



Immunogenicity Challenges Associated with Subcutaneous Delivery of Therapeutic Proteins

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Abstract

The subcutaneous route of administration has provided convenient and non-inferior delivery of therapeutic proteins compared to intravenous infusion, but there is potential for enhanced immunogenicity toward subcutaneously administered proteins in a subset of patients. Unwanted anti-drug antibody response toward proteins or monoclonal antibodies upon repeated administration is shown to impact the pharmacokinetics and efficacy of multiple biologics. Unique immunogenicity challenges of the subcutaneous route have been realized through various preclinical and clinical examples, although subcutaneous delivery has often demonstrated comparable immunogenicity to intravenous administration. Beyond route of administration as a treatment-related factor of immunogenicity, certain product-related risk factors are particularly relevant to subcutaneously administered proteins. This review attempts to provide an overview of the mechanism of immune response toward proteins administered subcutaneously (subcutaneous proteins) and comments on product-related risk factors related to protein structure and stability, dosage form, and aggregation. A two-wave mechanism of antigen presentation in the immune response toward subcutaneous proteins is described, and interaction with dynamic antigen-presenting cells possessing high antigen processing efficiency and migratory activity may drive immunogenicity. Mitigation strategies for immunogenicity are discussed, including those in general use clinically and those currently in development. Mechanistic insights along with consideration of risk factors involved inspire theoretical strategies to provide antigen-specific, long-lasting effects for maintaining the safety and efficacy of therapeutic proteins.

1 Introduction

1.1 Introduction to Immunogenicity of Therapeutic Proteins

Immunogenicity is the propensity of a therapeutic protein to induce unwanted immune response toward itself or endogenous proteins [1]. An anti-drug antibody (ADA) response can develop after a single dose and upon repeated administration of a therapeutic protein. ADA with neutralizing or binding capabilities directly or indirectly affect therapeutic protein efficacy, respectively [2]. Neutralizing antibodies targeting active site(s) on the protein can cause direct loss of efficacy. Several important examples underscore the impact of ADA against a therapeutic protein. Hemostatic efficacy

Key Points

Immune response toward subcutaneously administered proteins likely entails two waves of antigen presentation by both migratory skin-resident and lymph node-resident dendritic cells, which likely drive immunogenicity.

Subcutaneous route of administration as a factor of immunogenicity is intertwined with product-related risk factors including impurities, biophysical characteristics, aggregation, and subvisible particle concentration.

Some promising immunogenicity mitigation strategies in the investigative research stage are tolerance induction, T cell engineering, protein de-immunization and tolerization, use of chaperone molecules, and combination approaches.

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of factor VIII (FVIII) is compromised by development of anti-FVIII antibodies with neutralizing activity (termed ‘inhibitors’) in approximately 30% of severe hemophilia A (HA) patients [3, 4]. Neutralizing antibody development in mild to moderate HA patients led to spontaneous bleeding episodes due to cross-reaction with endogenous FVIII [5]. Clinical response to Pompe disease treatment is negatively impacted by sustained antibody development toward recombinant human acid- α glucosidase (rhGAA), which is more common in infantile-onset patients with negative status for cross-reactive immunological material [6]. Binding ADA can impact pharmacokinetics and pharmacodynamics (PK/PD) of therapeutic proteins by increasing clearance, and anti-adalimumab antibody response is associated with decreased adalimumab serum concentrations and diminished therapeutic response in rheumatoid arthritis patients [7, 8]. Anti-infliximab antibodies increase infliximab clearance, leading to treatment failure and acute hypersensitivity reactions [9].

Although less frequent, immunologically based adverse events have been associated with ADA development during replacement therapy, such as recombinant erythropoietin (EPO), thrombopoietin, interferon (IFN)- β , and factor IX [10–16]. Increased relapse rate during recombinant IFN β therapy has been observed for multiple sclerosis patients that develop neutralizing anti-IFN β ADA, and multiple studies have found neutralizing ADA against recombinant IFN β_{1a} and IFN β_{1b} are cross-reactive and neutralize endogenous IFN β [12, 17–20]. Other well-known examples include pure red-cell aplasia and thrombocytopenia development in patients receiving recombinant EPO or thrombopoietin, respectively, associated with detection of neutralizing ADA that cross-react with endogenous protein [13, 14, 21].

Food and Drug Administration (FDA) Guidance for Industry published in 2014 presents a risk-based approach for evaluation and mitigation of immune responses to therapeutic proteins that limit efficacy and negatively impact safety profiles [1]. Efforts to assess risk of immunogenicity have considered the currently known influential factors of immunogenicity, including a multitude of product-, treatment-, and patient-related factors. Examples of patient-related factors are age, immune status, genetic factors such as human leukocyte antigen (HLA) haplotype, and autoimmune condition [22]. Product-related factors include protein structure, stability, and dosage form, and intrinsic features of recombinant proteins can impact immunogenicity, such as sequence variation, post-translational modifications (PTM), immunodominant epitopes, and cellular expression system [23, 24]. Treatment-related factors include dose, duration and frequency of treatment, and route of administration [23]. Subcutaneous (SC) administration has unique immunogenicity challenges for some products compared to intravenous

(IV) administration that are likely due to differences in immune system exposure and antigen presentation mechanisms [25, 26]. Vaccine development elucidated the capacity of antigens to induce a more efficient and effective host immune response following SC administration compared to IV infusion, likely a consequence of frequent encounter by dynamic skin antigen-presenting cells (APCs) [26–29]. Understanding how route of administration and product-related factors impact immunogenic risk will be critical for mitigating immunogenicity and designing safer biologics for SC delivery.

1.2 Anatomy of the Subcutaneous Space and Skin-Resident Immune Cells

1.2.1 The Epidermis and Langerhans Cells

Human skin is composed of three main layers: the epidermis, dermis, and hypodermis or SC fat. In the epidermis, keratinocytes form a layer of stratified epithelium with tight junctions to provide water-impermeable barrier protection, and cytokine secretion by keratinocytes promotes inflammation during infection or injury [27, 30, 31]. Other featured cells are melanocytes producing melanin pigment, Merkel cells communicating with neurons, memory T cells, and infiltrating innate immune cells [32]. Langerhans cells (LCs) are key APCs in the epidermis that spread dendritic processes to probe for and recognize invading antigens [33]. LCs develop from yolk sac-derived progenitors and acquire a dendritic phenotype and morphology immediately following birth, then in situ proliferation and keratinocyte-derived interleukin (IL)-34 help maintain their population [27, 34, 35]. Tight cellular connections render epidermal stromal and immune cells primarily fixed in place, until LC motility upon maturation is prompted by downregulation of E-cadherin interactions with keratinocytes [27, 30].

Adaptive immune responses initiated by LCs are diverse. Upon maturation, LCs migrate to regional lymph nodes to induce T helper 2 (T_H2) polarization of naïve $CD4^+$ T cells via thymic stromal lymphopoietin (TSLP) signaling, as well as T helper 1 (T_H1) polarization to IFN γ -producing $CD4^+$ T cells [36, 37]. LCs are also involved in T follicular helper (T_H) differentiation and germinal center (GC) B cell responses [38]. A major role for LCs in cellular immunity is differentiation of naïve $CD8^+$ T cells into potent cytotoxic T lymphocytes (CTLs), but they have contrasting tolerogenic functions in the skin [37, 39]. LCs suppress contact hypersensitivity by interaction with cognate $CD4^+$ T cells in the context of IL-10 [40]. They induce multiple types of regulatory T (T_{reg}) cells during epicutaneous allergen immunotherapy in previously sensitized mice [41].

1.2.2 The Dermis and Dermal Dendritic Cells

The basement membrane regulates protein and cell movement between the epidermis and dermis [30, 42]. The major structural and functional protein components of the skin extracellular matrix (ECM) are produced by dermal fibroblasts [30, 43]. Intertwined collagen and elastin fibers provide structure and elasticity and facilitate migration of immune cells, such as dermal dendritic cells (DCs), along a 'highway system' to perform immunosurveillance [27, 30]. Compared to DCs, dermal macrophages have poor antigen presenting capacity and migratory activity but high phagocytic activity, thus they clean up debris to maintain homeostasis and facilitate wound repair/resolution [27]. Skin-resident macrophages arise from precursor pools established prenatally and from blood monocytes after birth, then reside in skin for long periods to provide early host defense [27, 44]. During immune response, dermal blood vessels facilitate recruitment and infiltration of circulating innate and effector immune cells into the skin. Endothelial cells regulate extravasation by production of cytokines, chemokines, and leukocyte adhesion molecules [30]. Macrophages also initiate infiltration of granulocytes into the skin, and perivascular macrophages are the main source of chemoattractants (CXCL1, CXCL2) in the dermis promoting neutrophil extravasation at post-capillary venules in response to bacterial infection [45]. Monocytes are recruited to the skin during homeostasis and in response to infection to differentiate into macrophages or myeloid DCs [30]. Effector cells recruited to the skin temporarily or that become skin-resident cells include CD8⁺ cytotoxic T cells, CD4⁺ T_H cells, and CD4⁺ T_{reg} cells [30].

