Video Article

Intralymphatic Immunotherapy and Vaccination in Mice

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Abstract

Vaccines are typically injected subcutaneously or intramuscularly for stimulation of immune responses. The success of this requires efficient drainage of vaccine to lymph nodes where antigen presenting cells can interact with lymphocytes for generation of the wanted immune responses. The strength and the type of immune responses induced also depend on the density or frequency of interactions as well as the microenvironment, especially the content of cytokines. As only a minute fraction of peripherally injected vaccines reaches the lymph nodes, vaccinations of mice and humans were performed by direct injection of vaccine into inguinal lymph nodes, *i.e.* intralymphatic injection. In man, the procedure is guided by ultrasound. In mice, a small (5-10 mm) incision is made in the inguinal region of anesthetized animals, the lymph node is localized and immobilized with forceps, and a volume of 10-20 µl of the vaccine is injected under visual control. The incision is closed with a single stitch using surgical sutures. Mice were vaccinated with plasmid DNA, RNA, peptide, protein, particles, and bacteria as well as adjuvants, and strong improvement of immune responses against all type of vaccines was observed. The intralymphatic method of vaccination is especially appropriate in situations where conventional vaccination produces insufficient immunity or where the amount of available vaccine is limited.

Video Link

The video component of this article can be found at https://www.jove.com/video/51031/

Introduction

Vaccines are typically injected subcutaneously or intramuscularly for stimulation of immune responses. The success of this procedure requires efficient drainage of vaccine to lymph nodes where antigen presenting cells can interact with lymphocytes for generation of T- and B-cell responses. In addition, the strength and the type of immune responses induced also depend on the density or frequency of such interactions as well as the microenvironment itself, especially the content of cytokines. As only a small fraction of a vaccine injected into a peripheral tissue reaches a lymph node, vaccinations of mice and humans by direct injection of the vaccine into lymph nodes, the site were immune responses are generated, have been performed. In man, the procedure is guided by ultrasound, a procedure also used for the administration of imaging agents for visualization and diagnosis in the lymphatic system. In mice, the procedure is invasive. Here, a small (5-10 mm) incision is made in the inguinal region of anesthetized animals[1], the lymph node is localized and immobilized with forceps, and a volume of 10-20 µl of the vaccine is injected under visual control; 10 µl is used for first injections and in young mice with small lymph nodes, whereas 20 µl can be injected into lymph nodes of older or already primed mice, which have larger lymph nodes. The incision can be closed with a single stitch using surgical sutures. By this method, mice have vaccinated with plasmid DNA[2,3,] messenger RNA[4], peptide[1,3,5,6], protein[7-10], particles[11], bacteria[12] as well as adjuvants[7,13], and strong improvement of immune responses against all type of vaccines have been observed. The intralymphatic method of vaccination is especially appropriate in situations where conventional vaccination produces insufficient immunity or where the amount of available vaccine is limited or very costly. In human, the intralymphatic method of immunization has been applied to allergy patients[14,15] or to patients with cancer[16-21]. Although the current notion is that the intralymphatic method is more invasive than other injectable methods such as intramuscular and subcutaneous injections, the pain perception is not higher than after a venous puncture[15]. It is expected that intralymphatic vaccination will become an alternative or complement to other methods of prophylactic and especially therapeutic vaccinations. This article describes in details how the procedure of intralymphatic vaccination is performed in mice. All procedures described, were approved by the Cantonal Veterinary Agency of Zurich and performed according Swiss federal guidelines and directives on the protection of animals used for scientific purposes.

Protocol

1. Anesthesia of Mice

1. Prepare the anesthetics by mixing ketamine (dissociative anesthetic) and xylazine (sedative and analgesic) in buffered saline. The concentrations of ketamine and xylazine in the final solution are 12.5 and 2 mg/ml, respectively.
2. Inject the anesthetics to the mice by intraperitoneal administration using a syringe with a 25-30 G needle. Use 0.1 ml/10 g of body weight.

3. Apply an ophthalmic ointment to the mice's eyes in order to prevent drying-out of the corneas.
4. Assure that the mouse is sufficiently anesthetized by pinching its foot or toe with the forceps. If the mouse does not react with reflexes to the pinch, proceed to surgery. If the mouse reacts to the pinch with reflexes or muscle contractions, wait 1-2 min and repeat the pinch test. If the mouse still reacts, replace the mouse with a new animal and repeat the procedures from step 1.2 onwards.

