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Designing CD8+ T Cell Vaccines: It's Not Rocket Science (Yet)

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Abstract

CD8+ T cells play important roles in clearing viral infections and eradicating tumors. Designing vaccines that elicit effective CD8+ T cell responses requires a thorough knowledge of the pathways of antigen presentation *in vivo*. Here, I review recent progress in understanding the activation of naïve CD8+ T cells *in vivo*, with particular emphasis on cross-priming, the presentation of protein antigens acquired by dendritic cells from their environment. With the rapid advances in this area of research, the dawn of rational vaccine design is at hand.

Introduction

From the time of Jenner's introduction of scientific method-based vaccination until the present day, vaccination for viral diseases has been based on administration of modified intact or fragmented viruses. For most important human viral pathogens, this empiric approach has been sufficient, as viral vaccines are one of the greatest successes of modern medicine. For a substantial number of pathogenic viruses, however, the empiric approach of vaccine design has failed. Moreover, it is becoming increasingly likely that therapeutic vaccination can play an important role in treating established diseases, particularly cancer, where the immunosurveillance theory has made a Lazarus-like reappearance[1].

Harnessing the full potential of the immune system to prevent and treat diseases will require rational vaccine design. Just as rocket science is rooted in Newton's laws of physics, engineering vaccines to precisely target pathogens and malignant cells requires establishing the laws of immunity. While there has been tremendous progress in understanding the immune system in all its complex glory, much remains to be learned before vaccines can be precisely engineered based on firmly established principles. Here, I review recent progress in understanding the induction of CD8+ T cell responses, which play important roles in clearing viruses and other pathogens, and in preventing and eradicating tumors. In keeping with the theme of this volume of *Current Opinion in Immunology*, I emphasize practical issues that impact vaccine design.

The Basics

CD8+ T cells typically express a clonally restricted αβ T cell receptor that recognize one of the “classical” MHC class I molecules bearing oligopeptides (normally 8 to 11 residues) in their binding groove. The cell presenting the MHC class I peptide complex (C_IPC) is termed the antigen presenting cell (APC). To activate naïve $CD8^+$ T cells to generate effector and particularly memory T cells, the goal of initial vaccination, APCs must express a combination of co-stimulatory cell surface and secreted molecules. In the absence of co-

stimulation, $CD8^+$ T cells are tolerized by interacting with cells expressing their cognate C_IPCs, providing anti-vaccination, but potentially useful in treating or preventing autoimmunity.

To avoid tolerance induction, naïve T cells limit their peregrinations to lymphoid tissues where they will exclusively encounter foreign antigen presented by "professional" antigen presenting cells (pAPCs), *i.e.* bone marrow lineage cells that express/secrete the appropriate co-stimulatory molecules for T cell activation. A key question in vaccinology are the identities of pAPCs in different vaccination/infection scenarios, and how differences between pAPCs influence $CD8^+$ T cell proliferation, effector function, and memory differentiation.

APCs can generate peptides from two potential sources: polypeptides they have synthesized on their own ribosomes, or polypeptides synthesized by other means. The former is termed direct presentation (or direct priming if the CD8+ T cell is naïve) while the latter is termed cross-presentation/priming. Gene based infectious agents/vectors are potentially presented by either route, while by definition, protein/peptide based immunogens are presented by cross-presentation. Peptides can be proteolytically processed for cross-presentation in two compartments: endolysosomes or cytosol. In the latter case, antigen is processed by the standard endogenous pathway proteases (including the proteasome) and liberated peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the former case, loading occurs in an endolysosomes compartment and presentation is TAP- and proteasome-independent.

What Do Vaccinologists Need to Know?

Rational vaccine design has two components.

First, what is the desired response? What specificities (*i.e.* class I peptide complexes) should be induced? What types of CD8+ T cells provide optimal functionality? What anatomic locations should be the focus of the response?

Second, how should these responses be generated? What immunogens should be used? What dose? What route and site of immunization? How many boosts?

