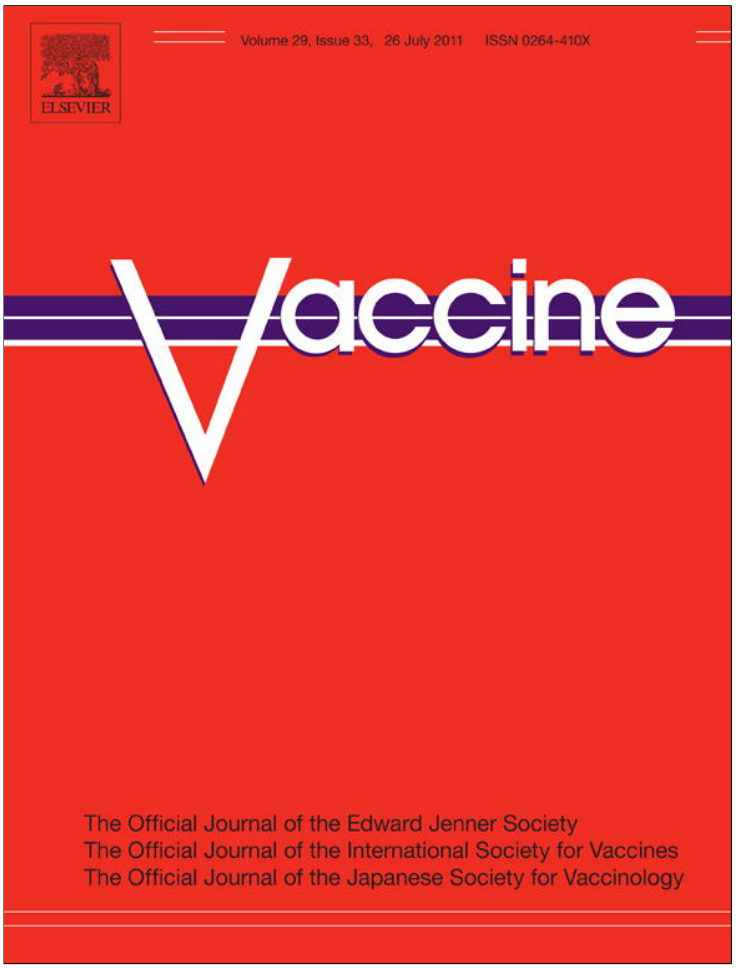

Vaccine 29 (2011) 5393–5398

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The influence of molecular adjuvants in the cutaneous response to antigen after topical vaccination

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ARTICLE INFO

Article history:
Received 4 March 2011
Received in revised form 18 May 2011
Accepted 23 May 2011
Available online 12 June 2011

Keywords:
Cutaneous
Immunity
Micro-emulsion
Vaccine
Adjuvant

ABSTRACT

A micro-emulsion (ME), previously shown to enable topical delivery of therapeutic amounts of protein, was used for immunisation of multiple strains of mice with tetanus toxoid (TT). Topical vaccination with TT alone induced low levels of serum antibody in the BALB/c and A/J strains, with C57Bl/6 the only strain capable of a significant TT-specific antibody response. Topical vaccination with TT in combination with murabutide and monophosphoryl lipid A adjuvant generated high humoral and cellular responses in both C57Bl/6 and the non-responsive strain, BALB/c, comparable to intramuscular injection with TT adsorbed to Alum adjuvant. High level immunity after topical administration with chemical adjuvants suggested that the poor response to TT alone in some strains was not due to a low bioavailability of protein. Weak immunity with TT alone may instead be related to passive absorption of antigen into skin that did not result in detectable inflammation or tissue damage. Immune mice given a booster vaccination also showed weak responses to topical TT alone; a further indication that the adaptive response to cutaneous antigen was highly dependent on adequate induction of innate immunity within local tissue. Our data supported the potential for high level adaptive immunity after cutaneous immunisation but only when combined with potent activators of the innate immune system.

