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Linking Autoantigen Properties to Mechanisms of Immunity

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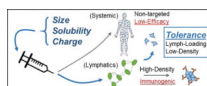
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Abstract

Antigen-specific immunotherapies (ASIT) present compelling potential for introducing precision to the treatment of autoimmune diseases where nonspecific, global immunosuppression is currently the only treatment option. Central to ASIT design is the delivery of autoantigen, which parallels allergy desensitization approaches. Clinical success in tolerizing allergen-specific responses spans longer than a century, but autoimmune ASITs have yet to see an FDA-approved breakthrough. Allergens and autoantigens differ substantially in physicochemical properties, and these discrepancies influence the nature of their interactions with the immune system. Approved allergen-specific immunotherapies are typically administered as water soluble, neutrally charged protein fractions from 10 to 70 kDa. Conversely, autoantigens are native proteins that exhibit wide-ranging sizes, solubilities, and charges that render them susceptible to immunogenicity. To translate the success of allergen hyposensitization to ASIT, delivery strategies may be necessary to effectively format autoantigens, guide biodistribution, and engage appropriate immune mechanisms.

Graphical Abstract



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1.13 Competing Interests

Authors JOS and CJB are co-founders of Orion BioScience, Inc., a company developing technologies for the treatment of autoimmune disease. The other authors declare no competing interests.

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1.3 Introduction

Autoimmune disease has long persisted as a difficult and costly problem in modern medicine. Indeed, the American Autoimmune Related Diseases Association estimates that these diseases such as Multiple Sclerosis (MS), Type 1 Diabetes (T1D), and nearly one hundred others collectively affect over 50 million Americans and cost upwards of \$100 billion to treat each year. A major criticism of this exorbitant treatment burden is that it does not adequately stifle disease presentations; contemporary therapeutics largely serve only to suppress symptoms without addressing the causative factors that drive autoimmunity^{1, 2}. In this review, we will explore molecular properties, biodistribution, and implications for immune mechanisms of antigen-specific immunotherapies (ASIT) and glean insights for designing safe and potent autoimmune treatments. We will begin by analyzing the physicochemical properties of several predominant allergens, as desensitization therapies have provided an analog for ASITs. We will highlight key differences between these properties and those of autoantigens to suggest design parameters for ASITs of the future to ultimately optimize clinical success.

Prevailing treatment strategies across many autoimmune diseases involve the administration of immunosuppressive drugs. These compounds broadly compromise healthy and aberrant immunity alike³. While the virtue of these approaches is a slowing of disease progression, a steep tradeoff demanded by these routes is the ablation of protective immune function as well⁴. Glucocorticoids have been widely applied to suppress inflammatory function against a plethora of autoimmune diseases, though they are marked by increased patient susceptibility to infection⁵. In MS, natalizumab is proven to significantly reduce relapse rates, however this monoclonal antibody is also well known for imposing the risk of progressive multifocal leukoencephalopathy (PML), a life-threatening result of the opportunistic JC virus⁶. Similarly severe risks exist with Rituxumab, a B-cell depleting immunotherapy that has been deployed for a plurality of autoimmune diseases such as Rheumatoid Arthritis (RA), T1D, and MS⁷.

The push for precision medicine is a major initiative that has built excitement and momentum especially in the arena of cancer, but it likewise captivates immense promise for autoimmunity^{8, 9}. Breakthroughs of precision approaches to cancer have come by way of leveraging the genetic anomalies that are hallmark of specific kinds of tumors. Capitalizing on these discrepancies has enabled the design of delivery systems that can specifically target and eliminate malignancies with potent chemotherapeutics, and these approaches have radically transformed previously hopeless prognoses^{10, 11}. Autoimmunity, however, has yet to see clinical success stories in the form of precision medicines. This setback is in part due to the fact that unlike cancers, the autoreactive cells that perpetuate disease can be phenotypically indistinguishable from healthy immune cells. The result has been an absence of targeted immunotherapies for autoimmune disease. Even Ocrelizumab, one recently approved drug for MS, works by broadly deleting B cells from patients¹². Although more targeted, this approach has already been plagued by the same kinds of adversities as its predecessors from past decades^{13, 14}.

Though autoimmune cells can appear similar to typical functional immune cell types, their detrimentality can be defined by a fundamental difference in the antigen receptor specificity^{15, 16}. The immune system serves to exact homeostasis by maintaining recognition of “self” and “non-self” antigens through highly specific T cell and B cell receptors (TCRs and BCRs, respectively)¹⁷. In the development of healthy immune cell progenitors, antigen receptors are selected by positive- and negative-filtering checkpoints^{18, 19}. These tolerance mechanisms typically serve to remove or inactivate autoreactive T and B cells, though they are indelibly compromised when autoimmunity ensues^{20, 21}. Antigen-specific immunotherapy (ASIT) invokes a meaningful stride toward precision in treating autoimmunity through the promise of selectively targeting cells that escape tolerance²².

ASITs, in the context of autoimmunity, direct immunomodulation to the autoreactive cells that propagate disease²³. This precision is accomplished by delivering formulations that incorporate the very same autoantigens responsible for the self-directed tissue destruction taking place. While the precision implications of ASITs are readily grasped from the cell-targeting that comes from delivering autoantigen, deeper mechanistic characteristics are critical to consider. Autoantigens and their constitutive epitopes vary widely in terms of physicochemical properties such as size, charge, and solubility, and these differences can vastly impact the way ASIT formulations are trafficked throughout the body and elicit effect²⁴. In this review, we explore these physicochemical drivers of effect that hold great importance for the design of effective ASITs with potential for treating authentic human disease.

1.4 Peripheral Autoimmunity is a Vicious Cycle

To understand antigen-specific approaches for the desensitization of aberrant autoimmunity, we must first review the underpinning mechanisms that propagate disease in the first place. In the cycle of autoimmunity, the faulty immune response is primed in secondary lymphoid organs and exacted in tissue compartments rich with cognate autoantigens (Fig. 1)²⁵. In the origination of autoimmune destruction, an initial or ongoing insult disrupts tissue (Fig. 1.1)²⁶. Damaged tissue both releases autoantigen and provokes innate arms of the immune system. Disrupted autoantigen passively or actively drains to lymphoid organs (Fig. 1.2a) by following transport phenomena that will be covered in greater depth later (Fig. 2). Also, innate arms of immunity are engaged as the tissue compartment evolves an inflammatory microenvironment (Fig. 1.2b). Tissue damage generates a host of immune-boosting cues including reactive oxygen species and damage-associated molecular patterns that stimulate sentinel cells such as dendritic cells (DCs), macrophages (mφ), and monocytes to take up antigen and become activated²⁷. With this activation comes a subsequent migration to regional lymph nodes; “active transport” implies antigen conveyed by this mechanism. (Fig. 4).

Once at the lymph node, autoantigen may undergo further processing to reach the T and B cell zones²⁸. In these locales, follicular antigen presenting cells are capable to present autoantigen to cognate naïve CD4⁺ T helper cells (Fig. 1.4). Naïve T helper cells will become stimulated if antigen presentation occurs with the simultaneous ligation of B7 pathway surface proteins²⁹. These proteins include CD80 and CD86 on the surface of

antigen presenting B cells and DCs which can bind CD28 on T cells to propagate their activation³⁰. Depending on T helper subtype and intranodal cues such as cytokines, these activated cells are compelled to proliferate and promote an effector-driven immune response through receptor and cytokine signaling (Fig. 4.5)³¹. While a plethora of finely-tuned antigen-specific effector responses can ensue, they can broadly be categorized by cellular or humoral responses in nature. Cellular immune responses are characterized by T helper 1 and 17 type responses which are recognizable by secreted inflammatory cytokines such as IL-2, IFN- γ , TNF- α , IL-23 and IL-17^{32, 33}. Responses of this nature are most commonly indicated in autoimmune disease, as they stimulate phagocytic innate immune cells such as $m\phi$ to engulf target cells or debris³⁴. Humoral immune responses recruit B cells into the effector response; these directive signals promoted through cytokines such as IL-4, IL-6, and IL-10 trigger antibody secretion by terminally differentiated plasma cells³⁵. The ensuing antibody production is efficient to bind and label free antigen. Humoral immunity is recognized to play an increasingly important role in some autoimmune diseases such as Neuromyelitis Optica and MS^{36, 37}.

