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Targeting Skin Dendritic Cells to Improve Intradermal Vaccination

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Abstract

Vaccinations in medicine are typically administered into the muscle beneath the skin or into the subcutaneous fat. As a consequence, the vaccine is immunologically processed by antigen-presenting cells of the skin or the muscle. Recent evidence suggests that the clinically seldom used intradermal route is effective and possibly even superior to the conventional subcutaneous or intramuscular route. Several types of professional antigen-presenting cells inhabit the healthy skin. Epidermal Langerhans cells (CD207/langerin^{+}), dermal langerin^{neg}, and dermal langerin^{+} dendritic cells (DC) have been described, the latter subset so far only in mouse skin. In human skin langerin^{neg} dermal DC can be further classified based on their reciprocal expression of CD1a and CD14. The relative contributions of these subsets to the generation of immunity or tolerance are still unclear. Yet, specializations of these different populations have become apparent. Langerhans cells in human skin appear to be specialized for induction of cytotoxic T lymphocytes; human CD14^{+} dermal DC can promote antibody production by B cells. It is currently attempted to rationally devise and improve vaccines by harnessing such specific properties of skin DC. This

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could be achieved by specifically targeting functionally diverse skin DC subsets. We discuss here advances in our knowledge on the immunological properties of skin DC and strategies to significantly improve the outcome of vaccinations by applying this knowledge.

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1 Modern Vaccine Science—Devising Rational Vaccines

Vaccinations in medicine are a success story. They are well established and well investigated. The traditional vaccines induce robust immunity against bacterial and viral microbes, thereby preventing the outbreak of infectious diseases. The commonly applied vaccines, which are used worldwide, were developed by microbiologists. Louis Pasteur discovered that distinct microbes cause diseases and that attenuated microbes can induce long-lived protection against a subsequent infection by the pathogenic, i.e., non-attenuated form of that organism. This was long before there was any clear understanding of cellular, let alone molecular mechanisms of vaccine immunity, such as the decisive role that dendritic cells (DC) have in this process (Steinman 2008b). The twentieth century brought major advances in our knowledge and understanding of the immune system. This initiated a new period of vaccine research that is based on our understanding and exploitation of key immune principles rather than on the empirical approach.

A vaccine can be defined as *a formulation that induces specific, non-toxic, and long-lasting immune responses to prevent or treat disease* (Steinman 2008b). Typically, this was, and still is, an infectious disease. Present vaccine research attempts to widen the spectrum of antigens, against which one could vaccinate, and include antigens specific for cancer, autoimmunity, or allergy (Pulendran and Ahmed 2006). Thus, in the future vaccines will not only serve to enhance immunity in the classical sense, but hopefully also to regulate or dampen it or even induce immunological tolerance in patients, as it would be desired in autoimmune diseases. DC are the prime inducers and regulators of immunity and tolerance. They are critical in designing of modern vaccines and are, therefore, being increasingly recognized in this context (Banchereau et al. 2009; Steinman 2008a; Steinman and Banchereau 2007). It is important to study these cells in vivo in order to move beyond traditional approaches and devise vaccines that directly take advantage of the specialized properties of DC to control immunity (Steinman 2008b). Thus, current vaccinology is characterized by the continuing use of the established and undisputed classical vaccines and by a wide open field of research that aims at rationally utilizing immunological knowledge to make vaccines helpful in a much wider spectrum of diseases than today.

2 Skin Dendritic Cells are Recipients of Intradermal Vaccines

Vaccines are commonly administered into the skin by injection. Most vaccines in humans, however, are deposited into the subcutaneous fat or into the muscle beneath the skin. Relatively few vaccines chose the route into the dermis (Nicolas and Guy, 2008). This comes a bit as a surprise to the dermato-immunologist, who has been studying for many years the prominent, though not completely understood, network of DC in the dermis and epidermis. These two layers of the skin are densely inhabited by different subsets of DC. In contrast, SC fat and muscle tissue (Casares et al. 1997; Dupuis et al. 1998; Hart and Fabre 1981) contain relatively few, not well-investigated DC. This conceptual discrepancy reflects

the above-described fields of vaccinology, namely, the traditional, empirical approach and the modern, rational approach. A recent example for an intradermal (ID) vaccine is a newly developed influenza vaccine that is administered into the dermis and that was shown to elicit good immune responses (Arnou et al. 2009). Less well-characterized and hardly applied clinically is the topical route, often called transcutaneous (Frech et al. 2008; Warger et al. 2007) or epicutaneous. Each of these routes of application (intramuscular, subcutaneous, ID, and epicutaneous) requires the presence of DC in the tissue that take up the vaccine, process it, transport it, and present it to T lymphocytes in the draining lymphoid organs. Different subsets of skin DC have been described over the years, starting from epidermal Langerhans cells already in the nineteenth century (Langerhans, 1868) to dermal langerin^{+} DC only few years ago (Bursch et al. 2007; Ginhoux et al. 2007; Poulin et al. 2007). For more in-depth reviews about skin DC, in particular Langerhans cells, the reader is referred to companion articles by Ginhoux et al. (2010) and Teunissen et al. (2010) in this issue of *Current Topics in Microbiology and Immunology*, to a few recent reviews (Dupasquier et al. 2008; Merad et al. 2008; Romani et al. 2008, 2010a; Zaba et al. 2009), and to an entire issue of *Immunology and Cell Biology* (Special Feature: Understanding the biology and function of Langerhans cells; volume 88 issue 4, 2010).

2.1 Langerhans Cells

The classical skin DC is the Langerhans cell (LC) of the epidermis (Romani et al. 2010a). This cell type has long been known and it is well characterized. LC form a network that spans our entire body (Fig. 1). They occur also in mucosae (Iwasaki 2007), including the oral/buccal (Cutler and Jotwani 2006) and nasal (Allam et al. 2006) mucosae, which occasionally serve as a site for vaccination. Examples are the well-known oral vaccination against polio or the less established intranasal vaccination against influenza (aerosol or powder administration) (Deans et al. 2010; Hickey and Garmise 2009). It is obvious that in these non-cornified epithelia LC most likely get in contact with the vaccine. Indeed, skin DC in the mouse, possibly including LC express CD155, the receptor for polio virus and CD155 knock-out mice, mount reduced IgG and IgA responses (Maier et al. 2007). This suggests that LC may play an important role in oral vaccination.