1. Introduction

The traditional method for the administration of antigen is injection into the muscle and requires trained medical personnel. Alternative methods of vaccination are frequently sought due to the limitations involved with needle injection such as potential for contamination, needle stick injury and reduced patient compliance. Vaccination by topical application to epithelial tissue holds several advantages, particularly ease of administration. The skin is thought to be an immune competent organ due to its function as a barrier to infection. The high density of Langerhans cells within the epidermis allow efficient antigen uptake and immune surveillance and support the idea that cutaneuos tissue plays a fundamental role in peripheral immunity [1]. Delivery devices that allow physical permeation of epidermal tissue such as jet injectors have been used in the past as an alternative to needle injection. The benefits of high pressure injectors do not include reduced tissue damage and studies report a significant increase in pain and inflammation compared to needle injection [2,3]. Epidermal powder immunisation uses a similar high pressure technique for the administration of vaccine powders or DNA-gold particles to the epidermis. Other physical techniques for assisted penetration of epidermal tissue include ultrasound, electroporation and microneedles.

Topical vaccination using passive methods for skin permeation such as colloidal carriers does not usually result in sufficient immunity [4]. Weak immunity may be the result of inadequate delivery of antigen or limited activation of Langerhans cells due to substantial reduction in vaccine related inflammation. The surface layers that make up the stratum corneum (StCm) of the epidermis are a complex structure of corneocyte clusters held together by desmosomes with extracellular passages tightly sealed by a lamellar lipid matrix. The StCm is normally impermeable to water-soluble macromolecules and passive carriers must allow partitioning and transport through the extracellular lipid matrix. Antigens that penetrate the StCm can be captured by Langerhans cells and immune induction is thought to involve migration of Langerhans cells to the draining lymph node followed by differentiation into mature dendritic cells [5]. The process of migration and maturation of skin Langerhans cells is believed to be a prerequisite for priming of

Abbreviations: ME, Micro-emulsion; TT, Tetanus toxoid; IM, Intramuscular injection; SC, Subcutaneous injection; StCm, Stratum corneum; M, Muramyl dipeptide; ML, Murabutide and monophosphoryl A.

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doi:10.1016/j.vaccine.2011.05.085

naïve T cells [6]. Cell activation in response to infection, irritation or physical trauma of the skin plays an important role in the migration of Langerhans cells out of epidermal tissue and expression of a wide array of cytokines such as interleukins, interferons, TNFα and chemokines are directly involved in the cutaneous immunological response [7].

In this report, we used a water-in-oil micro-emulsion (ME) for the topical delivery of tetanus toxoid (TT) to cutaneous tissue. In a recent report, the biodistribution of radiolabeled protein was used to demonstrate the high capacity of the ME vehicle for cutaneous delivery of water-soluble macromolecules in mice. Efficient epidermal permeation was further supported by therapeutic delivery of a protein-based anti-inflammatory drug [8]. The mechanism of ME-mediated penetration of water-soluble molecules may be related to the oil-permeable nature of the StCm lipid matrix. Partial extraction of lamellar lipids in the StCm by surfactant has also been shown to enhance skin penetration [9]. The oil/surfactant phase composes at least 90% of the ME and sustained skin absorption by this phase may allow penetration of the integrated aqueous phase, possibly through extensive deformation of nanometre sized aqueous droplets. Permeation by the ME does not appear to damage the epidermis since treatments are non-irritating as determined by repeat insult patch test of human skin (data not shown, Dermatest Inc., Sydney, Australia). Vaccination of mice using the ME vehicle permitted analysis of the cutaneous response to antigen when signals that regulate immune cell activation and migration are present at low levels. TT was used because it is highly immunogenic and commonly used in vaccines [10]. We found that topical vaccination with TT induced weak humoral responses in both naïve and previously immunised mice. Strong, antigen-specific immunity required incorporation of potent agonists for activation of cells through Nod-like and Toll-like receptors. Our results supported the growing recognition that appropriate activation of the innate immune system is essential for protective immunity by both initiating and amplifying antigen-specific immunity.