Nevertheless, when effector responses are triggered, these activated cells home back to antigen-rich compartments to cause autoreactive damage and continue the autoimmune cycle by renewing the production of immunogenic factors (Fig. 1.6)³⁸. The cyclic nature of autoimmune potentiation may illustrate the relapsing-remitting pattern followed by many of these diseases. However, this thematic scheme likewise demonstrates the potential for ASIT to influence the immune system. Antigen processing, presentation, and signaling occurs in secondary lymphoid organs, and these tissues represent a priority target in modulating the aberrant response. It is critical to target ASIT to the lymphatics not just for their directive role in propagating immunity, but also for the inherent probability of interacting with T and B cells that possess cognate antigen receptors. The body has an estimated 10^{12-18} discrete theoretical T and B cell receptor specificities³⁹⁻⁴¹. In peripheral blood, only 2% of all lymphocytes are estimated to be circulating at any given time⁴². Since an overwhelming majority of clonalities are present in the lymphatics, the importance of reaching these organs to evoke potent, successful effects is yet further emphasized.

1.5 Route and Size-Dependent Transport Dictates the Destination of Parenterally Delivered Compounds.

It is critical to understand the transport phenomena that must be harnessed for delivery to immunologically directive tissues. Particularly with delivered allergy and antigen formulations, many administration routes have been investigated, but each presents its own unique delivery considerations. In this review, we will refine our analysis to focus on parental routes administration, as non-injected delivery introduces complex facets of mucosal immunity that have been well-reviewed in the past⁴³⁻⁴⁵. Our scope includes interstitial and intravenous (IV) strategies, which have been clinically investigated for both allergy and autoimmunity (Fig. 2). Interstitial administration indicates formulations delivered directly into tissue parenchyma and envelops common routes such as subcutaneous (SC), intramuscular (IM), and intradermal (ID) injection (Fig. 2a).

Interstitial delivery is appealing for ease of administration and proven successes over a century-long history in vaccines and allergy desensitization^{46, 47}. This route can exploit extracellular matrix (ECM) properties and sentinel cell populations to deliver formulations to draining lymph nodes. The ECM largely consists of an interpenetrating network of collagen and glycosaminoglycan polymers, which hinders leakage from the injection site in a size-dependent manner. Both diffusion and convection can drive transport through the ECM. Albumin serves as an excellent reference (molecular weight 69 kDa, size 3.5 nm) in that compounds smaller than this protein can rapidly diffuse from the injection site and reach systemic circulation through the blood pool while the transport for larger entities is driven by convective flow⁴⁸. A negative pressure gradient exists in the interstitium that drains to the lymphatics. When particles are larger than the size of albumin, this convective flow dominates over diffusive transport and enables accumulation in lymph nodes. Importantly, however, when administered moieties exceed 100 nm in size, transport is restricted by the ECM (in humans)⁴⁸. Structures of this size persist at the injection site as depots and must rely on active transport by innate immune cells to reach lymphoid organs. An important note is that actively transported antigen presents challenges for immunosuppressive function, however, due to its particulate nature⁴⁹. We will discuss this perspective later in the review.

Indeed, entities between the size of 4–100 nm are generally able to reach lymph nodes from the interstitium by convective flow, but reaching these organs alone is not sufficient for affecting immunity. Lymph nodes are contained by a capsule that excludes much of the extranodal content that would otherwise intrude from systemic circulation. The T and B cell zones where immunity is potentiated lie within nodal sinuses that are protected by the subcapsular space and its resident macrophages and dendritic cells²⁸. Herein, a second barrier is embodied that must be considered for ultimately delivering ASITs via interstitial routes. Though antigen can be proteolyzed in the subcapsular sinus⁵⁰, conduits at this interface largely determine the penetration of free antigen into the nodal cortex with a 70 kDa cutoff⁵¹. The size of these channels once again represents a generalizable reference point similar to albumin, though shape and flexibility can confer access to larger compounds^{52, 53}. Entities between 10–100 nm reaching the subcapsular space are mostly excluded excepting transport by subcapsular innate immune cells⁵⁴. Most of the active internalization by these macrophages and dendritic cells is Fc-mediated, meaning antigen bound with antibody can reach the cortex. However, these complexes are poised to be immunogenic as part of natural immunological cues.

A set of delivery principles can also be surmised for the IV administration of antigen formulations as well (Fig. 2b). Compounds injected directly to the blood stream can be deposited by the spleen, renal elimination, or liver processing. The spleen is poised as an immunologic filter from which systemic circulation is policed⁵⁵. Delivery and penetration into this organ resembles the requirements for lymph nodes; moieties 4–200 nm in size are capable of reaching the spleen, but only those between 4 to 10 nm can traverse conduits into the cortex. Small compounds (<4–6 nm) are subject to renal excretion and do not persist to significantly influence immunity⁵⁶.

Though antigens between 4–200 nm are able to reach the spleen, blood flow highly favors liver processing. Blood flow through the spleen is typically 5–10% of cardiac output while

the liver draws up to 5-fold more blood⁵⁷. Liver delivery has drawn interest for ASIT delivery due to its immunologically privileged nature that is conferred by unique cell subsets including Kupffer cells and liver sinusoidal endothelial cells⁵⁸. For example, researchers have tuned properties such as size and charge to produce formulations that preferentially accumulate in the liver⁵⁹. This design is important for ASIT, because autoantigen relegated to the liver has been applied to enact tolerance against autoimmune mouse models^{60, 61}. Liver accumulation and retention is maximized with very large particles that exceed 200 nm in size, and persistence enables the longitudinal activation of tolerogenic mechanisms. In the case of 4–200 nm structures, exposure is less conducive to immunological effects because liver accumulation is not as pronounced.

1.6 Allergy Immunotherapies as a Reference for Antigen-Specific Approaches to Autoimmunity.

With an understanding of the delivery parameters that influence the ability of formulations to access and influence the immune system, we can now look to practical examples for how aberrant immunity is modulated. One ASIT strategy for addressing autoimmunity stems from historically successful applications in allergy desensitization therapies where hypersensitivity immune responses take place⁶². As such, we will assess historical underpinnings of allergy hyposensitization therapies and implications for ASIT applied to autoimmune disease. While recent ASIT strategies encompass a broad scope of approaches and delivery systems incorporating biomaterials, immunomodulatory drugs, and adjuvants, we have refined our focus here to the clinical delivery of allergen and autoantigen immunotherapies without co-delivery of drugs to enable the thorough analysis of physicochemical implications of these components. We aim to use this distilled approach to extrapolate a meaningful supposition about the clinical successes of allergen-specific immunotherapies in contrast with the continued elusiveness of autoantigen ASIT implementation. Through understanding the role of autoantigen contextualization in directing the immune response, we propose that next-generation ASITs can be formatted for optimized action against autoimmune disease.

