

Intralymphatic immunotherapy

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Purpose of review

IgE-mediated allergy can be treated by subcutaneous allergen-specific immunotherapy (SCIT). However, the percentage of allergic patients undergoing SCIT is low, mainly due to the long duration of the therapy and the risk of severe systemic allergic reactions associated with the allergen administration. Typically, SCIT requires dozens of subcutaneous allergen injections that stretch over 3–5 years. Over the last decade, sublingual immunotherapy has been established as an alternative to SCIT, but treatment duration and dosing frequencies could not be reduced. Recently, immunotherapy by direct administration of the allergen into lymph nodes [intralymphatic immunotherapy (ILIT)] has proven a promising alternative and this method is the focus of the present review.

Recent findings

Several studies on animals and on humans have shown that direct injection into lymph nodes enhanced immune responses to protein, peptide, and naked DNA vaccines. Moreover, ILIT strongly improved allergen immunotherapy, so that the number of allergen administrations as well as the allergen dose could be reduced. As ILIT was also well tolerated, practically painless, and easy to perform, patient compliance was improved as compared with SCIT.

Summary

Direct ILIT into a subcutaneous lymph node markedly enhances protective immune responses, so that both the dose and the number of allergen injections can be reduced, making ILIT safer and faster than other forms of immunotherapy, and most importantly, this enhances patient convenience and compliance.

Keywords

allergen, immunotherapy, intralymphatic, vaccination

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Introduction: the geographic concept of immunogenicity

Random rearrangement of T-cell and B-cell receptors early in lymphocyte development generates T and B cells carrying a diverse repertoire of receptors. Although this allows specific recognition of a nearly infinite number of antigens, the problem is that only one in 10^7 T and B cells can recognize a specific antigen. Therefore, antigens must be presented to millions of T and B cells to elicit a response. To make this possible, antigens must be drained or transported to sites where high numbers of T and B cells accumulate and recirculate, that is, into secondary lymphoid tissues such as lymph nodes and the spleen. Such lymphoid organs are highly organized with defined compartments for B and T cells, allowing them to rapidly and efficiently recirculate and interact with antigen-presenting cells. Only if an antigen drains to a lymph node or if it is transported there, an immune response can be elicited. If antigens stay outside secondary lymphoid organs, the chances for them to meet with a specific T or

B cell are minimal and, therefore, antigens that stay outside lymphoid organs are largely ignored by the immune system. More than a decade ago, we have called this the ‘geographic concept of immunogenicity’ [1–3]. In light of today’s complex views of immune regulation by different types of dendritic cells and T cells, this geographic concept may sound overly simple, but it should not be forgotten that the main trigger of any immune response still is the antigen and that the distribution and persistence of the antigen are the main regulators of the immune response.

Experiments on alymphoplastic (aly/aly) and spleenless (Hox11^{–/–}) mutant mice have confirmed the importance of secondary lymphoid organs, or at least of neo-lymphoid aggregates [4] in generating immune responses [5]. The importance of antigen drainage or transport from the periphery into lymph nodes via afferent lymph vessels has been unequivocally demonstrated by elegant early skin flap experiments performed by Frey and Wenk in 1957 [6].

Enhancing immune responses by targeting antigen to lymph nodes

Textbooks say that lymph vessels collect pathogens and substances from the interstitial fluid and drain them into regional lymph nodes. However, the efficiency of this drainage can be highly variable, the most effective drainage being that of pathogens. Viruses and to a certain extent small particles (<200 nm) freely drain from peripheral injection sites into lymph nodes quite efficiently, but only a few percent of the nonviable particles usually reach the lymph nodes [7]. Larger particles sized 500–2000 nm are mostly transported into lymph nodes by peripheral macrophages or dendritic cells [7]. In contrast, drainage of nonparticulate antigens from periphery to lymph nodes is typically very inefficient and only very small fractions (10^{−3} to 10^{−6}) of an injected dose reach the lymph nodes, as discussed below and summarized in Table 1 [8–10]. As many of today’s vaccines and immunotherapeutic agents are nonparticulate, their direct administration into a lymph node may, therefore, dramatically enhance antigen presentation and specific immune-response stimulation.

