Apoptosis in chronic inflammation, with specific reference to airway disease

Malin Müller

Stockholm 2006
Till Joni
Abstract

Mechanisms governing the normal resolution processes of inflammation in the lung are poorly understood, yet their elucidation may lead to a greater understanding of the pathogenesis of several pulmonary inflammatory disorders. The elimination of activated lymphocytes by apoptosis is one prerequisite for resolution, and knowledge concerning the role of apoptosis in chronic inflammation is still far from complete. A complicating fact is that apoptotic pathways seem also to be involved in cellular proliferation. The aim of this project was to study the relationship between apoptosis and inflammation with special regards to mitochondrial apoptosis and chronic inflammation in the airways. In the first two studies we showed that tributyltin (TBT) effectively induces mitochondrial apoptosis in resting human peripheral blood lymphocytes (PBL), by the release of cytochrome-c and caspase-3 activation. Interestingly, when peripheral blood T lymphocytes were anti-CD3 activated, we observed a time-dependent induction of caspase-3 activity in the absence of an apoptotic morphology. Moreover, at the observation point when the activity of caspase-3 had reached its maximum, an insensitivity against TBT induced apoptosis started to develop. Finally, co-culture with dexamethasone (DEX) inhibited caspase-3 activity as well as proliferation, suggesting a role for caspase-3 in the activation differentiation process in these cells.

Our goal in the third work was to investigate whether the increased airway inflammation, seen in individuals with asthma, exposed to airborne allergen, was associated with altered apoptotic phenotype of lymphocytes. Here we found a reduced sensitivity to apoptosis in broncho-alveolar lavage (BAL) fluid lymphocytes, following airway allergen exposure, which was accompanied by an increased amount of cells expressing the anti-apoptotic protein Bcl-2.

In a previous study, an increased apoptosis resistance was observed in lung T lymphocytes from sarcoidosis patients. Based on this, in the last study we further studied the mechanisms behind the observed apoptosis-resistance by investigating whether the sarcoidosis associated cytokines IL-12/IL-23 and IL-18 could influence the apoptotic phenotype of these lymphocytes. The results suggest a pro-survival role of IL-12 and/or 23 whereas IL-18 appear to have a pro-apoptotic function in the lungs of sarcoidosis patients. We also found a difference in apoptosis-inducing capacity between the anti-TNF-α drugs, infliximab and etanercept, with infliximab being the more effective in killing BAL fluid lymphocytes. This finding may be related to the differences in drug efficacy between these two compounds in the treatment of sarcoidosis.

In the present studies, we have shown that the activation state of the cell influences the sensitivity to apoptosis and that caspase-3 might participate in the early activation/proliferation machinery of T-cells. Moreover, allergen inhalation renders BAL fluid lymphocytes from asthmatic individuals more resistant to apoptosis, suggesting an association between the degree of inflammation and apoptosis. We have also identified cytokines affecting the apoptosis susceptibility of BAL fluid lymphocytes from sarcoidosis patients. Together these results might help to shed more light on the role of apoptosis in chronic inflammation.

Keywords: Apoptosis, asthma, chronic inflammation, lung, sarcoidosis, tributyltin (TBT)
List of articles

This thesis is based on the following publications:


IV. Müller M, Stridh H, Eklund A and Grunewald J. Influence of T-helper type 1 associated cytokines on the survival of bronchoalveolar lavage (BAL) fluid lymphocytes from sarcoidosis patients. *(Manuscript)*

Papers will be referred to by their Roman numerals.
1.7.1 Apoptosis in allergic asthma

1.8 Sarcoidosis
1.8.1 Apoptosis in sarcoidosis

2. Aims of this thesis

3. Methods

4. Results and discussion
4.1 Introduction
4.1.1 I and paper II
4.1.2 Interpretations of paper I and paper II
4.1.3 Paper III
4.1.4 Paper IV
4.1.5 Interpretations of paper III and paper IV

5. Conclusions

6. Summary and future perspectives

Acknowledgements

References

Paper I-IV
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAF-1</td>
<td>Apoptosis protease activating factor-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Broncoalveolar lavage fluid</td>
</tr>
<tr>
<td>BHL</td>
<td>Bilateral hilar lymphadenopathy</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>CD95</td>
<td>Fas / APO-1</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>c-FLIP_L/S</td>
<td>Cellular FLICE-inhibitory protein long/short</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DD</td>
<td>Death domains</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signal complex</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EN</td>
<td>Erythema nodosum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associating death domain protein</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume</td>
</tr>
<tr>
<td>FLICE</td>
<td>Caspase-8</td>
</tr>
<tr>
<td>G3PDH</td>
<td>Glucose 3 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte- colony stimulating factor</td>
</tr>
<tr>
<td>HSP27/70</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitors of apoptosis proteins</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>IL-23</td>
<td>Interleukin-23</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondrion-derived activator of caspase</td>
</tr>
<tr>
<td>TBT</td>
<td>Tributyltin</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethylester</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Cell death

1.1.1 Apoptosis

There are, at least, two forms of cell death; necrosis and apoptosis. Necrosis is an energy independent, disorganised event, which is a consequence of severe injury of the cell while apoptosis is an energy dependent, organised process. The necrotic process is rather fast and is characterised by the swelling and the eventual rupture of the cell with the subsequent release of intracellular contents that provoke an inflammatory reaction. Apoptosis, on the other hand, proceeds more slowly with distinct features that prevent an inflammatory response.

Apoptosis is a fundamental mechanism for normal development and tissue homeostasis in multicellular organisms. Apoptosis is an evolutionary conserved form of cell death, which is characterised by several biochemical and stereotypic morphological changes and proceeds through three distinct stages. The initial commitment phase includes the receiving of a signal and initiation of the apoptotic process. The execution phase, in which the cell dies, is characterised by the activation of different proteases followed by chromatin condensation, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation and finally the formation of apoptotic bodies (1, 2). The last stage involves the engulfment of the apoptotic cell by neighbouring phagocytes which recognise the dying cell by its exposure of phosphatidylserine (3). The clearance of apoptotic cells prevents an inflammatory reaction since spilling of intracellular contents is prevented.

1.1.2 Caspases

Caspases are a group of cysteine proteases that is thought to be responsible for initiation and execution of apoptosis, including the morphological and biochemical changes. At present, 14 different caspases have been characterised. All of them have human homologues except for caspase-11 and –12. Caspases are expressed as pro-enzymes (zymogens) and require proteolytic cleavage to become active. Upon activation, zymogens are cleaved into a large and a small subunit followed by the removal of the N-terminal pro-domain. Two large and two small subunits then associate into a heterodimer (tetramer) with two active sites. The proteolytic activity of caspases may result in the destruction of many vital cellular proteins, e.g. lamins, and the activation of proteins that contribute to cell destruction including DNA:ses. Caspases
recognise a short tetrapeptid within their target proteins, which can differ between the different caspases. They can undergo autoactivation or become activated by other pre-existing caspases as part of an amplification cascade.

The caspases can be divided into three sub-classes based on their physiological function. Group I, which includes caspase-1, -4, -5, -11, -12, -13 and –14 are thought to be primarily involved in the processing of cytokines. Caspase-2, -8, -9 and –10, are called initiator caspases and activate the effector caspase-3, -6, and –7, which are involved in the last step of the apoptotic process, where the cell dies. (4).

1.1.3 Caspase-3 and Caspase-8
Caspase-3, a member of the group II caspases, has uniformly been associated with apoptosis and no other function for this enzyme has been known. However, during the last couples of years new evidence has emerged which suggests additional non-apoptotic roles for caspase-3. Active caspase-3 has been proposed to participate in the differentiation process of such diverse cell types as neurones, muscle cells, monocytes and erythroblasts (5-8). Moreover, caspase inhibitors blocked proliferation, IL-2 processing and major histocompatibility complex (MHC) class II expression in activated T-cells, indicating that caspase-3 activation may be a physiological response to T cell triggering and that this family of enzymes might be involved in the early steps leading to lymphocyte proliferation (9-11). An additional report showed that caspase-3 can process pro-IL-16, a pro-inflammatory cytokine, into its active form (12, 13).Since a large number of proteins including procytokines have potential caspase-3 cleavage sites, future studies are needed to identify the substrates that contribute to cytokine processing following T cell activation.

Caspase-8 and possible caspase-10 are the principle caspases transducing the apoptotic signal induced by death receptor activation. However, recent work has shown that also caspase-8 have an additional role in activation and proliferation of T-cells where cFLIP, an inhibitor of pro-apoptotic caspase-8 associates with caspase-8 and this complex links TCR signalling to the activation of NF-κB (14).
1.2 Regulation of apoptosis

1.2.1 Apoptotic pathways

Apoptosis can be triggered by different signals, which seem to merge on a common pathway. At least two distinct initial pathways are at present known; receptor mediated, and non-receptor mediated apoptosis. Receptor mediated apoptosis involves primarily the ligation of death receptors (DR) belonging to the tumour necrosis factor (TNF) receptor super family. The DRs known so far, belonging to this family, are TNF-R1 (p55), Fas (CD95), TRAIL-R1, TRAIL-R2, and DR6 (15). The members of the TNF receptor family share amino acid homologies in their extracellular domains, which are cystein-rich, and in their cytoplasmic domains that contain a death domain. The Fas receptor is a widely expressed cell surface protein and it is the best-characterised death receptor (16). Within the immune system, Fas receptor engagement is involved in the down-regulation of immune reactions, as well as in T cell-mediated cell killing. Triggering of the Fas receptor can be performed either by its ligand (FasL) or by agonistic antibodies. The FasL is a homotrimeric molecule which promotes oligomerization of Fas receptors and causes the formation of a death inducing signal complex (DISC). The clustering of the DISC recruits the adapter proteins FADD which bind with its death domains (DD) to a DD in the cytoplasmic region of the receptors. FADD also contains a death effector domain (DED), which binds an analogous structure within pro-caspase-8, leading to the oligomerization of this caspase and its autoproteolytic activation. Active caspase-8 subsequently activates downstream-located caspases such as caspase-3 (17).