The first allergy immunotherapy was implemented with success in 1911 when Leonard Noon and John Freeman desensitized patients to grass allergy (also known as hay fever)^{63, 64}. In this seminal trial, the physicians subcutaneously administered grass pollen extract in regular intervals while increasing allergen dose over time to build resistance. This framework of repeated and escalating dose has overall remained essentially the same and informed allergy immunotherapies for the century since. Today there are 19 FDA-approved, standardized allergen desensitization regimens that are comprehensively administered by interstitial injection⁶⁵. These standardized regimens exclude oral and sub-lingual immunotherapies, though these approaches have gained momentum in recent years as well⁶⁶.

With such broad and repeated demonstrations of effective allergen-specific immunotherapy regimens, it is important to explore the underlying characteristics driving these successes to enable rationalization against the comparative lack of breakthroughs within autoimmunity.

Parameters such as size, charge and solubility each can play critical roles in directing the interaction between immunogens and the immune system, and as such we have organized these data among three prominent allergens for which FDA-approved, standardized desensitization regimens are available (Table 1)^{67, 68}. Properties for a comprehensive list of standardized allergens are compiled as supplementary information as well (Supp. Fig. 1), while statistical analysis of all of these quantities is presented later in Figure 3.

Somewhat conserved physicochemical properties are evident across sample allergens. In terms of molecular weight (MW), extract fractions average 25.8, 21.4, and 21.0 kDa for timothy grass, peanut, and domestic cat, respectively. MW does not exceed 65.4 kDa (Ara h 1) across all allergens, though the minimum MW bottoms out at 2.6 kDa (Ara h 6). Isoelectric points (PI) are generally found at or below physiological pH between allergens. A PI of 10.5 was the maximum observed in the set (Ara h 11), while values did not go below 3.84 (Phl p 7). Finally, grand average of hydropathicity (GRAVY) was used to roughly infer the water solubility of allergens through summing the hydropathy values of amino acids in the sequences. The GRAVY score describes the hydrophobicity of a given protein; larger, positive values generally mean the compound is insoluble while negative values imply hydrophilicity⁶⁹. Interestingly, allergen extracts are mostly water soluble, displaying negative GRAVY scores.

Together, allergen extracts paint a cohesive picture about conserved properties that may point to their utility for desensitization. For the most part, these aqueous extracts fall squarely in a size distribution between 10–70 kDa (Fig. 3a) – precisely the range which facilitates lymphatic drainage and penetration while likely being excluded from the blood pool when administered as subcutaneous or intramuscular injections (Fig. 2)^{48, 70}. Indeed, the desensitization regimens approved by the FDA enable the interstitial delivery of mixed extracts. Allergens are generally not extremely charged; these extracts possess PIs at or below physiological pH such that they carry a neutral or slightly negative charge (Fig. 3a). An absence of highly charged fractions enables innate immunogenicity as the result of complement, immune scavenger receptors, or cell membrane disruption to be generally circumvented^{68, 71, 72}. With these charge properties and net negative GRAVY scores, allergens avoid aggregation. These entities therefore remain at a size that facilitates lymphatic transport and prevents the formation of large, immunogenic aggregates.

1.7 Autoantigens Embody Physicochemical Profiles that Differ from Allergens

While allergens appear to present with similar characteristics, autoimmune antigens come with many different size, charge, and solubility properties appreciable across several disease pathologies (Table 2, Fig. 3a). Myelin autoantigens including proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP) are associated with MS. Their molecular weights are 30, 28.2 and 11.57 kDa, respectively, while each is positively charged at physiological pH. These autoantigens are found embedded within the fatty myelin sheath, and as such PLP and MOG display considerable hydrophobicity. Conversely, MBP presents with a highly water-soluble GRAVY score that is

likely conveyed by its significantly positive physiological charge. T1D presents glutamate decarboxylase (GAD), preproinsulin, and 60 kDa heat shock protein (HSP60) as conserved autoantigens. Among these proteins, a negative charge is presented at physiological pHs. GAD and HSP60 are both somewhat soluble in water, while preproinsulin is slightly hydrophobic. RA autoreactivity includes specificity for 40 kDa heat shock protein (HSP40), which is negatively charged and slightly soluble. The thyrotropin receptor autoantigen in Grave's disease is large (86.8 kDa), neutrally charged, and presents a positive GRAVY score. In Hashimoto's disease, a similar thyroid-directed autoimmunity, thyroglobulin is a very large (304.8 kDa), but hydrophilic protein. Aquaporin 4 in neuromyelitis optica is moderately sized at 34.8 kDa, neutrally charged, and considerably hydrophobic.

A number of clinical trials have been launched to investigate interstitial and IV delivery of autoantigenic epitopes within these proteins. Soluble antigen is reported as capable to skew immunity toward a regulatory phenotype and confer bystander immunosuppression effects. As such, most injected autoantigen therapies within human MS patients have used fragments of the soluble MBP protein. One phase III study investigated the immunodominant MBP_{82–98} epitope in 612 secondary-progressive MS patients possessing genetic dispositions for reactivity to the MBP protein in particular⁷³. Over the two-year period, the epitope was safe and well tolerated during twice-yearly bolus administration, but no clinical benefit was observed. An alternate dose escalation study assessed the efficacy of a cocktail containing four MBP epitopes (30–44, 83–99, 130–144, and 140–154). While only 16 weeks long, a decrease in gadolinium-enhancing lesions was evident⁷⁴.

In T1D, many insulin variants have been clinically evaluated. A 2002 study investigated the subcutaneous administration of insulin to first degree family members of T1D patients to assess its ability to prevent disease, though no success was realized⁷⁵. Likewise, an altered peptide ligand of the insulin B chain did not confer effect for newly-diagnosed patients⁷⁶. Another study followed newly-diagnosed patients over 12 months as they were administered a proinsulin peptide⁷⁷. Here, patients receiving the peptide did not exhibit significant adverse events and their glucoregulatory insulin requirements did not increase over the interval, suggesting some therapeutic benefit. Another T1D study administered a HSP460 peptide to patients⁷⁸. While safe and well tolerated, the study was retracted due to biostatistical misconduct that distorted clinical differences between the treatment and placebo groups. In patients with Grave's disease, two peptides were intradermally injected for a Phase I study lasting 18 weeks⁷⁹. While injection site swelling and pain were observed, the formulation was generally well tolerated and led to an improvement of disease-related biomarkers.

In previously outlining allergen properties, a consistent trend of moderately sized (10–70 kDa), slightly negatively charged (PI 5–7.4), hydrophilic (GRAVY < 0) fractions were observable (Fig. 3). 61% of allergen extracts embodied all three qualities (Fig. 3b). Such trends are not as evident in autoantigens, where only 20% of entities met all criteria. Many autoantigens are also moderately sized, but Thyroglobulin and Thyrotropin are well over the 70 kDa threshold. Charge properties are widely dispersed; unlike with allergens, autoantigens such as PLP, MOG, MBP, and HSP40 exhibit PIs greater than 8, conferring a positive charge at physiological pH, while others such as GAD and Preproinsulin resemble

more neutral, allergen-like charges. GRAVY scores are also highly variable. Ranges across autoantigens are dispersed from -1.013 (MBP) to 0.559 (PLP).

Further inference about allergen and autoantigen discrepancies can be gained by taking a perspective of MW, PI, and GRAVY scores holistically, rather than discretely. Very few autoantigenic proteins embody the full trifecta of size, charge, and solubility properties embodied by allergens. For example, while MBP is moderately sized (17.8 kDa) and theoretically water soluble (GRAVY -1.039), it carries an extreme positive charge.. Preproinsulin is likewise moderately sized (12 kDa) and its PI mirrors allergens (4.97), but it presents with hydrophobicity (GRAVY 0.193) that creates the possibility of aggregation that could likewise be immunogenic⁸⁰. Such unfavorable characteristics may pose safety and tolerability obstacles to the prospects of full-protein autoantigen administration. Clinical ASIT researchers have rather incorporated smaller peptide epitopes of immunodominant antigen regions to incorporate greater specificity to formulations. Peptide epitopes harnessed for parental interstitial and IV ASITs may neutralize safety concerns that may arise from whole-protein autoantigen. MBP epitopes present charges that are slightly attenuated over full MBP. Thytotropin peptides cut down on the very large size of the autoantigen. But is there a cost to electing these variants? What are the tradeoffs of size, charge, and solubility properties in the context of autoantigen delivery?