Designing the first vaccine component requires a thorough understanding of the interaction between the target agent and the host. For each system, it is necessary to understand what contributes to the effectiveness of the CD8+ T cell response. Many mysteries remain. Superficially, it would seem that vaccines should induce responses to as many immunogenic peptides as possible. But it appears likely that some C_IPCs provide superior targets; than others for CD8+ T cell effector activity [2, 3]. In this case, a narrower response is probably advantageous, since CD8+ T cells typically compete with each other in the priming, and particularly the boosting phase of vaccination [4]. In this regard, vectors that elicit the minimal response to vector antigens should be greatly preferred over more complex vectors. Poxviruses, with hundreds of open reading frames, dozens of which are immunogenic [5], are particularly ill suited as CD8+ T cell vectors. Despite their present popularity, they should ultimately be replaced with less ornate vectors, unless immunity to pathogenic poxviruses is an associated goal of vaccination.

Designing the second vaccine component requires a thorough knowledge of the immune system. Basic understanding of immunity will come largely from insights generated by mouse model systems. The power of mouse systems grows at a much faster rate than other systems, due to multiple factors including cost, ease of genetic manipulation, availability of reagents, and not the least, the capacity to study immune events in real time by intravital

imaging via multiphoton microscopy, which is being rapidly extended to microbial pathogens [6].

The half-joking phrase HIV vaccinologists employ to summarize decades of disappointments with animal vaccination models, “mice lie and monkeys mislead”, has a germ of truth, however. Mice and men are different creatures, and major differences in viral tropism and innate immune sensors, limit the predictive powers of mouse vaccine studies, particularly for cell-mediated responses. While studies in sub-human primates will provide a bridge to humans, human trials will remain just that: trials with a substantial chance of failure.

Routes of Natural Priming

A critical basic question in viral immunology with practical ramifications for vaccinology is the natural route of priming of anti-viral CD8+ T cells. Robust cross-priming of anti-viral CD8+ T cells is easy to demonstrate experimentally by simply injecting either protein immunogens or virus-infected cells incapable of producing infectious virions [7]. Further, the robust mouse CD8+ T cell response to mouse cytomegalovirus (MCVM) in the face of multiple efficient mechanisms to block direct presentation, argues strongly for the physiological relevance of cross-priming [8-10].

Despite mounting evidence, the physiological significance of cross-priming continues to be questioned (though even Zinkernagel and acolytes, once highly skeptical [11], appear to have accepted its relevance [12]). Kemball *et al.* [13, 14] reported that despite reaching enormous titers in multiple organs, Coxsackievirus B3 (CVB) induces barely detectable CD8+ T cell responses. CVB, like other picornaviruses, blocks exocytosis and thereby disrupts endogenous antigen presentation, which is dependent on export of C_IPCs from the ER [15]. Kemball *et al.* [13, 14] argued that given a potent mechanism to block direct priming, the poor immunogenicity of CVB undermines the physiological contribution of cross-priming to viruses in general.

Notably, however, the endogenous anti-CVB CD4+ T cell response was also deficient [13], and a transgenic TCR CD4+ T cell response was weak to an inserted antigen [14]. Since cross-priming is well established as the major mechanism in generating MHC class II peptide complexes, this suggests that CVB suppresses T cell responses independently of the route of antigen presentation. Further, the failure to detect transgenic TCR CD8+ T cell responses to inserted determinants, may be related to the strategy for expressing the determinants, whereby the determinants are rapidly released as oligopeptides from viral proteins, and likely to be rapidly degraded, and hence, poor at cross-priming (see below).

In any event, these intriguing findings with CVB findings stress the need to extend mouse studies from the handful of heavily studied model virus systems (LCM, vaccinia, influenza, herpes) to a wider variety of viruses. They also underscore the importance of using new approaches for discriminating the contributions of direct- *vs.* cross-priming *in vivo*.

Fortunately, a number of new approaches have been described for tackling this question.

1. *Modifying class I trafficking*. Lizee *et al* reported that modifying the cytoplasmic domain of class I molecules interferes with their trafficking to endosomal compartments, and when such class I molecules are expressed in transgenic mice, reduces cross-priming [16]. Although this would not affect cross-priming via cytosolic delivery, it provides a means for gauging the contribution of endolysosomal processing, which appeared play a major role in CD8+ T cell responses to vesicular stomatitis virus, and particularly, inactivated Sendai virus.