The conventional DC (cDC) class is highly abundant in the healthy dermis, with major human and mouse subsets being CD1c⁺ and CD11b⁺ cDCs, respectively [27]. Under resting conditions, cDCs acquire self-antigens in the periphery and undergo homeostatic maturation followed by migration to lymph nodes licensed by morphological and phenotypical changes, including upregulation of major histocompatibility complex II (MHC II) [27]. By presentation of skin-derived self-antigens to T cells, cDCs can eliminate autoreactive T cells to maintain peripheral tolerance [46]. Maturation of cutaneous cDCs upon pathogen stimulation is unique from homeostatic maturation where co-stimulatory molecules are upregulated, and cDCs migrate to lymph nodes to promote differentiation and proliferation of naïve antigen-specific T cells [27]. Dermal CD1a⁺ DCs in the upper human dermis can induce T_H2 polarization of naïve CD4⁺ T cells as well as differentiation of naïve CD8⁺ T cells into potent CTLs, although not as effective as LCs [37]. The CD14⁺ DC subset produces key anti-inflammatory cytokines, IL-10 and tumor growth factor- β (TGF β),

and a role for CD14⁺ DCs in B cell differentiation is suggested by their ability to induce CD4⁺ T cell production of T_H-associated chemokine CXCL13 [37].

1.2.3 The Hypodermis or Subcutaneous Fat

Underlying the dermis, the SC fat layer contains nerves, blood vessels, and lymphatic vessels, along with adipocytes that sequester potentially inflammatory lipids and produce proinflammatory cytokines upon stimulation [30]. Adipose tissue is separated into fat cell chambers by septa of connective tissue with heterogeneous structures in upper, middle, and lower layers of the hypodermis [47]. Connective tissue septa comprise the ECM and SC tissue architecture, which is composed of fibrous proteins and viscoelastic gel with the main components being collagen, elastin, glycosaminoglycans (GAGs), and proteoglycans [43, 48, 49]. Highly polar and negatively charged GAGs, including hyaluronic acid, are vastly abundant and contribute to the net negative charge of the ECM [50]. Along with high viscosity in the interstitium, collagen and hyaluronic acid constitute a major barrier to protein movement and dispersion in the SC ECM, and injection volume is limited [48, 51]. Binding of hyaluronic acid to water, creating a gel-like substance, and low hydraulic conductivity of the ECM consequently limit dispersion in the SC space [52, 53]. In the SC space, therapeutic proteins could encounter diverse cell populations including invading dermal DCs, LCs, or innate and effector immune cells recruited from circulation or lymph nodes.

1.2.4 Skin-Derived Immune Cell Migration

LCs, dermal CD1a⁺ DCs, and dermal CD14⁺CD1a⁻ DCs are skin-derived migratory DC subsets in human axillary lymph nodes that mediate transport and presentation of skin-derived antigens [54]. Upon exit to draining lymph nodes (DLNs), dermal DCs are of a mature phenotype, and their functional specializations, including T_H cell polarization and cross-presentation ability, remain unchanged by migration into lymph nodes [54, 55]. CCR7 signaling is required for DC migration under steady-state and inflammatory conditions. Through CCR7-mediated chemotaxis, migratory skin-derived DCs enter into lymphatic vessels in the skin in response to chemokine (CCL21) expression by lymphatic endothelial cells [56–58]. CCL17-deficient mice have demonstrated that CCL17 is strongly associated with LC migration to DLNs, and CCL17 also sensitized activated bone marrow-derived DCs in vitro for CCR7- and CXCR4-dependent migration [59]. Furthermore, T_H2 differentiation of naïve CD4⁺ T cells by CD11b^{high} migratory DCs required CCL17 expression, along with CCR7 upregulation, in response to TSLP signaling [60]. Mechanisms and stimuli

for cell migration out of the skin are important elements of the immune response to subcutaneously administered proteins.

1.3 'First-Pass' Interactions with Immune System Following Subcutaneous and Intravenous Delivery

Immunogenicity differences based on route of administration could arise from disparities in initial interactions between protein and the immune system as well as subsequent antigen processing and presentation mechanisms. First-pass interactions for SC proteins could occur in the injection site with immune cells, including skin-resident DCs, monocyte-derived DCs, and possibly innate or effector immune cells recruited into the skin during immune response [38, 61]. First-pass interactions could also occur later in the lymphatic system. Unlike IV administration, subcutaneously administered protein has to be absorbed from the injection site into the blood circulation [62]. Proteins or peptides less than 16 kDa in size can be transported from the SC injection site to systemic circulation by diffusing into blood capillaries; however, for larger molecular weight (MW) proteins, lymphatic uptake also plays a role in transport to systemic circulation [49, 63, 64]. A likely location for absorption is at initial lymphatics that start from 'blind stumps' and have leakier vessel walls than blood capillaries [64–66]. Under increased interstitial fluid pressure, stretching of connective tissue fibers creates tension on the anchoring filaments connecting endothelial cells to collagen, leading to opening of lymphatic lumen and intercellular channels [66, 67]. At this point, interstitial fluid containing water, macromolecules, and possibly therapeutic proteins, easily enters lymphatic capillaries with little protein exclusion [68].

Lymph drains into large lymphatic trunks then lymphatic collectors in the hypodermis that lead to the first DLN [49]. Lymph passes through at least one lymph node; thus, first-pass interactions between protein and immune cells could occur in DLNs, which constantly drain and monitor skin-derived antigens [65, 69]. Upon arrival in DLNs, lymph-borne protein antigen can encounter skin-derived lymph node-resident DCs located in close proximity to lymphatic vessel entry points, an ideal position for antigen uptake [69]. Thus, subcutaneously administered protein may encounter dynamic skin-derived APC populations that are highly specialized for antigen processing, presentation, and lymph node migration [70, 71].

Following IV administration, first-pass interactions between blood-borne protein and immune cells would occur more diffusely within systemic circulation and secondary lymphoid organs. IV administered albumin in mice had rapid distribution throughout the body, with accumulation in the liver area observed within minutes [72]. First-pass

encounters of blood-borne protein could be with soluble factors, such as preexisting ADAs or binding proteins [73]. Upon ADA binding, immune complex (IC) formation may initiate additional distribution pathways or accelerated clearance [74]. Blood-borne protein will likely encounter cells of the mononuclear phagocyte system (MPS), comprising circulating blood monocytes, DCs, and tissue macrophages that make intimate connections with endothelial and epithelial cells [75]. Following IV administration, biodistribution of aggregated fluorescently labeled mouse serum albumin revealed fluorescence hotspots in the liver, lungs, and spleen, suggesting entrapment in organs with the MPS [72]. The liver may be a key site for first-pass interactions with tissue macrophages, called Kupffer cells, that clear soluble proteins and aggregates from circulation and internalize antigen-antibody complexes using Fc receptor (FcR) and complement receptor (CR) recognition [76]. Beyond their role in phagocytosis and sequestration of antigen, thought to support hyporesponsiveness, Kupffer cells may be able to promote antigen-specific immunity [77]. Thus, circulating proteins, aggregates, or ICs are likely to be captured by Kupffer cells, but it is not entirely clear whether induction of immunity and/or tolerance responses would occur.

Noteworthy first-pass interactions could also occur in the spleen, a secondary lymphoid organ with lymph node-like structures (white pulp [WP]) and functions [78]. The spleen WP contains distinct lymphoid sheaths based on chemokine signaling: B cell populations reside in B cell follicles, while CCL19 and CCL21 attract CCR7⁺ T cells and DCs to the periarticular lymphoid sheath (PALS) or T cell zone surrounding the central arteriole [78–81]. Protein antigen is delivered into the spleen at terminal arterioles ending in sinuses in the marginal zone (MZ) (or perifollicular zone) surrounding the WP [82]. There, protein could encounter MZ-resident APCs, such as macrophages, DCs, and B cells, located in ideal positions to screen and respond to blood-borne antigens via T-independent and T-dependent mechanisms [80, 83]. MZ macrophages and B cells recognize antigen by CRs or pattern recognition receptors (PRRs), and FcR or CR interaction on spleen macrophages mediates clearance of protein antigen arriving as ICs bound to erythrocytes [82, 84]. Protein antigen may accumulate in splenic MZ following IV infusion; for example, FVIII accumulated in splenic MZ of HA mice co-localized with metallophilic macrophages and CD11c⁺ DCs [85]. When accumulated in the MZ or spleen parenchyma, known as red pulp (RP), blood-borne protein may be phagocytosed and processed by sinusoidal macrophages, pericapillary macrophages, or RP macrophages [75, 83]. RP macrophages commonly clear large particles (> 200 nm) that are retained longer in the RP, while smaller particles are primarily internalized by MZ macrophages [86].