1.8 Immunological Fates for Delivered Autoantigen are Determined by Format

The physical and chemical properties of delivered autoantigens are paramount in dictating distribution, kinetics, and immunological effect (Fig 2). These properties can together outweigh the primary intent of targeted interaction with autoreactive cells when they are not properly defined. The format of autoantigens can direct their distribution or retention in tissues, and these fates and ultimate immunological effects are dictated by physicochemical properties (Fig. 4).

1.8.1 Systemic Drainage

Small, hydrophilic autoantigens and epitopes are destined for loss to the blood pool and systemic circulation. Low lymphatic loading results. For this reason, safety can be maximized since antigen overstimulation and anaphylaxis can be avoided with low and infrequent dosing, however potency in desensitization is also reduced. Many autoantigen epitopes that have been clinically investigated are classifiable under this fate. One study using a peptide-based ASIT reported over 60% of patients experiencing 'treatment-emergent adverse events' resembling low to mid-grade hypersensitivity responses, which restricted dosing ranges⁷⁴. One of the main expressed benefits of ASIT is the improved safety profile over global immunomodulatory drugs; as such, the development of a new class of immunotherapy mandates safety as a main priority. The epitope-alone strategies reviewed here consistently fall well below the 10 kDa cutoff for lymphatic drainage. Clinical results from low dose interventions are favorable in early-phase studies as they are safe and well tolerated, however efficacy has yet to be fully carried over to a full FDA approval for autoantigen epitopes.

1.8.2 Lymphatic Transport

Lymph-draining formulations pose the opportunity to maximize autoantigen colocalization with directive immune populations. However, the resulting dose in lymphoid organs (such as the lymph nodes and spleen) is extremely important to consider in directing immunity, as parameters such as valency and density can highly impact the nature of the resulting immune response. Indeed, the seminal allergy desensitization work that led to autoimmune ASITs was based on a low, but progressively escalating dose over time. This regimen relates to the physicochemical properties of allergens; intermediately-sized (10–70 kDa), water-soluble allergen extract fractions are able to drain to and penetrate lymph nodes for the directed delivery of low valency antigen. Autoantigen physicochemical properties are not always conducive to the same routes. Immunogenic vaccines, for example, use adjuvants such as alum and MF59 to incorporate a high density of antigen in particulate formulations to trigger stimulatory processing and presentation by antigen-presenting cells⁸¹. The hydrophobic nature of some antigens such as PLP, MOG, and aquaporin 4 means these proteins are susceptible to aggregation after injection, which may have also led to their combination with adjuvants or particulate delivery systems. Large aggregates of autoantigen complexes can confer the same immunogenic effects as adjuvant delivery systems, causing antigen-presenting cells to present high densities of antigen at lymph nodes via active transport⁸⁰. Alternatively, in antibody-mediated autoimmunities, the aggregated autoantigen can invoke the formation of equally immunogenic immune complexes in the blood and interstitium⁸².

Unduly concentrated autoantigen loads pose danger and could lead to anaphylactic or adverse events. While hydrophobic aggregation is one mechanism of these unintended effects, amphiphilic autoantigens or peptide epitopes can cause fibrils that embody similar particulate properties⁸³. Even net hydrophiles are susceptible to the formation of particulates when regions of hydrophobicity are present⁸⁴. Each of these could favor innate arms of immune response near the site of administration.

1.8.3 Injection Site Retention

Some physicochemical properties may lead to the outright retention of formulations at the injection site. Human ECM restricts diffusion for entities that exceed 100 nm. While delivered autoantigens do not reach this threshold alone, significant aggregation or self-assembly can lead to macrostructures that are retained at the site of injection. Extreme positive charges can also retain autoantigen through electrostatic complexation with negatively-charged ECM components. Glatiramer acetate (Copaxone®) is a random chain of the four most prominent amino acids contained in MBP and is an FDA-approved injectable immunotherapy for MS patients⁸⁵. While this formulation's precise mechanism of action is unknown, we recently detailed that glatiramer acetate is highly retained at its site of injection through the electrostatic interaction of its many lysine residues with negatively-charged glycosaminoglycans such as hyaluronic acid⁸⁶. MBP, which is the autoantigen glatiramer acetate was initially designed to mimic, expresses an even more polarized PI of 11.57 and shows that charge should be considered even though it confers a favorable calculated hydrophobicity.

“Alum” salts such as aluminum hydroxide and aluminum phosphate have a long history as vaccine adjuvants, and have been tested as autoantigen delivery vehicles. Notably, formulations incorporating these adjuvants manifest as heterogenous microparticles between 0.5–10 μm in size⁸⁷, and as such they are widely reported to form nodules at the site of injection⁸⁸. These interstitial depots are thought to recruit innate immune cells, facilitate phagocytosis of autoantigen, and drive local responses^{88, 89}. Attempts have been made to harness these principles to treat autoimmunity, including a human trial delivering GAD-alum for the treatment of T1D⁹⁰. First demonstrations of the approach yielded positive results in a Phase I clinical trial⁹¹, though a follow up trial failed to hit its primary endpoint⁹². The group conducting this work has since moved to intranodal delivery of their formulation⁹³. Others have tethered proinsulin peptides to gold nanoparticles and injected intradermally (NCT02837094, data not yet reported), noting engagement of resident professional antigen presenting cells⁹⁴.

1.9 Strategies for Circumventing Delivery and Distribution Obstacles

1.9.1 Intranodal delivery

While the focus of our review is contained to the interstitial and IV administration of autoantigen-alone, several notable strategies exist to sidestep the inherent delivery constraints imposed by size, charge, and solubility. Interstitial injection may be favored over other routes for its ease and established history in allergy and vaccines, but intranodal injection directly administers antigen to target tissues. The principles governing the engineering of the lymphatic microenvironment have been thoroughly reviewed in the past⁹⁵ and may favor systemic immune tolerance⁹⁶. It has been maintained that biomaterial delivery systems are necessary to increase lymphatic retention and sustained kinetics after intranodal delivery. In one study, researchers administered 165 patients with alum-adsorbed grass pollen allergen either subcutaneously for 54 times over three years or just three intranodal injections over two months⁹⁷. Striking results were obtained where tolerance in the intranodal injection group was attained in just four months compared to the more intensive three-year plan. Further, these outcomes were achieved with just 1/1300 of the cumulative dose required for subcutaneous desensitization, and fewer adverse events were observed in the intranodal group. A study by the same group showed similar results for cat allergy. After just three intranodal injections over two months, nasal tolerance to cat allergen was increased 74-fold⁹⁸. These results highlight the importance of lymphoid organs as necessary target tissues in ASIT and illuminate the promise of direct intranodal administration.

