

Summary

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disorder, characterized by typically distributed eczematous skin lesions. The mechanisms involved in the initiation and maintenance of skin inflammation in AD are poorly understood. The characteristic AD skin lesions provide a model to study human cutaneous allergic inflammatory reactions. In this thesis the role of macrophages in acute (induced *in vivo* by the atopy patch test (APT) and chronic allergic inflammation in AD was studied. The *in vivo* macrophage distribution, phenotype and IgG receptor expression was studied in skin biopsies of AD patients. Monocytes, the precursor cells for both tissue macrophages and dendritic cells (DC), from AD patients were compared with healthy controls. For further functional analysis of dermal macrophages *in vivo* studies were performed in mice, and specific migratory capabilities of macrophages were studied. Moreover, the effect of immuno-toxin induced depletion of dermal macrophages showed a crucial role for macrophages in cutaneous inflammation.

In this section, first a brief summary and discussion for each Chapter will be given, followed by a general discussion placing the results from this thesis in a broader perspective.

In **Chapter 2** phenotype and distribution of dermal macrophages and DC were studied in non-lesional, APT, and lesional skin biopsies from AD patients. We found that during acute and chronic inflammation in AD, the number of macrophages was significantly increased, in contrast to the number of dermal DC, which remained unchanged. The macrophage and DC populations were phenotypically heterogeneous, showing even overlap of “specific” macrophage and DC markers on one cell.

The increased number of macrophages may be caused by an increased influx of monocytes, attracted into “primed” AD skin. In skin of AD patients adhesion molecules are upregulated on blood vessel endothelium and chemo-attractants are present in the dermis leading to an influx of immune cells. Local factors in the skin may be involved in preferential differentiation of monocytes into macrophages, rather than DC. One cytokine involved in macrophage differentiation, GM-CSF, is produced by keratinocytes in AD skin. This mediator is also involved in prolonging monocyte/macrophage survival, by decreasing apoptosis. The steady number of DC in the dermis may be the net result from both influx from blood and efflux by migration to draining lymph nodes.

In **Chapter 3**, the RFD1+RFD7+ supposed suppressor macrophage population was studied in detail. Previously, human alveolar macrophages, binding both RFD1 and RFD7, were

found to have a suppressive effect on T cell stimulation induced by DC . In allergic asthma a relatively reduced number of these suppressor macrophages is present and are thought to be involved in maintaining homeostasis in lung . However, the presence as well as involvement of these “suppressor” macrophages in cutaneous inflammatory reactions in AD has been unclear until now.

We found RFD1+RFD7+ “suppressor” macrophages in human dermis. The number of these macrophages is increased in non-lesional AD skin compared with healthy skin, and increased even further during inflammation. These results are in contrast to findings in lungs from allergic asthmatics . Changes in the balance between functionally different macrophage subpopulations during inflammation may dictate the outcome of local inflammatory reactions. An increased number of “suppressor” macrophages may thus result in decreased inflammation. However, in inflamed AD skin, the increase in RFD1+RFD7+ macrophages apparently does not result in decrease or resolution of inflammation. Therefore, we propose that the macrophages in human dermis are not comparable to the RFD1+RFD7+ macrophages in lung. As it proved difficult to isolate sufficient numbers of dermal macrophages from skin biopsies, functional testing of the RFD1+RFD7+ cells was not feasible. Differences in cytokine milieu exist between allergic inflamed lung and skin, i.e. in afflicted lung a Th2-cytokine milieu is present, whereas in chronically inflamed AD skin the Th1 cytokines predominate . Such differences may lead to a preferential differentiation of monocytes into RFD1+RFD7+ macrophages in skin. However, as described in **Chapter 3**, addition of IL-4 to culture medium of monocytes did not generate increased production of RFD1+RFD7+ macrophages. The development of macrophage phenotype is likely dependent on the cytokine milieu (shown by *in vitro* cultures), and tissue-specific factors, which remain unidentified.

The results from **Chapters 2** and **3** indicate dermal macrophage and DC populations to consist of an array of overlapping subpopulations. The “classic” macrophage and “classic” DC represent two ends of a spectrum of cells with great plasticity that in addition to their common ancestry, share many phenotypic and functional characteristics.