2. *Knocking out or modifying APC subsets*. Exploiting the selective shut down in cross-presentation that occurs upon DC activation *in vitro*, Wilson *et al.* showed that activating DCs *in vivo* by injecting TLR ligands blocks *in vivo* cross-priming to protein antigens [17]. DC pre-activation reduced CD8+ T cell responses to herpes simplex and influenza A viruses, supporting an important contribution of cross-priming to these viruses, and demonstrating the potential of this approach. Indeed, this approach was soon exploited to demonstrate the importance of cross-priming to a modified (vaccinia) virus Ankara (MVA) encoded antigen [18].

   Lin *et al.* reported that injection of cytochrome C selectively ablated cross-presenting DCs, due to the pro-apoptotic activity of cytochrome C following its delivery to the cytosol [19] This is potentially a powerful approach, but is limited to the extent that cross-presenting cells also participate in direct-priming.

   Hildner *et al.* described a novel knockout mouse (BATf3) that lacks development of CD8αα homodimer expressing (CD8α+) and CD103+ (langerin + CD8α-) -DC subsets [20], together to play a central role in cross-priming [21]. Depending on the importance of these DCs in direct priming, this could be a useful strain for weighing the contribution of direct *vs.* cross-priming.

3. *Drug-modulation of antigen processing.* Barnaba and colleagues reported that chloroquine enhances cross-priming by retarding antigen degradation in endolysosomes and thereby enhancing cytosolic delivery [22, 23]. Chloroquine-enhanced immunogenicity is therefore, potentially an indicator of cytosolic-based cross-priming. Importantly, from the practical standpoint, as chloroquine is a widely used and well tolerated drug, it has potential as an adjuvant for cross-priming vaccines.

   Conversely, Basler *et al.* reported that the proteasome inhibitor bortezomib (a.k.a. Velcade or PS341) reduces CD8+ T cell responses by blocking generation of C_iPCs (and not by interfering with T cell proliferation)[24]. Bortezomib-resistant immunogenicity is therefore, potentially an indicator of endolysosomal based cross-priming.

4. *Genetic modulation of antigen processing.* Saveanu *et al.* reported that knocking out mouse endosomal protease insulin regulated amino peptidase (IRAP) selectively interferes with cross-priming [25], implicating IRAP-trimming in endosomal dependent cross-presentation, and providing a new target for selectively modulating cross-presentation. Mice lacking ERAP (ER associated aminopeptidase, which trims TAP-transported peptides) are also available, but their antigen processing phenotype is complex [26-28].

5. *Caveat.* Antigen processing pathways are complex, and in addition to "non-classical" connections between theoretically distinct pathways, there can be considerable cross-talk between the pathways (*e.g.* knocking out TAP *decreases* numbers of peptide receptive molecules available for endolysosomal loading [29, 30].) It is essential, therefore, in given experimental scenarios to perform functional control experiments that document the specificity of the manipulation for the given pathways.

Mechanisms of Cross Priming

Protein vs. Peptide?

For gene-based CD8+ T cell vaccines the physical nature of the cross-priming antigen is of paramount importance, since it dictates antigen expression strategy. In 2004, three groups simultaneously reported that the relevant form of antigen for cross-priming are proteins (*i.e.*

proteasome substrates), rather than proteasome products [31-33], casting doubt on the importance of chaperoned peptides in cross-priming [34]. Subsequent studies in a number of different systems confirmed that antigen stability in cells is a determining factor in cross-priming potency [18, 35-38]. Lev *et al.* reinforced this conclusion by reporting that peptides, if metabolically stable, are capable of robust cross-priming [39]. Thus, the poor immunogenicity of peptides in cross-priming appears to be attributable to the law of mass action, *i.e.* their amounts in donor cells are typically too low to be immunogenic.

Whatever the role of chaperoned-peptides in physiological cross-priming, they still might be effective vaccines. Indeed, Oizumi *et al.* reported that expression of a secreted form of gp96-fused to the mouse IgG1 Fc domain enormously enhances the immunogenicity of a nominal antigen synthesized by the same cell [40]. These findings are partially consistent with those of Nicchitta and colleagues, who first reported the adjuvant effects of secreted gp96, but failed to detect and increase in immunogenicity of antigens from cells secreting gp96 [41]. Further, the evidence continues to mount that the immunogenicity of purified molecular chaperones is poor in the absence of contaminants introduced during their production and purification that trigger innate immune receptors and provide adjuvant activity[42-44].