1.9.2 Transdermal delivery

Another notable strategy for overcoming ASIT delivery constraints is the controlled release of autoantigen through transdermal patches. As previously mentioned, autoantigen epitopes below 10 kDa are likely to enter systemic circulation, thus bolus subcutaneous injections are not conducive to lymphatic loading and retention. Transdermal delivery offers a potential alternative to reformatting antigen for optimized trafficking. Here, the sustained release of epitopes can facilitate gradual lymphatic dosing. It is further hypothesized that transdermal administration can target resident Langerhans cells for active nodal transport⁹⁹. These

interventions have already shown some successes in the realm of autoimmunity. For MS, a study was conducted where patients were administered three immunodominant epitopes (MBP_{85–99}, MOG_{35–55}, and PLP_{139–155}) via the transdermal route¹⁰⁰. Over one year, patch-treated patients exhibited significantly fewer clinical signs of disease progression than the placebo-treated cohort, and researchers observed a 66.5% reduction in gadolinium-enhancing lesions over the interval.

1.10 Next-Generation Approaches to Formatting Autoantigen for Optimizing ASIT

Clinical explorations of allergen and autoantigen immunotherapies shed light on delivery considerations, which should be harnessed in aiming to desensitize autoimmunity. Lymphatic homing is paramount for evoking therapeutic effects. Size, solubility, and charge can each highly dictate the extent of lymphatic loading as well as the immunological results from delivery (Fig. 4). Physicochemical discrepancies between allergens and prominent autoantigens prompts the pursuit of next-generation ASITs that incorporate delivery systems and biomaterials to format the latter for optimal interfacing with tolerance mechanisms.

1.10.1 Soluble Delivery

Immunogens that are below 100 kDa and hydrophilic are purported to be naturally tolerogenic⁶⁷. As we reviewed, many clinical ASITs for autoimmunity have pursued peptide epitopes as autoantigens. Their small size is conducive to the size cutoff for tolerogenicity, but these autoantigen variants are not ideal for achieving high lymphatic loading and efficacy. Apitope lies at the forefront of clinical efforts to advance epitope delivery with their altered peptide ligands. Apitope's candidate ATX-MS-1467 has shown positive effects in MS⁷⁴ and the company has more recently promoted candidates for applications such as Graves' Disease and factor VIII intolerance^{79, 101}. These contemporary focal points may highlight an important niche for peptide epitopes in systemic inflammatory disorders.

Although formatting autoantigens and their epitopes to more closely mirror size and solubility properties for enhanced lymphatic delivery may amplify prospects of immune tolerance, inherent physicochemical autoantigen properties cannot be changed. Net molecular properties can, however, be modified using appropriate chemical modifications or carriers. Our group has employed polymers to solubilize antigen epitopes and control their valency properties to target lymphatic drainage and invoke immune tolerance pathways^{102–109}. The size adjustment conferred to autoantigens enables exclusion from direct absorption into circulation while the solubility and spacing of antigen engages mechanisms of tolerance.

1.10.2 Colloidal Delivery

One strategy for overcoming suboptimal physicochemical constraints for autoantigens has been to incorporate these handles into colloidal delivery vehicles. A vast majority of autoimmune-targeted ASITs have leveraged nanoparticulate delivery systems to deliver antigens and immunomodulatory drugs to lymphatics and the liver. Companies such as Cour Pharmaceuticals and Selecta Biosciences have embodied such approaches by encapsulating

antigen within polymeric carriers, although autoimmune ASIT initiatives have paused with focus shifting to disease states like Celiac (Cour)¹¹⁰ and anti-drug antibodies (Selecta)¹¹¹. Each platform technology has seen promising results in diseases involving responses to external factors that are encountered.

Colloidal delivery systems incorporate polymers, metals, or emulsions to facilitate interaction with tissue-resident pAPCs as well as drainage to lymphoid organs. Notably, many vehicle materials have been adopted from more mature fields such as vaccines or cancer immunotherapy. Such delivery systems can invoke innate immune responses that lend to applications where inflammation is desirable^{112, 113}. Thus, these have often utilized the ability of colloids to deliver drugs, such as immunosuppressants, to counteract any inherent immunogenicity and to direct downstream response pathways. Next-generation strategies for overcoming vehicle effects involves developing functional vehicles that are formulated using immunologically instructive signals as constitutive building blocks^{114, 115}.

1.10.3 Depot Delivery

Immunologically-directive microenvironments are a powerful tool for evoking either inflammatory or regulatory responses^{116, 117}. Several companies are leveraging microenvironments to bolster tolerogenesis in ASIT. Anokion is one example where liver or spleen homing functionalities are incorporated to enrich antigens in target organs and form ‘depots’, relying on resident immune cells to dictate responses¹¹⁸. Another strategy for autoimmune ASITs is to intentionally retain autoantigen at the site of administration. Localization of autoantigens at the site of administration recruits pAPCs and cognate immune cells, encouraging processing at a locus that can be engineered with immunomodulatory signals. Though not covered in this review, DNA vaccines such as Tolerion’s TOL-3021 candidate for T1D may also act in a depot manner by delivering instructions for the production of proinsulin at the site of intramuscular injection¹¹⁹.

Though not delivering autoantigen, recent work by the Hubbell group also showed the power of localized immunotherapies by homing anti-TNF- α antibodies to collagen-rich inflammatory microenvironments and suppressing a mouse model of RA¹²⁰. By skewing the inflammatory cues at the site of autoimmune response and autoantigen processing, disease was stifled. Depot-based autoantigen delivery systems may be further refined with the exploration of more immunological cues to control the inflammatory fates of autoantigen processing *in situ*.

1.11 Conclusions

ASIT represents a compelling step toward precision medicine to treat autoimmune diseases. To date, many formulations have sought to interrupt the vicious cycle of autoimmunity by delivering the same autoantigens and epitopes implicated in disease. To date, however, no comparable ASITs are available for treating autoimmunity. ASIT underpinnings could take a cue, from historical successes in allergy, where a plethora of FDA-standardized allergen desensitization regimens are available. The physicochemical properties of allergen extracts, however, differ substantially from those of autoantigens and their constitutive epitopes. Allergens are generally found between 10–70 kDa in size with neutral or slightly negative

charge and good water solubility. Autoantigens and their epitopes rarely express all three of these qualities, and thereby confound their local transport *in vivo*. Poor solubility, for example, might have driven the ASIT field toward particulate delivery systems. The physicochemical properties of autoantigens compel further engineering of molecular properties and delivery systems for developing ASITs to treat human autoimmune diseases. ASITs of the future will do well to modify physicochemical autoantigen properties to mirror those of clinically proven allergen extracts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1.1**Highlights**

- Current therapies for autoimmune disease broadly suppress the immune system. Antigen-specific immunotherapy (ASIT) seeks to introduce precision to these treatments by delivering the same autoantigens that are attacked by autoreactive cell populations.
- ASIT strategies parallel those employed by allergen desensitization regimens. However, many standardized desensitization regimens are clinically available for allergy, while no autoimmune ASITs have been FDA-approved to date.
- Allergens and autoantigens differ substantially in physicochemical properties including size, solubility, and charge. Each of these properties are paramount in determining immunological fates.
- Drug delivery and biomaterial design strategies are likely necessary to overcome detrimental autoantigen properties and optimally interface with the immune system.