2. Materials and methods

2.1. Animals and reagents

The C57Bl/6 J (H-2^b), BALB/c (H-2^d) and A/J (H-2^a) strains were obtained from the Animal Resources Centre, Perth, Australia. Ethics approval, housing and handling of animals was according to the guidelines of the New South Wales Department of Primary Industries. Precise formulation of the micro-emulsion oil/surfactant phase should be as previously described [8]. Briefly, medium chain oils consisting of glycerides of capric and caprylic acid (Croda International, Abitec Corporation) were added to polyoxyethylene sorbitan oleate and sorbitan oleate surfactants (1.5/1.0, w/w, Croda International), resulting in a final oil/surfactant ratio of 5.4/1.0, w/w. The ME solution for topical immunisation was prepared by diluting a solution of tetanus toxoid (TT, CSL Laboratories) with saline and addition to the oil/surfactant phase of the emulsion at a ratio of oil/surfactant:aqueous actives of 10:1 (v/v), giving a final vaccine dose of 20 μg. Chemical adjuvants were dissolved into the TT solution before addition to the oil/surfactant phase of ME to give a final vaccine dose of 10 μg muramyl dipeptide (M, Sigma–Aldrich) or murabutide at 30 μg and 20 μg monophosphoryl lipid A from Salmonella Minnesota Re 595 (ML, InvivoGen and Sigma–Aldrich respectively). Adsorption of TT to aluminium phosphate for IM injection was according to an established protocol [11]. For topical administration, precipitation of aluminium phosphate and adsorption of TT was performed within the ME vehicle by mixing three separate MEs containing aluminium chloride, trisodium phosphate or TT.

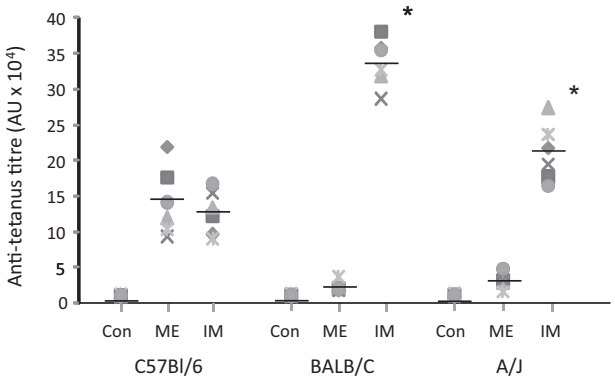

Fig. 1. Topical vaccination of mouse strains. Three strains of mice were immunised with 20 μg of tetanus toxoid by the topical route of administration (ME) or by intramuscular injection (IM) as indicated in the graph. Control mice (Con) were vaccinated by topical application of ME vehicle alone. Scatter plots show the tetanus specific serum IgG titres for the six mice within each group after three rounds of vaccination. Lines represent the mean value from each group. Statistical significance between groups marked $^*p < 0.01$.

2.2. Vaccination and immune assays

Topical vaccination was performed by administration of 30 μl of ME formulations onto a 2 cm^2 patch of shaved abdominal skin. For all topical applications, skin was shaved 24 h before treatment and mice were prevented from grooming for 30 min after administration by observation. Intramuscular (IM) vaccination was performed by injection of 20 μg TT in 0.05 ml into the quadriceps muscle with or without Alum adjuvant. Subcutaneous (SC) booster vaccination was performed by injecting 20 μg of TT in 0.1 ml into the dorsal skin fold. Topical immunisation with the ME vehicle alone was used as a negative control. Tetanus specific antibody was measured by serial dilution of serum and standard ELISA protocol using an alkaline phosphatase conjugated anti-mouse IgG antibody (Sigma). Pre-immune serum was assayed for TT specific serum antibody and results for all mice were equivalent to baseline levels for the ELISA assay (less than 1 AU). Footpad oedema was induced by injection of 20 μg of TT in 0.05 ml of saline into the left footpad. A constant pressure micrometer for determining paper calliper was used for footpad measurements. The level of oedema was measured as the increase in footpad thickness in the antigen injected left footpad relative to the right footpad injected with saline alone. Significance was determined using the independent student's *T*-test.