2.2 Dermal Langerin^{negative} Dendritic Cells

Healthy human and murine skin harbors dermal DC that do not express langerin/CD207 (Dupasquier et al. 2008). They were first unequivocally identified by studying human and murine skin explant cultures, a method to obtain *mature* skin DC, developed by the group of Jonathan Austyn 20 years ago (Larsen et al. 1990). Conspicuous cells emigrated from the explants over a culture period of 2–4 days. Their typical morphology (thin cytoplasmic processes, “veils”, see Fig. 2), their strong T cell stimulatory capacity in allogeneic mixed leukocyte reactions, and the absence of macrophage markers such as CD14, CD68, or F4/80 (in the mouse) proved their DC nature (Ebner et al. 1998; Lenz et al. 1993; Nestle et al. 1993). This clear picture contrasts with the much more complex situation in situ. There was, and still is, quite some uncertainty as to the relative proportions of DC and macrophages in the dermis. Important studies by the groups of Michelle Lowes in human skin (Zaba et al. 2007; Zaba et al. 2009) and Pieter Leenen in murine skin (Dupasquier et al. 2004, 2008) clarified the issue to a large extent. This work showed and emphasized that the healthy

dermis generally contains more macrophages than DC. In human skin, macrophages were identified by virtue of their expression of CD163, a scavenger receptor (Zaba et al. 2007). $CD163^+$ dermal cells did not co-express the DC-specific molecules CD1c or CD11c. High levels of autofluorescence are also useful to discriminate dermal macrophages from dermal DC (Haniffa et al. 2009). Macrophages in mouse dermis were characterized by expression of CD301, a galactose-/N-acetylgalactosamine-specific C-type lectin receptor (Dupasquier et al. 2004). Haniffa et al. have recently unraveled an important role for these dermal macrophages in sustaining graft-versus-host disease in human transplant patients (Haniffa et al. 2009). Phenotypical markers such as DC-SIGN/CD209, which were previously thought to be specific for (dermal) DC, are also expressed on macrophages (Granelli-Piperno et al. 2005; Zaba et al. 2007) and, therefore, confounded analyses for some time.

In spite of this progress the relationship between macrophages and DC in the dermis is still not entirely clear. As mentioned, there are distinct phenotypical differences between DC and macrophages in situ. In skin explant cultures, however, the population of migrated dermal DC appears relatively homogenous. The majority of these mature human DC express high levels of MHC class II, CD86, CD80, CD40, CD83, CD205, and CD208/DC-LAMP but no CD14 (Ebner et al. 2004). There is only a small subset of $CD14^+$ cells that is negative for CD205 and DC-LAMP. These are probably macrophages. With regard to CD14 and CD1a expression, these observations in skin explant cultures are similar to what was found in populations that had been directly isolated from human dermal tissue by enzymatic treatment. Dermal DC could be further subdivided into a quantitatively minor population expressing CD14 but not CD1a and a major population characterized by strong CD1a but not CD14 expression (Angel et al. 2007, 2009). In essence, this was already anticipated by Nestle et al. (1993). Whereas macrophages outnumber DC in the dermis of the mouse (Dupasquier et al. 2004) or are at least present in almost equal numbers in human dermis (Zaba et al. 2007) in situ, cells with a clear DC phenotype and morphology are more numerous in the migrant (“crawl-out”) populations. Thus, these proportions become inverted. This raises the question whether macrophages may be more firmly anchored in the dermal connective tissue and stay behind, as indicated in recent study by Haniffa et al. (2009). Alternatively, intrinsic differences in molecules involved in migration [chemokine receptors, matrix metalloproteinases (Ratzinger et al. 2002), etc.] could account for the discrepancy between in vivo and ex vivo proportions of macrophages and DC. Finally, the strong inflammatory milieu in these explant cultures may make some or many macrophages transform into DC. Such a transformation might happen as macrophages migrate across endothelial borders into lymph vessels, as it was shown for skin DC (Randolph et al. 2008; Romani et al. 2001). Transmigration across endothelial barriers of blood vessels can indeed mediate transformation of monocytes into DC in vitro (Randolph et al. 1998) and in vivo (Randolph et al. 1999). Very recent evidence further supports the transition from monocytes to DC in vivo: DC-SIGN/CD209-expressing DCs arise in mouse lymph nodes in response to LPS. They were shown to derive from monocytes (Cheong et al. 2010). What stands against the validity of this notion in the context of skin, at least at first glance, is the observation by Förster’s team, that in skin explant cultures many DC probably migrate into the culture medium without ever entering lymph vessels. This was inferred from skin explant cultures in CCR7 knock-out mice where no DC-filled lymph vessels [“cords” (Larsen et al. 1990;

Lukas et al. 1996; Weinlich et al. 1998)] could be found but yet, many more DC migrated into the culture medium (Ohl et al. 2004). However, these authors also noted that in the CCR7 knock-out mice migration of LC into the draining lymph nodes was severely inhibited indicating that in the intact organism the majority of cells migrate indeed via lymph vessels, and therefore, such a transformation could theoretically take place. In support of this hypothesis are unpublished observations from our lab. We found that cells expressing typical macrophage markers such as FXIIIa (Zaba et al. 2007) or CD68 became less in numbers during an explant culture indicating that they physically leave the dermis (Fig. 3). In conclusion, the interrelationship of macrophages and DC in the dermis is not yet resolved and clearly needs more study.

2.3 Dermal Langerin$^+$ Dendritic Cells

Few scattered langerin$^+$ cells have been observed in healthy human skin ever since antibodies against langerin were available, i.e., the antibody against the “lag” antigen (Kashihara et al. 1986), and antibodies against langerin/CD207 (Valladeau et al. 1999). Little attention was payed to these cells, mainly because they were so very few in numbers as compared to LC, but also because it seemed clear that they were epidermal LC in transit to the lymph nodes. Only recently this issue was revisited using modern methodologies such as bone marrow chimeric mice (Merad et al. 2008), LC ablation models (Kaplan et al. 2008), and langerin-EGFP transgenic mice (Kissenpfennig et al. 2005). It turned out that these langerin$^+$ cells in the dermis were, at least in part, a dermis-resident population, unrelated to LC and named “dermal langerin$^+$ DC” (Bursch et al. 2007; Ginhoux et al. 2007; Poulin et al. 2007). As opposed to the scarcity of dermal langerin$^+$ DC in situ, they can be readily detected in the migrated populations from skin explant cultures or, even more so, in the draining lymphoid organs. Their frequency in the dermis of mice is much lower than the frequency of langerin^{neg} dermal DC. The human counterpart for this very rare population is currently being investigated; there are hints that human dermis also harbors such a subset (reported in ref (Romani et al. 2010a) and discussed in a companion article by Teunissen et al. (2010).