The spleen and MZ macrophages play central roles in immune response toward therapeutic proteins delivered intravenously, demonstrated by splenectomy or depletion of splenic macrophages and CD11c⁺CD8 α ⁻ DC populations, which significantly reduced anti-FVIII immune response in FVIII-deficient mice [85]. Adaptive immune response requires antigen entry into the WP, and protein antigen greater than 60 kDa must be transported from the MZ across cellular borders by specialized DCs and MZ B cells [78]. CD8 α ⁻ cDCs that capture blood-borne protein antigen in the MZ or RP can initiate CD4⁺ T cell responses in the WP, otherwise endocytosed antigen can be transferred to CD8 α ⁺ cDCs in PALS for T cell activation [82, 83]. In conjunction with antigen uptake, activation of immature splenic DCs by innate immune stimuli can license DCs to mature, alter chemokine receptor expression, and migrate toward WP [87]. Thus, splenic DCs mirror migration patterns of tissue-resident DCs moving from peripheral tissue (i.e., spleen parenchyma) toward lymphoid tissue (i.e., splenic WP), and in both cases CCR7 upregulation directs migration into T cell areas [58, 87]. When compared to the splenic immune response following IV administration, more frequent interactions of protein with dynamic skin APC subsets that have highly efficient antigen processing and presentation ability, as well as migratory activity, could drive the immunogenicity of SC administration.

2 Immunogenicity of the Subcutaneous Route of Administration

2.1 Mechanistic Insights into Immune Response Toward Subcutaneously Administered Proteins

A two-wave mechanism of antigen presentation following SC injection of therapeutic proteins has been proposed previously, in which both migratory skin-resident DCs and lymph node-resident DCs drive immunogenicity (Fig. 1) [25]. Location of protein entry and subpopulations of skin DCs involved in first encounters could influence the nature of subsequent immune response following different routes of administration [30]. For example, the nonthreatening nature of entry of epidermal antigen is more likely to initiate a tolerogenic response by LCs. However, direct antigen encounter with dermal DCs may instigate a proinflammatory response because these cells are positioned to encounter pathogens, such as viruses, that would enter the dermis systemically or through skin disruption. Local inflammation generates host cellular components such as lipids, metabolites, or nucleic acids that are damage-associated molecular patterns (DAMPs) [88]. DAMPs activate intracellular and/or cell surface PRRs on DCs and provide dangerous context to protein antigen uptake that warrants proinflammatory

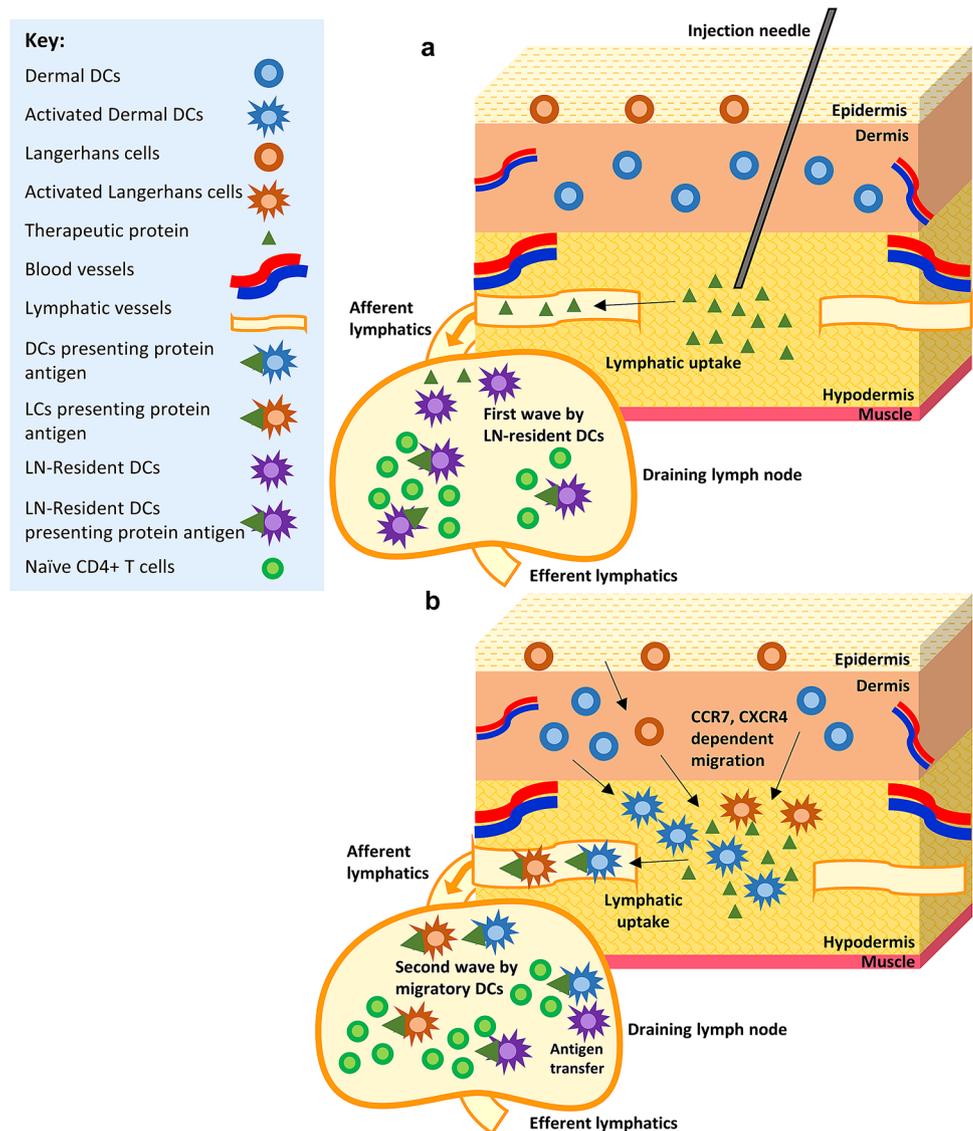
response [89]. Thus, they are danger signals that license skin-derived DCs for maturation, which upregulates antigen processing, presentation in the context of MHC II, costimulatory molecule expression, proinflammatory cytokine secretion, and migration [90]. Local skin inflammation can also generate small fragments or oligosaccharides of hyaluronic acid, which activate Toll-like receptor (TLR) 4 on DCs [91]. Furthermore, product-related attributes such as altered-self molecular patterns, impurities, host cell proteins, or aggregates have potential to serve as danger signals [24].

The first wave of antigen presentation following SC injection begins when skin-derived lymph node-resident DCs in DLNs are delivered lymph-borne protein antigen early post-injection [69]. The first wave continues for hours by lymph node-resident DCs containing intermediate levels of intact protein acquired in the lymph node [92]. Initial recognition of peptide antigen in the context of MHC II by naïve antigen-specific CD4⁺ T cells occurs within T cell areas of DLNs. These DCs display low levels of peptide:MHC II complexes and initiate CD4⁺ T cell responses toward protein antigen via T cell activation (CD69⁺ phenotype), IL-2 production, and clonal proliferation [69, 93]. Effector T cells are thus generated to mediate immune response in secondary lymphoid and non-lymphoid peripheral tissues [55]. Lymphoid-resident DCs also selectively retain antigen-specific lymphocytes in inflamed DLNs via MHC II expression and antigen presentation [93].

The second wave of antigen presentation occurs later, for example, 24 h post-injection, when skin-derived migratory DCs arrive in DLNs carrying large amounts of protein acquired at the injection site [69]. Cell migration to DLNs for the second wave is driven by receptor-ligand interaction of CCR7 and CXCR4 upregulated on mature dermal DCs with ligands expressed within lymphatic vessels [94, 95]. In addition to chemokine signaling, matrix metalloproteinase (MMP) enzymes are essential for movement of LCs and DCs through the skin. LC production of MMP-2 and MMP-9, in addition to CXCL12 signaling of CXCR4 on LCs, facilitates translocation of activated LCs through the basement membrane toward the dermis [90]. MMPs also degrade collagen, which could assist DC movement in the dermis toward initial lymphatics, and MMP9 induced by prostaglandin E2 during inflammation is essential for DC migration to DLNs [90, 96]. Proinflammatory cytokines, tumor necrosis factor (TNF)- α and IL-1 β , enhance lymphatic trafficking of migratory LCs and dermal DCs by upregulating vascular endothelial growth factor-C (VEGF-C), to increase lymphatic vessels in the inflammatory site, and reducing expression of adhesion molecule E-cadherin on LCs [90, 95]. Upon SC injection, mechanical injury to the skin could enhance and prolong LC and dermal DC migration [57].