3. Results

3.1. Immunisation by topical ME or IM injection

Measurements of the immune response in mice can be problematic since the type and level of response is dependent on many parameters that are independent of antigen delivery, in particular, the strain of mouse used. In order to account for different response types, we performed experiments on three strains of mice, C57Bl/6, BALB/c and A/J. The humoral response to topical application of TT in ME was compared to a standard method for antigen delivery, injection into the quadriceps muscle (IM). Three rounds of immunisation were performed to elevate the response after vaccination with TT protein alone. The results showed that all three strains produced significant levels of TT-specific antibody after IM vaccination. Serum IgG antibody from BALB/C was significantly higher than A/J mice ($p < 0.01$) and approximately 2.5 fold higher than C57Bl/6 (Fig. 1). In contrast, topical immunisation with TT resulted in high serum antibody levels, comparable to that of IM injection, in the C57Bl/6 strain only. The contrast between IM and topical

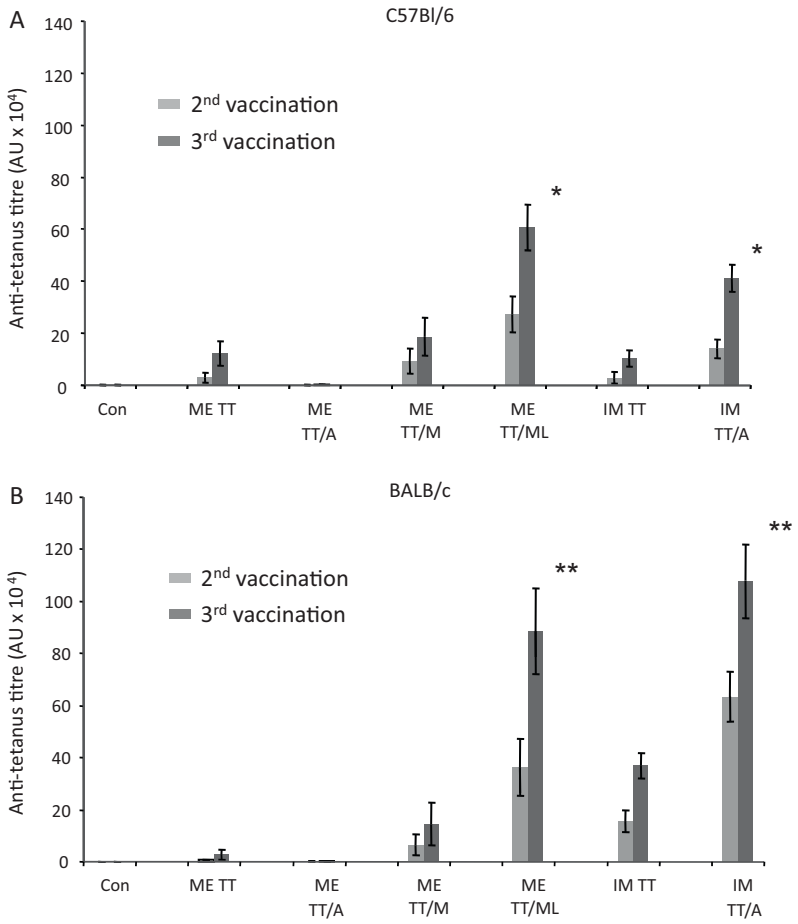

Fig. 2. Topical vaccination with adjuvant. Control mice (Con) were vaccinated by topical application of ME vehicle alone. Test groups were vaccinated with Tetanus toxoid alone (TT) or in combination with Alum (TT/A), muramyl dipeptide (TT/M) or murabutide with monophosphoryl lipid A (TT/ML) administered topically with ME or by IM injection in C57Bl/6 (A) or BALB/c (B) mice as indicated. Graphs show the level of tetanus specific serum IgG titres two weeks after either the second or third round of vaccination. Columns represent the mean and error bars the SEM of ten mice. The data is a representative sample of at least two independent experiments. Statistical significance between columns marked $^{*}p = 0.03$ and $^{**}p > 0.1$.

vaccination was most apparent in BALB/c mice since they were non-responsive to cutaneous administration of antigen in ME but displayed the highest level of TT-specific antibody after IM vaccination.