In **Chapter 4** phenotype and function of blood monocytes from AD patients and healthy non-atopic controls were studied. Moreover, differentiation of monocytes *in vitro* was studied under different culture conditions. Although macrophage populations were induced, induction of DC populations (monocyte-derived DC, moDC) was most clear-cut. Using monocytes and moDC from AD patients and healthy controls, phenotypic and functional characteristics were studied. No differences were detectable between AD and control

populations. Other groups reported similar findings concerning monocytes from allergic asthma patients . In contrast, yet another study reported increased functional capacity of differentiated monocytes from allergic asthma patients, compared with healthy controls . The differences between our data and the latter study may be attributed to differences in culture methods. Albeit that the phenotypes of freshly isolated monocytes from AD patients and normal controls were comparable. Our *in vivo* data of skin biopsies did show differences in number and phenotype of macrophages, in AD skin, compared with healthy controls.

To further investigate the role of macrophages in local immune responses in AD, we analyzed the expression pattern of IgG receptors (**Chapter 5**). IgE and IgE receptor expression is classically associated with atopic diseases, and has been studied in detail. However, AD patients exhibit increased serum levels of total IgG4 and antigen-specific IgG4 . During acute inflammation we showed expression of CD16 (Fc γ RIII) and CD64 (Fc γ RI) to be increased. In chronic inflamed lesional AD skin especially CD68+ macrophages express CD64 (**Chapter 5**). The increased expression of CD16 and CD64 on dermal macrophages is probably due to changes in local cytokine production (see Table II in Chapter 1). For example, IFN γ is known to potently induce CD64 expression. As chronically inflamed AD skin and 48 h APT skin is characterized by increased levels of IFN γ , the increase of CD64 expression in AD skin is likely to be due to this increased IFN γ production .

Both CD16 and CD64 trigger cell activation through an activation motif in the FcR γ -chain. Other IgG receptor members, such as Fc γ RIIb molecules, bear an inhibitory motif within their cytoplasmic tail. The different IgG receptor cytoplasmic motifs regulate signal transduction pathways in either positive or negative ways . Thus, IgG receptors can exert contrasting roles depending on the balance between “activating” and “inhibitory” intracellular signals. Changes in IgG receptor expression patterns on macrophages during inflammation may result in abundance of activating signals, leading to continuous macrophage activation.

Uptake of IgG-antigen complexes via CD64 on macrophages, enhances MHC class II restricted antigen presentation . As increased numbers of CD64+ macrophages are present in inflamed AD skin (**Chapter 5**), binding of IgG4 to CD64+ macrophages in the dermis of AD patients is not unlikely. In **Chapter 6**, we therefore studied IgG4 binding to dermal cells, and CD64-targeted antigen-presentation by moDC.

We found IgG4 to bind to dermal cells after IFN γ induced upregulation of CD64. Antigen-specific targeting to CD64 on moDC resulted in increased antigen-specific stimulation of T cells.

In Figure 2a, a hypothetical model is proposed for a role of antigen-specific IgG4 and CD64 positive macrophages in perpetuating local immune responses. CD64+ macrophages are capable of IgG4-antigen complexes uptake. After processing, these dermal macrophages may present antigens to local antigen-specific T cells. The T cells become activated and secrete cytokines, such as IFN γ , maintaining local immune activation. Furthermore, activated macrophages may produce an array of inflammatory mediators, thereby sustaining the local micro-milieu (see also Figure 2, Chapter 1).

Next to a combined role for macrophages, IgG4, and CD64 in chronicity of AD lesions, this model may also explain some contradictory results of specific immunotherapy (SIT) in allergic diseases. SIT has beneficial effects in treatment of insect venom allergy, allergic rhinitis and allergic asthma. However, SIT treatment of AD patients resulted in aggravation of symptoms . SIT treatment induces the production of (antigen-specific) IgG and IgG4 and increases IFN γ . Generally, in allergic diseases a local Th2-cytokine milieu predominates, directing the immune response by these IgGs towards a Th1-cytokine mediated cellular response. This may result in a beneficial Th2/Th1 cytokine balance. In chronic AD lesions, however, Th1 cytokines control the local immune response, and a possible push towards a cellular Th1 response may result in aggravation of the disease.

In **Chapter 7**, phenotype and migratory abilities of alveolar and peritoneal macrophages administered in skin, lung, and peritoneum were studied. Macrophages are renowned for their phagocytic capacities. This, combined with their anatomical localisation in peripheral tissues, implicates an important role when encountering a variety of antigens. Antigen translocation to draining lymph nodes is thought to be dominated by DC . As macrophages share functional capabilities with DC, translocation of antigens by migrating macrophages to draining lymph nodes may also occur *in vivo*. After labelling of alveolar and peritoneal macrophages with a lipophilic fluorescent dye, labelled cells were administered intra-tracheally, intra-peritoneally or intra-cutaneously, and their subsequent localisation was monitored.

