Eosinophils play a pivotal role in the pathophysiology of asthma, as was pointed out in the introduction of this thesis. Step by step, the mechanisms of activation and selective infiltration of eosinophils are now being unraveled. Important herein are the concepts of priming and activation. During priming the eosinophil becomes more prone to activation without actually producing damaging mediators, while during activation secretion of mediators actually takes place. It is important to realize, however, that both situations are often difficult to discriminate and a cell may be activated for one parameter and primed for the second at the same time. Eosinophils are heterogenic with respect to buoyant density and low density or hypodense eosinophils are considered to be involved in asthmatic inflammation. It was unclear, however, whether hypodense eosinophils reflect a primed or activated state. Furthermore, several studies have shown that elevated numbers of hypodense eosinophils are present in the peripheral blood of patients with asthma. The observed numbers of hypodense eosinophils, however, varied greatly between different studies. We started to doubt the observation of hypodense eosinophils as we noticed in pilot experiments that hypodense eosinophils were induced in vitro during frequently used purification steps. Based on these first pilot experiments, the studies described in this thesis were conducted to clarify the observation of hypodense eosinophils in asthma and to search for density related, more easily assessable, parameters. This culminated in the formulation of five specific questions put forth in the introduction. With the results of the experiments described in the previous chapters these initial questions and possible answers are discussed below.

I Presence of circulating hypodense eosinophils and priming in asthma

The peripheral blood of adults (chapter 3) and children (chapter 4) with allergic asthma does not contain more hypodense eosinophils than that of non-asthmatic controls. The differences reported in the literature may be the result of in vitro induction of low density by purification. However, we do not know whether the number of peripheral hypodense eosinophils in vivo may actually be increased during more severe asthma, possibly by elevation of peripheral interleukin levels. This possibility is illustrated by the fact that peripheral numbers of hypodense eosinophils are increased during treatment with IL-3 (chapter 7). After dextran...
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7). After dextran sedimentation, 25.3% of hypodense eosinophils were found in adult asthmatics compared to 10.6% in healthy controls. In children, these numbers were 15.4% and 8.8%, respectively. Using a direct method, without dextran sedimentation, no significant difference between asthmatics and controls were observed. This indicates that eosinophils of asthmatics are primed for density decrease by dextran sedimentation. These results were supported by a report from Agrawal et al. They have recently demonstrated that eosinophils from individuals with asthma are primed for density decrease induced by PAF or calcium-ionophore, although the effect may be underestimated because of the use of dextran sedimentation [430]. The percentage of hypodense eosinophils in asthmatic children we observed was slightly different from that in adult asthmatics. It is not possible to conclude that eosinophils from adult asthmatic are more primed than those from asthmatic children. The two studies are difficult to compare due to methodological differences such as differences in the osmolality of the used percoll solutions and different patient inclusion criteria.

A very interesting application for the number of hypodense eosinophils has been its use as a marker for asthma severity. Although we have shown that increased numbers of hypodense eosinophils are not present in the circulation, the in vitro induction by dextran sedimentation may reflect the extent of priming of eosinophils in vivo. This priming, in turn, is probably a result of ongoing inflammation in the lung. It is therefore interesting that we have found a significant correlation between the percentage of hypodense eosinophils after dextran sedimentation and FEV1 in adults (r = -0.60). However, the correlation between the number of eosinophils and FEV1 was stronger (r = -0.84), and the number of eosinophils may therefore be more convenient clinical parameter. In asthmatic children, no correlation was found between hypodense eosinophils induced in vitro and lung function. Total numbers of peripheral eosinophils correlated with FEV1/VC (%) but not with FEV1 or PC20. Thus, while a correlation was observed between lung function and total numbers of eosinophils, the correlation between lung function and priming was not found. One of the reasons may be that priming is likely to be a result of inflammation and may therefore correlate better with a parameter of airway inflammation, for instance the degree of bronchial hyperresponsiveness. Bronchial hyperresponsiveness in the adult study was tested with either histamine or methacholine for ten patients. For a good evaluation more patients may be needed in
which bronchial hyperresponsiveness is tested with only one of the two agents. A significant inverse correlation between the number of hypodense eosinophils and the PC_{20} for histamine in asthmatics individuals was observed by others [310,431]. In the study from Krouwels et al. [310], the use of dextran sedimentation was omitted based on our studies. Nevertheless, an effect of purification can not be ruled out completely, as we have shown that even with a direct method CD11b expression is increased compared to whole blood.