3.2. Effect of adjuvant on topical immunisation

Topical delivery with the ME vehicle was capable of inducing TT-specific serum antibody in the C57Bl/6 strain, suggesting that poor bioavailability of protein may not be the primary reason for the lack of response in other strains. The anatomical properties of the skin from different strains of mice do not vary significantly and an altered level of immune surveillance is a more probable reason for strain dependent immunity. The ability of various adjuvants to potentiate immunity after topical immunisation was investigated in the two strains that showed a differential response to cutaneous antigen, the high responder, C57Bl/6 and low responder, BALB/C. The level of ME-mediated immunity was compared to the standard vaccine protocol of IM injection of TT adsorbed to aluminium salts (Alum adjuvant) [12]. Topical vaccination with TT was performed after adsorption to Alum or in combination with chemical adjuvants, either muramyl dipeptide alone (M) or a mixture of murabutide and monophosphoryl lipid A (ML). Murabutide is a synthetic analogue of muramyl dipeptide and monophosphoryl lipid A is a detoxified derivative of lipopolysaccharide. Both have been shown to act as strong immune adjuvants but with reduced toxicity and vaccine-related side effects [13,14]. For topical vaccines, adsorption of TT to Alum adjuvant was performed within the ME vehicle itself to allow formation of submicron particles.

The results showed that vaccination with ME can induce a strong TT-specific serum antibody response when combined with potent activators of the innate immune system (Fig. 2). The addition of M adjuvant to ME allowed significant induction of antibody in the non-responsive strain BALB/c and increased the response in C57Bl/6 by 50% over topical TT alone. The humoral response in both strains was still significantly lower than IM vaccination with Alum adjuvant (TT/A). This enhanced response to TT/A injection was not seen when a more potent adjuvant was used during topical immunisation. ME vaccination with the TT/ML mixture resulted in levels of TT specific antibody that were slightly higher, C57Bl/6 ($p = 0.03$) or similar, BALB/c ($p > 0.1$) to IM injection with Alum adjuvant. The addition of a Toll-receptor agonist was essential for high level immunity after topical vaccination with the response to TT/ML 3.2 fold higher in C57Bl/6 and 6.1 fold higher in BALB/c compared to TT/M treatment (Fig. 2). Conversely, topical vaccination with TT adsorbed to Alum resulted in a significant reduction in the levels of specific antibody in both strains when compared to vaccination with TT alone.

3.3. Assay of cell mediated immunity

Delayed type hypersensitivity (DTH) after TT injection into the footpads of immunised mice was used as a measure of cell