2.4 Which Subset of Skin Dendritic Cells is the Major Recipient of an Intradermal Vaccine?

At first glance it would seem logical that dermal DC would pick up most of the ID injected antigen. It should be mentioned up front that this issue has not been studied systematically. Especially little is known in this regard about human skin. Nevertheless, some interesting pieces of knowledge have emerged from old and recent studies. Using an antibody (against MHC class II) as a protein antigen, Aberer et al. (1986) noted, somewhat surprisingly, that after intraperitoneal injection of the protein even epidermal LC had captured the protein. They detected this by simply labeling epidermal sheets from such treated mice with a fluorescently labeled secondary antibody. This underscored that a vaccine can easily reach LC “from within”. Recently, Flacher et al. (2010) addressed this question in an experimental setting that was more realistic with regard to clinical vaccinations. A protein antigen (again an antibody directed to an endocytic receptor on the surface of LC), which was injected into the dermis of mice (into the ear pinna), was readily taken up by epidermal LC, again emphasizing that LC are most likely involved in ID vaccination, even though the vaccine is not placed directly into the habitat of these cells. Similarly, this antigen also reached human

LC when placed into a skin explant culture (Flacher et al. 2010). The mechanism by which protein antigen crosses the basement membrane between the dermis and the epidermis was not studied. Presumably it is diffusion. However, an active mechanism whereby LC "reach out" into the dermis in order to fetch the antigen cannot be ruled out. This was shown for gut DC that extend their "arms" into the lumen of the gut to grab bacteria (Rescigno et al. 2001). It was also observed with LC that reach "up" into the horny layer (stratum corneum) of the epidermis where they might sample and take up microbes (Kubo et al. 2009). As expected, ID-injected protein antigen is readily taken up by both langerin^{neg} and langerin^{+} dermal DC (Flacher et al. 2010). Thus, it seems that in ID vaccination all subsets of skin DC gain access to the antigen and, therefore, contribute to the response.

There is one important caveat, though, to this conclusion. The experiments described above were performed with antibodies recognizing cell surface molecules on LC, i.e., MHC class II and the endocytic C-type lectin receptors DEC-205/CD205 and langerin/CD207. Control antibodies that did not have a binding partner on the surface of the LC, e.g., anti-langerin antibody 929F3, which recognizes only the intracellular domain of langerin, did not reach LC in detectable quantities (Flacher et al. 2010). On the one hand, this highlights the potential of targeting antigens selectively to receptors on skin DC [as discussed below and reviewed in ref. (Romani et al. 2010c)]. On the other hand, it leaves the question open, how LC would contribute when a conventional protein antigen, which is not a LC-binding antibody, is injected into the skin. Again, it would be expected that dermal DC take up this antigen. However, in a skin explant model where the fluorescently conjugated model protein ovalbumin (OVA) was offered in the culture medium, we could expectedly show uptake into langerin^{+} as well as langerin^{neg} dermal DC. But also LC in situ took up readily detectable quantities of OVA (Sparber et al. 2010). Compared to antigen conjugated to surface receptors on LC, however, at least 100-times more native protein needed to be given (Flacher et al. 2010). Also when injected ID did epidermal LC capture the protein antigen and carry it to the lymph node (Sparber et al. 2010).

Taken together, the evidence points to an involvement of all subsets of skin DC, including LC, in ID vaccination. The relative contributions of the different subsets, in particular of LC, may critically depend on the quantity of ID-injected antigen but also on the expression of receptors involved in internalization (see below).

3 Functional Repertoire of Skin Dendritic Cells

About a decade ago there was no doubt in the field that LC and dermal DC were always immunogenic in vivo. This conclusion was based on the manifold evidence that they are strongly immunostimulatory in in vitro experimental settings (Romani et al. 1989; Schuler and Steinman 1985; Stingl et al. 1980). LC were regarded as prototype DC that served as the "role model" for all other DC. This concept was frequently called the "Langerhans cell paradigm" (Girolomoni et al. 2002; Wilson and Villadangos 2004). This paradigm needed to be revisited in response to two important findings. (1) It was recognized that DC not only induce immunity but also serve to establish and maintain tolerance (Steinman et al. 2003; Steinman and Nussenzweig 2002). This occurs when they present antigen in the steady state, i.e., in the absence of full maturation. (2) Furthermore, herpes virus infection models and

transgenic antigen expression models in mice showed that LC do not under all circumstances present antigens that they have acquired in the skin in the draining lymph nodes in vivo; other DC including dermal langerin$^+$ DC can do the (cross-) presentation (Allan et al. 2003; Bedoui et al. 2009; Heath and Carbone 2009; Henri et al. 2010). Research into the in vivo functions of LC is presently in full bloom (Romani et al. 2010b), taking advantage of modern methodology, foremost of mouse models where langerin$^+$ cells can be selectively depleted from the living mouse [reviewed in (Kaplan et al. 2008)].