It appears that with few exceptions [45], the affinity for molecular chaperones for peptides is insufficient to preserve sufficient quantities for cross-priming in the natural setting [46]. The potency of chaperones-based vaccines is likely largely dependent on their affinity with their antigenic cargo. A general strategy to achieve high affinity interaction is to stably attach (via chemical cross-linking or producing a genetic/synthetic fusion protein) the immunogen to a high affinity ligand for the relevant chaperone [47, 48].

Antigen Acquisition for Cross-Priming

Cross-priming DCs can potentially obtain their antigen via multiple routes. For cell derived-antigens, antigen can be transferred to DCs by nibbling from live antigen expressing cells [49], or by phagocytosis from dead antigen expressing cells. It appears, however, that cross-priming antigen cannot be salvaged from every cellular compartment. Tewalt *et al.* reported that vaccinia virus encoded proteins sequestered in viral factories (*i.e.* viral assembly sites) are not available for CD8+ T cell cross-priming [50]. Since CD4+ T cell cross-priming was not inhibited, Tewalt *et al.* concluded that cross-priming DCs were able to acquire factory antigens, but could only process them in endolysosomes and not export them to the cytosol for class I processing.

Much remains to be learned about the DC antigen acquisition process, which is also critical in tolerizing T cells to self antigens in the absence of inflammatory signals. While there is considerable literature regarding the cross-priming immunogenicity of live *vs.* apoptotic *vs.* necrotic cells [51], many findings are contradictory and the message for optimizing gene-based vaccines is clouded. Plesa *et al.* generated recombinant rabies viruses that differ markedly in their cytopathic effect based on just two amino acid differences in an inserted protein, and found that cytopathicity increased cross-priming upon injection of infected cells [52]. There was little effect on immunogenicity of infectious virus, however, leading Please *et al.* to conclude that direct priming dominates in this system. Still, co-expression of death inducing/preventing gene products represents a viable strategy for optimizing cytopathogenicity in cross-priming of other gene-based vectors.

More broadly, manipulating cell death signals may also enhance protein based cross-priming. Sancho *et al.* identified CLEC9A as necrotic cell detector that enhances the cross-presentation by a still to be established mechanism [53]. This builds on Carminschi *et al.'s* original demonstration that CLEC9A is selectively expressed on mouse CD8α+ and

plasmacytoid DCs, and that targeting protein immunogens via CLEC9 greatly enhances their immunogenicity [54].

DCs can also acquire pre-formed C_IPCs from other APCs. "Trogocytosis" was coined by Joly and Hudrisier to connote the intercellular transfer of plasma membrane proteins during cellular interactions [55]. I coined "cross-dressing" to describe DCs trogocytosis of C_IPC from other cells [56] as a potential means for amplifying CD8+ T cell responses, as originally suggested by Fazekas de St Groth and colleagues [57]. Using a mouse tumor model, Dolan *et al.* initially described *in vivo* cross-dressing [58]. In a further, fascinating twist, Qu *et al.* demonstrated cross-dressing of priming DCs from monocytes that generated C_IPCs from phagocytosed antigen obtained from dead cells [59] (*i.e.* cross-dressing of cross-presented antigen!). (Note that this information transfer probably also extends to co-stimulatory and inhibitory molecules. Indeed, it appears the even TCRs can be exchanged between activated and naïve CD8+ T cells by trogocytosis, recruiting additional effector cells and adding an unexpected wrinkle to the clonal selection theory of lymphocyte function [60]).

More wonders abound: Neefjes and colleagues described peptide transfer to DCs from donor cells via gap junctions [61], and recently expanded the relevance to multiple systems [62, 63]. Gap junctions form between connexin 43-expressing DCs and other connexin 43-expressing cells. Connexins form six sided membrane channels that connect the cytosol of communicating cells, enabling the passage of flexible peptides and other small diameter molecules. The contribution of GAP junctional transfer to *in vivo* priming remains to be established. It is more likely to contribute to direct priming than cross-priming since direct priming in most cases generates greater quantities of peptides suitable for gap junctional transfer. In any event, it would be of interest to examine the adjuvant effect of connexin 43 expression in gene-based vaccines.