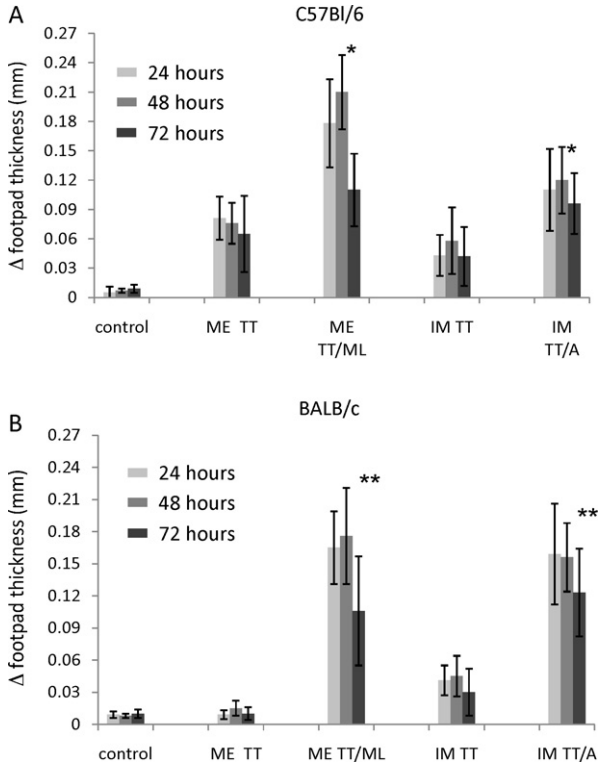

Fig. 3. Cell mediated immunity. Delayed type hypersensitivity was measured by injection of 20 μg of TT into the left footpad and saline into the right footpad of C57Bl/6 (A) or BALB/c (B) mice. Graphs show the change in footpad thickness in the antigen injected footpad relative to the saline injected footpad for each treatment group as indicated. The control group represents topical vaccination of mice with the ME vehicle alone. Columns represent the mean and error bars the SEM of ten mice. Statistical significance between columns marked $^{*}p = 0.008$ and $^{**}p > 0.1$.

mediated immunity [15]. Late timepoints post-injection were used to minimize the contribution of antibody-mediated footpad oedema. Footpad swelling due to cellular infiltration was measured after 24, 48 and 72 h post-injection. The results showed that DTH responses closely mirrored the antibody responses within vaccine groups and ML adjuvant was again required for induction of high cell mediated immunity to TT antigen. Topical vaccination with ME TT resulted in significant DTH responses in C57Bl/6 but failed to induce detectable footpad oedema in BALB/c. Both strains of mice produced strong DTH responses after immunisation with ME TT/ML, comparable to the levels seen after IM TT/A. Topical TT/ML vaccination resulted in higher DTH responses than vaccination with IM TT/A in C57Bl/6 mice ($p = 0.008$) and BALB/c mice showed no significant difference between vaccine groups ($p > 0.1$ Fig. 3).

3.4. Booster vaccination of immune mice

The mice from the topical application group, ME TT/ML and the injected group, IM TT/A showed high levels of humoral and cellular immunity after three rounds of vaccination as described above. These mice were used for analysis of the cutaneous memory response by ME-mediated booster vaccination of immune mice. Immune mice from the topical and IM groups showed baseline levels of TT-specific serum antibody ten weeks after their final immunisation. At this ten week time point, immune mice were given a booster vaccination of TT by topical application in ME or by subcutaneous (SC) injection. SC injection of TT was used for comparison to topical immunisation and not IM injection since IM injection results in a high degree of post-injection muscle damage and inflammation [16]. SC injection was expected to provide a high bioavailability of antigen with significant reduction in injection-induced tissue inflammatory mediators, providing a more useful comparison for the cutaneous memory response to antigen. Topical immunisation of immune mice resulted in induction of an unexpectedly low level of serum antibody with BALB/c mice again showing a weak response to ME treatment with TT alone. Immune C57Bl/6 mice gave higher responses to topical antigen but levels were still significantly lower than that seen with SC injection (Fig. 4).

4. Discussion

The large surface area of skin and dense network of Langerhans cells makes cutaneous immunisation an attractive substitute for needle injection. An essential component of the skin's immune response appears to be appropriate cell activation and subsequent migration of Langerhans cells into draining lymph nodes [17]. This requirement is consistent with the primary function of the skin as a barrier to environmental insults. If the skin is damaged, keratinocytes and Langerhans cells in the lower epidermis can become activated and both cell types are capable of secreting cytokines and modulating the immune response. Studies on topical immunisation with DNA vaccines have indicated that Langerhans cells rapidly leave the area of application and induction of adaptive immunity is largely independent of the site of immunisation. Topical immunisation with plasmids encoding green fluorescent protein via a gene gun clearly showed migration of cells to lymph nodes and expression of GFP [18]. Adoptive transfer of cells containing encoding plasmid was capable of generating an adaptive response *in vivo* [19]. Other experiments have shown that removal of the application site after DNA vaccination does not disrupt the development of antigen specific immunity [20].