The non-receptor mediated pathway is believed to operate via the mitochondria and is induced by cytokine withdrawal or cytotoxic stress, such as that imposed by γ-radiation or chemotherapeutic drugs. The triggering of apoptosis via the mitochondria induces the release of mitochondrial constituents such as cytochrome c into the cytosol. Cytochrome c binds to the apoptosis protease activating factor-1 (APAF-1) and, in the presence of dATP, associates with pro-caspase-9 into a giant protein complex called the apoptosome. A conformational change of the proteins within this complex leads to the activation of caspase-9. Once activated, caspase-9 is able to cleave and activate pro-caspase-3, which results in a cascade of caspase activation, cleavage of target proteins and cell death (18). The receptor- and non-receptor mediated pathways can, depending on cell type, be coupled (19, 20). For example ligation of FasL and the subsequent activation of caspase-8 can cleave and activate Bid, a member of the Bcl-2 protein family, which induces the release of cytochrome c and other pro-apoptotic which thereby
amplifies the caspase cascade. This scenario is believed to take place in type II cells where the activation of the receptor-mediated apoptotic pathway is not enough to push the cell into apoptosis whereas type I cells are characterised by their ability to undergo receptor-mediated apoptosis without the engagement of the mitochondria.

1.2.2 Proteins involved in the regulation of apoptosis

Different proteins regulate, directly or indirectly, the activation of caspases. The cellular FLICE-inhibitory protein c-FLIP belongs to a family of proteins known to inhibit apoptosis at the death receptor level (21). These proteins are also expressed by γ-herpes viruses, where they are called viral v-FLIPs. The v-FLIPS inhibit the activation of caspase-8 by binding to the DISC of the Fas receptor. In humans, two splice variants of c-FLIP are known, a short form and a long form, c-FLIPs and c-FLIPL (22). c-FLIPs structurally resembles v-FLIP whereas c-FLIPL has a similar domain structure as caspase-8 but lacks the active enzyme site, although both proteins are able to inhibit the generation of active caspase-8.

Figure 1: Death receptor mediated apoptotic pathway (black arrows) and apoptosis induced via the mitochondrion (blue arrows).
Proteins belonging to the Bcl-2 family have a major regulatory role in apoptosis. The Bcl-2 family consists of both anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, and pro-apoptotic proteins, like Bax, Bak, Bad and Bid (23, 24). These proteins are associated with apoptosis induced via the mitochondria where they are thought to enhance or prevent the release of pro-apoptotic molecules such as cytochrome c and Smac. However, the exact mechanisms behind the pro- and anti-apoptotic properties of the different Bcl-2 proteins are still a matter of much debate (24-27). The stress inducible heat shock proteins 27 and 70 (Hsp27 and Hsp70) have been proposed to regulate apoptosis downstream of the mitochondria. Hsp27 has been suggested to bind cytochrome c and thereby prevent apoptosome formation and the subsequent activation of the caspases (28) whereas Hsp70 is believed to associate with APAF-1, which prevents the recruitment and activation of caspase-9 (29, 30).

Further downstream of the apoptotic pathway are the inhibitors of apoptosis proteins (IAPs). IAPs regulate apoptosis as direct inhibitors of active caspase-3, -7 and –9 (31, 32) and are themselves inhibited by the mitochondria-released protein Smac (33).

1.3 The immune system - a brief overview

1.3.1 Innate immunity

Innate immunity is the first line of defence an intruding pathogen will encounter and the principle components of the innate immune system are antibacterial peptides, NK-cells, mast cells, granulocytes, macrophages and the complement system (34). Macrophages and neutrophils have phagocytic capacity and recognise pathogens by means of different cell surface receptors which upon pathogen ligation trigger a process where the cell membrane of the phagocytising cell surrounds and internalises the receptor/pathogen complex in a membrane bound vesicle called the phagosome. Most microbes engulfed into the phagosome get killed by the acid environment in this organelle or when the phagosome fuses with lysosomes, which are membrane-bound granules containing different anti-microbial compounds, to form the phagolysosome. Additional anti-microbial activities of macrophages and neutrophils includes the production of other bactericidal molecules such as nitric oxide (NO), the superoxide anion (O2-) and hydrogen peroxidase (H2O2), compounds which are very toxic to bacteria (35). The basophilic and eosinophilic granulocyte as well as mast cells do not phagocytose microbes, instead their anti-microbial activity depend on the extra-cellular release of toxic compounds, stored in intra-cellular granules, which will kill the pathogen. Another important member of the innate immune system is the complement system which is composed of different plasma proteins activating a
cascade of proteolytic reactions on microbial surfaces either by binding directly to the microbial surface (the alternative pathway) or to antibody/microbe complexes (the classical pathway). This proteolysis results in large amounts of protein fragments coating the surface of the microbe, which thereby get lysed and/or recognised by phagocytes, engulfed and destroyed(35). The pathogen recognition receptors of the innate immune system are not antigen specific, instead they recognise various sorts of molecular structures, commonly expressed by different pathogens, which are referred to as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are lipopolysaccharide, peptidoglycan, double stranded viral RNA and bacterial DNA. Receptors recognising PAMPs are called pattern recognition receptors and includes macrophage mannose receptor, scavenger receptors and Toll-like receptors (TLR). Some TLR (1, 2, 4,5 and 6) are expressed on the cell surface whereas other TLR (3, 7, 8 and 9) have intracellular localisations. TLRs are specialists in recognising PAMPs but are also able to get activated by endogenous material such as HMGB-1 (36-38) During the last few years, advances have been made in describing different activities of the Toll-like receptors and their possible roles in different inflammatory conditions. Activated by the microbe, neutrophils and macrophages initiate an inflammatory process with the production and release of large amounts of biologically active molecules such as cytokines and chemokines, which attracts more neutrophils and macrophages to the infected tissue (39, 40). NK-cells have a key role in the early defence against intracellular pathogens such as viruses by producing large amounts of pro-inflammatory cytokines and they kill infected cells by the release of cytotoxic granules. Expressed on the cell surface, NK cells have activating receptors and inhibitory receptors that recognise self MHC class I molecules, controlling their cytotoxic activity. If the NK cell do not get an inhibitory signal i.e. there is an absence of, or a reduction in MHC class I expression as is often the case in virus-infected or tumour transformed cells, it will kill the target cell. Many pathogens are cleared effectively by the innate immune system and will not cause disease. However, infections that are not resolved by this first defence require the activation of a second line of defence, the adaptive immune system.

1.3.2 Adaptive immunity
The activation of the adaptive immune system requires the recognition of the pathogen by TLRs expressed on the surface of tissue resident antigen presenting cells, most commonly dendritic cells (DCs), followed by the subsequent engulfment of the pathogen. The microbe loaded DCs will then travel through the lymphatic system to a lymph node and present pathogen-derived peptides for by-passing T-cells. CD8 positive T-cells express T-cells receptors (TCRs)
recognising peptides, presented by MCH class I molecules whereas CD4 positive T-cells have TCRs recognising peptide/MCH class II complexes(41). The TCR is made up by one α and one β chain, each composed of a variable (V) region and constant (C) region which are formed during the development of the T-cell by somatic recombination of discrete gene segments. The antigen recognition site of the TCR has less variable regions, the CDR1 and CDR2 loops, which mainly contacts the MHC molecule whereas the CDR3 regions, which forms the antigen peptide binding sites, are highly variable to be able to recognise unique peptides. The remarkable diversity of the TCR raises the probability that there is a matching TCR for virtually all existing pathogen derived peptides. Naïve T-cells with T-cell receptors recognising a specific peptide/MHC complex on the surface of the DC will get activated, expand clonally and develop effector functions. Naive CD8 cells that migrate from the thymus to the peripheral T-cell pool are predestined to become cytotoxic cells ready to kill infected cells while naive CD4 T-cells are able to, upon their first antigen encounter, differentiate into various T-cell populations such as Th1, Th2 and IL-17 producing T-cells. Although the exact mechanisms behind this development are not fully understood, factors that will influence the fate of the cell include the cytokine milieu, co-stimulators driving the response and the nature of the peptide:MHC complex(42). Cytokines that favour a Th1 response are principally the interleukins IL-12 and interferon-γ (INFγ) while a Th2 response is driven by IL-4. Fully differentiated Th1 cells are recognised by their production of INF-γ and IL-2 whereas Th2 cells express IL-4 and IL-5. The principle function of Th1 cells is to activate other cells such as macrophages to produce cytokines and kill pathogens whereas the main function for Th2 cells is to activate B-cells to produce antibodies, able to neutralise pathogen. The B-cell receptor is like the TCR highly diverse to be able to recognise all potential pathogens. Naïve B-cells recognise their specific peptide by the B-cell receptor, a membrane-bound immuno-globulin, and upon microbe ligation the whole Ig/peptide complex get internalised into the cell. The peptide is then presented by MHC class II molecules on the cell surface of the B-cell to antigen specific CD4+ T-cells which will get activated and release B-cell activating cytokines IL-4, IL-5 and IL-6. The B-cell will then proliferate and differentiate into antibody producing plasma cells. Activated B-cells migrate into primary lymphoid follicles where they continue to proliferate and form germinal centres where they go through several important modification steps to become producers of high affinity antibodies. These modifications involves somatic hyper-mutations, which alters the V-regions of the immuno-globulins, affinity maturation, resulting in the survival of those B-cells producing antibodies with the highest antigen affinity and isotype-switching which allows for the production of different antibody iso-types with distinct functions. Most of the cells that make up
the respective B-cell and T-cell clones, which have successfully defeated the pathogen, will die. However, a few B- and T-cells escape death and become long lived memory cells which upon re-infection with their specific pathogen, quickly get activated and produce high affinity antibodies or effectively kill infected cells.