2. Surgical Incision of the Inguinal Area of the Mouse

1. Place the mouse on its back.
2. Wet one inguinal region with disinfecting ethanol (70%). To provide better disinfection, the inguinal region may be shaved to remove fur prior disinfection with ethanol.
3. Take the hind leg and bend the hip joint to produce an approximately 90° angle of the hip joint.
4. Using a curved microdissecting forceps, take the mouse skin in the inguinal area where the hip joint is bent and pull the wetted skin up slightly.
5. While holding the skin up with the forceps, cut a small incision (<5 mm) through the skin using surgical scissors.
6. Place the tip of the closed scissors into the incision and further open the incision by opening the scissors while holding it inside the incision. This will cause the skin to tear and should produce a diameter of less than 10 mm. Note: A cold light source with flexible light guides can be used to improve visibility. In older mice, especially males, the identification of the lymph node may be difficult due to much fat tissue in the inguinal region.

3. Intralymphatic Injection

1. Prepare a syringe (0.5 ml or less) with a 28-30 G hypodermic needle; a short bevel may be preferable to a long bevel. Aspirate 10 µl of the vaccine to be injected and assure that the syringe is free of air.
2. Localize the inguinal lymph node with help of the curved forceps and the tip of the closed scissor. The lymph node will appear greyish within the more whitish fat tissue and can be further identified through afferent and efferent capillaries entering and leaving the lymph node.
3. Immobilize the lymph node by holding it between the branches of the curved forceps.
4. Take the syringe and insert the needle into the lymph node with the bevel facing up; making sure the whole bevel enters the lymph node.
5. Inject the vaccine (10 µl). If the lymph node is swelling (blowing up), the injection can be assumed successful. If the needle is not placed deep enough into the lymph node, some or the entire vaccine may leak out and not enter the lymph node. If the needle is inserted too deep, the injected vaccine will be released subcutaneously below the lymph node. In case of the latter, repeat the injection or replace the mouse with a new untreated mouse.

4. Closing the Incision by Suturing

1. Open a pack of sterile surgical suture.
2. Take hold of the suture needle with a needle holder at the distal portion of the needle body. Tighten the needle holder by squeezing it until the first ratchet catches.
3. Take hold of the skin at one side of the incision using forceps (depending on the surgeon's preference, toothed or un-toothed forceps or skin hooks may be used). Insert the needle *ca.* 2-3 mm from the edge of the incision, entering from the outside of the skin.
4. Loosen hold of the skin with the forceps and grasp the opposite side of the incision with the forceps. Insert the needle *ca.* 2-3 mm from the edge of the incision, entering from the inside of the skin.
5. Loosen the needle holder and grasp the needle end with the forceps. Pull the needle with the tread through the skin, leaving a convenient length of the tread outside the first needle-insertion point (if several mice are to be treated, leave a shorter end; 5-10 mice can be operated on with one suture needle/tread).
6. Make the preferred knot according to standard surgeon protocols. One stitch is sufficient if the incision is not larger than 10 mm. For larger incisions, make a second stitch.

5. Post-operational Treatment

1. Place the mouse in the cage and cover with tissues to keep warm. When treating multiple mice, place them close to each other to keep warm. Alternatively, place the mice on a warm pad until they have woken up.
2. Observe the mice until they wake up.
3. Observe the mice daily with regards to wound healing as well as other clinical symptoms such as wound infections. The wound is typically sufficiently closed within two days and healed within seven days of the procedure.

Representative Results

The procedure of intralymphatic injections in mice, despite the surgical nature, is straight forward and relatively fast. A trained person can perform the procedure in 3-4 min. The incision that is closed with one stitch typically heals within two days (**Figure 1**)

Intralymphatic vaccination or immunization has been performed with mRNA, plasmid DNA, peptides, proteins, virus and bacteria. **Figure 2** illustrates the antibody production after immunization with the protein phospholipase A2 (PLA2), the major bee venom allergen. CBA mice were immunized on days 0, 14, and 28 with 0.01, 0.1, or 10 µg PLA2 adsorbed on aluminum hydroxide by subcutaneous (sc) or intralymphatic (iln) injections. The PLA2-specific antibodies (IgG2a) were measured in blood after 8 weeks, and the results revealed that 0.01 µg PLA2 was sufficient to stimulate strong antibody responses, while 10 µg PLA2 was required when using the subcutaneous route[9].