Early investigations of LC-like (CD1a and langerin-expressing) and non-LC-like (CD14-expressing, interstitial type, dermal type) DC grown from human CD34$^+$ hematopoietic stem cells highlighted possible functional differences between the different types of skin DC for the first time. LC-like DC take up less endocytic tracers such as fluorescein isothiocyanate dextran or peroxidase. Another difference is the failure of LC-like DC to induce naive B cells to differentiate into IgM-secreting cells, in response to CD40 triggering and interleukin-2 (IL-2), as opposed to interstitial type DC (Caux et al. 1997). This was essentially verified with LC and CD14$^+$ dermal DC directly isolated from human skin. The former subset was superior in cross-priming CD8$^+$ T cells, and the latter subset was specialized to prime CD4$^+$ helper T cells that in turn induced B cells to become antibody producing cells (Klechevsky et al. 2008). As pointed out above, the CD14$^+$ subset of dermal DC comprises only about a tenth of all langerin^{neg} dermal DC (Banchereau et al. 2009). The majority of langerin^{neg} dermal DC, i.e., CD14^{neg}/CD1a$^+$ cells, appear to be functionally in between LC and the CD14$^+$ subset (Klechevsky et al. 2008). It remains unclear at this point how this could relate to ID vaccination, notably because the means of antigen uptake by DC in situ or by DC cultured ex vivo are likely to differ fundamentally. Nevertheless, these in vitro data correspond to in vivo observations in mice, where skin-derived dermal DC localized close to the B cell follicles in the outer paracortex of the lymph node. LC, in contrast, arrived and settled in the inner paracortex, intermingled with lymph node-resident langerin$^+$ cells (Kissenpfennig et al. 2005). It is not known, however, whether these dermal DC correspond to the human dermal DC subset that promotes the humoral response, i.e., the CD14$^+$ subpopulation. Yet, these findings highlight that a “division of labor” may indeed be operative in vivo.

At present, virtually all clinical trials that attempt to harness the immunogenic potential of DC are vaccination studies against cancer. They aim primarily at generating powerful cytotoxic T lymphocytes. Some recent observations are of importance in this regard. Several groups could show that LC, as opposed to dermal DC, are especially capable of inducing cytotoxic T lymphocytes. This was first shown with human LC-like DC derived from CD34$^+$ stem cells (Ratzinger et al. 2004). Importantly, these data were recently confirmed using human LC isolated from the epidermis; LC were indeed more potent to induce CD8$^+$ T cells that contained increased levels of lytic molecules (perforin, granzymes) and that efficiently killed tumor cell lines. LC-derived IL-15 appeared to be a critical factor (Klechevsky et al. 2008; Ueno et al. 2010). Of note, we found mature human LC, obtained by migration from cultured epidermal sheets, to induce substantial levels of IFN-γ secretion in naive allogeneic CD4$^+$ helper T cells (Ebner et al. 2007), surprisingly without detectable secretion of bioactive IL-12 p70 (Ebner et al. 2001). Also murine epidermal LC clearly have the capacity to elicit IFN-γ producing T cells (Koch et al. unpublished data; Table 1) in spite

of making almost no bioactive IL-12 (Heufler et al. 1996). Studies in a mouse tumor model confirmed and emphasized that LC (and possibly also dermal langerin$^+$ DC) are essential for anti-tumor immunity in vivo. Protection from an experimental tumor (B16 melanoma) was lost when LC and dermal langerin$^+$ DC had been ablated (Stoitzner et al. 2008) by means of the diphtheria toxin receptor knock-in technology (Kaplan et al. 2008).

In conclusion, it is important and promising to rationally address DC subsets of the skin, especially LC, for purposes of vaccination (Ueno et al. 2010). Depending on the subset that contributes most, the response will be dominated by cytotoxic T cells, and thus be useful for vaccinating against cancer, or by a humoral response and thus be beneficial for vaccination against infectious agents. LC appear to be responsible for the development of cytotoxicity, and CD14$^+$ dermal DC for the generation of an antibody response. Clearly, in reality the picture is not as simple as that. Yet, these data form a good basis to further explore the relative roles of skin DC in ID vaccination. One elegant approach to address this issue in vivo is to target antigens to one subset or another (see Sect. 4.1.5).

4 Harnessing the Distinct Properties of Skin Dendritic Cells for Intradermal Vaccination

4.1 Augment Lymphocyte Responses by Targeting Vaccine to Specific Antigen Uptake Receptors on Skin Dendritic Cells

4.1.1 What is “Antigen Targeting”?—DC are equipped with a wide range of receptors that facilitate the uptake of pathogens, including the so-called “C-type lectin receptors”. They recognize pathogen-associated molecular patterns (Figdor et al. 2002). Important examples are DEC-205/CD205, langerin/CD207 (Fig. 1), DC-SIGN/CD209, Dectin, and DCIR2. Groundbreaking studies from the groups of Steinman and Nussenzweig at Rockefeller University labs has revealed that immune responses can be dramatically enhanced when an antigen is delivered (“targeted”) directly and selectively to DC rather than being “only” injected into the dermis or under the skin (subcutaneously or intramuscular, as in conventional vaccinations or in footpad injections in mice). In other words, the vaccine obtains an “address tag” in the form of a specific antibody against a C-type lectin receptor. Thereby, the antigen or vaccine is guided directly and exclusively to the DC that expresses the respective C-type lectin receptor on its surface. This is achieved by coupling protein or peptide antigens to monoclonal antibodies against C-type lectin receptors. Researchers employ chemical conjugation methods or, preferrably, the genetic engineering approach. Antibodies to different C-type lectin receptors for conjugation are used as whole antibodies (Idoyaga et al. 2008) or as single chain fragments (Birkholz et al. 2010; Nchinda et al. 2008). This strategy is currently being extended beyond the widely used model antigen OVA to other antigens like hen egg lysozyme/HEL (Hawiger et al. 2001), or keyhole limpet hemocyanin/KLH (Tacken et al. 2005). Data from the OVA model must always be judged with caution, and premature generalizations must be avoided. OVA peptide-specific TCR transgenic T cells, in particular the CD8$^+$ T cells (“OT-I cells”) are extremely sensitive to TCR stimulation in an unphysiological manner: they already respond to tiny amounts of antigen in the picomolar range (Choi et al. 2009). Therefore, the supplementation of the experimental OVA model with other models is an indispensable

goal, even though the OVA model has yielded and is still yielding important insights. Importantly, a number of antigens relevant for clinical studies have now been successfully used in this approach, including HIV gag (Nchinda et al. 2008; Trumpfheller et al. 2008), a mouse melanoma tumor antigen (Mahnke et al. 2005) or the human tumor antigens mesothelin (Wang et al. 2009) and MAGE-A3 (Birkholz et al. 2010).