As we have shown that in vivo priming is revealed by purification, a comparison between hypodense eosinophils and another eosinophilic marker of asthma severity emerges: determination of serum ECP. This protein also turned out to be produced in vitro during coagulation of the blood, instead of being present in blood in vivo [150]. Similar to eosinophil density, it may be a a marker for ongoing inflammation provided the method used is highly standardized and in vitro effects are an integrated part of evaluating the results. The laborious standardization measures that have to be taken probably renders both procedures unsuitable within a routine clinical setting. Currently symptoms and lung function, together with bronchial hyperresponsiveness and eosinophil number as indirect markers of inflammation, may be the most appropriate markers for asthma and effects of treatment. Nevertheless, promising new methods of asthma monitoring, such as markers in induced sputum and measurement of exhaled NO, are emerging and may prove useful in the future.

The expression of CD11b by eosinophils and neutrophils from asthmatic children is also primed in vitro, as is described in chapter 5. This is in agreement with the results described in chapter 2, showing a concomitant decrease in density and increase of CD11b expression. The results of chapter 6 showed a direct relationship between eosinophil density and CD11b expression. In conclusion, our results suggest an in vivo priming of eosinophils in asthma that is similar for density decrease and CD11b increase.

II Modulation of density and receptor expression by purification procedures

Dextran sedimentation, frequently used as the first step in the purification of granulocytes to separate white and red blood cells, decreases density and increases CD11b expression of eosinophils and neutrophils (chapter 2). Density and CD11b expression are independent centrifugation procedures. However, when activation of eosinophils is induced by purified eosinophils and neutrophils, eosinophils may be stimulated to express CD11b. This renders the measurement of in vitro densities and of CD11b inactivation of these eosinophils sensitive. The results of chapter 6 showed a direct relationship between eosinophil density and CD11b expression. In conclusion, our results suggest an in vivo priming of eosinophils in asthma that is similar for density decrease and CD11b increase.
expression were changed even in the presence of EDTA, indicating that the effect is independent of extracellular calcium. Not only dextran sedimentation, but also centrifugation of cells decreased eosinophil and neutrophil density. By applying blood directly to the density gradient, the so-called direct method, the observed densities may be more similar to the actual densities of circulating eosinophils. However, even with this method, cells were shown to be modified since CD11b expression on neutrophils and eosinophils was increased compared to the directly fixed whole blood cells. The results of chapter 2 indicate that determination of activational or priming parameters of granulocytes must be carefully evaluated in the light of the sensitivity of these cells for modulation during purification. Especially when activational markers of purified eosinophils or neutrophils are being used for monitoring of disease activity, the influence of purification must be taken into account. Furthermore, the results demonstrated that even the use of calcium chelators will not prevent activation of cells by purification. Flow cytometric determination of markers in directly fixed whole blood cells may be an appropriate and sensitive alternative to measurements in isolated eosinophils, although pitfalls may be still present. Antibodies against CD16 can be used to discriminate eosinophils and neutrophils in a leucocyte suspension, since eosinophils normally do not express CD16 while neutrophils do. It has been reported, however, that CD16 is induced on eosinophils after treatment with cytokines such as IFN-γ [184], which renders this method less useful. When the method of De Grooth [342] is applied, additional antibodies are not necessary and it is therefore a very elegant procedure. The success of this method, however, may depend on the type of flow cytometer that is used.

The exact mechanism by which cell density decreases during purification steps is not completely clear. Degranulation is probably involved, because CD11b is released from intracellular compartments and also lactoferrin release was observed. In addition, the results of chapter 6 indicate a relationship between density and volume. Therefore, activation of membrane pumps and uptake of water is another mechanism that may be involved. The factors inducing low density during purification may be mechanical or soluble or mediated by cell-cell contacts.
III  Relationship between eosinophil density and membrane receptor expression

The previous chapters indicated that there is a common mechanism in priming and activation of density decrease and CD11b expression. The relationship between density and CD11b expression has previously been addressed by others, who were not able to confirm this relationship [181]. This may have been due to the considerable interindividual heterogeneity with respect to CD11b expression, a problem we minimized by the use of paired observations. In addition, we have utilized forward scatter as an indicator of cell volume and determined the expression of L-selectin because of its importance in the initial step in tissue infiltration. CD11b, L-selectin and forward scatter were related to eosinophil density. CD11b expression and forward scatter on both low density and high density eosinophils were significantly increased as compared to blood eosinophils. The results, together with those from chapter 2, indicate that eosinophils become hypodense by degranulation of CD11b containing compartments and by cell swelling, independently of extracellular calcium. Cell swelling, like increased CD11b expression and low density is indicative for activation. However, hypodense eosinophils are probably not fully activated since L-selectin was not decreased as compared to blood eosinophils.