The second wave of antigen presentation to CD4⁺ T cells by migratory DCs, expressing high levels of peptide:MHC II

Fig. 1 Schematic representation of the proposed two-wave mechanism of antigen presentation following subcutaneous injection of protein. **a** Lymph-borne antigen is delivered to skin-derived LN-resident DCs in DLNs, which begin antigen processing and presentation to antigen-specific naïve CD4⁺ T cells within T cell areas. **b** Skin-derived migratory DCs and LCs are recruited into the injection site to acquire antigen. Upregulation of chemokine receptors, CCR7 and CXCR4, on dermal DCs and LCs drives cell migration to initial lymphatics and DLNs. Antigen-loaded migratory DCs and LCs arrive in DLNs for the second wave of presentation to antigen-specific naïve CD4⁺ T cells, and migratory DCs also transfer antigen to LN-resident DCs [25]. *DC* dendritic cell, *DLN* draining lymph node, *LC* Langerhans cell, *LN* lymph node



complexes, is required to sustain T cell IL-2 receptor expression necessary for effector CD4⁺ T cell generation [69]. The CD4⁺ T cell activation program integrates signals from multiple encounters with antigen-bearing DCs before and after T cell commitment to cell division [97]. Subsequent contacts between recently activated CD4⁺ T cells and antigen-bearing DCs promotes T cell CD25 expression and IFN γ production. Increased opportunities for DC:T cell reencounter reinforces CD4⁺ T cell activation; therefore, the number of antigen-bearing DCs arriving in DLNs could be an important parameter for SC immunogenicity [97]. Lymph node-resident DCs may participate in the second wave of antigen presentation following antigen transfer by migratory DCs [98]. Migratory DCs and LCs also have an important role in T_H cell and GC responses within DLNs [38]. Physical removal of the injection site at 1 h post-intradermal antigen administration, thus preventing skin DC migration, significantly reduced GC B

cells and antibody-secreting cells as well as limited expansion of T_H cells. Even though nanoparticulate antigen efficiently transited to DLNs within 1 h (unaffected by injection site removal), the first wave of antigen presentation mediated by lymph node-resident DCs alone was not able to efficiently promote T_H cell and GC B cell responses. Although mechanistic understanding is incomplete, it is clear that both resident DCs in the DLNs and, more significantly, migratory skin-derived DCs could drive immunogenicity of subcutaneously administered protein.

Receptor-mediated uptake of proteins and mAbs by APCs could impact subsequent antigen processing and presentation. For example, mannose-sensitive receptor-mediated uptake into APCs could impact immunogenicity of recombinant human proteins with exposed mannose moieties. Averting uptake of FVIII by mannose-sensitive receptors reduced the ability of DCs to activate a human

CD4⁺ T cell clone specific for a non-glycosylated FVIII epitope, and mannose receptor (CD206)-expressing human splenic myeloid DCs were hypothesized to play a role in splenic anti-FVIII immune response [99]. Furthermore, SC immune response may involve mannose-sensitive uptake given that mannose receptor-expressing APCs have been identified in peripheral lymph nodes [100, 101]. Receptor-mediated uptake by neonatal FcR (FcRn) could also impact antigen presentation due to its expression by professional APCs and apparent association with MHC II-associated invariant chain in the endocytic pathway [102–104]. Monoclonal antibodies (mAbs) or Fc-fusion proteins that can be recycled by FcRn could be recycled out of APCs thus decreasing lysosomal processing and the probability of antigen presentation. FcRn binding can also direct the fate of monomeric and multimeric immunoglobulin G (IgG) ICs upon uptake; monomeric ICs are protected from degradation and recycled, whereas multimeric ICs are routed into degradative compartments where peptides can be loaded into MHC II [105, 106]. If IC formation between mAbs or between drug and ADA occurs prior to uptake by APCs, FcRn recognition of monomeric ICs could lead to recycling out of cells, while recognition of multimeric ICs could lead to lysosomal degradation and increased antigen processing and presentation. Fc γ receptor (Fc γ R) may initiate APC uptake of IgG ICs followed by hand off to FcRn in acidified compartments [105]. Furthermore, Fc γ RIII engagement is involved in the enhanced ability of ICs, compared to free antigen, to upregulate CCR7 expression and MMP-9 production by DCs *in vitro*, as well as boost skin-resident DC migration to DLNs following SC injection [107]. Complex interactions of proteins with lymph node-resident DCs and skin-resident migratory DCs could introduce immunogenicity challenges for SC delivery.

2.2 Evidence for Immunogenicity of the Subcutaneous Route

Some biologics formulated for SC delivery have demonstrated enhanced immunogenicity by this route of administration; however, this notion has been contrasted by a number of proteins that demonstrate comparable or higher immunogenicity by IV administration. Clinical evidence for immunogenicity is variable between products and individuals due to the multitude of product-, treatment-, and patient-related factors, but the SC route of administration is known to exhibit immunogenicity challenges. In order to compare therapeutic protein immunogenicity following SC and IV administration, available data should be examined where dosing by both routes was directly compared and ADA development was measured concurrently. However, there is not an extensive number of clinical trials that have

directly compared safety and efficacy of SC and IV dosing regimens for therapeutic proteins or mAbs.

2.2.1 Preclinical Evidence

Investigation into the impact of route of administration on immunogenicity of FVIII demonstrated that the SC route was more immunogenic than the IV route only in terms of total anti-FVIII titer, with no significant effect on neutralizing ADA (inhibitor) development [108]. It was hypothesized that modified epitopes of FVIII created upon proteolytic degradation at the injection site, with corresponding loss of conformational epitopes at the active site (likely inhibitor targets), could explain increased total anti-FVIII titers without increased inhibitors. Binding ADA are not inconsequential seeing as they could impact systemic exposure or clinical response rates by altering protein PK and clearance [109]. Because IFN α is administered by multiple routes clinically and induces ADA response in a significant patient population, impact of route of administration on IFN α immunogenicity has been investigated [110]. In BALB/c mice administered equivalent doses of IFN α , the SC route was most immunogenic followed by intraperitoneal (IP), intramuscular (IM), and then IV route based on anti-IFN α titers. Changes in IFN α half-life following SC administration along with exposure of a higher frequency of APCs to IFN α for longer times at higher concentrations could explain high titers induced at earlier times following SC administration [110]. Administration by the above routes is shown to impact kinetics and organ distribution of aggregated and monomeric albumin in mice; thus, administration by different routes could expose therapeutic protein to altered cell populations in lymphoid and non-lymphoid organs [72]. Furthermore, therapeutic proteins administered subcutaneously exhibit a relatively slower rate of absorption and prolonged terminal half-life compared to that observed following IV administration [64, 66].

Contrasting results for recombinant human IFN β identified the IV route to be most immunogenic upon administration to immune-tolerant, transgenic mice, proposed to be a result of high aggregate content in some IFN β products [111, 112]. Upon repeated IV administration, protein aggregates may have enhanced uptake and processing by splenic macrophages, compared to uptake of monomeric protein, with sustained activation of MZ B cells [111]. Similarly, murine growth hormone aggregates were immunogenic by IV administration, with higher IgG2c and IgG3 titers compared to SC delivery, suggesting involvement of T-independent type 2 response. However, IgG1 titers were high and comparable following SC and IV administration [113]. Aggregates can be considered an immunogenicity challenge for SC and IV administration, where mechanisms responsible likely differ.

2.2.2 Clinical Evidence

A mAb administered subcutaneously that has demonstrated considerable immunogenicity, where efficacy is impacted by ADA development, is adalimumab. In a long-term follow-up study for adalimumab in rheumatoid arthritis patients, 28% developed anti-adalimumab antibodies, 67% of which developed within the first 28 weeks of treatment [114]. Anti-adalimumab antibody development was associated with lower serum concentrations and lower likelihood of achieving minimal disease activity or clinical remission. However, without directly comparable clinical IV immunogenicity data, it is unclear whether the relatively high immunogenicity of adalimumab is due to the SC route or other intrinsic or extrinsic factors. Where available, comparative immunogenicity data, represented by incidence of total and neutralizing ADA response, within the same clinical trial have been collected, expanding on previous analysis by Hamuro et al. [73]. ADA incidence sourced from product labels or peer-reviewed publications are presented in Table 1 for ten currently approved biologics. Herceptin[®] (trastuzumab) formulated for SC administration has demonstrated enhanced ADA incidence following SC delivery. A higher incidence of anti-trastuzumab antibodies (16%) was observed following treatment with SC Herceptin Hylecta[™] (formulated with recombinant human hyaluronidase [rHuPH20]) compared to IV trastuzumab (10%) (Table 1) [115]. Additionally, 21% of patients treated with Herceptin Hylecta[™] developed anti-rHuPH20 antibodies—a common observation for products formulated with this permeation enhancer.

SC rituximab, which is much more concentrated than the IV formulation, is also formulated with rHuPH20 to facilitate larger injection volumes and improve antibody dispersion and absorption by temporarily hydrolyzing hyaluronic acid [52, 116]. Observed immunogenicity of rituximab in SC and IV formulations is low; treatment-induced/enhanced anti-rituximab antibody incidence in previously untreated patients with follicular lymphoma was 2.0% and 1.9% in the SC and IV groups, respectively [117]. SC rituximab treatment also induces or enhances levels of anti-rHuPH20 antibodies in 15% of patients. Pooled clinical trial results for SC trastuzumab, rituximab, insulin, and human IgG co-administered with rHuPH20 show an overall incidence of 1.7–18.1% for induced or boosted anti-rHuPH20 antibody development, plus a 3.3–12.1% incidence of pre-existing anti-rHuPH20 antibodies [118]. No neutralizing anti-rHuPH20 antibodies were observed, and adverse events were not associated with anti-rHuPH20 positivity regardless of boosting after rHuPH20 exposure. Antibody positivity to rHuPH20 has been found in 5.2% of a large cohort not previously exposed to rHuPH20, and rates were significantly higher in males

compared to females and varied with age [119]. The reasons for baseline prevalence of anti-rHuPH20 antibodies are not clear, but then rHuPH20 immunogenicity seems modest with no observed effects on adverse events or efficacy.

