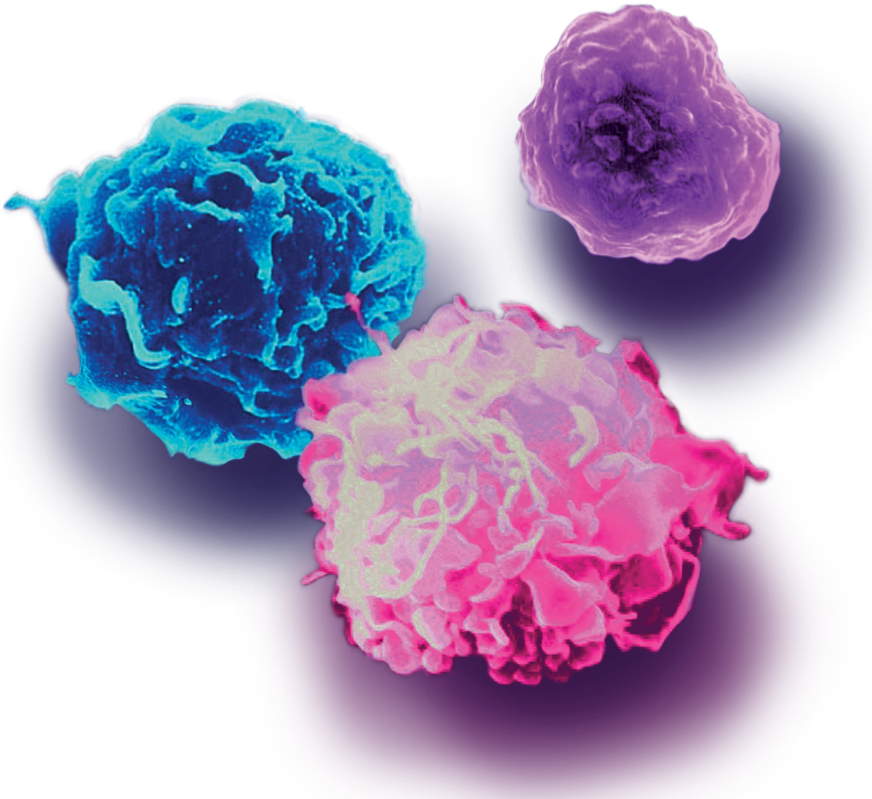
Chapter 8



General discussion

Scope

Through inhalation, the surface of the airways is continuously exposed to all different compounds, such as pollen, viruses, dusts, bacteria, chemicals and cigarette smoke. This requires an appropriate defense mechanism with an effective response to potential threads and ignoring of harmless compounds in order to maintain lung homeostasis. In asthma, the lungs respond to these harmless compounds (then called allergens) making the lungs of asthmatics hypercontractive[1]. After allergen exposure, the immune system is activated to fight the perceived dangers of the allergen with inflammation and subsequent expansion of macrophages to restore homeostasis[2,3]. Macrophages are important in maintaining lung homeostasis, because they have the ability by phenotype switching to regulate responses to homeostatic threats without impairing the functionality of the lung[4–6]. Unfortunately in asthma, this homeostasis is not achieved. It seemed likely that effective phenotype switching is impaired in asthma and that macrophages can then contribute to the pathogenesis of this disease. Therefore, the aim of this thesis was to investigate the role of macrophages in asthma and explore this as new avenue for the treatment of asthma.

Macrophage appearance and distribution in asthma

Depending on signals present in their environment, macrophages can polarize into many different phenotypes, which are hard to define in vivo[7]. Previously, macrophages were classified as M1 and M2 in an attempt to mirror the Th1/Th2 dichotomy[8]. The limitation of this nomenclature is that macrophages are not easily divided into just two groups based on similarities in protein expression, because their functional and physiological properties differ enormously within these two groups. For example, it was observed that macrophages that were classified in the M2 category were actually a collection of many functionally different subsets[9]. In fact, some of these populations more closely resembled M1 than M2 macrophages[10]. It also became clear that it was hard to match in vitro-generated phenotypes with their in vivo appearance as macrophages appear as a continuum rather than discrete entities in vivo[7,11]. A recently proposed nomenclature by a group of macrophage biologists advises researchers to describe macrophages according to the markers they express and/or the signals that induce them, avoiding the complexity of contrasting classifications and different definitions of activation[12]. The evolving field of macrophage nomenclature was something we struggled with during the years of this thesis. Therefore the nomenclature of different macrophage subsets changes from chapter to chapter as the field evolved.