Macrophages introduced into the trachea were found in lung tissue and in draining lymph nodes of the lungs. The localization of these macrophages in lymph nodes was typically in

the T cell area. In contrast, macrophages injected intra-cutaneously did not migrate to draining lymph nodes, but resided in the dermis. Typical markers present on alveolar macrophages, were also present on alveolar macrophages after injection into the trachea. However, injection of alveolar macrophages into the dermis resulted in loss of these markers. Whereas peritoneal macrophages, when introduced into the lungs, gained markers specific for alveolar macrophages.

The induction or loss of phenotypic markers shows that the “local milieu” is more important in shaping macrophage phenotypes than the tissue of origin. Alveolar macrophages migrate to T cell areas of lung draining lymph nodes, whereas dermal macrophages tend not to migrate to skin draining lymph nodes under non-inflammatory conditions. Migration of dermal macrophages may take place when an appropriate stimulus is given, e.g. phagocytosis, or inflammation. Macrophage migration to lymph nodes opens up a range of opportunities for these cells to intervene with immune responses. If macrophages take up antigenic peptides, and subsequently migrate to draining lymph nodes, they may present or transfer antigenic peptides to (follicular) DC, or may directly interact with T or B cells. Whether macrophages have a direct or indirect, inducing, suppressing, tolerizing or even a steering effect on T cells remains to be studied.

In Chapter 8 we assessed the effect of *in vivo* elimination of murine macrophages from inflamed skin. Cutaneous inflammatory responses lead to dermal infiltration of macrophages . Because macrophages are capable of antigen presentation and production of an array of inflammatory mediators, macrophage activation may lead to continuous T cell activation and maintenance of local inflammation (**Chapter 6**). Elimination of inflammatory macrophages during cutaneous inflammation may prove beneficial, as macrophages may be the culprits in sustaining chronic inflammation.

We performed our experiments in transgenic mice expressing the human high affinity IgG receptor (CD64). Upon intracutaneous injection of CD64-targeted immunotoxin, cutaneous inflammation, as determined both clinically and histologically, resolved within 24 h.

These experiments showed specific targeting of activated macrophages through CD64 to be achievable and beneficial. Elimination of macrophages from inflammatory sites resulted in resolution of inflammation, indicating an important *in vivo* role for macrophages in perpetuation of inflammatory responses in skin.

Macrophages & Atopic Dermatitis

Macrophages are ambiguous with respect to their function in the immune system. Under normal steady-state conditions macrophages are beneficial in elimination of pathogens, tumour cells etc. In lung, macrophages help maintaining homeostasis. Besides the beneficial effects exerted by macrophages, activated macrophages may sustain inflammatory responses under pathological conditions. Rheumatoid arthritis is an example of a chronic inflammatory disease where high levels of proinflammatory cytokines are produced by macrophages. These cytokines are thought to play a major part in the chronic rheumatoid inflammation, and in joint degradation .

The data from **Chapters 3, 7 and 8** indicate dermal macrophages to exert a different role from alveolar macrophages in regulating local immune responses. One reason for differences may be found in the different function of the various tissues. Lung tissue is constantly bombarded with foreign antigens. A suppressive “blanket” may then be beneficial as an “inflammatory” response to each encountered antigen would inadvertently lead to lung dysfunctioning. Under normal conditions, however, our skin is intact and when antigens penetrate into the dermis this is a “danger” signal. A prompt immune response is then necessary to eliminate the putative pathogen. Macrophages are one of the oldest immune cells with a broad spectrum of immunological activities, in contrast to more specialized effector cells. Macrophages, therefore, will react to all potential “danger” triggers, unlike specific effector cells.

It is likely that AD is not induced by deranged macrophages, albeit that a role for macrophages in chronic inflammation in AD is plausible. Already in healthy-looking skin from AD patients increased numbers of macrophages are present, and during cutaneous inflammation their number increases even further. Macrophages secrete many inflammatory mediators, and are capable of antigen presentation . We showed dermal macrophages to express increased levels of IgG receptors during inflammation (**Chapter 5**). In Figure 2, a model is shown for a possible role of macrophages in perpetuation of local inflammation in AD.

The macrophage has long been overlooked in research into allergic inflammation. This thesis has elaborated on the presence, distribution and role of macrophages in skin inflammation. Notably, macrophages proved suitable targets for immunotherapy in local inflammation. Using data from the studies described in this thesis, a clinical trial was developed aimed at elimination of activated macrophages from AD skin. Phase 1 studies

already indicate that depletion of activated macrophages will parallel clinical responses in a number of patients. Ergo, the results described in this thesis not only lead to a better understanding of macrophage potential in skin, but also to a new therapeutic strategy.