IV  Effect of in vivo administration of recombinant human IL-3 on density and membrane receptor expression

It has been suggested that priming of eosinophils in asthma is a result of circulating cytokines such as IL-3, IL-5 and GM-CSF. A trial in which ovarian cancer patients received rhIL-3 after chemotherapy gave us the opportunity to test this hypothesis. The results, described in chapter 7, show that during treatment with rhIL-3 the number of hypodense eosinophils increases even without using dextran sedimentation and eosinophils become activated and primed with regard to CD11b expression. This supports the in vitro effects that have been described for IL-3 regarding eosinophil density and CD11b expression. However, it became clear from the previous chapters that circulating eosinophils from patients with asthma are only primed and not activated for these parameters. Therefore, the serum levels of IL-3 as achieved by rhIL-3 treatment are probably higher than those of stable asthmatics. Moreover, although peripheral T-lymphocytes of patients with asthma
are capable of increased production of Th2 cytokines, the presence of these cytokines in serum has only been shown indirectly [63]. Thus, either the concentration of IL-3 and other cytokines in the circulation of asthmatics are very low and only sufficient to prime eosinophils, or these cytokines are not present at all in the circulation and eosinophils become primed at location distinct from the peripheral blood, possibly the vasculature of the lung. This may result from repeated contact with cytokines bound to endothelial cells of the lung vasculature.

V Inhibition of membrane receptor expression by cAMP increasing agents

Concentration-dependent inhibition of the PAF-induced CD11b expression and L-selectin shedding from neutrophils and eosinophils was observed with rolipram, cAMP, PGE2, and isoproterenol (chapter 8). This was tested in a whole blood system in the presence of other, possibly interfering, cells, thereby mimicking the in vivo situation. Each agent separately did not inhibit PAF-induced receptor expression more than 50%. However, with a combination of rolipram and isoproterenol or PGE2, up to 70% inhibition of PAF-induced L-selectin shedding from eosinophils was observed. A synergistic inhibition by rolipram and isoproterenol or PGE2 was expected because of potentiation of cAMP production found in previous studies [414]. Yet, only a cumulative effect was found which was possibly due to a masking effect by endogenously produced adenosine or because of the involvement of cAMP independent intracellular mechanisms. Despite the in vitro inhibition by the PDE type 4 inhibitor found in this and other studies and the in vivo effect in animal models, the clinical studies with inhaled PDE inhibitors have been rather disappointing [432]. In contrast, the non-specific PDE inhibitor theophylline has been successfully used for many decades and has been shown to abrogate eosinophil infiltration in patients with asthma [420]. One explanation for this difference may be the route of administration. The initial rolling step in eosinophil infiltration occurs in the pulmonary circulation and may be sensitive to inhibition by cAMP modulators. PDE inhibitors may therefore be more successful when applied via the circulation instead of via the airway lumen. Theophylline is administered orally and, in most animal studies, PDE inhibitors were administered systemically. Our results suggest that inhibition of the initial rolling of eosinophils may be a relevant subject for further investigation and therapeutic intervention,
especially since shedding of L-selectin seems to be a prerequisite for transmigration [264]. Not only the expression of adhesion molecules but also density decrease is, although to a lesser extent, inhibited by PDE inhibitors (Spoelstra et al., submitted). In addition, more selective PDE inhibitor are being developed [433].

**Hypodense eosinophils: a footnote in the history of eosinophil biology?**

In a recent editorial, Wardlaw posed that 'the enigma of the hypodense eosinophil appears destined to be relegated to a footnote in the history of eosinophil biology' [301]. Based on the experiments described in this thesis and reports from others, we agree that studies on the relationship between hypodense eosinophils and their functional capacity are affected by purification procedures, the source of eosinophils, the function being measured and the stimulus used. Expression of membrane receptors may therefore be a more controllable and clear cut tool to be useful as a clinical marker. Yet, hypodense eosinophils have been an interesting topic of investigation resulting in greatly enhanced knowledge on the pathophysiological role of eosinophils in general and in asthma in particular over the past years. Furthermore, results of the present studies indicate that hypodense eosinophils, at least after dextran sedimentation, may represent a specific phenotype between resting and fully activated eosinophils. This is supported by studies from others who were not able to show that hypodense eosinophils are more activated than normodense eosinophils in asthma by measuring parameters such as respiratory burst or LTC4 release (see chapter 1). The notion that eosinophils are not either resting or activated but that one or more states may exist in between may render evaluation of investigations on the pathophysiological role of hypodense eosinophils less confusing.