The first hints for the potential to enhance immune responses by addressing C-type lectin receptors came from the initial studies of the cell biology of antigen uptake into DC via such receptors (Mahnke et al. 2000). Mahnke et al. investigated the fate of immunoglobulin (Ig) binding to chimeric Fc receptors where the cytosolic domain of the Fc receptor was replaced by the cytosolic portion of two different C-type lectin receptors. When Ig was given to DC expressing the cytosolic part of the DEC-205 receptor, its uptake was strongly enhanced. Moreover, in contrast to targeting via the macrophage mannose receptor, antigens were specifically routed to late endosomes or lysosomes that contained abundantly MHC class II molecules. This resulted in a markedly (up to 100-fold) augmented T cell response in vitro (Mahnke et al. 2000). These seminal observations led to the series of in vivo *studies* described below.

4.1.2 Targeting Dendritic Cells in the Steady State—Immunization of mice with anti-DEC-205 antigen conjugates (OVA or hen egg lysozyme) in the absence of DC maturation stimuli (such as CD40 ligation or poly(I:C)), i.e., in the steady state, led to T cell unresponsiveness in both the $CD4^+$ (Hawiger et al. 2001) and the $CD8^+$ (Bonifaz et al. 2002) T cell compartment. Injection of the conjugates into the footpads under these experimental conditions induced an initial wave of T cell division. Importantly, proliferation was orders of magnitude greater than the proliferation in response to the free, unconjugated antigen. However, this proliferation was not sustained but, rather, T cell numbers dropped, and the remaining T cells did not respond any longer to a standard immunization protocol using complete Freund's adjuvant. This indicated that peripheral tolerance had developed in response to the steady-state administration of the antigen-antibody conjugate (Steinman et al. 2003). These findings were validated in a mouse model for type I autoimmune diabetes where both onset and progression of the disease could be inhibited by treatment with anti-DEC-205-conjugated antigen (Bruder et al. 2005; Mukhopadhaya et al. 2008). The underlying mechanism for tolerance induction appears to be not only deletion and anergy, as shown in the original study (Bonifaz et al. 2002; Hawiger et al. 2001), but also induction of regulatory T cells (Yamazaki et al. 2008).

4.1.3 Targeting Dendritic Cells Under Inflammatory/Immunogenic Conditions —In the above-described studies the outcome in terms of T cell responses changed dramatically when DC maturation stimuli were added at the time of immunization together with the anti-DEC-205–antigen conjugate. Again, T cell proliferation in vivo increased several orders of magnitude as compared with immunization with the same amount of unconjugated antigen, be it a peptide (Hawiger et al. 2001) or the whole antigenic protein (Bonifaz et al. 2002). Importantly, the augmented massive T cell proliferation translated into markedly improved anti-tumor immunity in an experimental model in vivo. The very infrequent naive antigen-specific T cells, which exist in a non-immune mouse, could only be

primed if the subcutaneously (footpad) injected antigen was coupled to an anti-DEC-205 antibody and a strong DC maturation stimulus (CD40 ligation) was co-administered. Uncoupled antigen plus/minus CD40 ligation, as well as immunization with antigen-pulsed cultured DC, did not lead to efficient priming of naïve T cells in this setting (Bonifaz et al. 2004). Alternatively, DC maturation and subsequent responses could be achieved by TLR agonists, such as poly(I:C) (Trumpfheller et al. 2008). This further highlights the potential of antigen targeting also for vaccinations in humans where vaccine-specific T cells would be equally scarce and difficult to be activated in sufficient numbers, enough to obtain clinical effects. Not unexpectedly, the increased stimulation of helper T cells by the anti-DEC-205-antigen conjugates led to amplified antibody responses in mice (Boscardin et al. 2006). These data emphasize that it is also feasible to harness antigen targeting for improving humoral immune responses in vaccinations.

4.1.4 Targeting Different Receptors on the Surface of Dendritic Cells—

Conceptually, two scenarios have to be considered. (1) A given subset of DC expresses simultaneously different targetable endocytic receptors. Immune responses may be influenced depending on which of these receptors is targeted. Examples are described below. (2) Subsets of DC often express different, non-overlapping targetable receptors. Immune responses may be influenced depending on which DC subset receives the antigen. This will be illustrated in Sect. 4.1.5.

The type of targeted endocytic receptor can influence the quantity and quality of resulting immune responses. This became evident in the original studies by Mahnke et al. (2000) who used mouse bone marrow-derived DC that expressed both DEC-205 and macrophage mannose receptor on their surfaces. Yet, antigen targeted to either of these two receptors was taken up differently, routed differently, and enhanced T cell responses were only observed with DEC-205-targeted antigen. A more recent, similar example was provided by Bozzacco et al. (Bozzacco et al. 2007) who compared targeting an HIV antigen (p24 gag) to human DC with anti-DEC-205 vis-à-vis anti-DC-SIGN antibodies. Both receptors are expressed on the same DC at similar levels (Ebner et al. 2004). Yet, the anti-DEC-205-conjugated antigen was more effectively cross-presented than anti-DC-SIGN-conjugated antigen, indicating different intracellular processing.

With special regard to the skin, we found pronounced differences in the handling of the model antigen OVA depending on whether it was offered to epidermal LC by ID injection as an anti-DEC-205/OVA conjugate or an anti-langerin/OVA conjugate. Both receptors are expressed on the surface of murine LC (Cheong et al. 2007; Inaba et al. 1995) and both receptors can be successfully targeted by the respective antibodies (Flacher et al. 2010)(Fig. 1). Idoyaga et al. observed previously that $CD4^+$ and $CD8^+$ T cells proliferated in vivo in the draining lymph nodes following targeting of DEC-205 or langerin receptors by SC injection of the antigen conjugates into the footpads of mice (Idoyaga et al. 2008). On the other hand, when we “loaded” LC in vivo with antigen via DEC-205 or langerin by ID injection into the ear, $CD4^+$ and $CD8^+$ T cell proliferation in vitro was only induced by LC that had taken up the antigen via the DEC-205 receptor. Targeting the antigen to the langerin receptor did not result in $CD4^+$ and $CD8^+$ T cell division. This unexpected discrepancy probably reflects the importance of other $langerin^+$ DC subsets present in the dermis or in lymph nodes draining

the immunization site (see below). These other subsets were *not* present in these experimental settings (Flacher et al. 2010).