1.3.3 Central and peripheral tolerance

Apoptotic cell death plays a key role in the development and maintenance of the immune system as well as in controlling an immune response. The selection process during intrathymic development of T-cells results in the production of mature T lymphocytes expressing T cell receptors (TCRs) which are self-tolerant and able to recognise foreign peptides bound to self-MHC molecules (43). Here, both thymocytes expressing TCR specificities that are unable to interact with self-MHC molecules during positive selection, and thymocytes with a TCR that recognises and bind self peptide:MHC complexes with high affinity are eliminated from the T cell repertoire by apoptosis (43). Only 2-3% of all T-cells survive the selection process in the thymus and can enter the peripheral T-cell pool. Developing B-cells expressing a B-cell receptor which recognises self antigens either die by apoptosis in the bone marrow or become anergic (44).

As mentioned earlier, potentially auto-reactive T-cells, i.e. those that bind strongly to self-peptide/MHC complexes are eliminated in the thymus during the development of the T-cell. However, all self peptides are not present in the thymus which makes it possible for potential auto-reactive T-cells to escape into the periphery. Auto-reactive T-cells are found in the peripheral circulation of healthy individuals but they do not normally cause autoimmune disease. In order for an antigen to elicit a T-cell response it has to be presented by professional antigen presenting cells (APC). The activation of the antigen-specific T-cells also requires the action of co-stimulatory molecules and if no co-stimulatory signals are provided, the T-cell can either become anergic or go into apoptosis. Autoimmunity does not normally develop because most self-proteins are expressed at levels too low to elicit an immune response and T-cells that can recognise such self-peptides are considered to be in a state of immunological ignorance (45, 46). Regulatory T-cells (T_{REG}) cells appears to play a central role in the maintenance of peripheral tolerance since it has been shown, in animal models, that the absence of these cells increases the development of autoimmune disease (47, 48). There are two types of T_{REG}s described, the natural arising, which are CD4^{+}CD25^{bright} and express the transcription factor FOXP3, and the inducible T_{REG}s, characterised by the production of anti-inflammatory mediators (49, 50). The naturally arising T_{REG}s are produced in thymus and regulate, by cell-cell contact, the proliferation and
function of activated T-cells, a regulation which appears to be antigen independent. In contrast, inducible T\textsubscript{REGs} require the TCR recognition of the correct peptide/MHC complex to obtain regulatory functions. The inducible T\textsubscript{REGs} can be further divided into Trl, which down-regulate an inflammatory response by secreting IL-10 and Th3 regulatory T-cells, producing TGF\(\beta\) (50).

### 1.4 Cytokines

Cytokines are small secreted peptides released by various cell-types and they induce responses by binding to specific receptors. They can act in an autocrine manner, affecting the cell which the cytokine came from or in a paracrine manner, where the cytokine influences neighbouring cells. Some cytokines can also affect distant cells in an endocrine way, although this requires that the cytokine can enter the bloodstream and that the half life of the molecule is not too short. The impact cytokines have on other cells are numerous including differentiation, proliferation and apoptosis.

#### 1.4.1 Interleukin-12

Interleukin-12 (IL-12) is a 75 kDa heterodimeric cytokine composed of two covalently-linked glycosylated chains, p35 and p40, which are encoded by distinct genes (51, 52) and the sub-units have to be expressed in the same cell to form the biological active cytokine. IL-12 is mainly produced by activated monocytes, macrophages and dendritic cells (DCs) and enhances INF-\(\gamma\) production, proliferation and cytolytic activity of both NK and T cells (53). Interleukin-12 is produced during the early phase of an infection/inflammation and sets the frame for the subsequent antigen-specific immune responses, favouring differentiation of Th1 cells while inhibiting the differentiation of Th2 cells (54-56). The cytokine is also important for the growth, INF-\(\gamma\) production and cell adhesion of already differentiated Th1 cells (57). Recently, IL-12 has been proposed to participate in the regulation of the apoptosis machinery. Studies in mice demonstrated that Th1 cells turned less sensitive to Fas-receptor mediated apoptosis upon IL-12 treatment (58). In another study, anti-IL-12 antibodies were administrated to mice, suffering from 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis, and this treatment led to a rapid resolution of the colitis, associated with apoptotic loss of T-cells in both colon and spleen (59).

The IL-12 receptor (IL-12R) is composed of two subunits, IL-12R\(\beta\)1 and IL-12R\(\beta\)2, and is primarily expressed on the surface of activated T and NK cells but also by macrophages and DC (60, 61). Co-expression of both subunits are required to form a high-affinity IL-12 binding site.
The IL-12Rβ2 subunit is preferentially expressed on Th1 cells while absent on Th2 cells, correlating with the unresponsiveness of Th2 cells to IL-12 (62, 63).

### 1.4.2 Interleukin 18

IL-18 belongs to the IL-1 cytokine family and is a key mediator in the defense against infections and tumors. A variety of cells including immune-cells such as macrophages and dendritic cells as well as several non-immune cells such as keratinocytes, intestinal epithelial cells and secretory gland cells express IL-18. IL-18 is produced as biologically inactive precursor and is transformed into its active form by enzymatic processing and, at least in macrophages, this proteolysis is performed by caspase-1 (64-67). However, other enzymes such as proteinase-3 and proteins belonging to the Fas/Fas ligand pathway have also been suggested to be involved in the activation of IL-18 (68, 69). The IL-18 receptor is like most cytokine receptors a hetero-dimeric complex composed of a cytokine binding chain, the α chain, and an inducible signaling chain, the β chain (68, 70, 71). The intracellular signaling of the IL-18 receptor is closely related to the signaling pathway of IL-1 with the recruitment of IL-1 receptor associated kinase (IRAK), the interaction with TRAF-6 and the subsequent translocation of NF-κB to the nucleus (72). The IL-18R is constitutively expressed at low levels on CD19+ B-cells, some CD8+ T-cell populations and NK cells while CD4+ T-cells requires activation to express the receptor (66, 73).

The biological effects of IL-18 are rather diverse and include the synergization with IL-12 to stimulate NK-cells, CD8+ T-cells and Th1 CD4+ T-cells to produce large amounts of INF γ as well as, in the absence of IL-12, stimulation of a Th2 response (74, 75). IL-18 also possesses pro-apoptotic properties by enhancing the cytotoxic activity of NK, NKT and CD8+ T-cells by inducing the Fas/Fas Ligand and/or the perforin/granzyme pathway (76, 77).

### 1.4.3 Interleukin-23

Interleukin-23 (IL-23) is a recently discovered heterodimeric cytokine, which resembles IL-12 in both structure and function. IL-23 comprises of a p19 sub-unit that dimerizes with the p40 sub-unit of IL-12 to form the biologically active cytokine. The IL-23 receptor is composed of the IL-23R, which associates with the β1 receptor sub-unit of the IL-12 receptor to form the heterodimeric receptor complex. IL-23 is, like IL-12, produced by DCs and macrophages and co-expression of the two sub-units in the same cell is also required for the formation of biologically active IL-23 (78, 79). The receptor for IL-23 is expressed on the surface of the same cells as IL-12 i.e. T- and NK-cells as well as macrophages and DCs. IL-12 and IL-23 activate similar JAK-
STAT– (Janus activated kinas-signal transducer and activator of transcription) signalling pathways, explaining the overlapping biological effects of these two cytokines. However, while IL-12 induces an enhanced phosphorylation of Stat4 and a relative weak phosphorylation of Stat3, IL-23 signalling stimulates the reversed phosphorylation pattern of these proteins(80). Although IL-12 and IL-23 have some biological functions in common, distinct effects of these two cytokines have emerged with time. Whilst IL-12 primarily acts on naïve T-cells and promotes a Th1 defence IL-23 appears to mainly affect memory T-cells and be important for the production and maintenance of IL-17 producing CD4+ T-cells which have been suggested to be involved in chronic inflammation (81). Recent studies have suggested that IL-23 rather than IL-12 is the critical cytokine in chronic inflammation and IL-23, not IL-12, has been shown to be the essential cytokine in the induction of experimental autoimmune encephalomyelitis (EAE) (82, 83).

