Chapter nine

Summary, Discussion and Future Prospects

9.1 Summary, Discussion and Future Prospects

Contact dermatitis (CD) is a common health problem, which affects both men and women and accounts for 85-90% of all skin diseases. Two main types of contact dermatitis can be distinguished, according to the pathophysiological mechanisms involved, i.e. allergic and irritant CD. Allergic CD (ACD) requires the activation of antigen specific (i.e. acquired) immunity leading to the development of effector T cells, which mediate skin inflammation [1,2]. Irritant CD (ICD) is due to inflammatory and toxic effects caused by exposure to xenobiotics activating an innate local inflammatory reaction [3,4].

Dendritic cells (DC) are professional antigen presenting cells (APC), which can efficiently stimulate T cell responses and are therefore important for the initiation and regulation of antigen- or hapten-specific immune responses [5-7]. In human skin, both epidermal DC (i.e. the Langerhans cells (LC)) as well as dermal dendritic cells (DDC) are involved in the initiation of ACD [8-10]. Upon capture of hapten, DC become activated and subsequently migrate to the paracortical area of the regional lymph nodes (LN) [11]. DC express several TLRs and are therefore a target for pathogen-associated molecular patterns (PAMP) [14]. Experimental studies have also demonstrated the involvement of Toll-like receptors (TLRs) in both the development and the control of allergic reactions [12,13]. In the skin, TLR expression is upregulated upon environmental assault [13]. Although the exact role of TLRs in the induction of contact hypersensitivity (CSH) remains to be elucidated, it has been reported that the development of CHS is abolished in mice lacking both TLR4 and TLR2 [12]. In addition, TLR2 deficient mice showed impaired infiltration of immune cells into the hapten-exposed area, indicative for a reduced CHS reaction [13]. It has been suggested that the TLR activation during ACD may possibly proceed via endogenous TLR ligands [12,13]. During the allergic reaction, TLR engagement on DC directs the polarization of the T cell response, while TLR2 and TLR4 may favor both Th1 and Th2 responses [15-17]. Human LC express a restricted set of functional TLR, namely TLR2, TLR6, and TLR3 [18]. The cytoplasmic counterparts of TLRs are nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) mainly present in the cytosol, which sense microbial molecular patterns that gain access to the cell. Like TLRs, the underlying mechanism(s) of activation of NLRs by haptens is as yet unknown, but may depend on the viral and bacterial environment of the host.

Certain NLR members can form a multimolecular complex, termed the inflammasome [19]. To date three prototypic inflammasomes have been described: the NLRC4, NLRP1, and NLRP3 inflammasomes [20]. In addition, intracellular crosstalk between TLR and NLR pathways might also occur and contribute to the formation of the inflammasome, which might lead to the synthesis of IL-1beta and other inflammatory cytokines, including IL-18 and IL-33 [21-23]. Next to DC, evidence exists that primary human keratinocytes also contain the necessary elements to form an inflammasome [24].

Interestingly, it was found that chemical allergens (TNCB), chemical irritants (SDS) and physical agents (UVB) could trigger inflammasome activation [25].This activation may depend on TNF-alpha and other non-microbial stimuli, including IL-1beta, which have been shown activate the inflammasome [26]. Activation of the inflammasome requires binding of TLR and/or NLR ligands, which mediate the activation of inflammatory caspase-1 [27]. Caspase-1 cleaves pro-IL-1beta into IL-1beta and also activates IL-18 and IL-33; in this manner, the enzyme controls the maturation of these inflammatory cytokines [22,28]. This mechanism results in secretion of IL-1beta by LC exposed to contact allergens, but not to irritants, which in turn stimulates epidermal keratinocytes to secrete TNF-alpha, and also IL-18. Strong evidence shows that these epidermis-derived cytokines are essential for the migration of LC out of the epidermis [29-31]. In addition, inflammatory cytokines such as TNF-alpha and IL-1 induce DC maturation [32]. Upon maturation, LC gain *de novo* expression of CCR7, which is essential in mediating migration of mature LC towards the lymphatics [33-40]. Once in the LN, DC present their hapten to circulating T cells, thereby initiating an immune response.

