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## Commentary

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# Immunogenicity of Subcutaneously Administered Therapeutic Proteins—a Mechanistic Perspective

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**Abstract.** The administration of therapeutic proteins *via* the subcutaneous route (sc) is desired for compliance and convenience, but could be challenging due to perceived immunogenic potential or unwanted immune responses. There are clinical and preclinical data supporting as well as refuting the generalized notion that sc is more immunogenic. We provide a mechanistic perspective of immunogenicity of therapeutic proteins administered *via* the sc route and discuss strategies and opportunities for novel therapeutic approaches to mitigate immunogenicity.

**KEY WORDS:** biotechnology; immunogenicity; mitigation of immunogenicity; protein therapeutics; subcutaneous administration.

## IMMUNOGENICITY OF PROTEINS GIVEN VIA SC ROUTE

The subcutaneous (sc) route of administration of therapeutic proteins is desired over the intravenous (iv) route due to patient comfort and compliance. In addition, it could also prolong half-life of the therapeutic in circulation (1,2). However, this route of administration could be problematic due to a perceived potential for unwanted immunogenicity (3). As most of the vaccines are given *via* the sc route, it is expected that the sc route is more immunogenic than the iv route. However, a recent comparative clinical study of sc *vs* iv administration of abatacept, a fusion protein of Fc of human IgG and extracellular domain of CTLA-4, showed that the efficacy and immunogenicity are comparable between the two routes of administration (4,5). However, the immunogenic potential of chronic administration and long-term effects are not often adequately addressed during clinical trials (6). Few preclinical studies have shown that the sc route of administration does not increase immunogenicity (7–9). For example, the relative immunogenicity of Betaseron, interferon beta, is less for sc administration compared to iv administration (9). Based on clinical experience of head-to-head comparison and a few preclinical studies, one could argue that the generalization that the sc route is more immunogenic than the iv route is not universally valid.

A comparative immunogenicity study of three brands of insulin in type 1 diabetics showed an increase in incidence of anti-insulin titer development, across brands, in patients self-

administering *via* sc route as compared to iv administration in hospital in the same cohort (10). In the therapeutic use of erythropoietin- $\alpha$ , incidence of immunogenicity increased with the change from iv to sc. It is appropriate to mention here that elimination of human serum albumin from the formulation as well as stopper material change (11) coincided with changes in route of administration, and thus, the effect of the sc route of administration on immunogenicity is not unambiguous for erythropoietin. There are several examples from preclinical relative immunogenicity studies that show the sc route is more immunogenic than the iv route. The sc administration of FVIII showed significantly higher total antibody titers compared to hemophilia A mice that were given FVIII *via* the iv route (12). A similar observation has been made for other therapeutic proteins such as interferon alpha and human growth hormone (3,13,14). In our recent relative immunogenicity studies in preclinical models, most of the mice that were given rituximab *via* the iv route did not produce antibodies to rituximab, whereas all sc administered animals responded with significant antibody titers (unpublished results). However, we could not make similar generalizations to other antibody therapeutics we tested in mice. Thus, the available immunogenicity data of therapeutic proteins supports as well as refutes the general notion that the sc route is more immunogenic.

## MECHANISTIC PERSPECTIVE

The immunogenic potential of sc space is a conundrum. This is partly due to lack of mechanistic understanding of factors that drives the immunogenicity of subcutaneously administered protein. Based on antigen trafficking studies in the field of vaccines and on pharmacokinetics and distribution of protein, we propose a mechanistic model to understand the immunogenic potential of the sc route for therapeutic proteins. Our aim here is not to suggest any preferred route of administration of therapeutic proteins, but rather to propose a possible

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mechanistic basis of steps involved in presentation and processing of proteins following sc administration. The mechanistic studies for antigen trafficking discussed here are primarily carried out in mice, and one must be cautious of its relevance to the human immune system. However, both species share similar subtypes of antigen-presenting cells such as dendritic cells in sc space (15,16).

### Primary Antigen-Presenting Cells Involved in Processing of Proteins Given *via* sc

In order to understand the mechanistic basis of immune response following sc administration, it is important to determine the primary antigen-processing cells involved in presentation and processing. The detection of peptide–MHC II complex using monoclonal antibodies provides an effective approach to track the fate of antigens and the cells that produce these complexes following different routes of administration. Germain and colleagues, followed by Reis D Sousa and colleagues, have shown that in the absence of endotoxin or adjuvant (situation similar to the administration of therapeutic proteins) B cells (that are not specific for the antigen in question) in the spleen and cutaneous dendritic cells (DCs) are the primary cell types that engage in antigen processing and presentation of the peptide–MHC II complexes following the iv and sc routes, respectively (17–19). Following iv administration of the hen egg lysozyme in mice, it has been shown that B cells not specific for the antigen in question rapidly take up the protein and present it in the spleen within 4 h of administration, and this is followed by presentation by dendritic cells after 24 h. Because antigen-unspecific B cells outnumber DCs in the spleen, the possibility of antigen-specific T cells encountering antigen presented by B cells is much higher than that which is presented by dendritic cells (19). In contrast, following sc administration, dendritic cells are the primary antigen-presenting cells that process and present the antigen (17). Dendritic cells are the primary initiators of T cell responses (20), and the cross talk between C-type lectin/Fc receptors and Toll-like receptors can mediate effective immune presentation (21).