Figure 2: The cytokines IL-12 and IL-23 with their receptors.
1.4.4 Tumor necrosis factor-α (TNF-α)

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, predominantly produced by macrophages, T-cells, NK cells and neutrophils. The biological effects of this cytokine are numerous, ranging from proliferation and differentiation to apoptosis.

There are two forms of TNF-α molecules, a cell membrane bound (mTNF-α) and a soluble form (sTNF-α), the latter being produced by enzymatic cleavage of the transmembrane form by TNF-α converting enzyme (TACE). Two receptors are responsible for the divergent actions of TNF-α, p55 (TNF-α RI), and p75 (TNF-α RII). They are co-expressed on most cell-types and both are able to transduce survival signals(84). Apoptosis is mainly triggered by ligand binding to TNF-α RI although cell death induced by the activation of TNF-α RII has also been reported (85). TNF-α increases the production of several pro-inflammatory molecules such as IL-1, IL-6 and vasoactive intestinal peptide (VIP) as well as the expression of adhesion molecules on the cell surfaces.

1.4.5 Anti-TNFα therapy in chronic inflammation

Infliximab and etanercept are two different anti-TNF-α drugs commonly used in the clinic. Infliximab is a chimeric anti-TNF-α monoclonal antibody with murine variable regions and a human IgG1 Fc part whereas etanercept is a soluble TNF-α receptor (a dimeric fusion protein composed of TNF-α RII linked to the Fc part of a human IgG1). Both infliximab and etanercept have been shown to be equally efficient in the treatment of RA. However, whereas infliximab treatment resolves the inflammation in patients with Crohn’s disease, which, like sarcoidosis, also is a granulomatous disease, etanercept has been shown to be largely ineffective in this condition. This phenomenon has also been observed when patients with sarcoidosis have been subjected to anti-TNF-α therapy, with infliximab being superior to etanercept (86–88). Since intestinal lamina propria T-lymphocytes in patients with Crohn’s disease are rather apoptosis-resistant has it been suggested that the observed discrepancy between the two compounds is related to the lack of apoptosis-inducing properties of etanercept (89). Infliximab induced apoptosis at a higher frequency than etanercept which is thought to be due to the stronger interaction of infliximab with the membrane bound form of TNFα, an interaction which is proposed to induce apoptosis in the target cell.
1.5 The immune system in the lung

The alveolar membrane is the largest surface of the body in contact with the outside environment. This means that the lungs, like the skin and the intestinal tract, are highly exposed to microbes of different kinds and a well functional defence against these possible pathogens is a prerequisite for life. Particles entering the conducting airways sediment onto the mucociliary surface were the upward force carries them up and out whereas smaller particles, a diameter of 1µm or less, which is the size of bacteria and viruses, reach the alveolar space. The airway fluid consists of various soluble anti microbial factors such as lysozyme which causes lysis of many different bacteria, IgA, IgG and proteins of the complement system, which neutralises and opsonises microbes, and antibacterial peptides like defensins.

Despite the constant low dose microbial exposure of the airways the alveolar space normally is a rather quiet place were inflammatory responses are strictly controlled. Two proteins, shown to be of importance in regulating the immune reactivity in the lung alveolar are the surfactant associated proteins SP-A and SP-D. These two proteins are able to opsonise bacteria, viruses and dying cells and stimulate the engulfment of these complexes by macrophages as well as to inhibit pro-inflammatory cytokine production (90).

In the alveolar space of a normal lung, macrophages account for approximately 90% and lymphocytes around 9% of the leukocyte population where the last percent is shared between granulocytes and mast cells. The airway macrophages are professional phagocytic cells which keep the alveolar space close to sterile by engulfing almost everything that enters.

Pathogens not cleared by this first line of defence will also in the lung, like everywhere else in the body, activate the adaptive immune system which requires the presentation of antigens by professional antigen presenting cells (APCs). The dendritic cells (DC), the principal APCs, are able to detect and ingest antigens in all parts of the lung, and then transport them to the regional lymph nodes. This requires a maturation process, induced by cytokines such as GM-CSF, TNF-α, IL-1, which are produced by epithelial cells and interstitial macrophages likely as a result of direct contact with antigens. DCs are also activated via engagement of the TLRs by bacterial or viral molecules. Antigen presentation by DCs in the regional lymph node results in the clonal expansion of antigen specific T-cells which will acquire their different effector functions and then travel back to the lung to combat the pathogen.
1.6 Apoptosis in the immune system

When an intruding pathogen is defeated is it necessary to decrease the amount of activated immune cells to avoid tissue destruction and auto-immune reaction. The innate immune system is fast but unspecific and cells involved in this first line of defense, especially the granulocytes, are specialists in killing pathogens quickly. These cells are filled with toxic compounds stored in granules, which kills microbes effectively and unfortunately, if released in to extra cellular space, also causes tissue injury. Hence it is of great importance that these cells can be cleared quietly when the pathogen has been defeated. The adaptive immune system on the other hand is slower but have a high specificity and when antigen specific T and B-cells become activated they expand clonally. When an infection is defeated the majority of the activated T- and B-cells die by apoptosis. However, some activated T and B cells are rescued and become long-lived memory cells. Thus, a definite and fine-tuned balance between apoptosis and survival during the development and in the activation process of immune competent cells is a fundamental mechanism for the homeostasis and an adequate function of the immune system. Insufficient apoptosis of immune-competent cells may interfere with clonal contraction and maintenance of tolerance, which could result in T cell accumulation contributing to chronic inflammation.

Apoptosis is one key mechanism in regulating T-cell homeostasis. T-cells which been activated through their T-cell receptor (TCR) die by apoptosis either by activation induced cell death (AICD) or activated T-cell autonomous death (ACAD). Activation of death receptors such as CD95 (Apo/Fas) is a prerequisite for AICD whereas the intrinsic pathway i.e. release of mitochondrial pro-apoptotic factors is crucial for the induction of ACAD (91). Which pathway that is going to be used depends on several factors e.g cytokines, growth factors, antigen presentation and the presence of correct co-stimulatory signals. Proteins regulating the mitochondrial induced pathway are the Bcl-2 family whereas the cFlip proteins have been suggested to regulate the death receptor induced pathway.

1.7 Allergic asthma

Allergic asthma is a chronic disease caused by an allergen-induced-inflammatory response in the airways. The disease is Th2 driven with elevated levels of IL-4 and IL-5, which results in increased concentration of IgE antibodies in the circulation and elevated numbers of eosinophils at the site of inflammation (92). The symptoms of asthma are shortness of breath, coughing and wheezing which are commonly treated with β2 receptor agonist and
glucocorticoids. Sensitised allergic individuals with allergic asthma develop, within 10-20 minutes after allergen exposure, an early asthmatic response (EAR), which normally resolves within one hour. Some individuals also develop a late asthmatic response (LAR) 3-4 hours after allergen encounter, which can persist for 24 hours or more. The EAR is characterised by bronchial constriction, increased vascular permeability and mucus production, symptoms which are caused by rapidly metabolised mediators such as histamines, prostaglandins and leukotriens released by cross-linking IgE on mast-cells. In contrast, the development of the LAR requires the synthesis and release of inflammatory mediators including leukotriens, chemokines and cytokines, which recruit other leukocytes such as eosinophils and T-lymphocytes to the bronchial mucosa (93). The inflammatory process in the airways of individuals with allergic asthma may eventually lead to remodelling of the airway tissue, which could cause a reduced pulmonary capacity. Thus, inflammation at the sites of target organs is a pathological feature of the disease process and among the various types of cells involved in tissue infiltration and damage are T lymphocytes, i.e. one of the main effector cells controlling the local ongoing allergic immune response. These cells are involved in the local recognition of allergens, acting through secretion of Th2-cytokines and thereby promoting IgE synthesis and the mucosal recruitment of other inflammatory cells such as neutrophils and eosinophils (94, 95). Although our knowledge of the initiation of the allergic response has rapidly expanded during recent years, little is known about how the inflammatory response develops into a chronic inflammation.

1.7.1 Apoptosis in allergic asthma

The increased amount of inflammatory cells observed in the airway mucosa during an asthmatic response is thought to be caused mainly by an enhanced recruitment of these cells. However, a decreased apoptotic rate is a factor, which could also add to the increased amount of cells observed. Previous studies have demonstrated that reduced apoptosis of T cells may play a role in asthma pathogenesis (96, 97). Apoptosis of lymphocytes was decreased in asthmatic subjects compared to normal controls and patients with chronic obstructive pulmonary disease(98). Additionally, T cells from asthmatic subjects fail to undergo the normal degree of apoptosis following Fas receptor ligation, and CD45RO+ T cells in the airway wall of patients with mild and severe asthma were less apoptotic compared to controls (99, 100). Thus previous data indicate alterations in the apoptotic phenotype of cells involved in asthmatic inflammatory processes. The observed reduced T cell apoptosis may interfere with the down-regulation of an immune response resulting in a T cell accumulation contributing to the chronic inflammation of asthma.
### 1.8 Sarcoidosis

Sarcoidosis is a granulomatous disease with unknown aetiology, affecting various organs in the body with the lung being the most frequently engaged organ. Other organs commonly affected are the eyes, nervous system, skin, heart and lymph nodes. The pulmonary symptoms of sarcoidosis can be shortness of breath, dry cough and chest pain whereas more general symptoms are fatigue, fever and anorexia. In early disease there is a mononuclear cell alveolitis, dominated by activated CD4+ T cells and macrophages. The coordinated interplay between these cells leads to the characteristic formation of granulomas which are structures composed of activated macrophages surrounded by CD4+ T-cells. Granuloma formation is a strategy of the immune system to battle certain microbes suggesting a possible pathogen-derived cause of sarcoidosis. The granuloma in sarcoidosis is non-caseating which means there is no necrosis, in contrast to the granulomas induced by *mycobacterium tuberculosis* where necrotic cells are present and hence called caseating. The rather odd term caseating (cheese-like) just describes how the two different types of granulomas appear in the microscope.