We speculate that the hypodense eosinophil after dextran sedimentation represents a significant phenotype of eosinophils just before starting to migrate into the airway tissue. This eosinophil is characterized by increased volume and degranulation of CD11b containing granules but not fully activated as indicated by an quantitatively unchanged L-selectin expression. Peripheral eosinophils in asthma are primed for this state of early activation. In addition, this low density state may facilitate transmigration of eosinophils into the airways of asthmatic patients. Although there is no direct evidence for this particular pathophysiological role of hypodense eosinophils in asthma in vivo, data on neutrophils, in which we have observed similar findings [304, 305], may provide support for this view. Furthermore, inflated hypodense neutrophils are probably not a random phenomenon, but play a role in the pathogenesis of asthma [306]. The increase in hypodense neutrophils may be the result of an increased migration of non-activated neutrophils due to an infiltrate in the airways [2]. This may also explain the finding that hypodense neutrophils are more activated than normodense neutrophils [170]. In conclusion, studies on hypodense eosinophils and neutrophils may play a role in the understanding of the pathophysiology of asthma and the evaluation of therapeutic strategies.
Migration increase is, however, 
observed similar effects of dextran sedimentation, support this hypothesis. In neutrophils, density decrease is also related to a state of activation and volume increase [304,306,334]. Especially volume increase has been more extensively studied in neutrophils than in eosinophils. Worthen et al. have shown in rabbits that during infiltration into airway tissue, neutrophils obtain twice their size in peripheral blood [372]. Similarly, BAL eosinophils display a much lower density, and therefore probably a larger volume, than peripheral eosinophils [360]. Furthermore, a direct relationship between the neutrophil volume and migratory capability has been found [300]. Migrating neutrophils in vitro increased their volume by about 50%. The increase appeared due to the action of the sodium/proton antiport, presumably through intracellular sodium chloride accumulation and water uptake. Migration was inhibited in experiments in which volume increase was prevented and enhanced by osmotic swelling. The mechanism by which cell volume effects migratory capacity, however, remains obscure. It has been suggested that osmotic forces drive protrusion of pseudopods in locomoting cells [434]. Alternatively, neutrophil and eosinophil volume may alter the balance between filamentous and monomeric actin, involved in cytoskeletal changes during cell locomotion and degranulation. A study using HL-60 cells describes an inverse relationship between resting cell volume and intracellular filamentous actin content [20]. Interestingly, it has recently been described that IL-5 signals for actin polymerization in eosinophils [435]. A role of actin polymerization in the decrease of eosinophil density is further supported by the fact that density increase is inhibited by pentoxyfillin, an inhibitor of actin polymerization (unpublished results). The apparent relationship between eosinophil density, adhesion molecule expression, volume and, possibly, migratory capacity opens an area of investigation which has not been extensively addressed in eosinophils yet.

Priming of these parameters may be a key factor in the pathophysiology of asthmatic inflammation. It is imageable that only after sufficient priming eosinophils become activated in the vasculature of the lungs. Rolling takes place under large forces induced by shear stress caused by blood flow. The volume increase may therefore help slowing the eosinophil down by letting it form a plug to retain it in the capillary vessel as has been suggested for neutrophils [436]. Thus, during these early steps, L-selectin down regulation and volume increase, may be an important prerequisite for infiltration. Together with the increased expression of
CD11b associated with density decrease these changes facilitate eventual eosinophil infiltration.

In summary, the hypodense eosinophil as a model for peripheral eosinophils just before infiltration into the airway tissue warrants further investigation instead of relegating it to a footnote in the history of eosinophil biology. Moreover, the first steps in infiltration and the underlying intracellular events may form an interesting target for new therapeutic interventions, which may be more effective than those acting when eosinophils have become fully activated after passing the endothelial layer.

Concluding remarks

In this thesis we attempted to elucidate the phenomenon of hypodense eosinophils in asthma. A great deal of the confusing results in the literature may have been caused by the fact that purification procedures change eosinophil density in vitro, thereby concealing the in vivo situation and resulting in greatly varying numbers of hypodense eosinophils in different studies. Therefore, one of the main conclusions from our studies is that elevated numbers of hypodense eosinophils are not present in the circulation of patients with asthma but that they are generated in vitro during dextran sedimentation. Furthermore, it became clear that density decrease is accompanied by CD11b increase and that peripheral blood eosinophils from asthmatic patients are primed for both parameters. In addition, a relationship was shown between density and cell volume, as measured by forward scatter. Because of this relationship between density, adhesion molecule expression and volume, we hypothesized that hypodense eosinophils reflect an early activated eosinophils, just before migrating into airway tissue. The hypodense eosinophil therefore constitutes an interesting piece in the puzzle of asthma, which may reveal important new clues for therapeutic intervention.