4.1.5 Delivering the Vaccine to Selected Dendritic Cell Subsets by Targeting Differentially Expressed Surface Receptors—As discussed above, antigen targeting allows to manipulate the antigen processing and presentation capacity of a given DC such that resulting T cell responses can differ in quantitative and qualitative terms. In the context of most vaccination schemes, including ID vaccination, the injected antigen can be sequestered by different subsets of DC. In the case of skin these are LC and langerin^{neg} and langerin^{+} dermal DC. Here, antigen targeting allows to address the vaccine to defined subsets of DCs. For instance, ID targeting via langerin would bring the antigen to LC and langerin^{+} dermal DC but leave out langerin^{neg} DC (Flacher et al. 2010). Targeting to a putative C-type lectin receptor expressed exclusively by langerin^{neg} dermal DC could be an alternative. Unfortunately, such a molecule has not been found yet. Future research will teach us about pros and cons for one or the other approach.

The importance of delivering vaccine antigens to defined DC subsets was highlighted in the first thorough side-by-side comparison of different targeting strategies by Dudziak et al. (2007). They looked at immune responses induced by immunization with antigens conjugated to antibodies against DEC-205 versus DCIR2 (dendritic cell inhibitory receptor-2; “33D1 antigen”) that are expressed by CD8^{+} and CD8^{neg} DC in the spleen of mice, respectively. Immunization via DEC-205 favored CD8^{+} T cell responses whereas DCIR2 targeting preferentially induced CD4^{+} T cell responses. Gene expression analyses indeed showed that CD8^{+}/DEC-205^{+} DC expressed more genes associated with processing for the MHC class I pathway (TAP, calreticulin, etc.) and CD8^{neg}/DCIR2^{+} DC expressed more genes associated with processing for the MHC class II pathway (cathepsins, etc.) (Dudziak et al. 2007). At another level, it was shown that the selective delivery of antigen to the CD8^{+}/DEC-205^{+} and CD8^{neg}/DCIR2^{+} DC subsets of spleen DC by means of the respective antigen-antibody conjugates in the absence of DC maturation signal, led to the de novo induction of FoxP3^{+} regulatory T cells and to the expansion of preexisting FoxP3^{+} regulatory T cells, respectively (Yamazaki et al. 2008; Yamazaki and Steinman 2009).

Finally, the development of monoclonal antibodies recognizing the extracellular domain of the langerin (CD207 (Valladeau et al. 2000)) molecule (Cheong et al. 2007) opened the way to study antigen targeting to this molecule expressed on important skin DC, particularly on LC. Given the pronounced properties of LC in the induction of cytotoxic responses (see above), targeting this receptor is of high interest. Initial analyses revealed many similarities in targeting properties to DEC-205 and only subtle differences between DEC-205 and langerin targeting (Idoyaga et al. 2008). The SC route (footpad) of immunization was used in these experiments, rather than the ID one. The splenic CD8α^{+} DC subset, which is the key subset responsible for cross-presentation in vivo (Heath et al. 2004), specifically co-expresses the langerin receptor (Douillard et al. 2005; Idoyaga et al. 2009; McLellan et al. 2002). It will be interesting to study antigen targeting to this important population of DC. Recent data suggest that, indeed, cross-presenting activity is restricted to that CD8α^{+}/langerin^{+} DC population in mouse spleen (Farrand et al. 2009).

4.1.6 Targeting Antigens Specifically on Skin Dendritic Cells—Which endocytic receptors on skin DC could be of importance for antigen targeting? For this it is important to determine in detail the expression patterns of the various endocytic receptors on skin DC. These patterns are still incompletely known.

Langerhans cells: They are phenotypically well characterized both in mouse and human skin, and their identifying receptor langerin and the prototype targeting receptor DEC-205 have been dealt with above. Both are expressed on the cell surface of LC (Cheong et al. 2007; Ebner et al. 2004; Inaba et al. 1995; Zaba et al. 2007) (Fig. 1). In addition, they express Dectin-1 (Ariizumi et al. 2000b) and Dectin-2 (Ariizumi et al. 2000a). Antigen targeting to both molecules was shown to elicit stronger responses than immunization with equal amounts of free antigen (Carter et al. 2006a, 2006b). The DCIR2 molecule is absent from murine LC (Witmer-Pack et al. 1987). For human LC this has not yet been determined.

Dermal DC: Most phenotypical data in the dermis do not (yet) take into account the newly described subsets of dermal DC. The “classical”, langerin^{neg} dermal DC express DEC-205 both in human (Ebner et al. 2004; Zaba et al. 2007) and in mouse skin (Henri et al. 2001; Lenz et al. 1993; Nagao et al. 2009). Interestingly, however, unequivocal cell surface expression on immature murine dermal DC has not been demonstrated, not least due to the trypsin sensitivity of this molecule (Inaba et al. 1995). Yet, two features became clear from these studies. DEC-205 is up-regulated on dermal DC, but even then, surface expression is lower than on LC. Finally, the ability to target dermal langerin^{neg} DC with anti-DEC-205 antibodies strongly argues for a functional level of surface expression (Flacher et al. 2010). The other subset of dermal DC, namely, langerin^{+} dermal DC expresses DEC-205 at levels similar to LC and can readily be targeted with anti-DEC-205 [Flacher et al. unpublished observations and (Flacher et al. 2010)]. DC-SIGN, which was initially regarded as a marker for dermal DC (Ebner et al. 2004), occurs more abundantly on macrophages in the dermis as identified by CD14 (Klechevsky et al. 2008; Turville et al. 2002) or CD163 (Zaba et al. 2007) expression. Thinking of vaccinations that aim at generating robust antibody responses, one could envisage to target antigen to dermal DC, namely, the CD14^{+} subset, in order to exploit the capacity of that subset to promote immunoglobulin production (Banchereau et al. 2009). CD36, the thrombospondin receptor and a scavenger receptor might be a candidate since this molecule is specifically expressed on dermal DC (Lenz et al. 1993), and there is evidence that targeting CD36 may also improve immune responses (Tagliani et al. 2008).

The expression on skin DC of additional interesting candidates for targeting such as Clec9 (Caminschi et al. 2008), Clec12 (Lahoud et al. 2009), Lox (Delneste et al. 2002), and others (reviewed recently by Caminschi et al. 2009) has not yet been investigated. Molecules beyond the field of C-type lectin receptors may also be interesting candidates. For instance, improved immune responses have been reported for antigens that were more broadly targeted to MHC molecules (by means of a novel construct based on an MHC-binding superantigen, not with antibodies) (Dickgreber et al. 2009).

