hypoxia [63]. We recently found that transfer of glioma cells from atmospheric (21%) to physiologic (5%) oxygen (for at least 2 weeks) led to a drastic shift in gene expression, bringing gene expression closer to the tumor *in situ* [64]. Lowering oxygen induced an increases expression of HER2, SOX2, EphA2 and IL-13Rα2, all glioma-associated antigens with characterized CD8 T cell epitopes [65]. Lysates from 5% oxygen cultures also primed CTLs with superior tumoricidal functions against gliomas *in vitro* using primary human samples and *in vivo* using murine models [64].

It is adaptive for stress-related molecules to increase during a hypoxic response, however, it was unknown what effect if any would be seen by decreasing oxygen from a supraphysiologic to a physiologic range. Lysates from 5% oxygen possessed unique adjuvant activity (that was absent in 21% lysates), inducing superior responses to exogenous antigens as measured by CD8 T cell proliferation and cytokine production [64, 66]. Weekly vaccination of 5% lysate-containing vaccines resulted in a 41% increase in median survival of glioma-bearing mice compared to vaccination with lysate from the same cells from atmospheric oxygen culture. 5% oxygen lysate co-delivered to splenic DCs with chicken ovalbumin also increased the number of peptide—MHC I complexes on the cell surface, indicating an enhancement of cross presentation (signal 1). Interestingly, these cells grown in 5%, 2%, and 1% oxygen led to a dose-dependent increase (followed by a decrease) in the number of peptide—MHC I complexes, suggesting this response could be further optimized (unpublished data).

These data formed the basis for allogeneic glioma cell vaccines expanded in 5% oxygen that are currently being tested in clinical trials in glioma patients. Still, a number of questions have yet to be answered. First, can oxygen increase the potency of vaccines for other tumor cell types? Second, 5% oxygen tension was chosen arbitrarily, although we argue that its use is more rational than atmospheric oxygen. In the case of oxygen as well as culture medium, trending in the direction of physiologic is an improvement over the *status quo*; however, may the most optimal condition not be truly physiologic? One example to learn from this principle is that proteasome inhibitors, an artificial pharmacologic intervention, applied to tumor cell vaccine cultures, change the quality and quantity of relevant antigens.

3.2 Autophagy

Antigen availability in tumor cell cultures depends on the relative stability of proteins during the life of a cell and the period between cell killing and vaccination. Autophagy and proteasomal degradation together govern protein recycling in tumor cells as much as non-neoplastic cells. The process of macroautophagy, characterized by the formation of double-membrane autophagic vesicles, can be induced upon stress, nutrient starvation, and chemical

treatment. Defective ribosomal products (DRiPs) typically make up a large portion of the MHC I—binding peptides but are quickly degraded by proteasomes. Proteasome inhibition therefore stabilizes DRiPs and short-lived proteins, rerouting them to the lysosomal—macroautophagosome route. Twitty and colleagues recently demonstrated that this inhibition can have a profound impact on tumor immunity. In their model of chemically induced sarcomas, vaccination with autophagosomes containing DRiPs and short-lived proteins recovered from autophagosomes increased the breadth of CD8 T cell responses [67]. Vaccination of mice bearing these chemically induced tumors with untreated cell lysates typically results in a tumor-specific immune response that fails to protect against other tumors induced by the same chemical. In contrast, vaccination with autophagosomes from cells pre-treated with bortezomib (proteasome inhibitor) primed CD8 T cells that reacted against self-tumor as well as the tumors of other mice. While the authors hypothesized that it is the DRiPs and short-lived proteins that result in this broadened response, additional experiments must be done to test this model. Nevertheless, these findings demonstrate that inhibition of protein degradation could be used to preserve labile antigens in allogeneic tumor cell vaccines to broaden their coverage [67].

While there is evidence that autophagy occurring before cell harvest regulates the cross priming ability of tumor lysates [68], our understanding of the mechanisms responsible for this process are still relatively immature. The mixing of cellular compartments differs depending on the type of cell death occurring, but a finite number of degradation mechanisms exist. A given alarmin's stability may therefore depend on its physiologic half-life. Proteasome inhibition of tumor cells shifts the recycling of all proteins to autophagy and enriches for three alarmins discussed later, HMGB1, calreticulin, and CLEC9A ligand [72].