In **Chapter 2** we investigated the initial phase of LC migration out of the epidermis upon topical exposure of freshly excised skin and epidermal sheets to contact allergens. $CD1a^+$ LC migrated out of the epidermis into the dermis upon topical exposure to the strong contact allergen 2,4-dinitrochlorobenzene (DNCB), the moderate contact allergen nickel sulphate ($NiSO_4$), and the weak contact allergen cinnamaldehyde (CA). We found that the initiation of LC migration into the dermis was triggered by the production and secretion of CXCL12 by dermal cells [41], thereby permitting their further journey to the draining LN via a two-step model [41]. This proposed two-step mechanism may reconcile a controversy that exists with respect to the essential or non-essential role of LC in skin immune responses and the relative contribution of dermal DC [41,42]. In this scenario, LC migrate into the dermis after hapten uptake. Once in the dermis they may either migrate on to the LN in a CCR7/CCL19-dependent fashion, or transfer hapten to surrounding DDC, which in turn migrate towards the LN and initiate a T cell response. Once arrived in the paracoritical T cell areas of the LN, DC activate an antigen/hapten specific T cell response. Interestingly, CXCL12 has been described as chemoattractant for peripheral $CD4^+$ T cells [43], thereby activating them [44]. Evidence exists for CXCL12/CXCR4 playing a role in homing T cells to the dermis in Sezary syndrome [45]. Since skin-localized antigen-presenting and T cell-inducing events are continuously taking place in ACD [46], CXCL12/CXCR4 interactions may play a dual role by mediating T cell homing to the skin in addition to initiating DC migration. Furthermore, CXCL12/CXCR4 engagement enhances DC maturation and survival [47].

In **Chapter 3** we investigated the migration of LC out of the epidermis into the dermis after topical exposure to irritants. Irritant substances are able to penetrate the epidermis, resulting in the release of both IL-1alpha and TNF-alpha [48,49], thereby inducing the production and secretion of CCL2 and CCL5 by dermal cells. The migration of irritant-exposed immature LC was facilitated by CCL2 and CCL5. In contrast to allergen exposure, irritant exposure does not induce upregulation of maturation-related CXCR4 and CCR7 expression on the surface of LC [38,41,50], thereby rendering LC unable to migrate towards the afferent lymphatics via the two-step CXCR4-CXCL12 and CCR7-CCL19/21 dependent mechanism [41,42]. Whereas allergen exposure induces the classical end-stage differentiation into fully mature LC, **chapter 4** describes an alternative end-stage differentiation of LC to $CD14^+$ macrophage-like cells upon irritant exposure [Ouwehand et al. submitted]. These $CD14^+$ macrophage-like cells could play an important role in the rapid removal of damaged tissue as a result of skin barrier disruption (e.g. in skin irritancy, tape-stripping, UV irradiation). Since, unlike LC, these macrophages lack the capacity to migrate to LN or prime naïve T cells [51], this CD1a-to-CD14 conversion might serve to maintain immunological ignorance and thus avoid the generation of collateral autoimmunity. In agreement with our previous findings, the anti-inflammatory cytokine IL-10 was shown to be responsible for this phenotypic switch of LC to $CD14^+$ macrophage-like cells [51]. Neutralizing antibodies against IL-10 have been shown to increase an allergic reaction by inactivating endogenous IL-10 [52] and sensitization studies in IL-10 knock out mice gave similar results [53]. Of note, the phenotypic switch of human LC into macrophage-like cells was also abrogated by i.d. delivery of neutralizing antibodies against IL-10 [**Chapter 4**]. IL-10 is secreted in the epidermis by keratinocytes or by melanocytes upon environmental stress [54-58] and in the dermis by activated macrophages [59,60]. IL-10 hampers the antigen-presenting properties of DC by reducing their expression of human leukocyte antigen (HLA) class II molecules, intercellular adhesion molecules (e.g., ICAM-1), co-stimulatory molecules (i.e., CD80/B7-1 and CD86/B7.2), and Th1-inducing cytokines (most importantly IL-12), which correlate with its ability to impair primary, alloantigen-specific T cell responses [61,62]. Beside an indirect impact via APC, IL-10 also exerts direct effects on T cells. In particular, inhibitory effects have been described on $CD4^+$ T cells. IL-10 inhibits the proliferation as well as the cytokine synthesis of these cells. The presence of IL-10 during the activation of $CD4^+$ T cells results in the development of a regulatory phenotype of these cells [63-66]. Regulatory T cells act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens [67]. Overall, we conclude that while irritancy leads to initial LC activation and migration, ultimately an IL-10 mediated

feedback loop induces their immunological decommissioning to macrophages with possible scavenger functions but lacking the ability to activate T cells.