Marginally higher incidence of immunogenicity following SC administration compared to IV is observed for peginesatide, mepolizumab, golimumab, and Phesgo[™] (pertuzumab, trastuzumab, and rHuPH20), although ADA incidence was approximately 5% or less (Table 1) [120–123]. Overall low immunogenicity of the protein itself seems to confound significant comparison of immunogenic risk between routes of administration in some clinical trials. Low and comparable immunogenicity of SC and IV administration has been observed for daratumumab and vedolizumab (Table 1) [124, 125]. In some examples, including tezepelumab (human anti-TSLP IgG2 λ) and inebilizumab (humanized, afucosylated anti-CD19 IgG1 κ), no ADA incidence was detected for either route of administration [126, 127]. The direct impact of B cell-depleting agents, rituximab and inebilizumab, on humoral responses may explain their observed overall low immunogenicity.

A phase IIIb clinical trial for the fusion protein abatacept, human IgG Fc plus extracellular domain of cytotoxic T lymphocyte-associated protein 4 (CTLA-4), demonstrated similar total ADA rates (anti-abatacept or anti-CTLA-4-T antibodies) between SC (1.1%) and IV (2.3%) administration [128]. However, in the long-term extension period where patients received SC abatacept, 23.2% were positive for anti-abatacept antibodies [129]. No correlations between anti-abatacept seropositivity and adverse events, infusion reactions, or efficacy changes have been observed [130, 131]. Similarly, for tocilizumab comparable efficacy and immunogenicity profiles are observed for SC and IV formulations [132–134]. ADA positivity rates in patients administered tocilizumab subcutaneously or intravenously were estimated to be 1.5% and 1.2%, respectively, based on a meta-analysis of 14 studies, indicating overall low risk of tocilizumab immunogenicity [135]. Although more ADA-positive patients who received tocilizumab subcutaneously had neutralizing ADA (85.1%) compared to ADA-positive patients who received tocilizumab intravenously (78.3%), none of these patients in either treatment group experienced loss of efficacy. Tocilizumab's low immunogenicity profile with limited ADA development may result from its suppression of IL-6-dependent B cell differentiation and T_H cell activity [136].

Comparative immunogenicity results for SC and IV administration are available for some mAbs currently undergoing clinical trials. In a phase I clinical trial for PF-06480605 (human anti-TNF-like ligand 1A [anti-TL1A] IgG1) conducted in healthy participants, high ADA incidence (81.8–83.3%) was observed for SC and IV

Table 1 Clinical immunogenicity of approved therapeutic proteins by SC and IV administration [73, 121, 122, 124, 125, 132, 213]

Brand name (molecule)	Molecule description ⁱ	Formulated protein concentration (mg/mL)		ADA incidence ^j (%)		Neutralizing ADA incidence (%)		Anti-rHuPH20 antibody incidence (%)
		SC	IV	SC	IV	SC	IV	
Actemra [®] (tocilizumab) ^a	Anti-human IL-6R humanized IgG1κ	180	20	0.8 or 3.5	0.8 or 0	0.8	0.8	N/A
Darzalex Faspro [™] (daratumumab) ^{b,c}	Anti-CD38 human IgG1κ	120	20	0	0.5	0	0	6
Entyvio [®] (vedolizumab) ^d	Anti-α4β7 integrin humanized IgG1	N/A	60	6	6	3	6	N/A
Herceptin [®] /Herceptin Hylecta [™] (trastuzumab) ^{c,e}	Anti-HER2 humanized IgG1κ	120	21	16	10	6	7	21
Omontys [®] (peginesatide)	Synthetic, pegylated 21 amino acid dimeric peptide, ESA	2–12	2–12	1.9	0.7	0.9	0.9	N/A
Orencia [®] (abatacept)	CTLA-4 modified human IgG1 Fc fusion protein	125	25	1	2	N/A	N/A	N/A
Nucala [®] (mepolizumab) ^f	Anti-IL-5 humanized IgG1κ	100	100	5	4	0	0	N/A
Phesgo [™] (pertuzumab + trastuzumab) ^{c,g}	Anti-HER2 humanized IgG1κ	60–80, 40–60	30, 21	4.8, 0.9	3, 0.4	0.4, 0.4	0.4, 0	0.9
Rituxan [®] /Rituxan Hycela [®] (rituximab) ^{c,e}	Anti-CD20 chimeric murine/human IgG1κ	120	10	2	1.9	0	0	15
Simponi [®] (golimumab) ^h	Anti-TNFα human IgG1κ	100	12.5	6	0	N/A	N/A	N/A

ADA anti-drug antibody, CD cluster of differentiation, CTLA-4 cytotoxic T lymphocyte-associated antigen 4, ESA erythropoiesis-stimulating agent, HER2 human epidermal growth factor receptor 2, IgG immunoglobulin G, IL-5 interleukin-5, IL-6R interleukin-6 receptor, IV intravenous, N/A not applicable or not available, rHuPH20 recombinant human hyaluronidase, SC subcutaneous, TNFα tumor necrosis factor-α

^aActemra[®] US Drug Label listed first; Ogata et al., 2014 listed second

^bMateos et al.[124]

^cSubcutaneous formulation contains rHuPH20 (2000 U/mL)

^dSandborn et al.[125]

^eTreatment-induced or enhanced ADA incidence listed

^fOrtega et al.[121]

^gTreatment-emergent ADA incidence; pertuzumab listed first, trastuzumab listed second

^hZhuang et al.[122]

ⁱProduct information sourced from US product labels accessed in Drugs@FDA database

^jADA incidence indicates % of reported ADA-positive patients sourced from product labels where not cited otherwise

administration in single-dose or multiple-dose groups [137]. Lower exposure of PF-06480605 was seen in participants positive for neutralizing ADA at lower doses, but not at higher doses, and ADA were not associated with adverse events. In a phase I trial for PF-06801591 (humanized anti-programmed cell death protein 1 [anti-PD-1] IgG4) conducted in patients with advanced solid tumors, ADA incidence was marginally higher in IV groups (9.5%) compared to the SC group (7.1%) [138]. No neutralizing ADA were detected. Clinical evidence for immunogenicity is variable between products and individuals, and although a range of products show comparable, low ADA incidence (< 5%), there are immunogenicity challenges for SC delivery of certain biologics.

2.3 Interplay Between Subcutaneous Immunogenicity and Product-Related Risk Factors

2.3.1 Biophysical Characteristics

Lymphatic transport of proteins, and thus absorption and distribution, are impacted by protein biophysical characteristics—molecular size and charge. Transit of proteins and peptides greater than 16 kDa through SC interstitium into lymphatic capillaries occurs by convection with limited diffusion [49]. Large MW proteins in interstitial fluid can enter highly permeable lymphatic vessels through intercellular channels opened under increased interstitial pressure

[68]. Protein will traffic through lymphatic capillaries and pass into lymph vessels draining into local lymph nodes, eventually reaching systemic circulation by draining through efferent lymph vessels into larger collecting vessels and the thoracic duct [49, 68]. Studies in sheep models have demonstrated direct correlation between protein MW and proportion of dose absorbed by lymphatic uptake, and large proteins (30–40 kDa and above) have demonstrated almost complete dose recovery in peripheral lymph [63, 139]. Insulin bioavailability following SC administration in sheep significantly involves lymphatic absorption; however, species differences caution against generalizing preclinical results [28, 49, 140].

Studies in mice have further demonstrated that rate of loss of protein from the SC injection site correlates inversely with protein MW in the range of 23–149 kDa, with 149-kDa protein cleared the slowest [141]. Also, lymphatic exposure, or fraction of injected dose recovered, at the draining, axillary lymph nodes following front foot pad injection increased proportionally relative to protein MW. Lymph node distribution and PK studies demonstrate arrival of subcutaneously administered proteins in DLNs within hours [25]. Mean t_{\max} value, or time to reach maximum protein levels, in draining, axillary lymph nodes obtained in mice was almost 3 h for 149-kDa protein following SC injection compared to shorter times just under or over 1 h for proteins 23, 44.3, and 66 kDa [141]. It is possible that slower rate of exit from the SC space and increased immunological exposure of larger MW proteins at the lymph nodes could translate into heightened immune response.

Very large protein and particulates could become trapped in the ECM due to convection limitations and steric hindrance. Furthermore, positively charged proteins sized 20–78 kDa appear in lymph at delayed times compared to negatively charged, size-matched proteins [142]. Biologics with slight positive charge at local physiological pH, due to an isoelectric point of 7–9, could interact with negatively charged GAGs that are highly abundant in the ECM [28, 49]. Protein molecular charges can be heterogeneous from deamination, isomerization, and PTM, and surface charge may change from surrounding pH variations in the SC space during transition and dispersion of the protein from the stable formulation state [28, 143]. An additional consideration is whether protein chemical stability pathways, for example, oxidation, could generate modified epitopes and impact immunogenicity [144–147]. Overall, increased retention time due to charge interactions, or steric hindrance, could slow absorption and prolong exposure of therapeutic protein to invading LCs and dermal DCs (Fig. 2). The presumed mechanism of vaccine adjuvants demonstrates how SC immune response can be enhanced via facilitation of phagocytosis and slowing antigen exit from the injection site to promote uptake and trafficking of antigen by migratory DCs [57, 148].