Although the most important enigma of sarcoidosis, ie its aetiology, remains unsolved, in the past few years knowledge has emerged regarding general immunologic and molecular aspects of the inflammatory mechanisms leading to granuloma formation. During the sarcoid inflammatory process, several cytokines are secreted at sites of disease activity; in addition, high affinity receptors for cytokines important for the granuloma development have been identified. The lung accumulated CD4+ T cells display activation markers, such as HLA-DR, and produce high levels of the Th1 cytokines IL-2 and IFN-γ. IL-12, a key cytokine for the shift towards a Th1 immune response, and IL-18, which enhances the Th1 stimulating effect of IL-12, are both elevated in BAL fluid of sarcoidosis patients. Additionally, the pro-inflammatory cytokines IL-1, IL-6 and TNF-α are all found at high levels at the site of inflammation. Interestingly, in one study it was shown that patients with a good prognosis had an increased expression of TGF-β. This particular cytokine has been suggested to inhibit the production of IL-12 and IFN-γ(104). The cytokine profile generated by activated macrophages and lymphocytes in the chronic inflammation of sarcoidosis will have varying effects on both differentiation and death of cells, and influences the final outcome of the disease state. One factor that predicts the disease course in sarcoidosis is the HLA type. Scandinavian sarcoidosis patients expressing a particular HLA molecule, DRB1*0301, often have an acute form of the disease, frequently with Löfgrens’s syndrome i.e bilateral hilar lymphadenopathy (BHL) on chest x-ray, fever, erythema nodosum (EN) and/or arthropathy (105, 106). In the lungs of sarcoidosis patients positive for DRB1*0301
is an expansion of CD4+T-cells expressing the T-cell receptor Vα2.3 gene segment (107, 108). Patients with the acute form of sarcoidosis often have a good prognosis and will commonly recover spontaneously in one to two years whereas patients with a slower onset, in particular those positive for DRB1*14 and DRB1*15, can acquire a more chronic form of sarcoidosis. Patients with the more severe chronic form of sarcoidosis are also at higher risk to develop fibrosis and permanently impaired lung function (109).

The use of bronchoscopy and bronchalveolar lavage (BAL) has made it possible to study cells from the site of inflammation in sarcoidosis patients, and sarcoidosis can be considered to be a good model for studies of other pulmonary inflammatory disorders.

1.8.1 Apoptosis in sarcoidosis

Most studies describing the survival of immune-cells involved in the granuloma formation in sarcoidosis have showed reduced apoptotic events, although there are conflicting reports. Both the death receptors TNFR1 (p55) and Fas are expressed at high levels on alveolar macrophages and, at least, the Fas receptor expression is high on BAL fluid T-lymphocytes, from sarcoidosis patients (110, 111). Moreover, elevated levels of soluble Fas ligand (FasL) has been measured in BAL fluid from these patients (112). However, despite the presence of these pro-apoptotic molecules reduced apoptosis has been observed in macrophages in granulomas from the skin and in lung T-cells from sarcoidosis patients (113, 114)suggesting other tasks than apoptosis for these proteins in the sarcoid granuloma.
2 Aims of this thesis

The aim of this project was to study the relationship between apoptosis and different aspects of the immune system with special regards to mitochondrial induced apoptosis and on chronic inflammation in the airways. The specific aims were:

I. To evaluate the effect of tributyltin (TBT), an agent which specifically triggers apoptosis via the mitochondrion, in mature resting and anti-CD3 stimulated lymphocytes regarding apoptosis and caspase-3 activity.

II. To further investigate the association between the T-cell activation process, caspase-3 activity and apoptosis sensitivity and the influence of dexamethasone (DEX) in anti-CD3 activated T-lymphocytes.

III. To study whether the increased airway inflammation, seen in asthmatic individuals exposed to airborne allergen, was associated with an altered apoptotic phenotype of blood and lung lymphocytes.

IV. To investigate the influence of the cytokines IL-12, IL-18 and TNF-α as well as IL-23 on apoptosis susceptibility of bronchoalveolar lavage (BAL) T-cells of sarcoidosis patients.
3 Methods

Cells and study subjects (Paper I-IV)
All the experimental work was performed using human immune cells from the blood or the lungs except in paper I were the initial experiment were performed using Jurkat T-cells (human T-cell lymphoma cell line). The blood lymphocytes were from healthy blood donors (paper I-II) and the lung cells were obtained by bronchoalveolar lavage (BAL) from 12 patients with mild atopic asthma (paper III), 18 sarcoidosis patients with pulmonary manifestations and 6 control patients (paper IV). The diagnosis for three of the control patients were pulmonary infiltrate, two suffered from lung fibrosis and one had alveolitis. The sarcoidosis was classified as active according to the WASOG criteria (115) in all patients. None of the patients was treated with corticosteroids at the time of the BAL (paper III- IV) and the subjects in paper III controlled their asthma, when needed, solely with β2-agonists. In paper III the study population was was composed of 9 females and 3 males, aged 22-46 years (median 27) and they were all never-smokers except one who was an ex-smoker. The study population in paper IV was composed of six females and 12 males, with a median age of 45 years (range 25 to 71) where 3 were smokers, 5 ex-smokers and 10 were never smokers. In the control patient group (paper IV) was four females and two males, with median age of 54 years (range 40 to 71), the smoking habits in this group was as follows, 2 were ex-smokers and 4 had never smoked..

All studies were approved by the Ethical Committee.

Bronchoalveolar lavage (BAL) (Paper III and IV)
BAL was performed under local anaesthesia the flexible fibreoptic bronchoscope wedged in a middle lob bronchus and sterile PBS solution was instilled in five aliquots of 50 ml. After each instillation the BAL fluid (BALF) was gently aspirated and collected in a siliconised plastic bottle kept on ice(116).

Handling and separation of cells (Paper I-III)
The recovered BALF was strained through a Dracon net, centrifuged, and the pellet was resuspended in RPMI 1640 medium and kept on ice until use. The viability was determined by Trypan Blue stain Heparinised blood from healthy blood donors (Paper I-II) or from atopic patients (Paper III) was centrifuged at 400g over Ficoll-Paque gradients and the phase containing peripheral blood mononuclear cells (PBMC) was collected, washed in PBS and
centrifuged at 400g. The PBMC pellet was re-suspended in RPMI 1640 medium and kept on ice until use.

**Flow-cytometric analysis of changes in the mitochondrion membrane potential (Paper I)**
Mitochondrial alterations were determined using the dye TMRE. Reduction of the membrane potential leads to loss of TMRE from mitochondria and consequent quenching, which is reflected by a decrease of the overall fluorescence. Fluorescence of TMRE was quantitated by flow cytometry. Lymphocytes were identified by size and complexity.

**Cell culture conditions and neutralising of cytokine activity. (Paper I-IV)**
Blood and BAL fluid cells in all experiments were cultured in RPMI-1640 containing 5% FCS, 2mM L-Glutamine, penicillin (100U/ml) and streptomycin (0.1mg/ml) referred to as complete medium (CM) (Paper I-IV). PBMC were seeded out on 24-well tissue culture plates, pre-coated with anti CD3- monoclonal antibodies (mAbs) and stimulated for up to 168 hours (Paper II) or 300 hours (Paper I). CD3-stimulated PBL were co-cultured with dexamethasone (DEX) for up to 168 hours (Paper II). BAL cells were cultured in round-bottomed 96-well plates and in the presence of monoclonal antibodies neutralising IL-12Rβ1 (1µg/ml), IL-18 (1µg/ml) or IL-23 (5µg/ml) respectively or in the presence of isotype-matched control mAbs at the same concentrations (Paper IV). Two different compounds were used to block the biological effect of TNF-α, infliximab or etanercept (Paper IV). Infliximab is a chimeric anti-TNF-α monoclonal antibody with murine variable regions and a human IgG1 Fc part whereas etanercept is a soluble TNF-α receptor (a dimeric fusion protein composed of TNF-α RII linked to the Fc part of a human IgG1). BAL fluid cells were cultured in CM supplemented with LPS (1µg/ml) for 48 hours in the presence of infliximab or etanercept at a concentration of 10µg/ml.

**Induction and detection of caspase-3 activity (Paper I-II)**
Type II caspase activity (caspase-3 and 7) was measured by two different methods. Caspase activity of cell extracts (Paper I) was measured using the DEVD-AMC cleavage assay and analysed by fluorometri. Caspase activity in intact cells (Paper II) was measured, using the cell-permeable caspase-3 substrate PhiPhilux® containing the consensus sequence DEVDG. Fluorescence was measured in the FL1-channel of a flow cytometer. Lymphocytes were identified by size and complexity.
Induction of apoptosis

Tributyltin (TBT) was used to induce mitochondrial apoptosis since this compound has been shown to effectively induce apoptosis via the mitochondrion (117-120).