A New Cross-Presentation Compartment

It is beyond the scope of this review to discuss the recent progress in unraveling the cell biological mechanisms that enable cross-presentation, except for a recent set of findings with important implications for protein-based vaccines. Kurts and colleagues described a novel endosomal compartment accessed by the mannose receptor that participates in cross presentation in bone marrow derived CD8α+DCs and in cross-priming *in vivo* [64-66]. Remarkably, TLR-activation recruits TAP to endosomes, providing a pathogen-specific mechanism for regulating cross-presentation. Presentation of a model antigen was proteasome dependent, and evidence suggested the export of the antigen to proteasomes associated with the cytosolic face of endosomes, with TAP-mediated re-import of peptides into the originating endosome, where they associate with peptide receptive class I molecules. A similar process was previously proposed for phagocytosed material [67]. Such a mechanism could greatly increase the efficiency of cross-presentation by targeting locally generated, pathogen-derived peptides to TAP and limiting competition from cellular peptides. Peptides might also be directly generated in the mannose receptor compartment, particularly since IRAP is reported to be present [25].

If a similar compartment exists in human DCs that cross-prime *in vivo* [68], targeting protein antigens to this compartment through interaction with the mannose receptor (or potentially other yet to be discovered receptors), could enable efficient protein-immunogen based cross-priming.

Who's Priming *In vivo?*

DCs rule (for now)

A large number of experiments in mice point to the central importance of DCs in both direct and cross-priming. Cross-priming appears to be particularly dependent on CD8α+ and CD103+ DCs (whose relative importance likely varies with circumstances of immunization and between immune organs [69]), and, it will be important to see if future studies with the Batf3 knockout mice affirm this conclusion. CD8α+DCs have a number of adaptations that may account for their enhanced cross-priming ability. These include optimizing endolysosomal pH and composition [70] and CLEC9 regulated-cross-presentation[71]. The importance of CD103+ DCs in cross-priming has only recently become clear [72 73], and much remains to be learned about their special adaptations for cross-priming. CD103+ DCs are a migrating subset that transports antigens from the periphery to the draining nodes, and they are likely to be of central importance in cross-priming of immunogens delivered by local injection with limited access to the circulation.

To translate the mouse model findings to human immunology it is critical to establish whether there is are equivalent DC subsets in humans (which do not have a CD8α+ DC subset) particularly adept at cross-priming [74]. Galibert *et al.* provided the initial evidence for the equivalence of human BDCA3+ DCs with mouse CD8α+ DCs [75], and the conserved expression of CLEC9 [54] and CLEC12 [76] supports the relationship. It is of obvious importance to characterize the functional characteristics of BDCA3+ DCs, and to determine whether they are present in sub human primates, where experimental *in vivo* manipulation is possible.

pAPC Wannabes

Although thousands of studies point to the central importance of DCs in cross-priming CD8+ T cells, other cell types have been identified that are capable of efficient cross-priming, including in recent years, plasmacytoid DCs [68], interferon producing killer dendritic cells [77] and neutrophils [78]. None of these cell types are thought to be present in significant quantities when and where priming occurs in immune organs, undermining their potential participation in priming. On the other hand, although it is assumed that priming exclusively occurs in draining lymph nodes and spleen, effective (though delayed) anti-viral CD8+ T cell priming can occur in lymphotoxin-α -/- mice, which lack lymph nodes and have a disorganized spleen [79] (indeed, the gp96-Ig fusion protein discussed above highly immunogenic in these mice [40]).

Intravital Microscopy to the Rescue

In any event, conclusive definition of naturally priming APCs must include anatomical evidence for the interaction between naïve CD8+ T cells and the priming APCs. The recent advances in intravital microscopy, coupled with the generation of transgenic mice and microbial vectors expressing fluorescent proteins under tissue specific promoters, provide an unprecedented opportunity for characterizing immune activation in real time in something approaching to natural conditions [6]. This enabled Hickman *et al.* to demonstrate that direct priming to viruses introduced subcutaneously occurs in a newly defined anatomic region in the draining lymph node, termed the peripheral interfollicular region [80]. Intravital microscopy also revealed that lymph-borne viruses are transported from the subcapsular sinus into the lymph node parenchyma by macrophages that sample the subcapsular fluid [81].