Intralymphatic vaccination has also been used to stimulate cytotoxic T cell responses, *e.g.* the peptide gp33 (KAVYNFATM) from the LCMV glycoprotein, which bind MHC-class-I in the context of H-2Db for stimulation of CD8 T cells. While a single subcutaneous injection of TCR318 mice with gp33 triggered cytotoxic CD8 T-cell responses, the effect was approx. six orders of magnitude stronger after intralymphatic injection (**Figure 3A**); the TCR318 mice are transgenic for the CD8 T-cell receptor specific for gp33[1].

CD4 T-cell functions were also tested in a tumor challenge assay. Again C57BL/6 mice were immunized subcutaneously or intralymphatically with the peptide np52 (SDLRGYVYQGLKSG) from the VSV nucleoprotein, which bind MHC-class-II for stimulation of CD4 T cells, and the peptide was mixed with ODN CpG 1826. When the mice were challenged intravenously with np52-expressing syngeneic EL4 tumor cells, the mice developed systemic lymphoma (**Figure 3B**). Mice that were immunized by the intralymphatic route showed much longer survival times (>75 days) than mice immunized by the subcutaneous route (<22 days).

The method of intralymphatic immunization has also been performed in man, especially for the treatment of allergies such as hay fever. The method is currently known as ILIT (Intra Lymphatic Immune Therapy). In one study on 165 patients with hay fever, 3 ILIT sessions over 2 months with grass pollen allergens was equally efficient as conventional therapy with 54 subcutaneous injections over 3 years[15]. Moreover, the dose could be reduced by more than 1,000-fold. Similarly, in a study on 20 patients with cat fur allergy, 3 sessions with intralymphatically administered cat fur allergen was sufficient to cause improved tolerance to later allergen exposure[14].

One explanation of the improved efficacy of intralymphatic immunization as compared to subcutaneous immunization could be illustrated in man. Using a protein labelled with radioactive 99mTc and as illustrated in **Figure 4**, subcutaneous injections were observed to drain poorly to lymph nodes while of course direct intralymphatic injection delivery the whole vaccine content to the lymphatic system, hence, making it available to immune cells and for immune response stimulation[22]. Similar results were obtained in mice injected with radioactive proteins[9].