Apart from draining pathogens into lymph nodes, lymph vessels also transport dendritic cells. This migration, however, is rather an active process by the dendritic cells themselves. When comparing intralymphatic to other routes of dendritic cell vaccination, it may, therefore, not be surprising that, likely depending on the quality and maturation stage of the dendritic cells used, certain studies find that intralymphatic immunotherapy (ILIT) enhances vaccination, whereas others do not. In the following, we will review the method of intralymphatic administrations for the purpose of improving immunotherapies.

Intralymphatic immunotherapy with dendritic cells

The optimal route of administration of antigen-pulsed dendritic cells to induce T-cell responses remains controversial. Intravenous administration of dendritic cells leads to accumulation of cells in lung, liver, and spleen,

Table 1 The enhancing effect of intralymphatic immunotherapy inversely correlates with pharmacokinetics of the vaccine

Type of vaccine	Dose required (SCIT/ILIT)	Stability <i>in vivo</i> and drainage into lymph node
Naked RNA ^a	>10 ⁶	–
Oligopeptide [8]	10 ⁶	+
Naked DNA [9]	10 ⁴	++
Protein [10]	10 ³	+++
Live virus ^a	1	++++

ILIT, intralymphatic immunotherapy; SCIT, subcutaneous allergen-specific immunotherapy.

^aUnpublished results (T.M.K.).

whereas delivery into skin leads to homing of dendritic cells preferentially to draining lymph nodes [11–13]. However, the vast majority of dendritic cells injected subcutaneously or intradermally remain at the injection site [11,14–19], where they appear to stay for at least 48 h [20]. Therefore, intralymphatic or intranodal delivery of antigen-pulsed dendritic cells has been attempted [21–23]. Antigen-pulsed dendritic cells injected into the lymph node localize to the paracortex [15,24,25]. Clinical trials have suggested that intralymphatic administration of dendritic cell vaccines enhanced immune responses [16]. However, other clinical trials found no advantage of intranodal delivery over intradermal delivery of adenovirus-transduced dendritic cells, as animals in both groups responded to vaccination with essentially the same frequency of IFN-γ-secreting T cells [24]. Similarly, in a study comparing intravenous, intradermal, and intralymphatic routes of injection of dendritic cells in human prostate cancer patients, intradermal and intralymphatic delivery generated stronger but comparable T-cell responses [26].

Intralymphatic immunotherapy with tumor cells

The lymph node is a highly immunocompetent environment in which macrophages, dendritic cells, and lymphocytes are present at very high densities, which again allows very efficient antigen presentation, costimulatory signaling, and bystander effects that finally lead to T-cell and B-cell activation. We have found that nonprofessional antigen-presenting cells such as a fibrosarcoma tumor cell line can efficiently induce antigen-specific CD8 T-cell responses when injected into the lymph nodes [1]. These findings have been confirmed in other tumor models [27], and intralymphatic immunization using tumor cells has been tried in both human cancer patients and dogs with indication of success [28–32].

Intralymphatic immunotherapy with major histocompatibility complex class I-binding peptides and plasmid DNA

Direct administration of major histocompatibility complex (MHC) class I-binding peptide vaccines into lymph nodes or the spleen has been demonstrated to enhance dramatically CD8 T-cell responses that protected mice against viral and tumor growth [8]. Furthermore, intralymphatic injections have also been applied to enhance vaccination with antigen-encoding plasmid DNA in mice [9,33]. Indeed, ILIT using DNA priming followed by a peptide boost produced the strongest CD8 T-cell responses ever reported in mice [34•]. Although experiments on dairy cows did not reproduce these observations, clinical trials on ILIT using a Melan-A/MART-1 DNA plasmid vaccine in stage IV melanoma patients generated

significant immune responses that correlated with anti-tumor responses [35^{••},36].