Intravital microcopy is poised to rewrite the rules of immunogenicity, and surprises regarding the APCs involved and the anatomic sites of T cell priming are likely. A key challenge in the future is to adapt intravital microscopy to non-human primate models.

Practical cross-priming –optimizing antigen, route, conditions

Gene Based Immunogens

For gene-based priming, if the route of priming is known, there is a simple rule for selecting the form of the expressed antigen. Direct priming is optimized by expressing rapidly degraded polypeptides (exception: some minigene products are degraded so rapidly that immunogenicity is reduced relative to even the full-length source polypeptide [82]). Conversely, cross-priming is optimized by expressing long lived antigens. If the route of priming is uncertain, it is best to express long-lived antigens. This will optimize cross-priming, and will be reasonably effective in direct priming, since even directing 100% of nascent antigen to proteasomes only increases peptide production by two- to three-fold, due to the efficiency of the DRiP pathway in generating peptides from stable proteins [83].

Polypeptide based Immunogens

Extended Peptides—Optimally sized synthetic peptides (for class I affinity) are generally ineffective immunogens in humans. Wei and Sherman reported that adding just 3 residues to the amino terminus of three different optimal 8- or 9-mer peptides greatly increased their cross-presentation capacity [84]. Presentation was TAP-dependent, implying cytosolic delivery, and the enhancing effect was dependent on the trimer extension sequence, an important consideration in designing such peptide immunogens. Relating these findings to Lev *et al.'s* report of stable cytosolic peptides [39] could provide great insight into the intersection of cross-priming and direct priming pathways, and shed light on the metabolic stabilities of oligopeptides in the cytosol.

DC cross-presentation of proteins [85] and extended peptides [86] lasts for days after antigen acquisition, due to the storage of antigen in a lysosome-like depot compartment in matured DCs [86]. The increased immunogenicity of extended peptides is likely due to two factors: resistance to protease destruction (any proteolysis will drastically diminish binding of minimal peptides to class I molecules), and restricting presentation to cross-presenting DCs, since minimal peptides will generate large number of C_IPCs on nearly every cell type they accesses, leading to tolerance induction [87].

Success!—Melief and colleagues have spectacularly demonstrated the vaccine potential of long peptides. Immunizing cervical carcinoma patients with a incomplete Freund's adjuvant containing a mixture of 13 different 20-30+ mer peptides corresponding to human papilloma virus 16 transforming proteins, they found remarkable clinical responses related to induction of anti-viral CD8+ T cell responses [88, 89]. A key advantage of this approach is that multiple HLA class I and II allomorphs are likely to be covered by the mixed peptide immunogen.

Encapsulated Antigens—Advances in material science offer great promise for polypeptide based vaccines introduced in particulate form. It will take time to optimize the parameters that influence immunogenicity, including route of immunization [90] method of antigen attachment, particle size [91] and composition (synthetic [92, 93], or self-assembling, *e.g.* virus like particles [94]). A general principle is that immunogenicity is greatly enhanced by delivering antigen and TLR-(or other innate immune receptor) activating substances in the same particle [95 96]. In one virus like particle system, the increased immunogenicity was due to enhanced DC co-stimulatory capacity, and not antigen presentation [97]. Evidence suggests that innate immune activating substances should directly or indirectly activate the type I IFN receptor, which enhances both cross-presenting DC-co-stimulation and T cell activation [98]. Combining two approaches, it would be interesting to target particulate immunogens to optimal human DC subsets by coupling antigen containing particles to subset-specific ligands (*e.g.* anti-CLEC9 antibodies).

Conclusion

Practical people, like vaccinologists and Presidents ("Give me a one armed economist"… attributed to Harry Truman, weary of receiving "on the other hand.." answers) prefer clear advice. The immune system, however, is even more complicated than the economy, and more insight into the workings of the immune system is necessary for vaccinology to become rocket science, and for antigen processingologists to shed an arm.

Fortunately, a combination of successes (T cell cancer immunotherapy [89, 99]) and failures (HIV vaccines) provides a strong carrot and stick argument for robust funding of basic research into antigen presentation and T cell activation. Perhaps the words of Churchill best describe the current state of affairs regarding rational vaccine design.

> Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

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