Additional findings on autophagy and its role in immunogenic cell death *in vivo* hint at additional potential for autophagy to boost cross priming *in vitro*. Michaud and colleagues discovered that autophagy was required for anthracycline-mediated "cryptic vaccination"—induction of tumor cell death *in vivo* that subsequently led to CD8 T cell-mediated tumor elimination. Interestingly, secreted ATP was the alarmin responsible for enhancing cross priming by its ability to bind P2X purinergic adenosine receptors of DCs, resulting in IL-1β production. Moreover, the authors also discovered that a P2XR hypomorphic mutation was associated with poor response to chemotherapy [69, 70]. The implications of these studies for tumor cell vaccines remain unexplored. The principle questions to answer are whether ATP secretion can be controlled until the optimal time for release in the setting used, and whether the resulting ATP pool is capable of assisting in cross priming in the setting of tumor cell vaccines.

3.3 Cell Rupture and Necrosis

Cells that undergo death through autophagy, specifically macroautophagy, share many alarmins with cells that die through necrosis and apoptosis. A starting point for determining the optimal cross priming source is comparison between necrosis (often from instant cell rupture) and apoptosis. Since studies have reported that necrotic tumor cell lysates are superior at maturing DCs [71], it is important to consider that the difference in cross priming may be from differential expression of alarmins, or degree of autophagy, rather than differential expression of antigens. Teasing apart the antigen versus alarmin contribution requires testing the intrinsic adjuvant activity of cancer cell vaccines, in which an exogenous surrogate antigen is used as we have done in murine and human systems [64]. Although we currently know little about how killing affects antigen availability, we know more about how this process affects the availability of alarmins present in tumor cell vaccines.

Early experiments on alarmins in tumor cells tested whether killing cells by various means resulted in differences in cross priming. Cancer cells lysed by rapid freeze-thaw exhibited equal cross priming potency regardless of pre-treatment with protein synthesis inhibitors, demonstrating that alarmins were pre-made and released upon loss of cell integrity [29]. Alarmin release also occurred after secondary necrosis (lysis during apoptosis). Since then, reports have found that necrotic cells release alarmins from the three major cellular compartments and bind pattern recognition receptors to spark danger signals.

High-mobility box protein-1 (HMGB1) is a classic alarmin and a highly complex molecule. HMGB1 has been reported to bind to at least four receptors (TLRs 2, 4, and 9, and RAGE) and is secreted/upregulated under many conditions. While it can translocate from the nucleus to cytoplasm under conditions of stress, passive release occurs during primary necrosis. Release from tumor cells can be elicited through chemo- and radiotherapy induced killing [72]. Its ability to boost cross priming and mediate treatment effect may stem from its multiple TLR activity or its ability to complex with other TLR agonists [73]. HMGB1 gained further fame after a report that breast cancer patients bearing a hypomorphic allele for TLR4 have lower response rates to anthracycline therapy. Such studies document HMGB1-dependence for curative tumor chemotherapy in mice [74], yet it is still not well established whether HMGB1 binds target receptors alone or in a larger complex. Evidence exists, however that TLR4 activation differs between LPS stimulation and reportedly pure HMGB1 stimulation [75].

While cytoplasmic alarmins are released upon membrane rupture (Table 1), the nucleus also provides a potent alarmin—hypomethylated DNA. Unlike apoptotic cells, necrotic cells can supply large amounts of hypomethylated DNA to DC to enhance cross priming through TLR9. Mammalian DNA bearing CpG sequences binds TLR9 in endosomes of human plasmacytoid dendritic cells (pDCs), and these interactions are implicated in autoimmune conditions such as lupus erythematosis and psoriasis [76]. While pDCs are not likely to cross prime, they can assist in supplying signal 3 to nearby CD8 T cells through secretion of type I interferons. It is also possible that cytokine release from B cells, which also express TLR9, could provide maturation signals to nearby DCs. While less well characterized, there is still potential for single-stranded RNA (ssRNA) to activate PRRs and enhance cross priming. Guanosine/Uridine-rich ssRNA can bind TLR7 and 8 in DCs. TLR8, unlike TLRs 7 and 9, is expressed in conventional DCs and may therefore directly assist in signals 1 and 2 [77].