Intralymphatic immunotherapy with protein-based vaccines

Before recombinant technology and peptide synthesis revolutionized the production of protein, the limited amounts of antigens made researchers look for more efficient methods for antibody induction. Here, intralymphatic injections offered an opportunity [37] and as little as nanogram quantities of a protein antigen were sufficient to elicit immune responses when injected into the secondary lymphatic tissue [38].

Macaques were immunized with simian immunodeficiency virus (SIV) envelope gp120 and core p27 vaccine by targeted lymph node immunization, as well as via the intradermal, nasal, and intramuscular immunizations. Only targeted lymph node immunization induced protection against the infectious virus challenge [39]. Similar results were obtained in a study of macaques immunized with p27 alone [40] or with HSP70 linked either to SIVgp120 or p27 adsorbed to aluminum and challenged with SIV [41], as well as using HSP70 conjugated to HIV gp120, SIV p27, and CCR5 peptides [42,43], a particulate SIVp27 protein vaccine [44], or SIVp27 virus-like particles [45].

Targeted lymph node immunization using 29mer peptide vaccine against feline immunodeficiency virus was also successful in cats [46]. Finally, immunization of cows by injection in the area of the supramammary lymph node was found superior to intramammary vaccination using *Staphylococcus aureus* extract as a vaccine [47].

Intralymphatic immunotherapy with bacterial vaccines and adjuvants

In one report only, the application of a bacterial vaccine by intralymphatic injection has been described. Juillard and Boyer [28] compared intralymphatic with intradermal vaccination with *Bacillus Calmette-Guerin* (BCG) and found that a positive tuberculin test could be observed earlier upon intralymphatic (11–23 days) than after intradermal vaccination (>45 days).

ILIT does not only enhance the immune response to antigens, but also enhances the efficacy of adjuvants. We could show that intralymphatic administration of CpG required 100 times lower doses than subcutaneous administration [48], thus avoiding unwanted systemic adverse effects of the adjuvant [49]. This is in line with reports of better safety profiles and enhanced efficacy of CpG when targeted to lymph nodes using particles

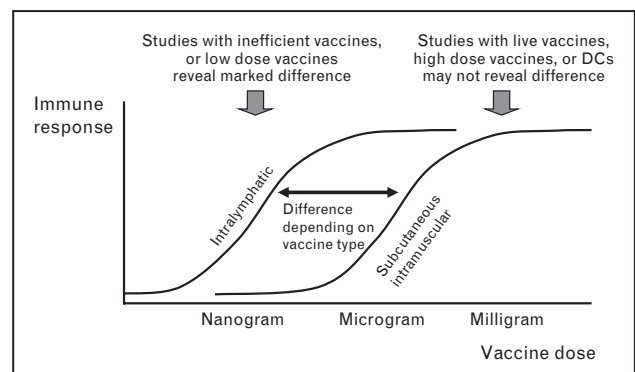
[50,51]. Moreover, HIV formulated in a PR8-Flu immune stimulating complex (ISCOM) adjuvant was used to immunize rhesus macaques against HIV via intranasal or targeted lymph node immunization. The latter generated significantly stronger cytokine, lymphoproliferative, and antibody responses than intranasal application [52].

Which vaccines profit the most from intralymphatic administration?

For more than a decade, we have injected various types of vaccines directly into subcutaneous lymph nodes and compared the doses required to those for subcutaneous injection (Table 1). There appears to be an inverse correlation between the benefit of intralymphatic administration and the stability of the administered vaccine. Using ILIT, we could induce specific CD8 T-cell responses even by injection of naked mRNA encoding for lymphocytic choriomeningitis virus (LCMV) glycoprotein, which could not be achieved even with much higher subcutaneous doses of mRNA (T.M.K., unpublished). This is not surprising, as RNA in tissues is immediately degraded by RNase. In contrast, by injection of live LCMV, no benefit of ILIT was observed in terms of T-cell responses (T.M.K., unpublished). This may also readily be explained by the optimal size of viral particles for drainage into lymph nodes, the stability of the viral particle, and because LCMV, once in the lymph nodes, replicates there.