2.3.2 Protein Aggregation

Protein molecules acquire a strong propensity to aggregate when externally applied stresses induce changes in protein conformation or partial loss of native structure that increase surface exposure of hydrophobic domains [149, 150]. Bio-processing stresses include high concentration, high temperature, changes in pH or ionic strength, shear stresses, and air–liquid or liquid–surface interfaces [24]. Even though classification systems are in place to determine aggregate features that confer immunogenic potential, there is an overall lack of understanding of the type and size of therapeutic protein aggregates universally implicated in immunogenicity [151–153]. Filipe et al. endeavored to correlate type and amount of stress-induced IgG aggregates with immunogenic potential, and not all aggregates had the same propensity to induce an immune response [152]. FDA Guidance for Industry recognized subvisible aggregates or particulates (0.1–10 μm) to have a strong potential to be immunogenic, but preclinical studies present contrasting results [1, 154].

Submicron-sized mAb aggregates (100–1000 nm) were demonstrated to be most immunogenic upon SC administration compared to soluble oligomers (< 100 nm) or micron-sized aggregates (1–100 μm) [155]. Conversely, native-like soluble oligomers (< 100 nm) induced higher antibody response in mice following SC administration compared to native mAb monomer or micron-sized non-native aggregates [153]. Subvisible aggregates of single-chain variable fragment (scFv) and ovalbumin induced significantly higher IgG2a titers compared to monomeric protein by SC injection in BALB/c mice, although total IgG and IgG1 titers were comparable. Skewing towards T_H1 -type immune response by aggregates was also suggested by cytokine profiles in DC co-culture experiments [156, 157]. Additionally, T_H1 -type immune response was observed for bevacizumab heat-triggered aggregates in a human artificial lymph node (HuALN) model, where delayed immune reactions can be monitored by long-term exposure of the system up to 28 days [158].

Human IgG aggregates induced by stirring and micron-sized particles coated with IgG induce B cell-mediated immune response in an immunologically tolerant murine model [159]. Thus, IgG-coated particles with multivalency were able to transiently break immunological tolerance upon SC immunization. The particulate nature of aggregates may be responsible; via presentation of repetitive surface antigens, multivalent protein aggregates may be uniquely capable of cross-linking B cell receptors, leading to antibody production without T cell help [160]. Also in human IgG transgenic mice, human IgG oligomers with chemical amino acid modifications from light stress were able to break tolerance and induce ADA recognizing native IgG, the mechanism of which depended on T cell help and presumably involved generation of ‘neo-epitopes’ [161]. Notably,

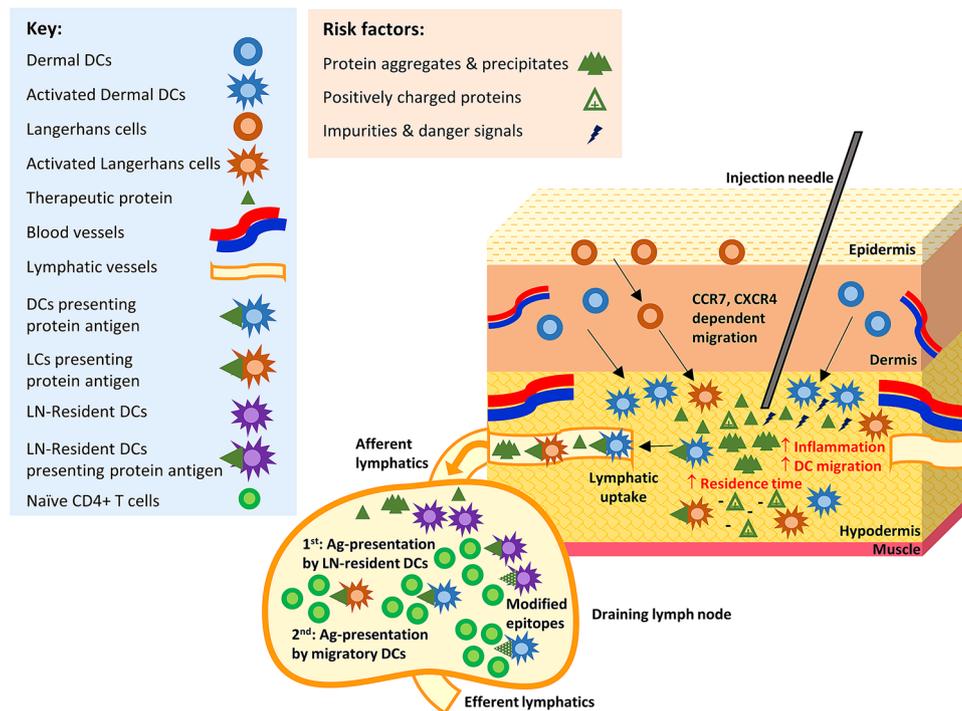


Fig. 2 Product-related risk factors for immunogenicity of subcutaneously administered therapeutic proteins. Structural or conformational modifications related to instability pathways or proteolytic degradation could generate new/modified epitopes. Protein aggregates or precipitates present in the formulation or formed post-injection can have longer SC retention time. Charge interactions between slight positive charge on mAbs at local physiological pH and negative charge density in ECM may increase SC retention time. Enhanced retention time

of protein could confer immunogenic risk by increasing opportunities for encounter with invading dermal DCs and LCs post-injection. Innate immune stimulation by adjuvant-like drug product impurities (e.g., host cell proteins, leachates, and endotoxins) at the injection site can trigger maturation and migration of dermal DCs and LCs. Ag antigen, DC dendritic cell, ECM extracellular matrix, LC Langerhans cell, LN lymph node, mAb monoclonal antibody, SC subcutaneous

IgG oligomers generated under process- or pH-related stress conditions were immunogenic in wild-type mice but did not break tolerance in the immune tolerant mouse model. Breaking of tolerance was also observed in mice following SC administration of recombinant murine growth hormone (rmGH) aggregates with a presumed T_H1 -type antibody response [113]. IV administration of the same aggregates, however, showed a mixed T_H1/T_H2 -type response with possible T-independent type 2 response. Encounter of IV-injected aggregates with splenic MZ B cells may make T-independent type 2 response more likely following IV administration.

Upon SC administration, submicron- to micron-sized aggregates can experience longer retention in the SC space, enrichment in DLNs, and slower clearance than monomeric protein [72, 162, 163]. Removal of albumin aggregates from the SC injection site in mice is slower than monomeric protein, and complete clearance was not observed after 48 h [72]. Retention of protein aggregates in the SC space or enrichment in DLNs could prolong interactions with skin-derived DCs and heighten ADA response (Fig. 2). Furthermore, the complex, unpredictable nature

of protein aggregates alludes to potential for changes in composition and size distribution once introduced to the body. Upon dilution in human serum, subvisible IgG aggregates formed by pH-related stress immediately became smaller and more numerous; then, incubation at 37 °C led to formation of large, subvisible aggregates, with evidence suggesting association of serum components [164]. The type or composition of aggregates, host immune tolerance status, and immunogenicity of the monomer itself could impact immunogenic risk [165]. Because a minute amount of aggregates could enhance immune response and current analytical techniques could overlook these particles, it will be important to prevent or moderate aggregate formation [166, 167].

2.3.3 Dosage Form Considerations

Presence of product impurities or contaminants can exacerbate immune response following SC administration by introducing danger signals (Fig. 2). Host cell proteins, leachates, and endotoxins are adjuvant-like contaminants that could enhance DC migration and antigen presentation,

demonstrated experimentally by lipopolysaccharide (LPS) [24, 168, 169]. Even a small amount of residual impurities present in the drug product may activate the innate immune system, for example, via TLR stimulation, to promote immunogenicity [170, 171]. Immune response could also be exacerbated by pre-existing inflammatory/autoimmune conditions that increase sensitized APCs or if patient skin-resident DC populations are activated by DAMPs from tissue inflammation/injury [73, 88]. An additional dosage form consideration for SC immunogenicity is the presence of proteinaceous or non-proteinaceous particulates, possibly enhanced by use-related stress. Adalimumab samples shaken in siliconized syringes contained a significant concentration of silicone oil droplets and soluble to subvisible particles; these samples were more immunogenic upon SC administration to BALB/c mice compared to unstressed or non-siliconized syringe stressed samples [172]. Besides agitation, freeze–thaw cycles or continuous low temperature storage (96 h) increased subvisible particle concentration in multiple TNF α inhibitors [173]. The potential immunogenic risk of subvisible particle concentration in therapeutic protein preparations requires further investigation.