In both **Chapter 2** and **Chapter 3** we have proposed an important role for dermal cells (most likely fibroblasts) in the initiation of LC migration to the dermis through the release of chemoattractants. This release is most likely effected by cross-talk with epidermal keratinocytes. LC are anchored within the epidermis by E-Cadherin (CD324) to keratinocytes [68]. As the outermost cells of the skin, keratinocytes have a major defensive function, as these cells undergo terminal differentiation into a resistant and impermeable cornified layer. Upon barrier disruption keratinocytes become rapidly activated, resulting in the production and release of various signalling factors such as cytokines, growth factors, and pro-inflammatory mediators [69]. The pro-inflammatory cytokine IL-1alpha is stored as a depot in the stratum corneum and intracellularly within keratinocytes, and is released very quickly upon epidermal injury [49,70]. Within two hours after chemical exposure, a peak in IL-1alpha protein release can be observed, serving as the initial alarm signal of epidermal damage [71]. Keratinocytes express the IL-1 receptor I at their surface [72], through which they may activate themselves in an autocrine fashion. In addition, the IL-1 receptor II is also detected on keratinocytes, but does not transmit signals due to the lack of an intracellular protein domain. The proposed function of the IL-1 receptor type II is to protect cells from overwhelming IL-1 responses by acting as a decoy receptor [73]. Next to keratinocytes, IL-1alpha also stimulates fibroblasts located in the dermis to produce more IL-1alpha and other primary pro-inflammatory cytokines IL-6, IL-8, and tumor necrosis factor (TNF)-alpha [71,74,75]. Moreover, TNF-alpha release by keratinocytes is also transiently induced after exposure to irritants, independent of the release of IL-1alpha [76]. Upon their release from the epidermis these cytokines diffuse into the dermis and trigger dermal cells (e.g.: fibroblasts, macrophages, and endothelial cells) to secrete chemokines that stimulate the initial trafficking of LC as well as DDC [41,71].

Using the knowledge of **chapter 2 and 3**, wherein CXCL12 was identified as a potential chemoattractant for maturing LC and CCL5 as a potential chemoattractant for irritant activated LC, in **chapter 5** we described a technique to discriminate sensitizers (i.e. maturing factors) from non-sensitizers (non-maturing factors). The acute myeloid leukemic-derived MUTZ-3 cell line was used in a transwell migration assay. This readily available cell line displays the unique ability to differentiate into LC (MUTZ-LC) in a cytokine-dependent fashion [77]. These MUTZ-LC resemble their *in vivo* counterparts, in that they exhibit characteristic expression of Langerin and contain Birbeck granules [78]. Furthermore, immature MUTZ-LC express the receptors for CCL5 (e.g. CCR1, CCR5), which are downregulated upon maturation. In addition, CXCR4 is upregulated upon stimulation with maturing factors, such as allergens. These characteristics of MUTZ-LC were used to mimic LC migration characteristics in a transwell migration assay. MUTZ-LC, either exposed to sensitizers or to non-sensitizers, were labelled with CSFE and allowed to migrate towards either CXCL12 or CCL5. Analysis of fluorescence showed that significantly more allergen exposed MUTZ-LC had migrated towards CXCL12 as compared to CCL5, while non-sensitizer exposed MUTZ-LC had preferentially migrated to CCL5. As demonstrated in **Chapter 5**, we have thus developed a highly physiologically relevant *in vitro* assay, with predictive value for the discrimination between sensitizers and non-sensitizers.

In **Chapters 6-8** we have described the development of an *in vitro* skin model to study LC biology in more detail. In order to develop approaches for the evaluation of skin sensitization that do not depend on animal models, we focused on the development of three-dimensional keratinocyte-LC co-culture systems. These models not only allow for topical application of chemicals, but also integrate all events occurring during the initial phase of sensitization (barrier function of the skin, bioavailability of chemicals, keratinocyte-DC interactions, and DC maturation and migration), that can be potentially addressed in future assays. If the chemical is a sensitizer, the chain of events should result in DC maturation and migration towards the LN, which in turn will initiate a T cell response.