In closing, it should be emphasized once more that virtually all studies hitherto used the subcutaneous route of immunization rather than the ID one. Given the likely differences in

DC subset composition between the subcutis and the dermis, it will be necessary to study in detail targeting the various endocytic receptors in ID vaccination.

4.2 Augment and Broaden Responses by Including Innate Lymphocyte Responses

Antigen targeting via endocytic receptors on DC aims at improving $CD4^+$ and $CD8^+$ T cell responses. Cytotoxic T lymphocytes are undoubtedly the immune system's strongest weapon against cancer. However, there are other important innate lymphocytes that are also critically involved in immune defense reactions and that should also take part in vaccine-induced immune responses. NKT cells are important in two ways. First, they can serve as potent cytotoxic effector cells (Cerundolo et al. 2009; Godfrey et al. 2010; Neparidze and Dhodapkar 2009). Second, the mutual cross-talk between NKT cells and DC leads to activation and full maturation of both cells types. The glycolipid alpha-galactosylceramide (alpha-GalCer) is the molecular link between the two cell types. It is presented by DC on their CD1d molecules and thereby mediates the activation and numerical expansion of NKT cells (Fujii et al. 2002). These activated NKT cells, in turn, bring about full maturation of DC (Fujii et al. 2003). These cellular interactions are the likely basis for the observed clinical benefit when using alpha-GalCer as an adjuvant in the treatment of cancer (Chang et al. 2005; Neparidze and Dhodapkar 2009). This approach appears so promising that in a recent review article Fujji and Steinman "*urge development of the DC-NKT axis to provide innate and adaptive immunity to human cancers* (Fujii et al. 2007)".

How can ID vaccination harness the potential of NKT cells? We have recently studied this question in some detail in the mouse (Tripp et al. 2009). The initial observation was that cutaneous DC express and upregulate CD1d on their surface in situ and ex vivo. At least half of the LC and dermal DC (including dermal $langerin^+$ DC) express CD1d on their surface upon emigration from skin explants. In the skin-draining lymph nodes nearly all DC derived from the skin were positive for CD1d besides all lymph node-resident DC subsets. Corresponding to this finding, skin and lymph node-derived DC were able to present the synthetic glycolipid alpha-GalCer to an NKT cell hybridoma in vitro. Moreover, ID-injected alpha-GalCer was incorporated and presented by migratory DC. The adjuvant effect of ID-injected alpha-GalCer was not via a migration stimulus or the induction of maturation markers on lymph node DC, but rather led to an enhancement of CD40 expression on distant, more immature spleen DC. Furthermore, alpha-GalCer activated B and T cells in lymph node and spleen to upregulate CD69. The effects observed in the spleen indicate that ID-injected alpha-GalCer exerts its effect systemically, at least in the small body of a mouse. However, the stimulation of antigen-specific $CD8^+$ T cells happened in a locally restricted way. ID immunization activated cytotoxic T cells only in the lymph nodes, in contrast to the intravenous route which mainly provoked T cell responses in the spleen. Most importantly, ID treatment of mice demonstrated that the combination of a protein antigen plus alpha-GalCer can improve the survival of mice bearing B16 melanoma tumors. Thus, alpha-GalCer proved to be a promising adjuvant not just for intravenous immunization but also for the ID route. A surprising finding of this study was that migratory skin DC are not mandatory to mediate the effect of ID immunization with protein antigen plus alpha-GalCer. When we prevented the participation of migratory skin DC by depletion of $langerin^+$ DC subsets (using the diphtheria toxin ablation technique (Kaplan et al. 2008)) or by removal of

the immunization site within 4 h, activation of cytotoxic T cells was still induced. This indicates that most of the ID-injected antigen and adjuvant diffused through lymph and blood to lymphatic organs where resident DC could stimulate cytotoxic T cell responses. In an attempt to increase T cell responses even more, we compared side-by-side unconjugated and anti-DEC-205-conjugated antigen in combination with alpha-GalCer. We observed that 1,000-times less of the conjugated antigen was sufficient to achieve equivalent anti-tumor responses in the B16 model (Tripp, unpublished observations). This is of importance in regard to the immense costs of producing antigens in good-manufacturing practice (GMP) quality (Ueno et al. 2010). With regard to ID vaccination in man, it is important that dermal DC in human skin express CD1d and could, therefore, be targeted with alpha-GalCer expressed on dermal dendritic DC (Gerlini et al. 2001). In conclusion, the synthetic glycolipid alpha-GalCer proved to be a potent adjuvant for ID immunization with protein antigen and might be a promising adjuvant for targeted immunotherapies.

5 Concluding Remarks

ID injection of an antigen is an old technique as illustrated by the classical tuberculin test. Novel influenza vaccines that are ID injected have only recently been introduced on the market (Deans et al. 2010). They were developed in the classical way, mainly based on empirical knowledge. Researchers are now trying to improve ID vaccination rationally by harnessing the specific properties of skin DC as they become known bit by bit. Targeting of antigens to defined, functionally distinct subsets of skin DC and addressing innate lymphocytes as additional, powerful effector cells are two promising strategies to further establish this route of immunization and, in the more distant future, also tolerization. This should ultimately lead to more effective protective as well as therapeutic vaccines.

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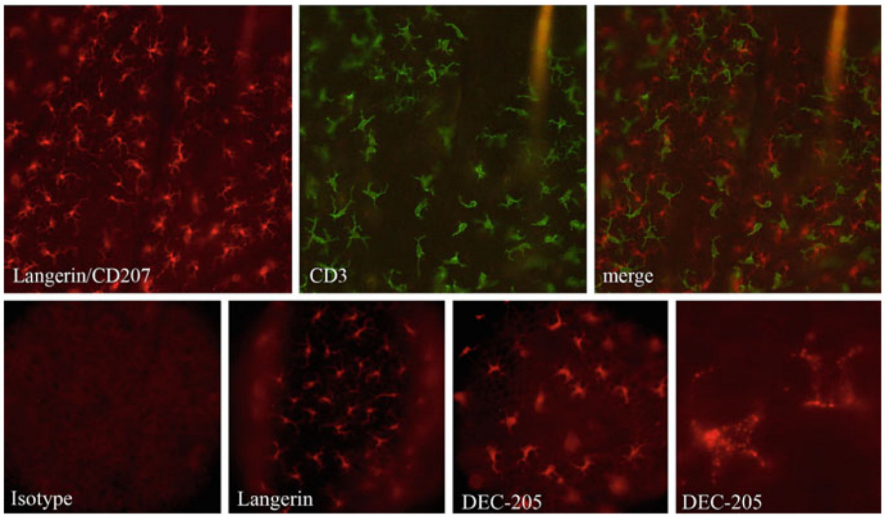

Fig. 1.