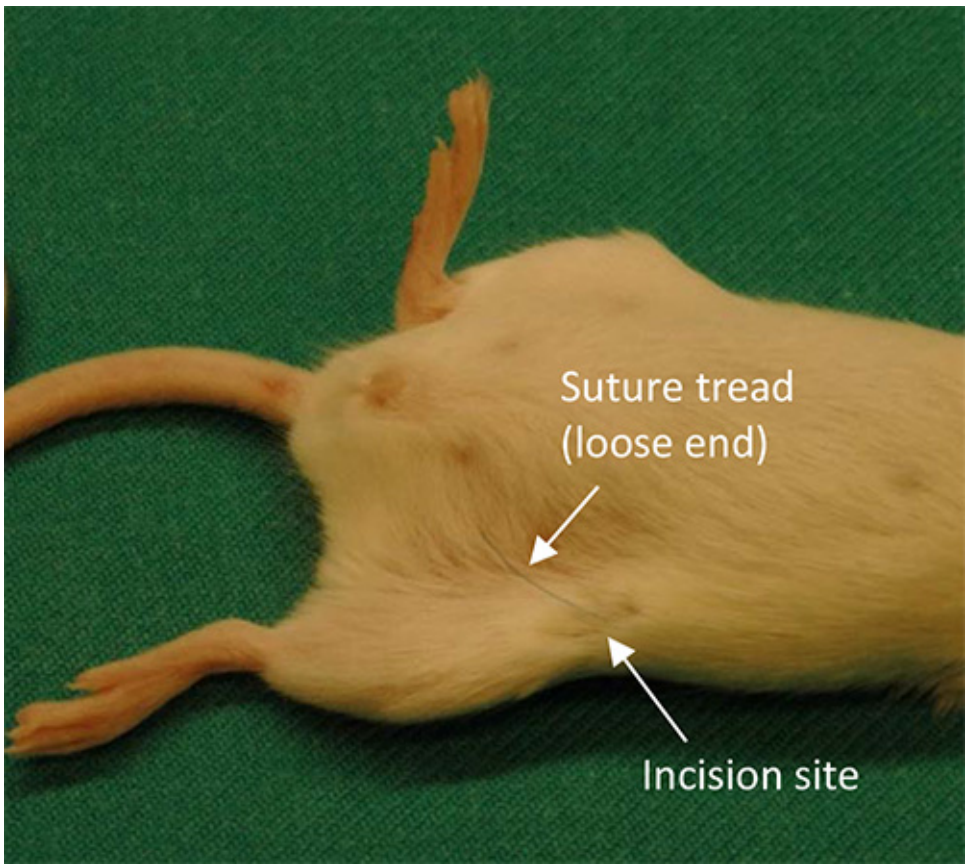

Figure 1. Healing of surgical wound after intralymphatic injection. Female BALB/c mice were immunized by intralymphatic injection. The incision was closed with single suture stitch. Two days later, the site of surgery and injection were photographed for management of the wound healing.

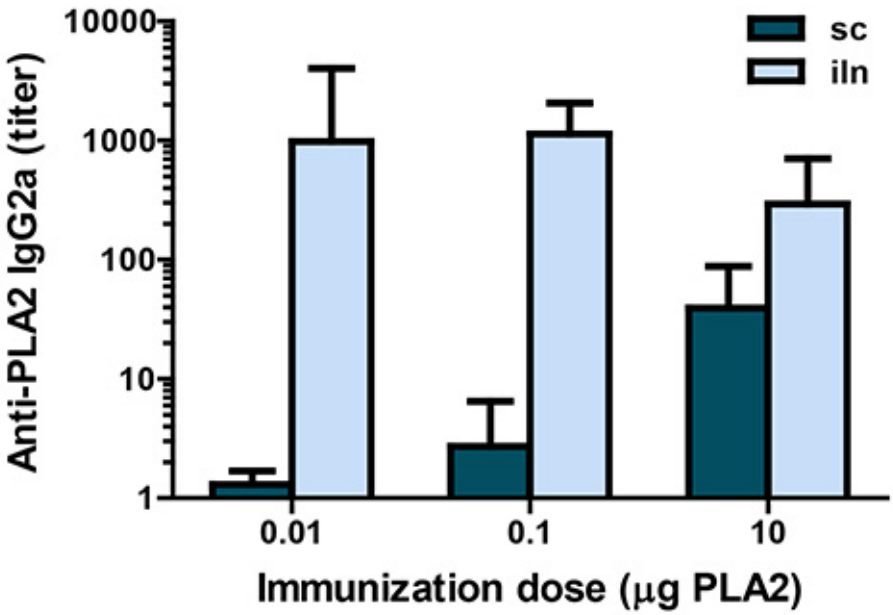

Figure 2. Intralymphatic immunization with protein for stimulation antibody responses. Female CBA mice were immunized on days 0, 14, and 28 with the indicated dose of phospholipase A2 protein adsorbed on aluminum hydroxide. The vaccine was administrated subcutaneously (sc) by injection of 50 µl in the scruff of the neck or 10 µl in an inguinal lymph node: the left lymph node on days 0 and 28 and the right lymph node on day 14.