Heat shock proteins (HSPs) are a class of chaperone proteins that are upregulated in many cells during stress. Tumor cells often overexpress HSPs, bind misfolded mutant proteins, and release HSP complexes upon death. HSPs were originally thought to induce TLRs but are now thought to be chaperones for antigens that do not possess intrinsic TLR activity [78, 79]. Despite doubts on TLR activity, HSPs may still have non-redundant functions in bringing antigen to DCs in large doses. One such delivery method involves enabling uptake of mutant proteins/peptides to DCs through endocytic receptors such as CD91 [80]. Recent evidence also indicates that HSP90 can transfer antigen from endosomes to the cytosol—a crucial step in the routing of antigen DCs for cross presentation [81]. HSP90 upregulation could be implemented in tumor cell vaccines through pre-treatment with bortezomib, which has been shown to mobilize HSP90 to the surface of myeloma cells and enhance anti-tumor cross priming [82]. Currently, gp96—antigen peptide complexes are being tested as purified components in clinical trials for melanoma and glioma [83, 84], but optimizing HSP release from tumor cells can increase the cross priming ability of whole tumor cell vaccines as well.

Shi and colleagues found that uric acid in crystal form (monosodium urate, MSU) was capable of enhancing T cell killing of a surrogate antigen expressing cell and increasing DC

surface expression of CD80 and CD86 [85]. Other alarmins were likely present within cells, as elimination of MSU only mildly decreased the ability of DCs to cross prime [85]. How cross priming is enhanced by MSU is still only partially understood, despite identification of one pattern recognition receptor for MSU, NLRP3 [85]. It has yet to be proven that NLRP3 is required for the enhancement of cross priming by MSU, although inflammasome activation (of which NLRP3 is often a part) mediates enhancement of cross priming [69].

C-type lectin 9A (CLEC9A, or DNGR1) has sparked the interest of DC biologists and synthetic vaccine producers as a potent mediator of cross priming. One ligand for CLEC9A, filamentous actin, is released upon primary or secondary necrosis, through rapid freeze-thaw cycles, anthracycline treatment, fixation and permeabilization, ultraviolet radiation, and serum-deprivation [86-88]. CLEC9A ligand was found to be released from cytoplasm in primary and secondarily necrotic cells but a smaller portion was seen on the cell surface during secondary necrosis. As the most potently cross presenting DCs ($CD8_{\alpha}^{+}$ murine and $BDCA\text{-}3^{+}$ human subpopulations) selectively express CLEC9A [41, 42] this ligand displays tremendous potential as a DC subset target. Even though recent studies show the potential of anti-CLEC9A—antigen conjugates in tumor immunotherapy [89, 90], our knowledge of CLEC9A is still limited. C type lectin receptors are traditionally seen as scavenger receptors that impact uptake, but there is no difference in phagocytosis between $clec9a^{+/+}$ and $^{-/-}$ bone marrow-derived $CD8_{\alpha}$ - like DCs [86]. Despite equivalent uptake, cross priming against necrotic cell-associated antigens was boosted by CLEC9A. Zelenay and colleagues recently reported that antigen routing within the DC is altered upon CLEC9A ligation. This process involves mechanisms that are partially dependent on CLEC9A's SYK recruiting domain known as hemi-ITAM [91]. Previous studies also suggest SYK kinase activation can lead to NFκB activation, as occurs with TLR activation [92]. Moreover, dectin-1-induced SYK activity led to enhanced IL-12p70 secretion by DC, suggesting signal 3 increases from SYK-linked CLRs (93). Future studies should investigate the mechanisms behind CLEC9A ligation and methods of tumor cell culture and killing that increase F-actin availability.