In the *upper row*, LC are visualized within epidermal sheets from murine skin by immunolabeling with anti-langerin antibody (*red fluorescence*). Dendritic epidermal T cells are identified by anti-CD3 antibodies (*green fluorescence*). The *bottom row* demonstrates how well murine LC can be targeted with antibodies injected into the dermis of the ear (anti-langerin, anti-DEC-205, isotype control). Epidermal sheets were prepared 4 days after the injection and stained with a fluorochrome-coupled anti-rat Ig antibody. Note that an unrelated antibody (isotype) does not bind to the LC whereas anti-langerin and anti-DEC-205 antibodies readily find their way into the epidermis and are taken up by LC. The picture to the far right is a higher magnification of DEC-205-targeted LC in situ. Intracellular vesicles containing the targeting antibody can be appreciated

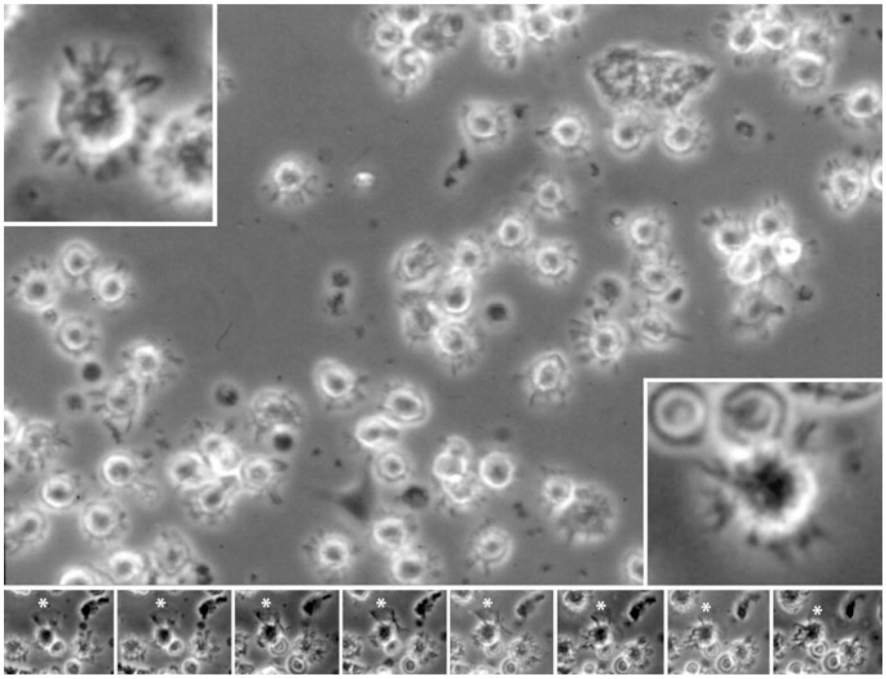

Fig. 2.

Human dermal DC obtained by emigration from dermal explants. This means that epidermis and dermis were separated from each other *before* the onset of culture. Phase contrast photographs of cells that migrated out of the explants into the culture medium over a period of 3 days. Note the typical morphology of mature DC with thin cytoplasmic processes ("veils"), best visible in the two inserts. The processes are motile as can be seen in the bottom row of photographs that were taken about 15 s apart from each other. Please note the shape change of one exemplary "veil" under the *white asterisk*

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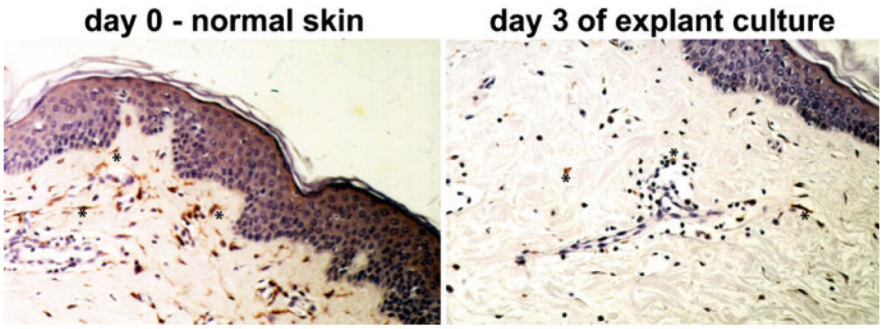

Fig. 3.

Human skin before (*left*) and after a 3-day skin explant culture (*right*). Dermal macrophages were visualized with an immunoperoxidase technique using antibodies against Factor XIIIa, a marker for these cells (Zaba et al. 2007). Positive cells can be identified by the brown reaction product (few examples marked with an *asterisk*). Note that after 3 days of culture the numbers of dermal macrophages are markedly reduced

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Table 1

Induction of a Th1 cytokine secretion pattern in T lymphocytes by murine Langerhans cells

	10,000	3,000	1,000	300	LC/well
	15,900	10,900	7,130	4,450	IFNγ (pg/ml)
Anti-IL-12	1,060	940	800	390	IFNγ (pg/ml)
	55	61	40	45	IL-4 (pg/ml)
Anti-IL-12	81	62	55	55	IL-4 (pg/ml)

Resting T lymphocytes of C57BL/6 mice were stimulated by graded doses of mature epidermal Langerhans cells (LC) from BALB/c mice in an allogeneic mixed leukocyte reaction. Resulting T cell blasts were restimulated for three additional rounds with fresh batches of mature LC. Supernatants of the forth allogeneic mixed leukocyte reaction were tested for the presence of secreted T cell cytokines by ELISA. Note that LC induced strong secretion of IFN-γ but virtually no IL-4. IFN-γ production was inhibited when anti-IL-12 antibodies were continuously present in the cultures (Koch et al. unpublished data)