### Anatomy of the Skin and sc Space

The anatomy of the sc site contributes to effective presentation of the protein to lymph node-resident and cutaneous dendritic cells (Fig. 1). The most superficial layer of the skin is the epidermis, and Langerhans dendritic cells (LCs) reside in the epidermis. The next layer is the dermis and is separated from the epidermis by a membrane that supports the vascular network for nutrient supply to the epidermis. This layer contains dermis dendritic cells that are functionally distinct from LCs (22). The third layer of the tissue is called the hypodermis and is also called sc connective tissue. The proteins deposited in this area trigger the uptake, processing, and maturation of cutaneous dendritic cells and LCs in the epidermis and dermis that subsequently migrate into draining lymph nodes and secondary lymphoid tissues (23). The intensity of adaptive response depends upon the transport of proteins by dendritic cells to lymphoid organs for effective presentation to T cells. Further, proteins distribute in lymph nodes (determined by their pharmacokinetics and

lymphatic transport), where these proteins are processed by lymph node-resident dendritic cells (24). This is consistent with two waves of antigen presentation following sc administration of a protein antigen.

### Two Waves of Antigen Presentation

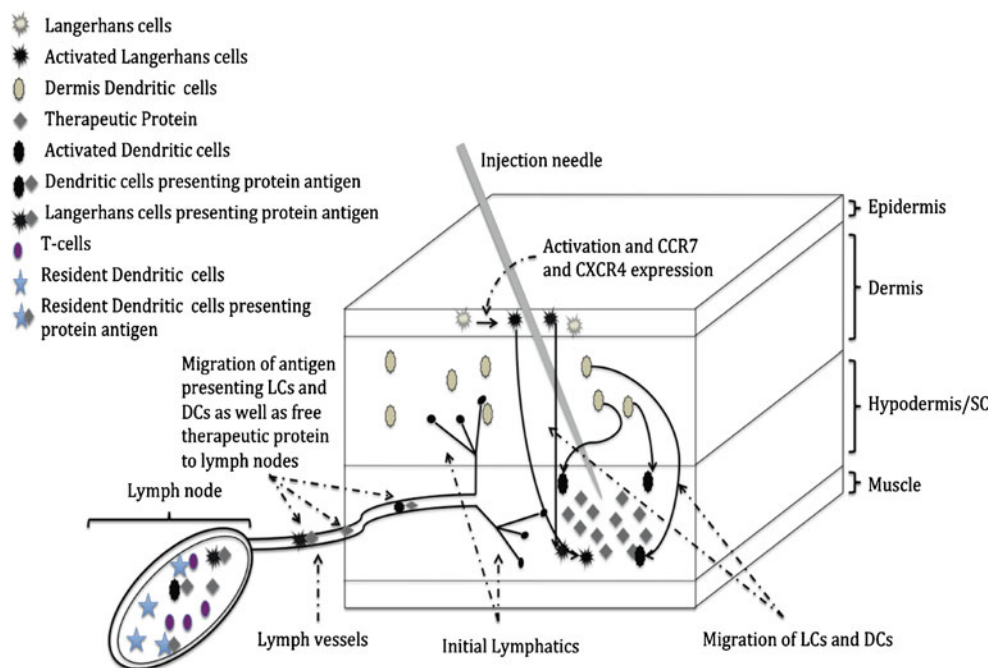
Using the peptide–MHC II complex of a fluorescent antigen and CD11c/CD40 markers, evidence of two waves of antigen processing and presentation was observed. This approach has been used to identify cells that present the antigen to CD4+ T cells following sc administration of antigen (25,26). The protein deposited in the sc space is presented by resident DCs in lymph nodes first and later by DCs that migrate from the epidermis and dermis.

#### *First Wave and Lymph Node Distribution*

The first wave of antigen presentation is influenced by pharmacokinetics and lymph node distribution of the protein. It has been demonstrated that following sc administration, lymphatic transport of the protein leads to its distribution in lymph nodes (27). Our PK and lymph node distribution studies showed that proteins deposited in the sc space arrive within hours to the lymph node (data not shown). These proteins and their fragments are processed by lymph node-resident DCs and subsequently present the antigen to T cells in peripheral lymph nodes. CD11c<sup>high</sup>/CD40<sup>high</sup>, increased E-cadherin expression, and Birbeck granules characterize this antigen presentation. This set of DCs arrives within hours of administration of the antigen and is accompanied by IL-2 production and effective proliferation of T cells.