In this thesis we decided upon defining three main subsets based on described functional characteristics (proinflammatory, anti-inflammatory and wound healing) and throughout the thesis we used similar markers to identify these three subsets. We characterized macrophages with enhanced microbicidal capacity (also known as proinflammatory, classically activated, or M1) by IRF5 expression or high MHC-II expression. IRF5 is directly involved in the polarization towards a proinflammatory state of macrophages by inducing transcription of MHC-II, IL-6, IL-12 and IL-23 while suppressing the transcription of IL-10[13]. The expression of YM1 and CD206 were used to identify macrophages in mice that are associated with wound healing (formerly known as alternatively activated or M2). To identify these macrophages in humans, CD206 expression was used[14]. To identify macrophages with anti-inflammatory activity, we studied the expression of the immunosuppressive cytokine IL-10, which is the most important characteristic of this macrophage phenotype[7].

Our data showed that many macrophages in healthy lungs are characterized by expression of IL-10 and few are characterized by expression of IRF5 or YM1 and/or CD206. In both allergic and nonallergic lung inflammation the balance in macrophage phenotypes was shifted. Both types of inflammation were accompanied by lower numbers of IL-10+ macrophages, but in our farm dust extract model of nonallergic inflammation IRF5+ macrophages dominated, while in our house dust mite (HDM) model of allergic inflammation YM1+ macrophages dominated compared to healthy (chapter 2 and 3). This suggests that the balance of macrophage phenotypes is an important determinant of the different types of inflammation in each model. This was confirmed by our experiments inhibiting alternative activation with cynaropicrin, which shifted the inflammation from eosinophilic with YM1+ macrophages to neutrophilic with IRF5+ macrophages (chapter 6).

A similar subset distribution was found in bronchial biopsies of asthma patients versus healthy controls. Higher numbers of both IRF5+ and CD206+ macrophages and lower numbers of IL-10+ macrophages were found in asthma patients, with relatively more IRF5+ macrophages in males and relatively more CD206+ macrophages in females (chapter 5). Predominance of one macrophage phenotype may contribute to the heterogeneity of asthma, especially observed between the sexes[15].

Where do they come from?

As we have shown in this thesis that lung tissue contains more IRF5+ and CD206+ macrophages during allergic airway inflammation, another remaining issue was the

question of where these cells come from. Are they the result of local proliferation of resident macrophages, are they being recruited from blood monocytes that differentiate into macrophages on site, or do they develop from other resident macrophages through phenotype switching? During homeostatic conditions, it was shown that macrophages have embryonic progenitors and are maintained throughout life by local proliferation[16–23]. These groundbreaking findings do not necessarily exclude blood monocytes as precursors. Studies in which depletion of lung macrophages was followed by adoptive transfer of bone marrow or $Gr1^{low}$ monocytes showed that lung macrophages could be replenished from bone marrow during homeostatic conditions[24,25]. This led to studies investigating the origin of macrophages during allergic lung inflammation[26,27]. It has been shown that maintenance of the alveolar macrophage pool mostly depends on local proliferation of resident macrophages, but immediately after allergen exposure macrophages might develop from recruited monocytes. This is in accordance with our data indicating that immediately after allergen rechallenge, alveolar macrophages may be supplemented with macrophages derived from infiltrating monocytes. At later time points though, we showed that resident macrophages proliferate to maintain the alveolar macrophage pool. We showed that these resident macrophages could either be locally proliferating alveolar macrophages and/or interstitial macrophages that switch phenotype (chapter 4). This is consistent with previous studies that showed replacement of alveolar macrophages by interstitial macrophages after LPS exposure or during a bacterial infection[28,29]. In addition, in steady state conditions it was reported that alveolar macrophages are the longer-lived macrophages in lungs of mice and rhesus macaques[30], while interstitial macrophages exhibited a high turnover rate and were rapidly replenished with blood monocytes in lungs of rhesus macaques[31]. These findings suggested that alveolar macrophages develop from interstitial macrophages, which in turn derive from blood monocytes. It is, however, still possible that interstitial macrophages are also from embryonic origin as was also shown for alveolar macrophages, since we were not able to directly demonstrate that monocytes are precursors of interstitial macrophages.