Solubility challenges are a key dosage form consideration for SC administration due to requirement for high protein concentration in small injection volumes [28]. Protein crowding and aggregation are concerns for high concentration formulations, and excipients and stabilizers are added to maintain conformational and colloidal stability [28, 174, 175]. Low solubility within skin ECM is also an issue, and precipitation at the injection site is a possible consequence. mAbs with poor solubility at neutral pH and formulated at high concentrations could spontaneously precipitate due to pH change after SC dosing [176]. Following SC administration of such a mAb, precipitated antibody was retained at the injection site, but cellular immune response within lymph nodes and ADA development were not enhanced. Precipitation in this case may be reversible upon dilution *in vivo* due to concentration-dependent solubility as the driving factor. Also, precipitated antibody could be cleared by phagocytic cells without inducing a strong immune response; co-localization of the mAb with CD68⁺ cells (likely macrophages or monocytes) in the skin was observed along with no increase in systemic cytokine response [176]. No correlation between immunogenic risk and protein precipitation after SC delivery was established. To avoid solubility challenges, depot formulations with hyaluronidase (rHuPH20) and protein stabilizers can facilitate administration of increased injection volumes [177]. The use of hyaluronidase could address slow and incomplete absorption of proteins to limit immunological exposure, and pre-existing or induced anti-rHuPH20 antibodies have not impacted efficacy or safety in tested products [73, 118].

3 Existing and Future Strategies to Minimize Subcutaneous Immunogenicity

3.1 Immune Suppression and Lymphocyte Manipulation

Conventional strategies to mitigate immunogenicity of biologics, whether dosed subcutaneously and/or intravenously, have varying degrees of success clinically and rely on immune suppression using small molecule drugs, such as methotrexate, rapamycin, bortezomib, and cyclophosphamide (Fig. 3) [7]. The immunomodulatory drugs azathioprine and methotrexate have been used in combination with TNF α blockers infliximab and adalimumab [178]. Kishnani and colleagues have combined rituximab with methotrexate and IV gamma globulin to successfully prevent and reverse anti-rhGAA antibody response in infantile Pompe disease patients [179, 180]. Anti-rhGAA titer development has been prevented by anti-CD3 antibody treatment in preclinical models, which also provided modest reduction of pre-existing titers [181]. This non-FcR-binding anti-CD3 ϵ F(ab')₂ fragment protects HA mice from total and inhibitory anti-FVIII antibody formation, the mechanism of which involves increased CD25 expression on peripheral effector CD4⁺CD25⁻ cells [181, 182]. The importance of antigen-specific T_{reg} cells (CD4⁺CD25⁺) inspired a strategy to transduce FVIII-specific CD4⁺ T cells with forkhead box P3 (FoxP3), thus imparting a T_{reg}-like phenotype [183]. In addition to preventing inhibitor formation upon adoptive transfer, combination with anti-murine CD20 (anti-mCD20) antibody provided modest reversal of pre-existing inhibitors. However, inhibitors rise upon discontinuation of anti-mCD20 therapy. Note that important CD20⁻ cell populations survive anti-CD20 therapy, particularly long-lived plasma cells [184]. To achieve FVIII-specific B cell depletion, cytotoxic CD8⁺ T cells have been engineered with B cell antibody-targeting receptor (BAR) expressing immunodominant FVIII A2 and C2 domains [185]. Lymphocyte manipulation strategies should ideally deliver a long-lasting, antigen-specific protective effect.

3.2 Inhibition of Skin-Derived Immune Cell Migration

Theoretical mitigation strategies for SC immunogenicity could co-administer inhibitors or function-blocking antibodies directed against chemokine receptors CCR7 and CXCR4 to interrupt cutaneous DC migration to DLNs during the second wave of antigen presentation [25]. The small molecule drug cosalane, identified by high-throughput screening, inhibits CCR7-mediated mouse and human cell chemotaxis *in vitro* in response to CCL19/CCL21 stimulation [186]. CD11c⁺ DC maturation markers and CCL21-directed

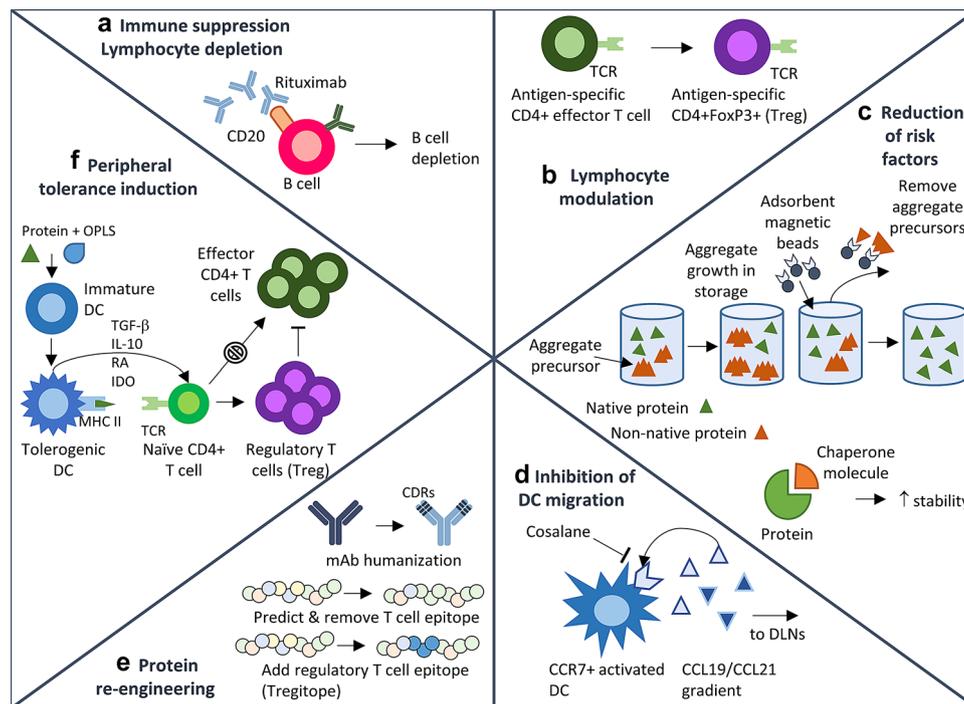


Fig. 3 Selection of existing and theoretical strategies to minimize immunogenicity of subcutaneously administered therapeutic proteins. **a** Conventional strategies rely on nonspecific immune suppression using small molecule drugs, such as methotrexate, rapamycin, bortezomib, and cyclophosphamide. A combination approach uses the lymphocyte depletion agent rituximab (anti-CD20) with methotrexate and intravenous Ig. **b** Example lymphocyte modulation strategies are anti-CD3 antibody, engineered antigen-specific FoxP3⁺ T_{reg} cells, and cytotoxic BAR CD8⁺ T cells. **c** Reduction of product-related factors by ex vivo human cell-based assay screening, removal of non-native IgG aggregate precursors by specific adsorption to AF.2A1 magnetic beads, and chaperone molecules to improve protein stability. **d** Inhibition of CCL19/CCL21 directed DC migration to lymph nodes by the small molecule CCR7 inhibitor cosalane (anti-HIV agent). **e** mAb

humanization by incorporation of fully human content apart from complementarity-determining regions. In silico prediction of T or B cell epitopes on proteins to perform de-immunization or incorporation of T_{reg} cell epitopes (Tregitopes). **f** Peripheral tolerance induction by co-administration of OPLS with therapeutic protein subcutaneously to induce tolerogenic DCs and antigen-specific T_{reg} responses [7, 178, 179, 183, 185, 186, 191, 192, 197, 209]. *BAR* B cell antibody-targeting receptor, *CDR* complementarity determining region, *DC* dendritic cell, *DLN* draining lymph node, *FoxP3* forkhead box P3, *HIV* human immunodeficiency virus, *IDO* indoleamine 2,3-dioxygenase, *Ig* immunoglobulin, *IL* interleukin, *mAb* monoclonal antibody, *MHC* major histocompatibility complex, *OPLS* O-phospho-L-serine, *RA* retinoic acid, *TCR* T cell receptor, *TGFβ* tumor growth factor-β, *T_{reg}* regulatory T cell

migration in vitro are also impaired by etanercept (TNF inhibitor) [187]. Collagen-induced arthritic mice treated with etanercept had significantly reduced myeloid and plasmacytoid DCs in lymph nodes compared to controls while peripheral blood DC numbers remained unchanged, and CXCR4 downregulation partially explains reduced DC migration [187]. IL-4 also modulates DC migratory capacity. DCs matured by TNFα in the presence of IL-4 have reduced CCR7 expression and impaired CCL19/CCL21-directed migratory activity [188]. Interrupting DC migration from the injection site will not impact antigen presentation by lymph node-resident DCs, thus ADA development could still occur.

3.3 Reduction of Product-Related Risk Factors

Reduction of protein aggregates and impurities, such as residual host cell proteins or endotoxins, could alleviate potential product-related immunogenic risk factors [168, 169]. Protein samples can be screened in human ex vivo cell-based assays for impurities and contaminants [189]. Prediction of aggregation propensity and susceptible sites for PTMs would help mitigate immunogenic and hypersensitivity risks of aggregates and non-human glycosylation [189]. Sequence-based prediction tools can be employed to identify potential aggregation-prone sequence and structural motifs on proteins [190]. Prevention of aggregation during

long-term storage is ideal, but small aggregate precursors are not removed by 0.22- μm filtration during manufacturing. A specific approach for IgG formulations removes non-native IgG molecules and small aggregate precursors by adsorption to magnetic beads conjugated with AF.2A1 protein [191]. Addition of chaperone molecules to protein formulations could prevent aggregation; a chaperone molecule (miglustat) for rhGAA that improves PK and protein stability in circulation is under investigation as a replacement therapy for Pompe disease [192]. FVIII is also prone to form aggregates with high immunogenic risk; however, onset of aggregation is delayed by the stabilizing interaction of O-phospho-L-serine (OPLS) with the aggregation-prone, immunogenic region on FVIII [193, 194]. Moreover, FVIII-OPLS complex was significantly less immunogenic than free protein upon SC administration.