The extent of immune surveillance in normal epidermal tissue and its capacity to elicit an adaptive response in the absence of potent cell activation signals is an important factor in the design of topical vaccines or for understanding the origin of chronic inflammatory disorders of the skin such as psoriasis. One barrier to the study of immune function in normal tissue is permeation of foreign antigen through the StCm without causing damage to the lower epidermis. It is becoming increasingly evident that cell damage results in the release of endogenous adjuvants that enhance immune surveillance and the adaptive response to antigen [21]. The ME vehicle appeared to allow significant permeation and spread of antigen within skin [8] and may provide insight into the efficiency of immune surveillance in intact, non-inflamed tissue.

The significance of the animal model in the analysis of skin was demonstrated by the differential response between C57Bl/6 and BALB/c mice. These mice represent distinct response types and can be distinguished by their cytokine expression profiles, responsiveness to allergens and susceptibility to infections [22]. C57Bl/6 mice are highly resistant to cutaneous infection by Leishmania major when compared to BALB/c and this resistance would be consistent with an increased level of immune surveillance [22]. Parasites lack many of the pathogen associated molecular patterns (PAMPs) carried by other infectious agents and Leishmania species are capable of nonspecific immune suppression that often results in antigen-specific anergy [23,24]. Our data indicated that C57Bl/6 skin had a higher capacity than BALB/c for eliciting antigen specific responses when activation of the innate immune system by infectious or endogenous immune potentiaters is minimal.

Altered immune surveillance between C57Bl/6 and BALB/c mice is further supported by differences in the number and expression profiles of immune cells in the epidermis. BALB/c mice have a very low level of dendritic epidermal T cells ($\gamma\delta^{+}$) compared to C57Bl/6 and these cells are known to play a role in cutaneous

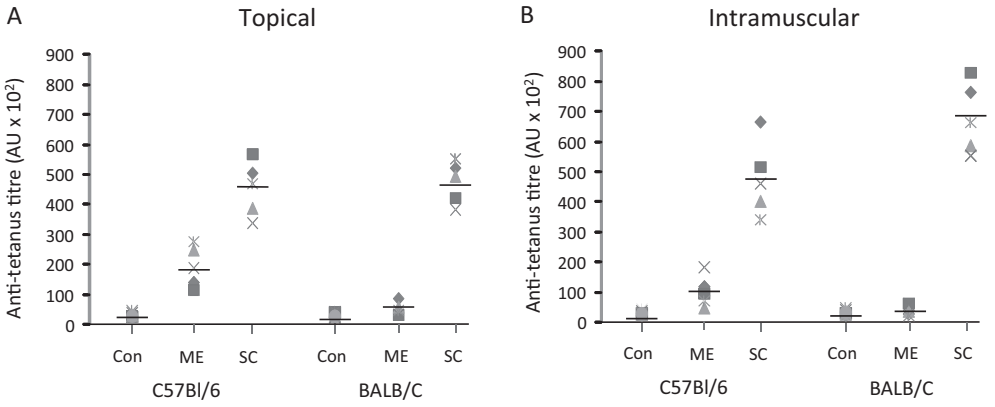

Fig. 4. Booster vaccination of immune mice. TT was administered topically with ME or by subcutaneous (SC) injection in C57Bl/6 or BALB/c mice as indicated. Booster vaccinations were performed on immune mice previously immunised by three successive inoculations with ME TT/ML (Topical, A) or IM TT/A (Intramuscular, B). Control mice (Con) represent the level of serum antibody prior to booster vaccination. Scatter plots show the tetanus specific serum IgG titres for each group of five mice. Lines represent the mean value from each group.

immune surveillance [26]. Conversely, tail skin from BALB/c mice was shown to have twice the number of Langerhans cells than skin from C57Bl/6 [25,27]. BALB/c mice, however, show a considerable higher proportion of CD8α^{high} Langerhans cells and this subset has been shown to be associated with allograft tolerance [25,28].

An elevated cutaneous response can be protective against infection but constant immune surveillance may lead to inflammatory or autoimmune disorders of the skin through repetitive exposure to a sensitizing agent. One theory for the high incidence of psoriasis (2–3% of the population in Northern Europe) is that a hyper-response to antigen provides a selective advantage by increased resistance to cutaneous infection [29]. A balance must be maintained between the capacity to respond to infection and the need to minimize immune hypersensitivity. A key element is the immune mechanisms that activate the innate immune system specifically in response to wounding and infection.