Some recent and older studies have highlighted the importance of resident alveolar macrophages in maintaining lung homeostasis and immigrating monocytes in contributing to allergic inflammation[26,27,32,33]. The picture that arises is one of fast recruitment of monocytes after allergen exposure to fight the perceived dangers of the allergen with consequently inflammation and subsequent expansion of alveolar macrophages in an attempt to restore homeostasis.

Role of YM1+/CD206+ macrophages in asthma

The inducers (IL-4 and IL-13) of YM1+/CD206+ macrophages are abundantly present in allergic asthma and therefore these macrophages have been more extensively studied than other macrophage phenotypes. High numbers of CD206+ macrophages lungs of asthmatics have been reported by us and others previously [34–37], and in this thesis (chapter 5). In addition, we showed that in several models of HDM-induced asthma the number of YM1+ macrophages positively correlated with severity of airway inflammation (chapter 2). Our previous adoptive transfer study of in vitro differentiated IL-4/IL-13-stimulated macrophages into the lungs of allergic mice showed that these macrophages actively contribute to the exacerbation of the disease and are not just bystanders as a result of Th2 inflammation[15]. These findings were later confirmed by two other studies. Both studies found enhanced allergic inflammatory responses in lung tissue. However, the role of YM1+ macrophages in asthma is the subject of ongoing debate. In contrast to the previously mentioned studies, it was later demonstrated that YM1+ macrophages are not necessary for allergic airway disease and are only a consequence of elevated Th2 responses[38]. In this study the contribution of YM1+ macrophages to acute and chronic HDM-induced allergic lung inflammation was investigated by using $LysM^{cre}$ mice with abrogated IL-4Rα signaling on macrophages. The investigators observed that airway hyperreactivity, Th2 responses, mucus hypersecretion, eosinophil infiltration, and collagen deposition were not significantly affected by decreased development of M2 macrophages. Recent data, however, showed that these $LysM^{cre}$ mice successfully abrogate IL-4Rα signaling on mature tissue resident macrophages, but fail to delete this on more immature macrophages arising from proliferation or from recruited monocyte precursors[39]. In addition, it was found that YM1+ macrophages derived from monocytes or from tissue macrophages are phenotypically and functionally distinct[40]. Combining these findings, it seems likely that mature YM1+ resident macrophages do not contribute to allergic lung disease, but YM1+ macrophages that are newly derived from either proliferation or recruited monocytes may be the active contributors to the disease. In this thesis we treated mice during the induction of allergic inflammation with cynaropicrin, a substance that inhibits alternative activation of both newly derived and resident macrophages. Indeed, we found that these mice developed less severe eosinophilic lung inflammation and less collagen deposition around the airways (chapter 6). Our findings confirmed previous studies in which YM1+ macrophages and TGM2-expressing macrophages were inhibited[41,42]. Strikingly however, we found that inhibition of alternative activation shifted inflammation towards more IRF5+ macrophages, neutrophilia and development of more severe airway hyperresponsiveness. The apparent disconnect between airway hyperresponsiveness and the amount of collagen around the airways demonstrates

that collagen deposition appears to protect the airways from even worse contractility. As airway hyperresponsiveness is traditionally thought to be caused by airway smooth muscle hyperplasia and collagen deposition around the airways[43–45], our data may indicate we have to rethink the role of collagen deposition. As YM1+ macrophages are associated with fibrosis, the decrease in collagen deposition may be directly linked to the inhibition of alternative activation[14,46].