Detection of apoptosis (Paper I-IV)

In paper I, apoptosis was determined by morphological evaluations by staining cells with a mixture of chromatin dyes: the membrane permeable dye H-33342 and the membrane impermeable dye Propidium Iodide. Necrotic cells (damaged plasma membrane, non-condensed nuclei) and apoptotic cells (condensed nuclei) were identified by their difference in the uptake of these dyes and scored in a fluorescence microscopy. In paper I-IV, apoptosis was determined by Annexin V and Propidium Iodide staining and analysed by flow cytometry. Annexin V binds to exposed phosphatidyl serine (PS), which is translocated from the inside to the outside of the cell membrane during the early phase of the apoptotic process. Propidium Iodide binds to nuclear acids in cells with broken cell membrane. Cells that are Annexin+/Propidium- are defined as early apoptotic whereas cells that are Annexin+/Propidium+ are defined as late apoptotic/secondary necrotic cells. Live and dead lymphocytes were identified by by size and complexity.

Preparations of cytosols (paper I)

For analysis of mitochondrial-released cytochrome c, cytosolic fractions (lacking the heavy organelles) from cells was prepared by digitonin permeabilization.

Proliferation assay (Paper II)

The cell proliferation of CD3-stimulated PBL was measured by Methyl-3H Thymidine incorporation. The 3H thymidine activity was measured on a scintillation counter using UltraTerm III version 1.5 software.

Western blot (paper I-II)

We analysed the expression of different apoptosis-related proteins by western blot. Protein extracts were loaded onto 15% SDS-polyacrylamide gels, electrophoresed and then trans-blotted, all according to standard laboratory protocols. The membranes were probed with antibodies against cytochrome c, G3PDH, HSP70 and 27, Bcl-2, DFF 45 and PARP (paper I) or antibodies against XIAP, G3PDH and Bcl-XS/L (paper II) followed by a secondary peroxidase-labelled antibody, and then visualised by ECL.
**Analyses of the cell surface molecules (Paper I-III)**

Surface molecules on lymphocytes were detected after double or triple staining with mAbs against different cell surface markers, followed by flow cytometric analysis. Viable lymphocytes were identified by size and complexity. The analysed cell surface markers were CD3, CD4, CD8, CD45RO, CD95, CD69, HLA-DR, IL-12Rβ1 and IL-18R.

**Study design (Paper III)**

The study was performed out of the pollen season. Patients were blinded and administered either saline or an allergen for which they had a positive history and RAST result. Peripheral blood and bronchoalveolar lavage (BAL) samples were obtained 2-3 weeks before (baseline) and one day following the allergen/saline challenge. The type of allergen used for challenge, cumulative allergen dose, peak drop in FEV$_1$ during the EAR and the PD$_{20}$ values are presented in **paper III**

In brief, the allergen challenge was initiated by inhalation of diluent. Provided that the FEV$_1$ did not change more than 10%, allergen was inhaled every 15 minutes with half-log increments of the cumulated dose until the FEV$_1$ dropped 20% or more from the post-diluent baseline value.(121). The occurrence of any late asthmatic reactions (LAR) were examined by measurements of peak expiratory flow (PEF) rates every waking hour during the first 24 hours following challenge. The patients were instructed to make additional recordings if any airway symptoms occurred.

**Immunocytochemistry staining (Paper-III)**

Detection of the Bcl-2 protein in BAL fluid cells was performed using immunocytochemistry. BAL fluid cells on cytospins were fixed with paraformaldehyde 4% and made permeable with methanol permeabilised. After blocking unspecific binding sites, cell smears were incubated overnight at 4°C with primary antibodies, washed, incubated with secondary antibody and finally exposed to the enzyme conjugate of choice (Alkaline phosphatase or peroxidase) and visualisation was performed using the alkaline phosphaase substrate Fast Red or the peroxidase substrate DAB (3,3’-Diaminobenzidine).

**Analysis of secreted cytokines by cytometric bead array (CBA) (Paper-IV)**

To measure secreted cytokines we used a method called cytometric bead array (CBA). This method has the advantage of measuring several proteins at the same time. The CBA
technique is based on beads with distinct fluorescence intensities, each coated with antibodies to a particular protein. The bead/antibody complex, big enough to be detected by flow cytometry, bind to the specific protein and is detected using a fluorescence-conjugated antibody recognising another epitope of the bound protein. Since the beads have distinct fluorescence intensities and the fluorescence is of another sort than the detection antibodies is it possible to detect different soluble proteins simultaneously. A commercially available CBA kit was used for detection of secreted cytokines in BAL cell culture supernatants. The cell culture supernatants were saved and frozen until cytokine analysis and were concentrated 10x using Amicon Ultra-15 Filters the day before cytokine testing was performed. The Human Inflammation Kit was used, which allowed detection of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α. The assays were performed according to the manufacturer's instructions and the data were analysed on a FACS-calibur flow cytometer using the BD Cytometric Bead Array software.

**Intracellular staining for IL-6 in BAL fluid lymphocytes (Paper-IV)**

To inhibit cytokine secretion cells were treated with Brefeldin (1µg/ml) 5 hours prior to harvest. Intracellular staining were performed as follows: cells, were fixed with paraformaldehyde 4% for 10 minutes in room temperature and permeabilised with PBS containing Saponin 0.1% and 0.05 w/w NaN₃. Fixed and permeabilised BAL cells were incubated with antibodies against IL-6 at a concentration of 1µg/ml in the dark, at room temperature for 45 minutes and analysed by flow cytometry.
4 Results and discussion

4.1 Introduction

The aim of this work is to study apoptotic cell death of activated T-cells and how this event may be related to inflammation in the airways. In Paper I and II, we used, as a model for T-cell activation, T-cells activated through their T-cell receptor (TCR) by plate bound anti-CD3 antibodies. In Paper III and IV we investigated the apoptotic phenotype of T-cells activated in vivo i.e. obtained from the site of inflammation, the lungs of patients with allergic asthma and sarcoidosis, respectively.

4.1.1 Apoptotic phenotype of anti CD3-activated PBL (Paper I and II)

Mitochondrial-induced apoptosis and caspase-3 like activity in naïve T-cells (Paper I)

Mature resting peripheral blood lymphocytes (PBL) were exposed in vitro to the mitochondrion toxin TBT for 8 hours at concentrations between 0.001-10µM and analysed for apoptosis and activation of caspase-3 at 10 different time points during the incubation period. Concentrations between 0.1µM and 2µM of TBT induced a time and dose-dependent activation of caspase-3 as well as apoptosis in these cells. The optimal TBT concentration for inducing caspase-3 activity in resting PBL appeared to be 2µM where a 30-fold increase in the activity of the enzyme was seen after 2 hours of TBT exposure, although already after 20 minutes a considerable rise in the activity of caspase-3 was observed. However, no caspase-3 like activity could be detected in PBL exposed to the highest concentration of TBT (10 µM) (Fig. 3). The caspase-3 activity was accompanied by classic apoptotic features such as cell membrane blebbing and nuclear fragmentation. No apoptotic cells

Figure 3: Kinetics of TBT induced caspase-3 like activity at different concentrations and exposure times.
were observed at TBT concentrations below 0.01 µM during the observation time (8 hours). Exposure of the cells to 2 µM TBT for 60 min resulted in approximately 55% apoptotic cell death and prolongation of the exposure time did not increase the apoptotic rate, instead the apoptotic cells underwent secondary necrosis. However, no signs of apoptosis was detected in the presence of 10 µM TBT, correlating well with the absence of caspase-3 activity at this concentration. Instead, an associated breach in cell membrane integrity indicative of necrosis was seen in these cells (Figure 4a, b).

Figure 4:  
a. TBT induced apoptosis in PBL  
b. TBT induced necrosis in PBL
Western blot analyses of cytochrome c in cytosolic extracts from PBL exposed to 2 μM TBT for 5 or 10 minutes were performed to confirm the involvement of the mitochondrial pathway in TBT induced caspase-3 activation and apoptosis in these cells. Cytosolic release of cytochrome c was seen at 5 minutes and was increased after 10 minutes of TBT exposure which is early enough to be responsible for the activation of caspase-3 and the subsequent apoptotic cell death (Fig. 5).

![Figure 5: Cytochrome c release into the cytosol after TBT treatment.](image)

**Caspase-3 activity and apoptosis in anti-CD3 activated T-cells and in anti CD3 activated T-cells treated with DEX (Paper I and II)**

Activation of naïve PBL with plate-bound anti-CD3 antibodies resulted in a rise in caspase-3 activity, which peaked after 72-150 hours of activation and then declined. Caspase-3 activity was measured in cell-lysate in paper I. However, since granzyme B can activate caspase-3 and granzyme B is expressed at high levels in CD8+ T-cells and therefore would also be present in cell lysate is it possible that the observed increase in caspase-3 activity was due to granzyme B activity. To exclude this we also used a method were caspase-3 activity could be measured intracellularly in whole viable cells were compartmentalisation was intact (Fig.6a and b). The increased caspase-3 activity was not accompanied by apoptosis, instead, at the time-point when the activity of caspase-3 peaked, a resistance against TBT induced apoptosis started to develop which increased during the observation period (Fig. 7).
Co-culturing anti-CD3 activated PBL with the synthetic glucocorticoid DEX (1μM) blocked caspase-3 activity at 24 and 72 hours while a small increase was seen at 168 hours which was also accompanied by an increase in apoptotic cells (Fig 7b and data not shown). Interestingly, DEX did not change the anti-CD3 induced TBT apoptosis in these cells (data not shown).