Our studies suggested that cutaneous tissue is not normally hyper-responsive to antigen since topical vaccination appeared to require strong adjuvants for high level immunity. Although the C57Bl/6 strain responded to TT antigen alone, significant induction of serum antibody required three rounds of vaccination and was six fold lower than vaccination with ML adjuvant. In our earlier experiments, topical muramyl dipeptide failed to act as a strong adjuvant when compared to IM injected Alum and this limited capacity to potentiate immunity after topical vaccination warranted an adjuvant that acted through multiple receptors. The strong adjuvant effect from the ML combination compared to muramyl dipeptide alone suggested that monophosphoryl lipid A was a critical immune potentiator and future experiments will be needed to determine if it can act as a strong adjuvant on its own. Topical vaccination of the BALB/c strain was almost entirely dependent on use of the ML adjuvant. This requirement did not appear to involve MHC dependent differences in TT antigen presentation since the serum IgG antibody response in BALB/c was significantly higher than C57Bl/6 after topical vaccination with ML adjuvant (Fig. 2). Higher humoral immunity in BALB/c with either IM or topical TT/ML vaccination was an indication that the ability of C57Bl/6 to respond to topical TT alone was due to differences in constitutive immune surveillance within normal skin. The poor performance of topical DNA vaccines in humans compared to mice highlights the need for potent adjuvants in the cutaneous immune response. Decreased immunity in humans is thought to involve a reduction in the capacity of CpG DNA to act as an adjuvant, due to the relatively low expression of Toll-like receptor-9 in human skin [30]. The apparent need for activation of both Nod-like and Toll-like receptors to generate immunity comparable to the positive control, IM injected TT/A, further supported the hypothesis that skin may not act as a preferential immune organ.

Topical vaccination with TT and Alum adjuvant failed in both strains. This result may be due to Alum interference with skin penetration, possibly due to volume to surface constraints from salt precipitates that interfere with droplet deformation. A reduction in bioavailability due to Alum adsorption is supported by a large reduction in humoral immunity when compared with topical vaccination by TT alone (Fig. 2). Although the aqueous droplets in the ME are nanometer in size, we believe that effective penetration through the intercellular passages of the StCm still requires extensive deformation and elongation of the droplet. The integration of the aqueous phase into a skin permeable oil/surfactant phase may be the driving force behind this mechanism of penetration.

Booster vaccination of immune mice by topical application in ME induced a surprisingly weak antibody response. Antigen presentation in skin-draining lymph nodes results in expansion of a phenotypically distinct subset of memory T cells. These cells can selectively exit into skin from circulation by expression of cutaneous lymphocyte antigen and an undefined group of chemokine receptors [31]. The strong immunity elicited from previous vaccinations indicated that immune mice should have an elevated memory T cell response and augmented immunity after booster vaccination. The relatively weak response was evidence that appropriate immune activation signals may still be required for efficient activation of immune cells within cutaneous tissue. These signals may be present at low levels with passive exposure to antigen by administration in ME. In a separate study, an eight week treatment of mouse skin with ME resulted in no evidence of skin damage or inflammation by skin sampling and histological analysis (data not shown). The effect of adjuvant on booster vaccination remains to be determined and extended time points for serum antibody measurements post-vaccination may be needed to properly evaluate the memory response after topical immunisation; however, this extension did not fit within the ethics and scope of the study.

ME-mediated delivery of proteins into living epidermal tissue could provide a useful tool in the study of skin diseases. Cytokines or therapeutic agents could be administered and their role in the disease process determined with minimal interference from collateral tissue damage or release of inflammatory mediators. This study underlined the value of specific chemical adjuvants in topical vaccination and similar studies could be performed to evaluate other immune potentiators or suppressors either for induction of protective immunity or tolerance. These experiments could take

advantage of the ability of C57Bl/6 and BALB/c to act as distinct animal models for study of the cutaneous response to antigen.

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