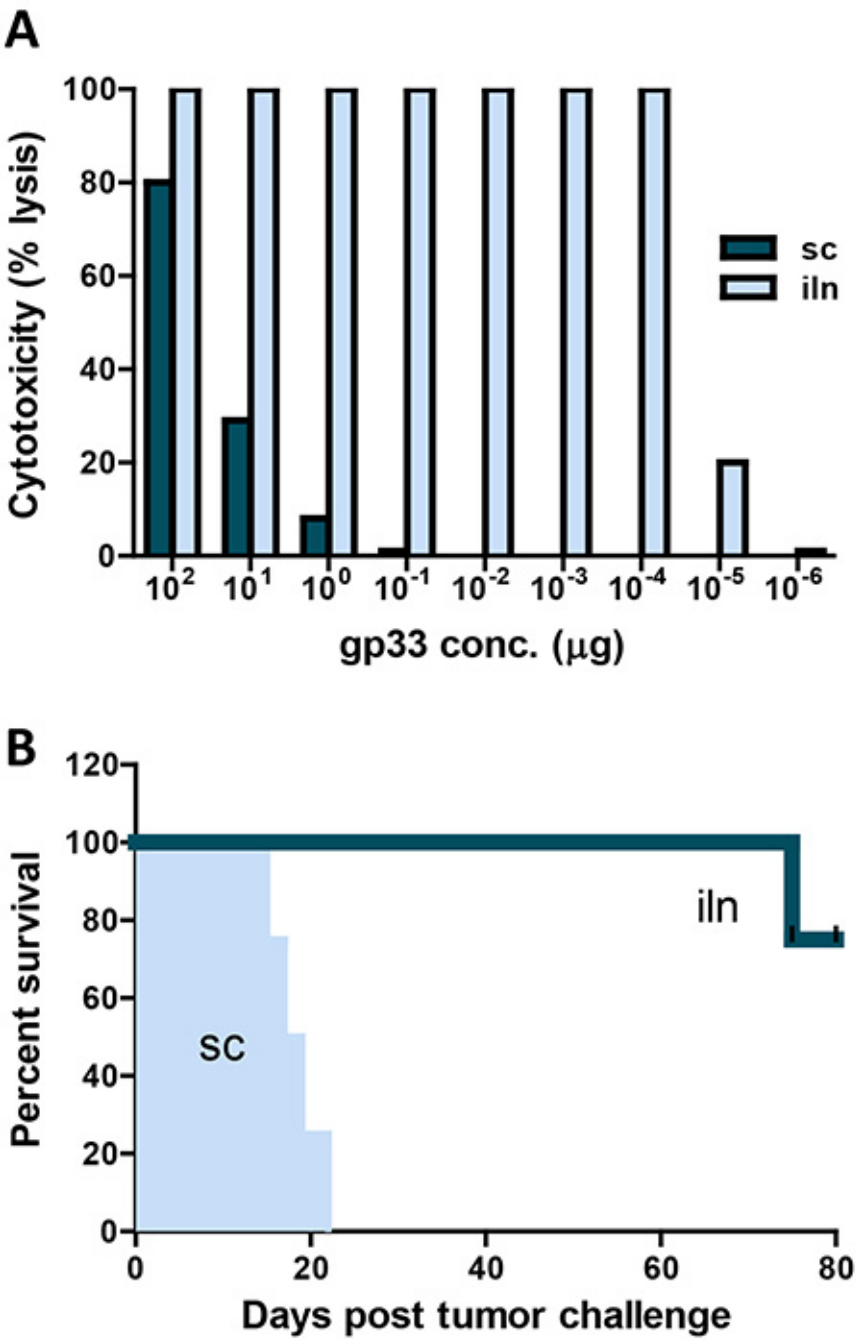

Figure 3. Intralymphatic immunization with peptide for stimulation cytotoxic CD8 T-cell responses. (**A**) Female TCR318 mice were injected with gp33 peptide into the inguinal lymph node (iln) or subcutaneously (sc) into the hind footpad. After 24 hr, the draining lymph nodes were harvested and analyzed for gp33-specific CTL activity at an effector/target- (E/T) cell ratio of 30:1. (**B**) Female C57BL/6 mice were immunized on days 0, 2, and 4 with 10 μg np52 by subcutaneous (sc) or intralymphatic (iln) routes of administration. Mice were then challenged with a syngeneic lymphoma cell (EL-4) expressing VSVnp52, and the survival of mice was illustrated with Kaplan-Meyer curves.