#### *Second Wave and Migration of Cutaneous Dendritic Cells*

The sc injection of proteins also triggers the migration of cutaneous dendritic cells. The use of CD11c/CD40 markers showed that both DCs from the epidermis and dermis migrate to peripheral lymph nodes *via* the G-protein-dependent mechanism following the subcutaneous deposition of antigen in about 1–3 days (25). These migratory cells display CD11c<sup>int</sup>CD40<sup>high</sup> and much lower expression of E-cadherin. This presentation produces a distinct function and is associated with a sustained expression of the IL-2 receptor and delayed hypersensitivity responses. The migration of the cutaneous DCs and molecular process that drives this migration are well characterized. Following sc administration of a protein antigen, the migration of LCs from epidermis and dermis DCs is triggered by inflammatory cytokines such as IL-1 beta and TNF alpha, and these cytokines up-regulate VEG-F C that in turn increases the number of lymphatic vessels at the inflammatory site (23). The migration of dendritic cells is also triggered by up-regulation of two receptors CCR7 and CXCR4 (23,28). The ligands for these receptors are expressed in lymphatic vessels, and the receptor–ligand interaction drives the migration of cutaneous DCs to draining lymph nodes. These migratory dendritic cells can also transfer the antigen to lymph node dendritic cells (29). Overall, the immunogenicity of subcutaneously administered protein antigen appears to be driven by migratory cutaneous dendritic cells and by lymph node-resident dendritic cells. Certainly, investigations are necessary to test several hypotheses based on



**Fig. 1.** Schematic representation of the sc space and mechanism involved in immunogenic response against sc administered therapeutic proteins

this mechanistic model to completely understand the immunogenicity of subcutaneously administered proteins.

### Strategies to Mitigate Immunogenicity

This understanding could be useful in developing strategies to mitigate immunogenicity of therapeutic proteins given *via* the sc route. A few possible strategies are discussed. An important step in the mediation of immunogenicity of therapeutic proteins given *via* the sc route arises from migration of cutaneous DCs (23,28). The migration of DCs is initiated by the inflammatory stimuli at the injection site. Thus, reduction in impurities such as LPS and aggregates in the injected proteins would be expected to ameliorate the immunogenic effect of the sc injection. The presence of LPS has been shown to increase the migration and presentation of antigen by dendritic cells to T cells (17,19). Further, our studies showed that presence of native-like aggregates in sc administered FVIII produced a much higher antibody response and possible secondary memory response compared to native FVIII and nonnative aggregates (30). Thus, avoiding inflammatory impurities and aggregates in the formulation is critical for decreasing the immunogenic effects of the sc administration. Further, the DC migration is accompanied by expression of surface receptors such as CCR7 and CXCR4, and thus, an effective approach could be use of molecules such as function-blocking antibodies specific for these receptors or formulating therapeutic proteins with molecules capable of inhibiting these receptors. These inhibitors or antibodies against CCR7 or CXCR4 can also interfere with receptor binding to ligands that are expressed in lymphatics following antigen administration, and this inhibition could reduce migration of DCs into the secondary lymphoid tissues. These approaches may limit the

processing and presentation of antigens by migratory DCs, but contribute very little to control effective presentation to lymph node-resident dendritic cells.

The challenge of immunological exposure to dendritic cells for immunogenicity also provides an opportunity for mitigation approaches. Induction of immunological tolerance using dendritic cells is one of the effective ways to mitigate immunogenicity (31). It has been established that the presentation of an antigen to dendritic cells in a tolerogenic manner can promote peripheral tolerance *via* induction of regulatory T cells (32). We have shown that administration of therapeutic proteins such as FVIII in the presence of an immune-modulatory lipid phosphatidyl serine (PS) reduces development of inhibitory antibody responses against FVIII and promotes a hyporesponsiveness by tolerogenic presentation to dendritic cells (33–36). As the preexposure of FVIII in the presence of PS induced tolerance/hyporesponsiveness, we proposed a novel strategy called reverse vaccination to induce tolerance and thereby reduce immunogenicity of therapeutic proteins given sc. Unlike conventional vaccination, this approach induces antigen-specific hyporesponsiveness. The success of this vaccination strategy depends on effective exposure of the antigen and PS to dendritic cells, and the sc route of administration is very attractive from the “immunological exposure” perspective.

### Conclusions

Overall, the immunogenicity of subcutaneously administered protein is dependent upon antigen presentation and processing by lymph node and migratory cutaneous dendritic cells in the sc space. A better understanding of this process provides opportunities to design strategies to reduce antibody responses to sc delivered therapeutic proteins and for the

development and use of novel approaches to modify proteins that make them less immunogenic or tolerogenic.

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**Conflict of Interest** The authors declare that there is no financial conflict of interest.

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