**Figure 6:**

a. Caspase-3 like activity in anti-CD3 activated T-cells

b. Caspase-3 like activity in anti-CD3 activated T-cells co-culture with DEX 1μM

**Figure 7.** Sensitivity to TBT induced apoptosis, at different time points, in anti CD3 activated PBL.

**Apoptosis-associated proteins in anti-CD3 activated T-cells and in anti-CD3 activated T-cells treated with DEX (Paper I and II)**

In an attempt to understand the mechanisms behind the apoptosis resistant phenotype of CD3 activated T-cells we investigated the expression of the apoptosis associated proteins Bcl-2, Bcl-XL, bcl-XS and XIAP as well as the two heat shock proteins Hsp70 and 27 by western blotting. The expression of Bcl-2, Bcl-XS, XIAP, and HSP70 was constant throughout the observation
period whereas we did not detect any expression of the anti–apoptotic proteins Hsp27 and Bcl-X_L proteins at 0 hr. However, after 24 -72 hours of activation was Hsp27 expressed and the amount of the protein increased throughout the observation period. When the cells had been activated for 72 hours Bcl-X_L expression was detectable and stayed expressed throughout the observation period. Co-culturing with DEX inhibited the expression of detectable amounts of Bcl-X_L while the expression pattern of Bcl-X_S, XIAP and HSP27 was not affected by the Treatment.

**Figure 8:** Expression pattern of different apoptosis associated proteins in anti-CD3 activated PBL and in anti-CD3 activated PBL co-culture with DEX 1μM.

**Proliferation of PBL in response to anti-CD3 and DEX and expression of activation and phenotype markers (Paper II)**

Since the dogma for activation through the TCR is activation, proliferation and differentiation and a possible role for caspase-3 in the early activation and proliferation machinery of T-cells has been proposed, we wanted to investigate if the observed caspase-3 activity in anti-CD3 stimulated T-cells correlated with activation markers and proliferation in these cells. Dexamethasone blocked, as expected, anti-CD3 induced proliferation whereas the proliferative activity of cells activated in the absence of this steroid peaked at 72 hours and thereafter declined.

After 24 hours of anti-CD3 activation there was, as expected, an increase in the ratio of cells expressing the early activation marker CD69. At this time-point, approximately 70% of the T-cells expressed CD69 on their cell membranes compared to 0% at 0 hours and no substantial
difference in the expression pattern of the protein between CD4 and CD8 positive T-cells could be detected (Fig. 8a). Approximately 5% of resting T-cells expressed the late activation marker HLA-DR and after 72 hours of CD3 stimulation were approximately 75% of the CD8+ and 65% of the CD4+ T-cells HLA-DR positive. The fraction of CD8+ T-cells expressing HLA-DR stayed at the same level at 168 hours whereas a decrease in the amount of CD4+ T-cells expressing the protein was seen at this time-point (Fig 8b). The amount of CD4+ and CD8+ T-cells positive for the memory cell marker CD45RO increased along the observation time and at the last observation point all lymphocytes expressed this protein on their cell surface (Fig. 8d). Around 50% of resting T-cells stained positive for the death receptor CD95 (FasR) and by 72 hours, all T-cells expressed the receptor and stayed positive throughout the observation period (Fig. 8c) Co-culturing with dexamethasone, on the other hand, inhibited any increase in CD4+ and CD8+ T-cells positive for -HLA-DR, CD45RO and CD95 (FasR) whereas the compound had no effect on the expression of the early activation marker CD69.

4.1.2 Interpretations of the findings in Paper I and II

TBT appears to be a good tool for the activation of caspase-3 and for the induction of mitochondrial apoptosis in PBL. The increased caspase-3 activity seen in T-lymphocytes after activation with anti-CD3 antibodies was not associated with apoptosis. Instead, when the activity of caspase-3 peaked, these cells were rather insensitive for TBT induced apoptosis. These results are in line with previous studies suggesting caspase-3 to be involved in T-cell activation as well as differentiation of other cell types (5-8). Questions naturally arising are which intracellular pathways induce this non-apoptotic caspase-3 activity, how is the cell protected against the pro-apoptotic actions of this enzyme and what is the substrate/s for caspase-3 which promotes survival instead of death? We detected an up regulation of Bcl-XL after 72 hours of anti-CD3 activation, which is in line with the observed apoptosis resistance against TBT at this time-point. However, a problem that emerges is how these two events are related since the anti-apoptotic function of Bcl-XL involves the inhibition of the release of mitochondrial pro-apoptotic factors into the cytosol which triggers the activation of caspase-3. But, in these cells caspase-3 is already activated when detectable amounts of the Bcl-XL protein are expressed. So if Bcl-XL has a role in protecting the activated T-cell from apoptosis it would apparently not be by preventing the induction of caspase-3 activity. One explanation model could be that caspase-3, activated to be a part of the proliferation/differentiation machinery, requires the cytosolic presence by one or several mitochondria associated factors to turn pro-apoptotic, an event which could be prevented by, for example, Bcl-XL. However, the elevated caspase-3 activity declined after 72 hours of
CD3 stimulation and was back to baseline by 168 hours although the insensitivity to TBT induced apoptosis, seen after 72 hours, did not decline. Instead the cells were even more resistant against apoptosis at the last time-point, suggesting that the caspase-3 activity and the apoptosis resistance does not necessarily have to be related.

Glucocorticoids are commonly used in the treatment of many inflammatory disorders and induction of apoptosis is one of many biological effects of these proteins. They act by binding to a specific receptor (GR) that, upon activation, translocates to the nucleus and either increases (transactivates) or decreases (transrepresses) gene expression (122).

In this study we co-cultured the anti-CD3 stimulated T-cells with dexamethasone, a synthetic glucocorticoid, to investigate if this compound was able to induce apoptosis in these cells. Dexamethasone, at a concentration of 1 μM, was not able to induce apoptosis in anti-CD3 activated cells during the observation period. Instead, the glucocorticoid inhibited anti-CD3 induced caspase-3 activity, proliferation and the expression of late activation markers as well as the differentiation of these cells into CD45RO+. Moreover, anti-CD3/DEX activated T-cells did also develop an apoptosis insensitive phenotype, however in the absence of Bcl-XL. This suggests either another mechanisms behind the apoptosis resistance in anti-CD3/DEX activated T-cells, distinct from the one operating in anti-CD3 stimulated cells, or that Bcl-XL is not involved, or a combination of these two.

As mentioned earlier, active caspase-3 has been suggested, together with caspase-8, to participate in the early steps of the activation and proliferation machinery. To investigate if the caspase-3 activity correlated with activation of the T-cell we analysed the presence of different activation markers on CD3 and CD3/DEX stimulated T-cells. We found that DEX inhibited the anti-CD3 induced increase in HLA-DR and CD95 expressing cells while the anti-CD3 induced rise in CD69 was left unaffected. The CD69 protein is an early marker of activation, appearing on the cell surface 2-3 hours after activation. Caspase-3 activity correlated with the increase in the late activation marker HLA-DR but appeared not to be related to the expression of CD69 since DEX inhibited caspase-3 but not CD69 expression. DEX treatment also inhibited anti-CD3 induced proliferation but not CD69 expression, which is in line with reports where CD69 was shown not to be required for the activation and proliferation process (123).

It is still unclear how cells with high caspase-3 activity could be rescued from the proteolytic degradation machinery that is normally active in an apoptotic cell. There must be a tight regulation of the apoptotic machinery in activated cells in order to prevent caspase-3 from completing its commitment to degrade vital proteins. How such protection is achieved is not
known as yet, but compartmentalisation, conformational changes of the target proteins or protection via chaperones are possible mechanisms.

4.1.3 The impact of allergen on the apoptotic phenotype of lymphocytes from the blood and airways of patients with allergic asthma (Paper III)

Clinical parameters
All patients airway challenged with allergen, but not those receiving saline, had an early asthmatic reaction (EAR) defined as a 20% or more decrease in FEV\textsubscript{1}. One of these patients also had a late asthmatic reaction (LAR), defined as >15% decrease in FEV\textsubscript{1} during 3-24 hours post-challenge.

BAL fluid and blood cell data
BAL fluid recovery and cell viability did not differ in samples retrieved at baseline and after allergen or saline challenge. The differential count of BAL cells revealed a significant increase in percent eosinophils following allergen challenge (p<0.05). However, both allergen and saline seemed to influence the number of eosinophils in the lungs, since 3/5 patients challenged with saline and 5/7 patients challenged with allergen had an increase in the proportion of BAL eosinophils compared to baseline levels. A tendency to a relative increase in BAL fluid lymphocytes was observed in patients challenged with allergen. Here, the differential count showed an increase of lymphocytes in 6/7 patients challenged with allergen compared to only 1/5 challenged with saline. The differential cell counts of whole blood revealed no change in the cellular composition at baseline and after allergen or saline challenge (data not included).