3.4 Protein Modification and Re-engineering

Less immunogenic therapeutic proteins could be designed through de-immunization or tolerization. Protein modification is especially relevant for immunogenicity driven by non-self recognition, for example, replacement therapies in patients that lack central tolerance to endogenous protein or proteins with non-human sequences [195]. Humanization incorporates fully human sequences into mAbs without changing the complementarity-determining regions, but inadequacy of humanization is revealed by unfortunate ADA rates for fully human adalimumab [114, 196, 197]. Zurdo et al. reviewed quality by design methodologies and early risk assessment for immunogenic potential of proteins in development [189]. De-immunization or T cell epitope removal should limit high-affinity, long-lived ADA development by abrogating T cell responses [189]. De Groot and Martin developed web-accessible tools to perform *in silico* protein re-engineering and screen then rank candidate protein sequences for immunogenic potential [198]. Less immunogenic, but efficacious, versions of FVIII and immunotoxin LMB-2 (anti-CD25) were engineered by de-immunization of immunodominant T cell epitopes [199, 200]. Additionally, B cell epitope modification is expected to interfere with binding of pre-existing ADA or memory B cells [201]. Beyond de-immunization, sequences known to be regulatory T cell epitopes, i.e., Tregitopes, can be introduced into the protein to promote tolerogenic responses [197].

3.5 Tolerance Induction

Tolerance induction to therapeutic proteins would avoid severe issues associated with immune suppression, such as susceptibility to secondary infections [7]. Strategies can take advantage of natural peripheral tolerance mechanisms involving antigen presentation by migratory DCs to

antigen-specific CD4⁺ T cells in the context of MHC II but with low co-stimulatory signals [202–206]. Regulatory mediators produced by tolerogenic DCs, such as IL-10, TGF β , IL-35, and indoleamine 2,3-dioxygenase (IDO), induce the generation of antigen-specific T_{reg} cells [206]. Retinoic acid, another important mediator of T_{reg} induction, is produced by skin CD103⁻CD11b⁺ DCs in mice [207]. Strategies for antigen-specific tolerance induction, including those employing SC administration, have been reviewed by Luo et al. [208]. Certain skin-resident migratory cell populations, including dermal CD103⁺ DCs and LCs, have been targeted in such strategies to induce or expand T_{reg} cells [205]. A reverse vaccination strategy employs SC pre-exposure to low-dose protein in the presence of OPLS to induce tolerance, and mice were rendered hyporesponsive upon re-exposure to protein alone [209, 210]. OPLS co-administration generates DCs with a tolerogenic profile including high secretion of regulatory TGF β and normal migratory capability (Fig. 4). SC co-administration of OPLS and rhGAA in a mixed formulation induced hyporesponsiveness to rhGAA in Pompe disease mice [211]. Furthermore, reverse vaccination by SC pre-administration of Lyso-phosphatidylserine (Lyso-PS)-containing nanoparticles loaded with FVIII significantly reduced anti-FVIII antibody response during re-exposure to FVIII intravenously, the mechanism of which involved a specific PS receptor, TIM-4 [212].

4 Conclusion

The SC route of administration provides convenient and non-inferior delivery of therapeutic proteins compared to IV infusion, but unwanted ADA response can occur upon repeated administration. Based on available preclinical and clinical studies, there is evidence both supporting and refuting the notion that the SC route of administration increases risk of immunogenicity. Mechanistic insight into molecular and cellular contributors that may drive immunogenicity of subcutaneously administered therapeutic proteins is critical to rationally develop safe and efficacious protein-based therapies. A key contributor may be the large population of dynamic APCs in the skin with high antigen processing efficiency and migratory activity. Product-related factors of immunogenicity are also particularly relevant to SC formulations. Molecular characteristics, presence of protein aggregates, and impurities have the potential to increase SC retention time, upregulate immune cell migration, and/or enhance local inflammation. Current mitigation strategies for immunogenicity are lacking antigen-specific, long-lasting effects. Mechanistic insights and risk factors for SC immunogenicity inspire future approaches to prevent or reduce immunogenicity, which merit further investigation.

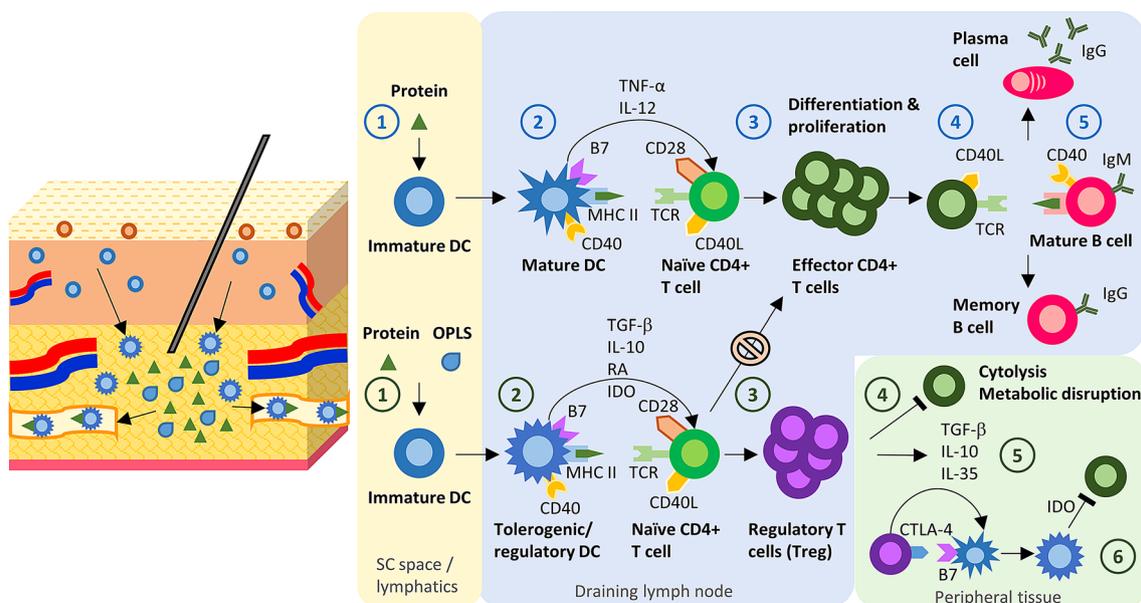


Fig. 4 Immune tolerance induction using SC co-administration of OPLS and therapeutic protein to mitigate immunogenicity. *Top*: (1) Uptake and processing of protein by skin-derived immature DCs licenses DC maturation and migration to DLNs. (2) DCs present peptide:MHC II complexes to naïve CD4⁺ T cells in T cell areas and induce (3) differentiation and proliferation of effector CD4⁺ T cells. (4) B cell activation and differentiation in germinal centers generates (5) memory B cells and plasma cells producing ADA (e.g., IgG). *Bottom*: (1) FVIII and OPLS are mixed immediately before SC administration. Uptake and processing of FVIII in the presence of OPLS by skin-derived immature DCs induces tolerogenic DCs, with down-regulation of proinflammatory cytokine production and co-stimulatory molecule expression, but similar MHC II expression compared to mature DCs. (2) Tolerogenic DCs retain migratory activity and reach DLNs to present peptide:MHC II complexes to naïve CD4⁺ T cells in T cell areas. (3) Antigen presentation in the context of regulatory mediators with low co-stimulatory signals induces T_{reg} cells. (4)

T_{reg} cells can suppress effector T cell function via metabolic disruption, adenosine A_{2A} receptor activation, cytolysis, and deprivation of crucial cytokines (e.g. IL-2). (5) T_{reg} cells produce key regulatory cytokines. (6) Interaction of T_{reg} CTLA-4 with DC co-stimulatory molecule B7 induces tolerogenic DCs that suppress effector T cells via regulatory mediators [202–204, 209]. Note that steps 4–6 (bottom) represent general pathways of tolerance induction by T_{reg} cells; the mechanism of OPLS-mediated hyporesponsiveness beyond tolerogenic DCs requires further investigation. ADA anti-drug antibody, CD40L CD40 ligand, CTLA-4 cytotoxic T lymphocyte-associated protein 4, DC dendritic cell, DLN draining lymph node, FVIII factor VIII, IDO indoleamine 2,3-dioxygenase, Ig immunoglobulin, IL interleukin, MHC II major histocompatibility complex II, OPLS O-phospho-L-serine, RA retinoic acid, SC subcutaneous, TCR T cell receptor, TGFβ tumor growth factor-β, TNFα tumor necrosis factor-α, T_{reg} regulatory T cell

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Declarations

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Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and material Not applicable.

Authors' contributions NLJ performed the literature search and drafted the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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