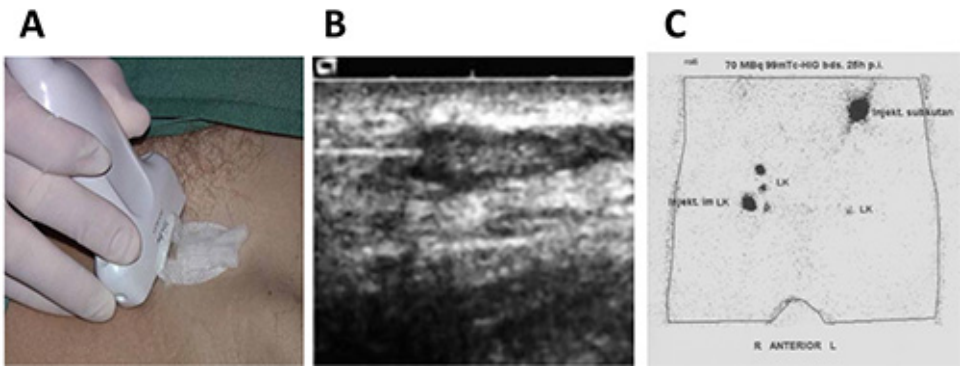

Figure 4. Intralymphatic vaccine administration in humans. Intralymphatic vaccine administration in humans is done by allocating subcutaneous lymph nodes with the aid of ultrasound. The hypodermal needle is inserted into the paracortex of the lymph node, and the injection can be controlled by observation of immediate lymph node swelling in the ultrasound image. (**A**) The intralymphatic injection is guided by ultrasound. (**B**) Ultrasound image showing needle (straight whitish line) inserted into the paracortex of the lymph node. (**C**) Biodistribution after injection of radioactively labelled protein into an inguinal lymph node ("Injekt im LK"; right anterior) and subcutaneously ("Injekt subkutan"; left anterior) in man. Click here to view larger image.

Discussion

Intralymphatic immunization and immunotherapy have been shown to be appropriate for stimulation of both antibody responses and T-cell responses. As demonstrated in this video-article, the intralymphatic procedure of vaccination is a quick and easy method for stimulating strong immune responses in mice. A trained surgeon can perform the procedure during 3-4 min. The session can also be shared between two surgeons where one is typically doing anesthesia and the suturing and the second surgeon is doing the incision and the injection. The efficiency of the procedure is more operator dependent than other immunization method, especially if the surgeon is not yet well trained. However, an experienced operator is able to reproduce experimental results also quantitatively as monitored by immune-response analysis. A modification of the method is to inject the vaccine into the spleen[1,2] . The preparation is similar to that of the intralymphatic injection, but the size of the incision is typically larger and two stitches are made to close it. The major advantages of such intrasplenic immunization are that the spleen is easier to find and to hit than a lymph node, larger injections volumes and more frequent injections can be applied, and while any damage of a lymph node caused by needle insertion or injection would involve a major area of the lymph node, the damage of the spleen would involve relatively less of the organ and be more of local nature.

The procedure of intralymphatic immunization in mice can be repeated (*e.g.* vaccine boosting injections) after a recovery time of 3-4 weeks before second injection in the same lymph node is done. If more frequent injections are planned, both left and right inguinal lymph nodes should be used. At least two injections per lymph node can be made, but further studies are required to define this potential limitation more precisely. Further injections may be difficult due to scar formation both in the lymph node and in the skin, as well as potential granuloma formation caused by adjuvants. On the other hand, immunization will cause transient lymph node inflammation, and the increased lymph-node size will simplify subsequent localization and injections. Indeed, the localization of the lymph node is a major limitation of the method. While young mice (less than 5 weeks) may have very small lymph nodes, older and especially older male mice may have so much subcutaneous fat, that localization is difficult. Optimal conditions are obtained with female mice of 6-12 weeks. Indeed, one of the most critical steps of the procedure is the injection into the lymph node itself. An injection into the adjacent area of the node will be equivalent to a subcutaneous injection with much weaker triggering of immune responses than after direct intralymphatic injection. The reason is that the rather solid capsule that surrounds the node is not permeable to large molecules. For an antigen to get into a lymph node, it has to drain with the lymph. This journey typically starts in the initial lymphatic vessels in the skin, continues in the larger ducts in the deeper layer of the skin, which drain the lymph to collecting vessels in the subcutaneous tissue, from where the lymph is driven by peristalsis towards the next draining lymph nodes[23,24] . The further downstream an antigen is deposited along this path, the less of it will get access to the afferent lymph.