In **Chapter 6** either MUTZ-3 progenitor cells or MUTZ-LC were placed in the lower well of a transwell culturing system. In the upper well, a fully differentiated epidermis was cultured. These epidermal equivalents were exposed to a panel of chemicals, which resulted in an increased CD86 and CD54 surface expression on MUTZ-3 or MUTZ-LC. Changes in surface expression of CD86 and CD54 were indicative of chemical penetration through the reconstructed epidermis. Since this two-compartment co-culture model only allowed cross-talk between keratinocytes and DC through soluble mediators, a model in which LC were directly integrated into the reconstructed epidermis was generated. Currently, only two groups have succeeded in introducing LC into an epidermal equivalent [79-82]. However, these models require fresh blood-derived precursor cells as source of LC. This renders them susceptible to donor variability in their response to chemicals, and also logistically difficult to construct. DC donor variability has been described to have a large impact on whether DC will respond to a chemical *in vitro* [83]. As further outlined in chapter 6, we therefore reconstructed an epidermis containing MUTZ-LC, on a human acellular dermis. Langerin^{+} MUTZ-LC are observed distributed throughout the epidermal layers in a similar manner to that observed *in vivo*. As chapter 2 and 3 revealed an important role for dermal fibroblasts in the initiation of LC migration out of the epidermis into the dermis, we next set out to introduce fibroblasts into the dermis of our Skin Equivalent (SE) model. In **Chapter 7** we described the immigration of MUTZ-LC into the epidermis of a full thickness skin equivalent. An autologous human full-thickness skin substitute was constructed, essentially as we described previously [84]. This skin model was used to investigate chemokines involved in trans-dermal MUTZ-LC immigration into the epidermis.

There is a constant turnover of LC, both in the presence and absence of inflammatory signals. Several LC-precursors have been identified which are involved in the repopulation of the epidermis with LC. It has been postulated that the CCR6-CCL20 axis regulates epidermal homeostasis and homing of immature LC [85-88]. Beside CCL20, also CCL5 [89-91] and CXCL14 [92] have been shown to attract immature LC precursors into the epidermis. In **Chapter 7** two pivotal chemokines, CCL5 and CCL20, were identified to be involved in trans-dermal migration of MUTZ-LC into the epidermis of the human full-thickness SE. The incorporation of MUTZ-LC into the epidermis of the SE model through migration over the dermal layer, showed a relatively low success rate. This was found to be most likely due to divergent chemokine secretion by epidermal keratinocytes as a result of both inter- and intra-donor variability. In order to develop a robust and reproducible culture methodology, another dermal scaffold was used in **chapter 8**. The model described in this chapter consists of fibroblasts incorporated into collagen gels as described by Spiekstra et al. [71]. The reconstruction of the human SE containing MUTZ-LC was achieved by co-seeding MUTZ-LC with keratinocyte cultures onto the fibroblast-populated collagen gels. Exposure of this full-thickness skin model to the sensitizer $NiSO_4$, resulted in maturation and migration of MUTZ-LC out of the epidermis. Maturation of MUTZ-LC was confirmed to occur by gene expression of the maturation-inducible pro-inflammatory cytokine IL-1beta and the chemokine receptor CCR7, both known to be induced in LC upon their activation.

Taken together, all these data demonstrate the complexity of the skin immune system. Within a network of cross-talk between epidermal and dermal cells, LC guide a resourceful army against intruding foreign agents (e.g. pathogens and haptens). *Ex vivo* skin explants and *in vitro* SE models as described in this thesis provide essential tools to study these interactions and immune mechanisms in a physiologically relevant 3-D setting.

9.2 Future prospects

With our daily exposure to a plethora of chemicals (e.g. in cosmetics), the risk of developing contact dermatitis increases. Therefore, the development of reliable tests is warranted to predict the sensitizing potential of chemicals. Currently all potential chemical sensitizers are tested with the aid of animals. The results from the chapters listed above could be of great importance in the future, as they support the replacement and reduction of animal tests. In particular, the *in vitro* tests described in **chapter 5** and the *in vitro* skin

models described in **chapter 6-8** could be very helpful to discriminate potential sensitizers from non-sensitizers.