These findings revealed an important dual role for YM1+ macrophages of allergic lung inflammation. YM1+ macrophages contribute to induction and progression of eosinophilic lung inflammation, but protect against development of neutrophilic lung inflammation and worsening of airway hyperresponsiveness.

Role of other macrophages in asthma

Although the inflammatory process in asthma is dominated by a Th2 inflammation, it was suggested that IRF5+ macrophages are also involved in this disease[47–49]. Their role in asthma, however, is complicated because of their many faces in different aspects of the disease. During the induction of HDM-induced asthma, we found that numbers of IRF5+ macrophages are high in a short, less severe, model as compared to control mice and lower with longer exposure and more severe inflammation, while for YM1+ macrophages the opposite was observed (chapter 2). Interestingly, functional studies have shown that IFNγ-stimulated macrophages act preventive in the onset of allergic airway inflammation in mice and suppressed DC maturation[50]. This would suggest that these macrophages are induced as a counterregulatory mechanism to dampen inflammation. On the other hand, we found that higher numbers of IRF5+ macrophages in bronchial biopsies of asthma patients were associated with more severe airflow obstruction (lower FEV_1/FVC) (chapter 5). In addition, products and numbers of TNFα+ macrophages correlated with asthma severity, suggesting that IRF5+ macrophages play a contributing role in severe asthma as well[51–53]. Altogether, these data imply that IRF5+ macrophages can help preventing allergic sensitization, but can also promote the development of a more severe phenotype in established disease. This is consistent with the findings of a study that investigated the role of the cytokine IL-12 during the development allergic airway inflammation in mice. They showed that neutralization of IL-12 during the sensitization phase aggravated development of allergic airway inflammation but neutralization of IL-12 during challenges abolished the symptoms of allergic airway inflammation. Through IL-12, IRF5+ macrophages may have a dual role in asthma: they act preventive during Th2 sensitization, but they contribute to severity of allergic airway disease in established disease[54].

Considering their function, IRF5+ macrophages appear to be needed directly after allergen exposure due to their enhanced phagocytosis and antigen presentation capabilities to accomplish microbial clearance[55,56], while YM1+ macrophages are instructed at a later stage by the damaged tissue for repair purposes. Since we showed that in vitro farm dust extract exposure induces classical activation of macrophages (chapter 3 and [57]), it is tempting to speculate that IRF5+ macrophages are directly involved in the initiation of a neutrophilic immune responses. If exposure to a certain trigger persists in the context of low levels of Th2 cytokines, neutrophilic inflammation prevails, while if Th2 cytokines are high eosinophilic inflammation is chosen. Indeed, in lungs of mice exposed to farm dust extract we found neutrophilic inflammation with increased numbers of IRF5+ macrophages and low levels of Th2 cytokines (chapter 3).

IL-10+ macrophages appear to be predominant in homeostasis and this suggests that once microbial or allergen clearance is accomplished, a shift towards an anti-inflammatory IL-10-producing phenotype should take place to achieve resolution of inflammation. The finding that anti-inflammatory macrophages were lower in both HDM- and farm dust extract-exposed mice compared to control mice supports this thought (chapter 2 and 3). In asthma patients, both IRF5+ or the YM1+ macrophages dominate the lungs with a loss of IL-10+ macrophages (chapter 5). This may explain why resolution is not achieved in asthma. The role of IL-10 in the immune system has been studied intensively and it was found to be an important mediator in the resolution of (airway) inflammation[58,59]. In addition, in bronchial biopsies of asthma patients we showed that ICS treatment was accompanied by higher numbers of IL-10+ macrophages and these higher numbers were associated with better lung function (chapter 5). This indicates that induction of these IL-10+ macrophages may be an interesting novel therapeutic avenue to explore.

Are macrophages a new avenue for asthma treatment?