Taken together, it appears that vaccines which work only poorly by subcutaneous or intramuscular administration profit the most from intralymphatic delivery, whereas vaccines which already induce strong immune responses after subcutaneous or intramuscular administration may not be further enhanced if injected intralymphatically (Fig. 1).

Figure 1 Expected immune response as a function of immunizing dose and the route of administration



DC, dendritic cell.

Allergen-specific immunotherapy by intralymphatic injections

IgE-mediated allergies, such as allergic rhinoconjunctivitis and asthma, have become highly prevalent and affect up to one third of the population in westernized countries [53–56]. The gold standard treatment is subcutaneous allergen-specific immunotherapy (SCIT), the administration of gradually increasing quantities of an allergen vaccine to an allergic patient, which is an effective treatment for allergic rhinitis, conjunctivitis, asthma, and allergic reactions from stinging insects [57–59]. SCIT confers long-term benefit even after discontinuation [60,61], interrupts expansion of allergic sensitization [62] and progression from allergic rhinitis to asthma [63]. From a medical perspective, SCIT is, therefore, superior to symptomatic treatment, but patient compliance is compromised due to the long duration, frequent allergen injections, and risk of allergic side effects, including anaphylaxis and fatalities [64–66].

SIT acts by modulating the allergen-specific immune response. SIT stimulates the production of allergen-specific and high-affinity IgG antibodies, especially IgG4 [67] and it shifts the balance of T-lymphocyte subsets from a Th2 toward a Th1 phenotype with preferential IFN- γ and decreased IL-4 and IL-5 production [68,69]. Also, production of allergen-specific IL-10 by CD4⁺CD25⁺ T-regulatory cells is observed and found to be important for the protection against allergic reactions in [68–70]. Which of these immunological mechanisms is most responsible for ameliorating the allergy symptoms is a matter of debate, and it is probably a concerted action of all effects.

Intralymphatic immunotherapy induces overall stronger Th1, Th2, and Treg responses

In light of the above, we have been investigating whether allergen immunotherapy could be enhanced by intralymphatic administration. We demonstrated that ILIT with a variety of allergens, for example, bee venom phospholipase-A2, ovalbumin, and allergen extracts from grass pollen, birch pollen, and cat dander, stimulated antiallergic and protective B-cell and T-cell immune responses [71,72]. Direct intralymphatic injection also significantly enhanced the efficiency of immunization as compared with subcutaneous injections. Typically, allergen-specific IgG2a antibody responses were 10–20 times higher with only 0.1% of the allergen dose, and ILIT enhanced IL-2, IFN- γ , IL-4 and IL-10 secretion as compared with subcutaneous injection [10]. The latter suggests that ILIT did not polarize the response to the allergen, but rather generated overall stronger Th1, Th2, and Treg responses.

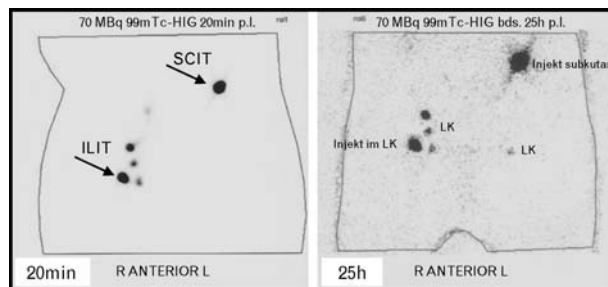
Biodistribution after intralymphatic and subcutaneous injections

Further studies on mice revealed that approximately 100-fold higher allergen doses were found in lymph node after ILIT as compared with the subcutaneous injection of the same dose in the draining region [10]. Similar results were obtained in humans using radiotracing. On the right abdominal side, a ^{99m}Tc-labeled protein was injected directly into a superficial inguinal lymph node. Contralaterally, the same dose was injected subcutaneously, 10 cm above the inguinal lymph nodes. As shown in Fig. 2, only a small fraction of the subcutaneously administered protein reached the lymph nodes after 24 h. In contrast, the intralymphatic injection allowed the protein to drain into the deep subcutaneous lymph nodes and further into one pelvic lymph node within 20 min. Thus, intralymphatic injection efficiently pulsed five lymph nodes with the full amount of the protein.