3.4 Apoptosis and Antigen Uptake

Programmed cell death is a cascade of enzymatic activation that leads to differential translocation and degradation of molecules within and on the surface of the cell. Intrinsic apoptosis occurs in response to many stressors that can be induced *in vitro*, including nutrient deprivation, x-ray or ultraviolet radiation, temperature extremes, and DNA damage. Whereas apoptosis resistance *in vivo* presents one of the key hurdles to success of cancer therapies, this process is easily induced in cultured cells for vaccine production. Confusingly, apoptosis in cells used for cross priming can be a benefit or detriment depending on the experiment [94, 95]. A few factors influencing this impact include the method of induction, previous culture conditions, phenotype and genotype of the specific tumor cell, and prior stresses influencing other factors such as preceding autophagy or ensuing secondary necrosis. Poor understanding of the complexity of this heterogeneous process has led investigators to empiric tests of whether apoptotic tumor body vaccines achieve tumor immunity. Ongoing identification of alarmins involved in this process will enable improvement of methods for maximizing adjuvanticity through apoptosis in the future. In particular, DC-mediated antigen uptake emerges as an avenue for improving tumor cell vaccines, as it is both a crucial step [96] and an attractive target for enhancement of cross priming [97].

Recently the Zitvogel and Kroemer groups discovered the mobilization of calreticulin (CRT, also known as cC1qR) to the tumor cell surface upon apoptosis-inducing endoplasmic reticulum stressors such as UV-C light, radiation, or anthracycline treatment. CRT was required for phagocytosis of dyeing tumor cells by DCs and for curative anti-tumor immune responses from chemotherapy [98]. CRT, a quality-control chaperone protein in normal

physiology, is shuttled to the outer cell membrane before phosphatidyl serine exposure during early apoptosis. It is known that binding to chaperone protein receptor CD91 [99] on DCs enhances cross priming. As suggested by its target receptor CD91, CRT has additional potential to function as a chaperone for tumor antigens (as discussed previously in section 3.3). When considering CRT and its ability to enhance cross priming, it is important to note that CD91-mediated phagocytosis by macrophages in certain contexts can also result in the clearance of apoptotic bodies, resulting in tolerance. As phosphatidyl serine blockade led to a decrease in phagocytic competition from macrophages; tumor cell harvest during the early stages of apoptosis may result in the selective phagocytosis by DCs [100]. This and many other important aspects of apoptosis-induced alarmin signaling through PRRs await testing.

4. A Framework for Testing Tissue Culture/Killing Techniques in Vaccine Production

We have a toolbox of endogenous adjuvants already encoded within tumor cells for use in tumor cell vaccines. Conditions in which to induce these molecules in order to enhance the activity of DCs await optimization and empirical testing. While each alarmin likely follows subtly different requirements for enrichment, we use oxygen as an example of how to prioritize future studies to further improve this vaccine in patients.

4.1 Clinical Questions in Cross Priming

We are currently testing glioma lysate vaccines from 5% oxygen in a phase I clinical trial, but many outstanding unknowns call for additional experiments. These empiric findings will face challenges similar to all clinical trials of immunotherapy. Consider that of response predictors. To predict efficacy of low oxygen-derived vaccines, the crucial antigens and alarmins must be identified. It may then be possible to predict responses based on expression levels, and to more accurately measure the degree of inter-tumor heterogeneity in the oxygen effect. In an autologous tumor vaccine strategy, this knowledge would enable measurement in culture before vaccine production. Upon noting a relative lack of the appropriate alarmins, one may appropriately titrate a patient's tumor culture alarmin levels to known effective levels by decreasing or increasing oxygen. Alternatively, functional tests could predict responders versus non-responders. Testing adjuvant effect on a patient's own peripheral blood monocyte-derived DCs could test alarmin functions of lysates from several oxygen concentrations. To be feasible even on a medium scale, we must determine the minimum time required for culture at a given oxygen tension for alarmin expression changes. As well, sufficient tumor material must be present for a fast turnaround time and oxygen titration testing. Although currently limited, our identification of alarmins could help us improve vaccine efficacy with a less empirical, more mechanistic framework.