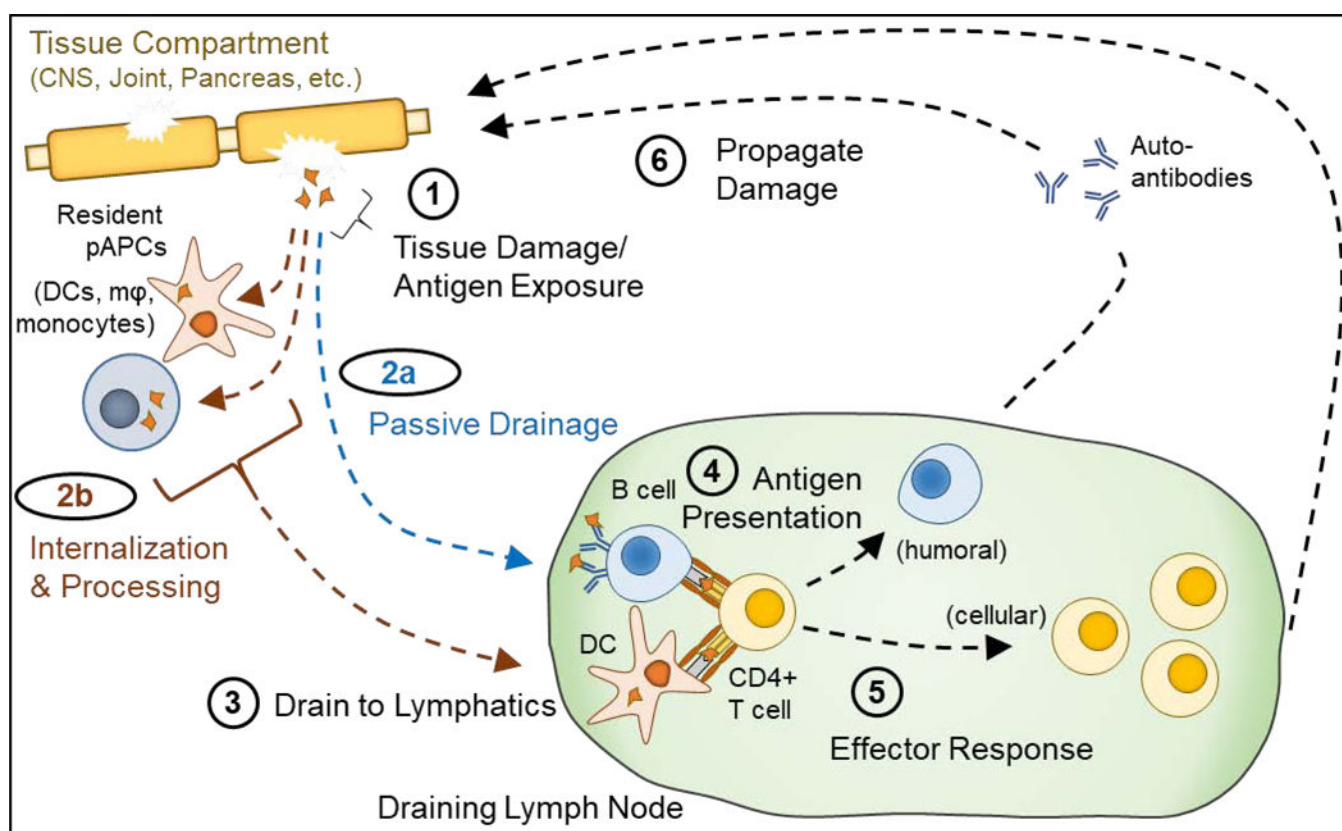


Figure 1.

Mechanisms of peripheral autoimmunity. Damaged tissue releases autoantigen (1) that either drains directly to lymphatics (2a) or is internalized by resident professional antigen presenting cells (pAPCs, 2b and 3). In the secondary lymphoid organs, follicular pAPCs process and present antigen to naïve T helper cells in the presence of costimulatory signals (4). Naïve T cells become stimulated to proliferate and differentiate a cellular or humoral effector response (5). These activated effectors egress from lymphatics and eventually home to antigen-rich tissue compartments once more, where more damage can occur and the cycle of autoimmunity is renewed (6).

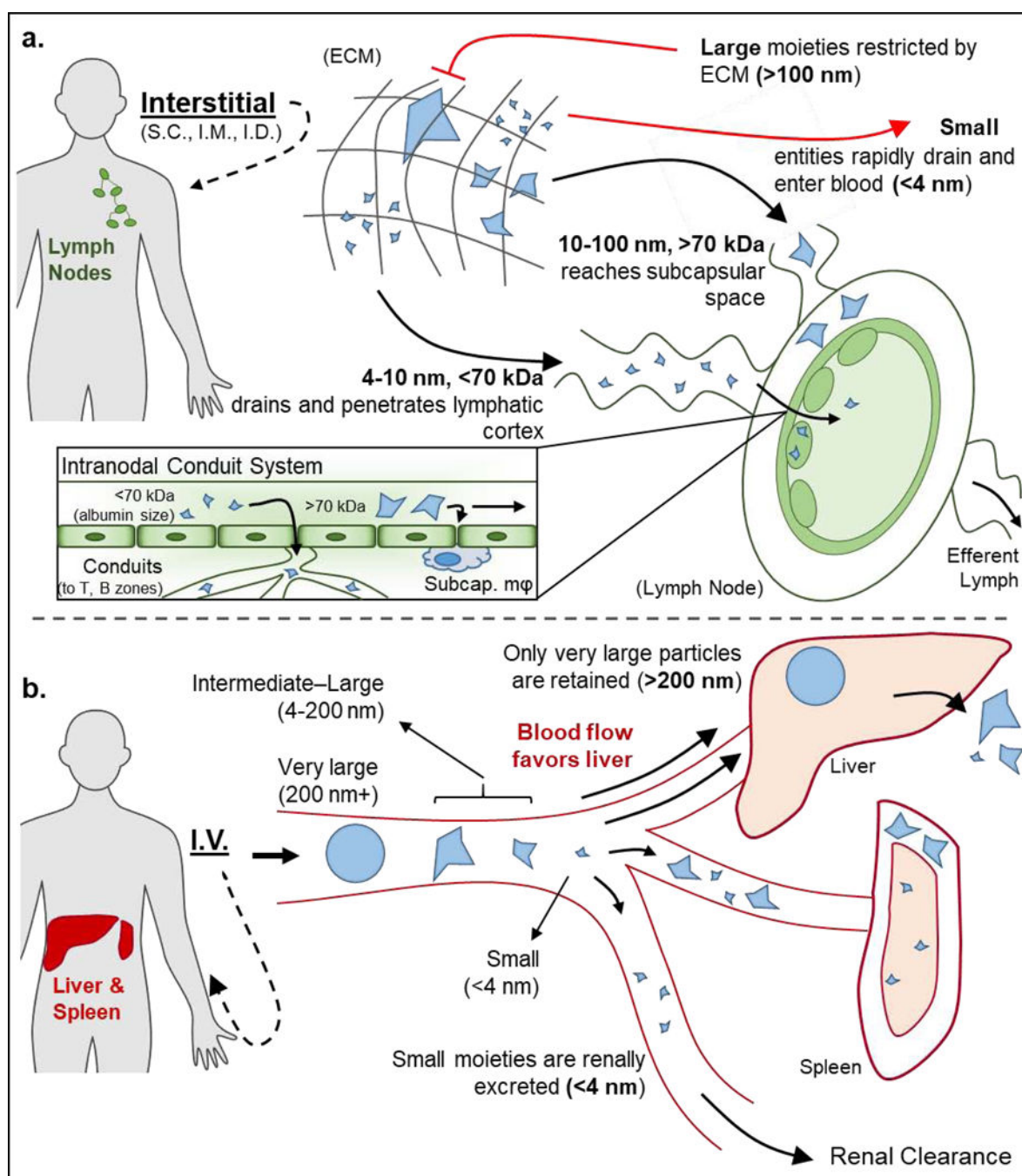
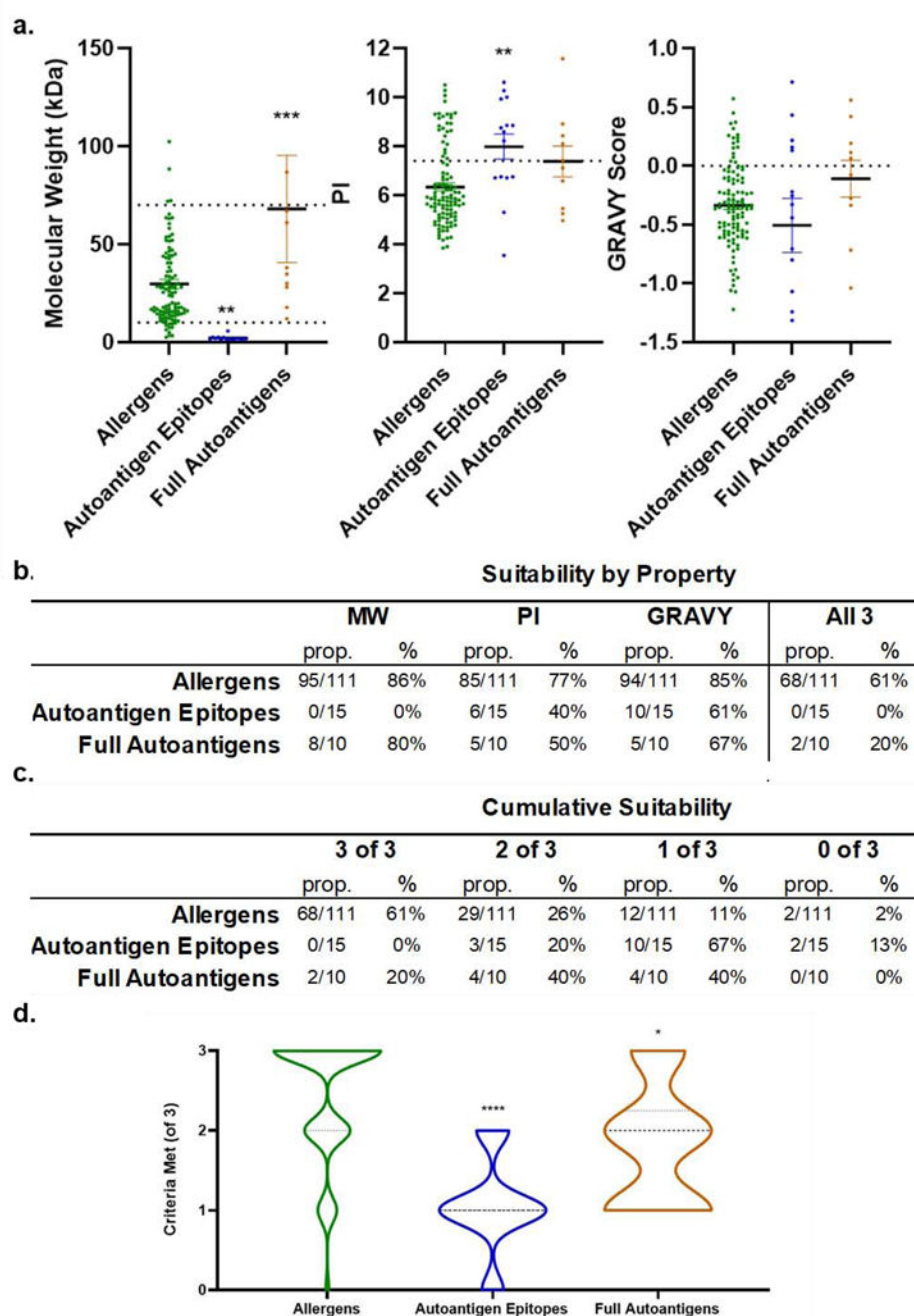