In addition, perhaps surprisingly, the findings described in this thesis could also be of importance for the development of novel cancer treatments. Cancer is the second leading cause of death in the western world [93]. Although improvements have been made in the treatment of cancer, still a large number of patients die of the disease. Currently, several immunotherapeutic approaches have been developed, which can be classified as either passive or active immunotherapy. Passive immunotherapy is based on the transfer of effector components to patients, like specific antibodies or antigen-specific T-cells [94-96], which will result in a fast anti-tumor response without inducing a memory response. Active immunotherapy aims at the induction of a long-lasting anti-tumor immune response e.g. by vaccination with autologous or allogeneic tumor cells [97], tumor antigen-presenting DC [98-101], or genetically engineered viral vectors encoding tumor antigens [102]. As orchestrators of the adaptive immune response, DC are main therapeutic targets, utilized to induce a memory response against tumor cells. However, the effectiveness of DC-based vaccines is hampered by the immune-suppressive microenvironment within most tumors.

Tumors escape immune surveillance by disturbing the pro- and anti-inflammatory cytokine balance, resulting in an immune suppressive environment [103]. A strong overlap in the immune suppressive environmental conditions and their effects on cutaneous DC maturation between irritant reaction and tumor development could be observed. This is in line with intriguing findings that repetitive exposure to contact irritants induce tumor development [104-108]. As discussed in chapter 4, IL-10 could effect an alternative end-stage differentiation of LC into macrophage-like cells upon irritant exposure. Such a mechanism was also proposed in tumor-conditioned environments, containing high levels of IL-10 [51], and could explain the absence of a specific T cell response during both tumor development and irritant contact dermatitis.

A challenge with the hapten DNCB after tumor resection was found to hold prognostic significance. In contrast to patients with a good prognosis, patients with poorer prognosis did not develop a specific immune response against DNCB [109]. These results are indicative for a failure of the immune system in cancer patients with poor prognosis. As the microenvironments during tumor development and skin irritation are highly alike, current knowledge in skin irritation and sensitization could be used in the fight against cancer, in order to convert the immune-suppressive environment of the tumor or skin vaccination site (akin to an irritant reaction) into an immune-stimulating environment (akin to an allergic reaction). A good example of this is the haptenization of tumor antigens to enhance their immunogenicity and induce an effective anti-tumor immune response [110,111]. Based on the work described in this thesis, the potency of DC-based immunotherapy could be greatly improved through formulation with either neutralizing antibodies against IL-10 or with recombinant chemokines (e.g. CXCL12) resulting in improved activation and migration of DC/LC. Indeed, our proposed two-step migration model for LC (chapter 2) implies that it should be possible to mobilize LC (e.g. through epicutaneous application of a skin sensitizer or Toll-Like Receptor-Ligands [TLR-L]) and through i.d. delivery of a CXCL12 depot have them accumulate in the dermis. Preliminary experiments indeed show that already 8h after combined cutaneous sensitization and i.d. CXCL12 injection, accumulation of $CD1a^+$ LC in the dermis is evident (Ouwehand *et al*., unpublished observation). The use of liposomes or nanocontainers to achieve a steady release of CXCL12 over longer periods of time, might permit targeting of dense LC accumulations in the dermis and thus potentiate DDC/LC-targeted vaccines. Further activation of the dermally mobilized DDC and LC (e.g. through co-administered cytokines an/or TLR-L, possibly combined with neutralizing IL-10 antibodies) would then effect subsequent CCR7-mediated migration to the LN for T cell activation.

In conclusion, the novel LC-incorporating SE models described in this thesis may not only prove useful in allergen vs irritant prediction, they also provide a powerful tool to study human LC biology in the context of its 3-D tissue microenvironment. This may yield novel insights that could lead to LC-based therapies for contact dermatitis or even cancer. The incorporation of other immunologically active components in the SE models ($CD4^+$ and $CD8^+$

T cells being the most obvious next candidates) would be an important next step in the long journey towards this ultimate goal.

9.3 Reference List

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