Currently, the most widely used therapeutics to inhibit inflammation in asthma are inhaled corticosteroids. Long-term use of corticosteroids, however, may lead to side effects and a subset of asthma patients, mostly with severe disease, do not respond to this treatment[60,61]. Therefore, development of more specific and effective therapeutics is needed. With the emerging role of CD206+/YM1+ macrophages in asthma pathogenesis, they seemed to be an interesting target for a new therapy. However, the results of this thesis suggest otherwise, since inhibition of alternative activation during HDM exposures results in more classical activation of macrophages with neutrophilic inflammation (chapter 6). Modulating development of IRF5+ macrophages

probably is not a valid therapeutic option either as this may shift the inflammation towards more eosinophilic inflammation. It seems that the asthma phenotype is determined by a balance between the two predominant macrophage subsets IRF5+ and YM1+, with a loss of the beneficial anti-inflammatory IL-10+ macrophages. This finding suggested to us it would perhaps be possible to re-instate homeostatic behavior of macrophages by boosting the anti-inflammatory function of macrophages in vivo in lung tissue and thereby treat lung inflammation. We found that PGE2 was the best candidate to induce IL-10 production in macrophages (chapter 7). PGE2 is a well-known anti-asthmatic compound that has been shown to prevent allergen-induced bronchocontriction, to inhibit airway hyperresponsiveness and inflammation. These anti-asthmatic effects were shown to be mediated through E prostanoid receptors 2 and 4 (EP2 and EP4) on wide range of target cells[62–67]. Of course this presented us with a challenge trying to show anti-inflammatory effects of PGE2 specifically through macrophages. As was shown in many studies before[68,69], we confirmed that free PGE2 instilled in the lungs had anti-inflammatory potential. As it is unlikely that this was due to a specific effect on macrophages, we studied a macrophage-specific approach by treating macrophages ex vivo with PGE2 and then adoptively transferred these IL-10-producing macrophages into the lungs during the induction of allergic lung inflammation with HDM. Interestingly, this approach was more effective than treatment with free PGE2 and was independent of macrophage origin, as we investigated both macrophages with a hematopoietic and an embryonic origin (chapter 7). These findings indicate that the lower numbers of the IL-10+ macrophages we have found in asthma are important in the development of allergic inflammation because re-introducing these macrophages into lung tissue has obvious beneficial effects. Whether these PGE2-treated macrophages are also effective in established disease is an important question that needs further studies. If so, this could open up a whole new therapeutic perspective for the treatment of asthma.

Future perspectives

In conclusion, the balance in macrophage phenotypes appears to be tightly regulated in order to reach homeostasis every time lungs are exposed to allergens. We showed that this balance is dysregulated in asthma and can determine the type of inflammation, indicating that macrophages play an important role in asthma pathogenesis. This thesis provided some clues on the exact role of the different macrophages and the most important message is to focus on the good qualities of the macrophages, i.e. their ability to quickly change phenotype. We used this approach to re-introduce anti-inflammatory macrophages to restore lung homeostasis and indeed found inhibition

of HDM-induced lung inflammation. Therefore, it would be interesting to further study the mechanism by which anti-inflammatory macrophages inhibit lung inflammation. We tested whether the anti-inflammatory effects of PGE2-treated macrophages were mediated through modulation of dendritic cell behavior and found no evidence for this hypothesis (chapter 7). It is therefore likely these anti-inflammatory macrophages have a local effect either through inhibiting local adaptive immune responses in lung tissue or through other anti-inflammatory effects. An interesting new paradigm for the control of inflammatory responses by macrophages was recently presented[70]. It was shown that anti-inflammatory effects of PGE2-treated macrophages can be mediated through increased transcellular delivery of vesicular SOCS (suppressor of cytokine signaling) proteins to epithelial cells. Elucidating these mechanisms further may not only give valuable information on how macrophages control inflammation in asthma, but may also help to resolve other forms of lung inflammation induced for instance by infections or smoking. This knowledge may also be applicable beyond the respiratory field. Dysregulated macrophage responses have been shown in many organs, among which for instance fibrotic liver diseases[71–74]. Thus, these future studies may result in the identification of new targets for resolution of chronic inflammation in many different organs.

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