Figure 2.

Parental routes of administration for influencing immunity. **a.** Interstitial antigen transport is dictated by two major barriers. First, the ECM can serve to restrict the trafficking of large entities greater than 100 nm in size. Small antigens less than 4 nm are subject to systemic circulation. Compounds between 4–100 nm can travel by convective transport to the lymph node, but only molecules between 4–10 nm are capable of traversing conduits into the directive T and B cell zones of lymph node parenchyma. **b.** IV administration poses the opportunity for antigen to reach the liver, spleen, or kidneys. Particles greater than 200 nm

preferentially accumulate and are retained in the liver, but 4–200 nm particles can also be processed via this route. The same 4–200 nm particles can reach the spleen, and penetration into its cortex is dictated by the same principles as the lymph nodes. Finally, antigens less than ~4 nm are subject to clearance by renal excretion.

**Figure 3.**

Comparative analysis of molecular weight, PI, and GRAVY score between standardized allergens, clinically tested autoantigen epitopes, and full, native state autoantigen proteins. **a.** Molecular weights are reported for each entity (left). Dotted lines represent the 10 (bottom) and 70 kDa (top) cutoffs for entities to evoke optimal lymphatic action. Data points for one allergen (Api m 12) and one full autoantigen (Thyroglobulin) are omitted from the graph but included for statistical analysis. PI is also reported (middle), where the dotted line indicates physiological pH at 7.4. GRAVY scores (right) are mapped as indicators of water solubility.

A dotted line at score 0 generally delineates solubility (negative scores) against insolubility (positive scores). **b.** Allergens and autoantigen variants were assessed for “suitability”, defined by entities in the class falling within highlighted physicochemical boundaries. The molecular weight window was defined to be 10 to 70 kDa. The PI threshold was established for entities at or below pH 7.4. GRAVY scores less than 0 were considered suitable. Results are expressed both in terms of proportion (prop.) of the total class pool, as well as percent suitable. **c.** Analysis was also performed to determine the number of “suitable” properties for each class of immunogen. **d.** Cumulative suitability is graphically represented by violin plots to illustrate the distribution of suitability among entity classes. Statistical analyses were performed by ordinary one-way ANOVA, comparing against the allergens class as a control column and using Dunnett’s multiple comparison correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