Beyond the fundamental question of identifying the molecules responsible for increased cross priming and further optimizing oxygen levels, it makes sense to test it in combination with other compatible methods of antigen/alarmin preservation. One could easily culture cells at low oxygen and treat them with proteasome/autophagy inhibitors before harvest, for example. We predict that alarmin and antigen abundance would further augment breadth and strength of CD8 T cell tumoricidal activity. While our preliminary results of low oxygen-derived vaccines are promising, we still know relatively little on the ability of alarmin and antigen induction methods to synergize.

In a time when approaches for immune checkpoint inhibition have commanded the attention of tumor vaccinologists, we must not forget other hurdles in cross priming tumoricidal CD8 T cell responses that lower overall response rates. The rapidly expanding understanding of

alarmins, mechanisms of DC maturation, and tumor cell malleability in culture demand further investigation in order to improve the clinical efficacy of tumor cell vaccines.

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Table 1

Alarmins Capable of Enhancing Cross Priming

Alarmin	Induction Method	Receptor(s)	Signals/Processes Enhanced	Citation
F-Actin	Necrosis, Apoptosis	CLEC9A	SYK Cross Presentation (Antigen Routing)	86-90
Calreticulin, gp96, HSP70	Apoptosis	CD91*	NF B, Uptake Routing Costimulation Cytokine Secretion	77-83, 97,98
HMGB1	Necrosis, Apoptosis	TLR2/4/9*	NF B/MAPK Cross Presentation Costimulation Cytokine Secretion	71-74,77
DNA	Necrosis	TLR9	NFkB/MAPK Cross Presentation Costimulation Cytokine Secretion	72,73
Uric Acid	Necrosis, Apoptosis	NLRP3	Caspase-1, Cytokine Secretion	84
ATP	Autophagy, Nutrient Deprivation	P2XR	Caspase-1, Cytokine Secretion	68,69
?	Oxygen Decrease	TLR2/4*	NFkB/MAPK Cross Presentation Costimulation Cytokine Secretion	Unpublished Results

* Denotes the need to establish the dependence of cross priming on a given receptor

Table 2

Human Dendritic Cell Subsets and Their Abilities to Cross Prime

Subset	Markers	PRR Expression	Cross Priming	Location	Citation
BDCA3$^+$	CD11c$^+$ CD1c$^-$ CD141 (BDCA3)$^+$	CLEC1, Langerin, Dectin-1, CLEC10A, MRC-2, CLEC9A TLR1,2,3,6,7,8,9,10	+++	Blood, Spleen, LN, Peyer's Patches	40-42,44
BDCA1$^+$	CD11c^{hi} CD1c$^{\alpha}$ (BDCA1)$^+$ BDCA3$^{+/-}$	DCIR, CLEC10A, MRC-1, DC-SIGN$^{+/-}$ SIRPα TLR 2,3,4,5,6,8	+	Blood, Spleen, LN (dermal, see CD1a$^+$ below)	40-42
Blood (CD16$^+$)	CD11c^{hi} CD14$^+$ CD1a$^-$	Langerin$^{+/-}$ TLR2,4,5,7,8	+/-	Blood	39,40,42,44
Plasmacytoid	CD1c$^+$ CD11c$^-$ BDCA2$^+$ CD123$^+$	CLEC4C TLR 7,9	+/-	Blood, Spleen, LN	38,40,41
Langerhans	CD11c$^+$ Langerin$^+$ CD1a$^+$	Langerin, SIRPα TLR 1,2,3,5,6,10	++	Epidermis, Cutaneous LN	43,44
Dermal (CD1a$^+$)	CD11c^{hi} Langerin$^-$ CD1a$^+$ BDCA1$^+$ CD1c$^+$	DCIR, CLEC10A, MRC-1, DC-SIGN, SIRPα TLR 2,4,5,6,8	+	Dermis, Cutaneous LN	39, 41,44