Clinical trials on intralymphatic immunotherapy with bee venom

In a first clinical trial, eight patients with bee-venom allergy grade III received three injections of 10 μ g bee venom allergen extract directly into the inguinal lymph node; these patients would normally receive SCIT with approximately 70 injections of 100 μ g allergen extract. In this proof-of-concept trial, seven out of the eight treated patients were protected against a subsequent bee sting challenge, and complementary and correlating data have recently been generated in a larger multicenter clinical trial in which 66 grade III and IV bee venom allergic patients were treated by ILIT (Senti *et al.*, manuscript in preparation).

Figure 2 Biodistribution of ^{99m}Tc-labeled human IgG after intralymphatic (left abdominal side) and subcutaneous (right abdominal side) injections



The radiotracing was made by gamma-imaging 20 min (left panel) and 25 h (right panel) after injection. The arrows indicate the site of injection. ILIT, intralymphatic immunotherapy; SCIT, subcutaneous allergen-specific immunotherapy.

Clinical trials on intralymphatic immunotherapy to treat pollen-induced hay fever

In a monocentric open-label trial [73^{••}], 165 patients with grass pollen-induced rhinoconjunctivitis were randomized to receive either 54 SCIT injections with grass pollen allergen extract over 3 years [cumulative allergen dose 4031540 standardized quality units (SQ-U)] or three intralymphatic injections with 4-week intervals (cumulative allergen dose 3000 SQ-U). The patients were evaluated after 4 months, 1 year, and 3 years. Three low-dose intralymphatic allergen administrations increased tolerance to nasal provocation with pollen already within 4 months. The tolerance was long-lasting and equivalent to that achieved after standard 2–3 years of SCIT. ILIT ameliorated hay fever symptoms, reduced skin prick test reactivity, decreased specific serum IgE, caused fewer adverse events than subcutaneous immunotherapy, and enhanced patient compliance as compared with SCIT. In conclusion, intralymphatic allergen administration enhanced safety and efficacy of immunotherapy and reduced treatment time from 3 years to 8 weeks.

Future directions

ILIT brings the antigen or the allergen into close proximity to dendritic cells and other antigen-presenting cells. To further improve SIT, intracellular translocation sequences and sequences further targeting the MHC class II pathway may represent potential tools. Accordingly, using allergens fused to a HIV-Tat-translocation peptide and to a part of the invariant chain, we were able to target the MHC class II molecules in the endoplasmatic reticulum [74,75]. As this circumvents the inefficient pinocytosis process and enzymatic degradation in phagolysosomes, immunogenicity was significantly enhanced [10,76^{*}]. Finally, a double-blinded placebo controlled trial on ILIT using this new generation of therapeutic allergen molecules has recently proved this concept (Senti *et al.*, manuscript in preparation).

Conclusion

In conclusion, intralymphatic SIT (ILIT) allows reducing markedly both the number and the dose of allergen injections, thus making the SIT shorter and, as allergic side effects are proportional to the allergen dose, safer. From a physician's point of view, ultrasound-guided injection into a subcutaneous lymph node in the groin area is performed within minutes, and has proved to be practically painless, the pain arising solely from penetrating the skin, whereas lymph nodes are purely innervated [73^{••}]. In conclusion, as ILIT renders allergen-specific immunotherapy faster and safer, it enhanced patient compliance and should have the potential to

make SIT more appealing to a larger number of allergic patients wishing to treat their illness but not willing to go through years of conventional subcutaneous treatment.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 576).

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