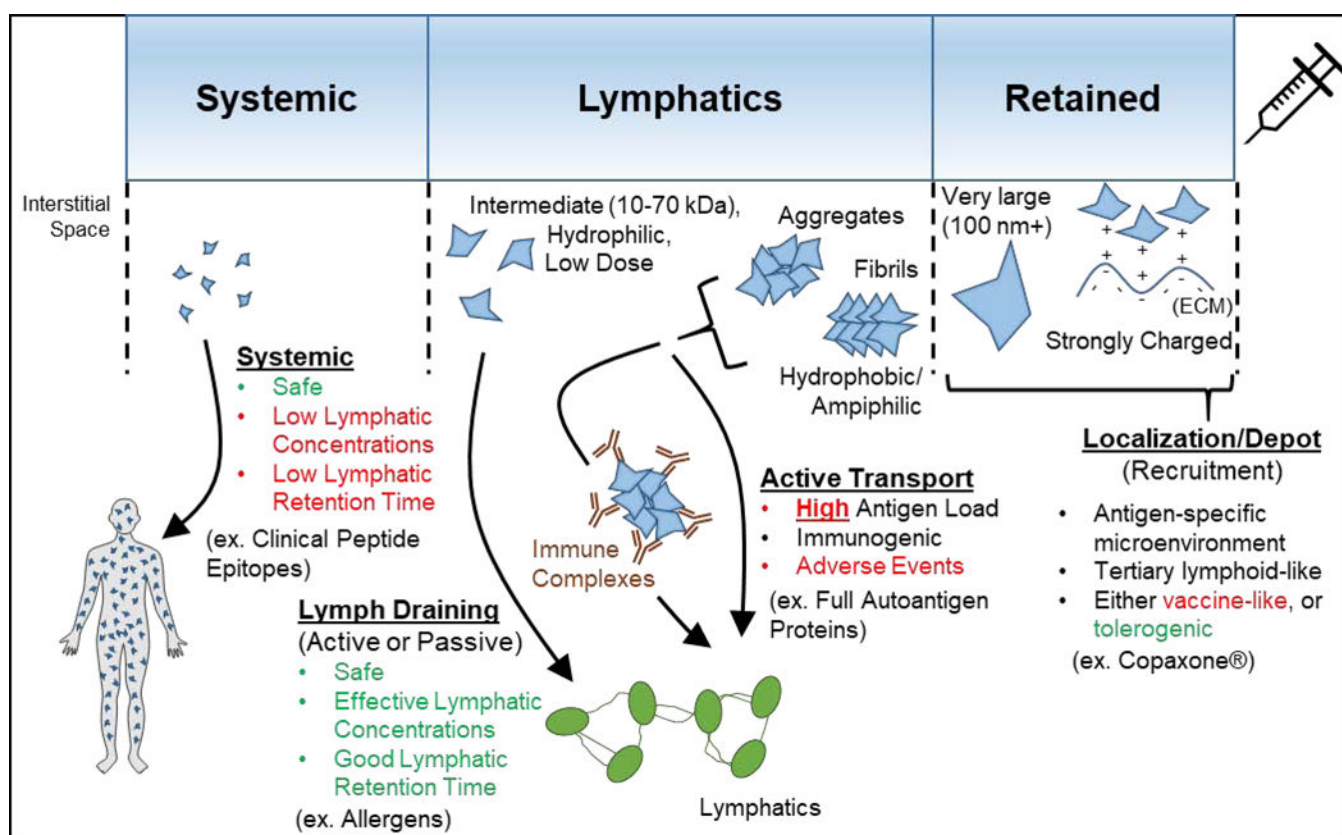


Figure 4.

Autoantigen format dictates transport and can direct the resulting immune response. Small, hydrophilic entities that are delivered interstitially may replicate allergen regimens at low doses, but the peptide epitopes used are typically too small, and thus subject to systemic exposure. As a result, low lymphatic loading and retention occurs, and efficacy becomes elusive. Conversely, lymphatic-draining formulations can be harnessed with intermediately-sized (10–70 kDa), hydrophilic moieties that most closely resemble the allergen extracts reviewed above. Antigens with this format are excluded from the blood and can achieve loading into lymph nodes in a low-density format that may be conducive to immune tolerance. Conversely, antigens that are net-hydrophobic or prone to fibril formation form immune complexes or are actively transported to the lymphatics, which entices an immunogenic response that introduces risks for autoimmune ASITs. Finally, large entities exceeding 100 nm are restricted by the ECM and retained near the site of injection. Likewise, entities with a strong positive charge can be retained through electrostatic interactions with the negatively charged ECM. When formulations are retained, the innate immune system is recruited, and the microenvironment (stimulatory or tolerogenic) directs the immune response.

Table 1.

Physicochemical properties of model allergens. Molecular Weight (MW), Isoelectric Point (PI), and Grand Average of Hydropathy (GRAVY) are reported for protein extract fractions where full amino acid sequences are available. Allergen extract fractions were identified by searching allergen.org for the major allergen (ex. *Felis domesticus*). MW and PI were calculated using the PepCalc.com online tool. GRAVY score was determined using the calculator at gravity-calculator.de.

Allergen	Extract Fraction	GenBank ID	MW (kDa, calculated)	PI (calculated)	GRAVY Score (calculated)
<i>Phleum pratense</i> (Timothy Grass)	Phl p (avg)		25.8	5.90	-0.179
	Phl p 1		28.5	6.12	-0.433
	Phl p 2	P43214.1	13.4	4.28	-0.124
	Phl p 4	ABB78007.1	58.2	9.37	-0.127
	Phl p 5	CCD28287.1	31.1	7.5	0.195
	Phl p 6	CAA81608.1	13.9	5.08	0.041
	Phl p 7	CAA76887.1	8.7	3.84	-0.283
	Phl p 11	Q8H6L7.1	33.7	6.23	-0.562
	Phl p 12	CAA54686.1	19.1	4.78	-0.141
	Phl p 13*	CAB42886.1	30	7.33	-0.364
<i>Arachis hypogaea</i> (Peanut)	Ara h (avg)		21.4	7.35	-0.316
<i>Arachis hypogaea</i> (Peanut)	Ara h 1	AAB00861.1	65.4	6.91	-1.07
	Ara h 2	AAN77576.1	13.4	5.91	-1.22
	Ara h 3	AAD47382.1	54.1	5.29	-0.891
	Ara h 5	AAD55587.1	17.8	4.26	-0.078
	Ara h 6	ABQ96216.1	2.6	6.1	-0.821
	Ara h 7	AAD56719.1	19.6	5.8	-1.015
	Ara h 8	AAQ91847.1	17	4.73	-0.365
	Ara h 9	ABX56711.1	11.6	9.15	0.572
	Ara h 10	AAU21499.2	9.5	10.07	0.153
	Ara h 11	AAZ20276.1	10.8	10.5	0.451
	Ara h 14	AAK13449.1	18.4	10.28	0.259
	Ara h 15	AAU21501.1	16.9	9.22	0.238
<i>Felis domesticus</i> (Domestic Cat)	Fel d (avg)		21.0	5.09	-0.170
	Fel d 1	AAC37318.1, AAC41616.1	17.8	4.5	0.265
	Fel d 2	CAA59279.1	38.3	5.27	-0.340
	Fel d 3	AAL49391.1	7.7	4.37	-0.584
	Fel d 4	AAS77253.1	15.9	4.62	-0.050
	Fel d 7	ADK56160.1	20.2	4.56	-0.521
	Fel d 8	ADM15668.1	25.9	7.24	0.209

Table 2.

Size, charge, and solubility properties for autoimmune antigens and interstitial/IV epitope treatments that have been clinical investigated. Property values were calculated using the same methodologies as in Table 1.

Disease	Epitopes	GenBank ID/ Reference	MW (kDa, calc.)	PI (calc.)	GRAVY Score (calc.)	Clin. Resp.
MS	Proteolipid Protein	AAA60117.1	30	8.11	0.559	
	Myelin Oligodendrocyte Glycoprotein	CAA52617.1	28.2	8.43	0.114	
	Myelin Basic Protein	AAC41944.1	17.8	11.57	-1.039	
	82–98	73	2	6.75	-0.441	–
	30–44	74	1.7	9.94	-0.800	++
	83–99	74	2	8.85	-0.329	++
	130–144	74	1.7	10	-1.067	++
T1D	140–154	74	1.6	8.59	0.160	++
	Glutamate Decarboxylase	AAB59427.1	67	7.11	-0.334	
	Preproinsulin	AAA59172.1	12	4.97	0.193	
	Insulin	75	5.8	5.3	0.217	–
	Insulin B (9–23)	76	1.7	6.71	0.433	–
	Proinsulin peptide	77	1.9	8.75	0.133	++
	Heat Shock Protein 60	P10809.2	61.1	5.46	-0.076	
RA	DiaPep277	78	2.4	3.54	0.713	*
	Heat Shock Protein 40	BAA12819.1	38	8.91	-0.715	
Grave's	Thyrotropin Receptor	AAA36783.1	86.8	6.58	0.066	
	5D-K1	79	2.5	10.61	-1.314	+
	9B-N	79	1.3	6.71	-0.253	+
Hashimoto's	Thyroglobulin	CAA29104.1	304.8	5.25	-0.276	
Neuromyelitis Optica	Aquaporin 4	AAH22286.1	34.8	7.41	0.420	

Here, clinical responses are denoted with the following identifiers:

(–) no therapeutic outcome

(+) biomarker improvement

(++) clinical improvement.

* The DiaPep277 study was retracted due to biostatistical misconduct.