The method of intralymphatic immunization is particularly interesting for administration of antigens that are weakly immunogenic when administrated by conventional methods such as intramuscular or subcutaneous routes. Intralymphatic vaccination has been performed with mRNA, plasmid DNA, peptides, proteins, virus and bacteria[22,25-27]. While peptides and proteins have no in-build adjuvants, mRNA plasmid DNA virus and bacteria exert intrinsic adjuvant effects by providing danger signals (replicating virus and bacteria) or by stimulating Toll-like receptors (TLRs) which can sense pathogen associated molecular patterns (PAMPs) such as single-stranded RNA (TLR7 and TLR8), double stranded RNA (TLR3), un-methylated cytosine and guanine oligonucleotide clusters (TLR9), bacterial flaggelin (TLR5), lipopolysaccharide (TLR2 and 4) as well as other viral and bacterial molecules[28]. Hence, especially peptide- and protein-based vaccines would benefit from the immune potentiation of injecting the vaccine directly into the lymph node. Indeed, it has been demonstrated that B- and T-cell responses are increased after intralymphatic peptide and protein vaccination in mice[1,8,9]. Moreover, genetic vaccines, especially mRNA-based vaccines, are very unstable for which reason they would surely profit from an administration method that enabled short passage times in extracellular tissues. Indeed, intralymphatic vaccination with antigen-encoding RNA elicited potent prophylactic and therapeutic immunity in mouse tumor models[4]. Similarly, intralymphatic administration of adjuvants such as CpG enabled a reduction of toxicity due to the lower dose required, and an increased adjuvant effect was observed due to the direct and undiluted effect on the immune cells[13]. Also the performance of other adjuvants or antigen delivery systems such as polymeric nano- and microparticles as well as liposomes could be improved by intralymphatic administration[11]. Another major advantage of intralymphatic over conventional vaccination routes is that much lower doses are typically required. For proteins and for stimulation of antibody responses, the dose could be reduced by 100-1,000-fold[9], while the dose of DNA for stimulation of cytotoxic CD8 T-cell responses could be reduced with approx. 10,000-fold[2].

In contrast to subunit antigens or dead vaccines, live viral and bacterial vaccines are normally not expected to benefit from intralymphatic administrations. The reason for this is in part that live microbes typically drain well to lymph nodes without any adjuvant help. Moreover, infectious microbes can cope with antigen dilution effects in peripheral tissues by replication. Hence, the antigen dose reaching the secondary lymphatic system will typically be sufficient to generate immunity. However, where the existing live vaccines are producing insufficient immunity, intralymphatic administration could provide the correction necessary to make the vaccine effective. It was recently demonstrated that immunity after vaccination with the currently only available tuberculosis vaccine *M. bovis* BCG could be significantly improved by direct injection of BCG into the inguinal lymph nodes of mice[12]. Intralymphatic administration also allowed a reduction of the inoculated amount of BCG by 100-1,000 fold compared to subcutaneous administration. This represents a safety benefit of intralymphatic vaccination, especially in immune compromised persons.

Intralymphatic immunization and immunotherapy in human has so far been applied to cancer patients and to allergy patients. The method in human is noninvasive, but best results and quality control is obtained by using ultrasound to guide injection, which may limit application to hospitals or larger care units. In cancer studies, prime-boost vaccination with plasmid DNA and peptide for the treatment of metastatic melanoma revealed immunogenicity and evidence of disease control in a defined patient population [29]. Also intralymphatic autologous vaccination with dendritic cells has been performed in cancer patients. While some trials enhanced immune responses[30,31], other trials failed to demonstrate the benefit of intralymphatic administrations[32,33]. The procedure of intralymphatic administration has proven to be especially beneficially in the immunotherapy of allergy patients. By changing to the intralymphatic method, the number of injections for complete and successful immunotherapy could be reduced from approximately 50 subcutaneous injections over 3 years to only 3 injections over 2 months[14,15]. Since both injection numbers doses can be strongly reduced by doing allergen-specific intralymphatic immunotherapy, and since lymph nodes do not contain histamine-releasing mast cells, the methods represents a safety benefit, and with the short treatment duration, patient compliance is improved. Over the next years, allergy is perhaps the indication that would see most immediate benefit of this new method of vaccination and immunotherapy.

Disclosures

TMK is named as inventor of patents dealing with intralymphatic immunotherapy in man. TMK has been scientific advisor and has received travel expenses from ImVisioN GmbH, Cytos Biotechnology, MannKind Corporation, and XBiotech USA Inc. PJ has no conflict interest to disclose.

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