Mitochondrial-induced apoptosis and caspase-3 activity in PBL and BAL fluid cells at baseline and after airway saline or allergen challenge.
Paired BAL fluid lymphocytes and PBL from all individuals, at baseline and after allergen or saline inhalation, were tested for their apoptotic sensitivity by incubating the cells with 2μM TBT for 1 hour. Airway exposure to allergen, but not to saline, resulted in a significant decrease in TBT induced apoptosis of BAL fluid lymphocytes. Only 23.5% (range 15.3-42.4%) of the allergen exposed cells became apoptotic upon TBT treatment compared to 42.2 % (range 33.9-62.5%) at baseline (Fig.7). However, neither allergen nor saline inhalation affected the sensitivity of PBL to TBT induced apoptosis. The increased
apoptosis resistance among BAL fluid lymphocytes from lungs exposed to allergen was associated with a higher proportion of T-cells expressing the anti-apoptotic protein Bcl-2 compared to BAL fluid lymphocytes at baseline.

Since T-cells, which have migrated to the airway mucosa, are more activated than the majority of T-cells in the blood (124), we expected these cells to have a higher caspase-3 like activity compared to PBL. Indeed, an elevated caspase-3 like activity was recorded in BAL fluid lymphocytes at baseline compared to PBL. We then hypothesised that allergen inhalation would activate allergen specific lymphocytes and thereby increase the caspase-3 activity in these cells. However, allergen airway challenge did not affect the activity of the caspase-3 enzyme in either cell group. Interestingly, in PBL, TBT-induced apoptosis was accompanied by an significant increase in caspase-3 like activity whereas in BAL fluid lymphocytes, apoptosis induced by this compound was not associated with a higher activity of caspase-3.

Figure 9: Sensitivity of BAL fluid lymphocytes to TBT induced apoptosis at baseline and post airway challenge with a allergen and b saline.
4.1.4 Impact of Th1 associated cytokines on apoptosis of BAL fluid lymphocytes from sarcoidosis patients (paper IV)

Background
Previously we found that BAL fluid lymphocytes from sarcoidosis patients were rather apoptosis-insensitive and since the sarcoidosis associated cytokines IL-12, IL-18 and TNF-α all have been shown to affect survival of T-cells, we hypothesised that these cytokines also could influence the survival of lymphocytes in the lungs of sarcoidosis patients. To investigate this in vitro we cultured BAL fluid cells from sarcoidosis patients in the presence of antibodies neutralising the biological effect of IL-12/IL-23 or IL-18. To neutralise TNF-α we used different compounds, infliximab or etanercept.

Opposing effects by blocking the IL-12/23β1 receptor sub-unit and IL-18
BAL fluid lymphocytes from 16 sarcoidosis patients were cultured for 48 hours in the presence of neutralising antibodies against the IL-12 β1 receptor sub-unit. Blocking the β1 sub-unit for 48 hours resulted in a significant increase (p<0.05) in the proportion of necrotic/late apoptotic BAL fluid lymphocytes. At this time point 41% (16-58.5) of the anti-β1 sub-unit treated lymphocytes were dead compared to 34% (14-57.5) of the iso-type treated control cells. Moreover, blocking of the β1 sub-unit resulted in a tendency to an increased amount of BAL fluid lymphocytes in early apoptosis (p=0.057). To neutralise IL-12 we used antibodies that previously have been shown to block the biological activity of IL-12. However, since the β1 receptor sub-unit also can associate with the IL-23 receptor sub-unit to form the IL-23 receptor complex is it possible that the combination of these two antibodies block the IL-23 signalling as well. To test this we cultured BAL fluid cells, from 5 sarcoidosis patients for 48 hours in the presence of antibodies, specifically neutralising IL-23. Anti IL-23 treatment did not affect the viability of

![Figure 10: Cell death in BAL fluid lymphocytes from sarcoidosis patients after blocking IL-12/IL-23 β.](image)
BAL fluid lymphocytes from these 5 patients, suggesting that the lack of IL-12 and not IL-23 is responsible for the observed increase in cell death. However, since the study population is rather small, is it not possible to rule out an survival effect of IL-23 on these cells.

On the contrary, neutralising IL-18 for 48 hours resulted in a significant decrease in necrotic/late apoptotic BAL fluid lymphocytes (p<0.05). At this time point were 38% (24.5-49.0) of the lymphocytes dead compared to 43% (26.0-56.0) of the iso type treated lymphocytes. However, no difference in the amount of BAL fluid lymphocytes in early apoptosis could be detected between anti-IL-18 and iso-type IgG treated cell cultures.

![Late apoptosis graph](image)

**Figure 11:** Cell death in BAL fluid lymphocytes from sarcoidosis patients after IL-18 neutralisation.

**Release of pro-inflammatory cytokines after blocking IL-12/IL-23 and the IL-18 cytokine**

Next we wanted to investigate how the cytokine profile was affected when IL-12/IL23 and IL-18 were blocked. BAL cell culture supernatants from the β1 sub-unit and IL-18 blocking experiments were subjected to CBA analysis for the presence of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α protein. Blocking the β1 sub-unit for 48 hours increased the amount of IL-6, compared to iso-type control, in three patients out of four patients analysed and this was also shown in BAL fluid cells using intra cellular-staining for IL-6 in one additional experiment whereas

![IL-6 expression graph](image)

**Figure 12:** IL-6 expression in BAL fluid lymphocytes from sarcoidosis patients after IL-12/IL-23 β1 receptor blocking. Dotted black line control and black line anti-IL-12/IL-23 β1.
no effect was found on the levels of the other cytokines and neutralising IL-18 had no effect on the levels of any of the secreted cytokines analysed.

Neutralising TNF-α

BAL fluid cells from 7 patients with pulmonary sarcoidosis and 6 patients with other, non-sarcoid, pulmonary inflammatory diseases were cultured for 48 hours, with or without LPS (1 µg/ml) and in the presence of infliximab or etanercept, both at 10 µg/ml. There was no difference in apoptotic rate between infliximab and etanercept treated cells cultured in the absence of LPS. However, in those cultures were LPS had been added infliximab was significant more potent in inducing apoptosis, since 23.0% (11.0-38.0) of the infliximab treated lymphocytes were apoptotic compared to only 15% (9.0-34.0) of the cells exposed to etanercept (p<0.05).

Figure 13: Apoptotic BAL fluid lymphocytes from sarcoidosis patients and control patients after culturing with infliximab or etanercept.

4.1.5 Interpretations of Paper III and IV
In **Paper III**, BAL fluid lymphocytes from asthmatic subjects exposed to allergen inhalation challenge were significantly more resistant to TBT-induced (mitochondrial-mediated) apoptosis compared to non-allergen exposed BAL lymphocytes from the same subjects. These results are in line with other reports showing a decreased sensitivity among the immune cells participating in the allergic inflammation in the airways (96, 97, 99, 100). This decreased sensitivity to apoptosis was accompanied by an increase in the proportion of BAL fluid lymphocytes expressing the Bcl-2 protein. However, airway challenge with saline did not alter the sensitivity of BAL fluid lymphocytes to TBT-induced apoptosis and neither did allergen or saline provocation influence the sensitivity of PBL to mitochondrial-mediated apoptosis. These data suggest that airway provocation with allergens triggers an inflammatory response that results in the alteration of the apoptotic phenotype of lung lymphocytes but not of peripheral blood lymphocytes. The observed discrepancy in apoptosis-sensitivity between BAL and PBL following allergen challenge may reflect the different activation status of the cells in these two compartments; it is known that airway allergen challenge can cause an accumulation of activated allergen-specific lymphocytes in the airways (94, 125, 126). These results concord with the findings in **Paper I and II** where we found an increased apoptosis resistance in lymphocytes activated *in vitro*. The caspase-3 activity in BAL fluid lymphocytes was elevated compared to blood lymphocytes which might be related to the higher activation status of the lung lymphocytes. Airway challenge with allergen did not, however, increase the elevated caspase-3 activity further suggesting that the observed increase in apoptosis resistance following inhalation of allergen was not related to any change in the activity of caspase-3. Interestingly, in PBL, TBT induced apoptosis in PBL was associated with a rise in caspase-3 activity whereas in BAL fluid lymphocytes from nine of the twelve subjects underwent TBT induced apoptosis in the absence of an increased caspase-3. Possible explanations for these observations could be either that an additional, caspase-3 independent apoptotic pathway is operating in lung lymphocytes or that active caspase-3 in its non-apoptotic state is kept compartmentalised, away from pro-apoptotic substrates and when a pro-apoptotic signal arrives, perhaps a mitochondrion associated factor, caspase-3 becomes pro-apoptotic and the cell dies.

In **Paper IV** we show that *in vitro* blocking of the β1 sub-unit of the IL-12 and IL-23 receptor on BAL fluid lymphocytes from sarcoidosis patients resulted in increased lymphocyte apoptosis while neutralising the biological activity of IL-23 alone had no effect. In contrast, inhibition of the biological activity of IL-18 resulted in a slight decrease in apoptotic lymphocytes and a significant decrease in the overall lymphocyte death. Thus, these data
suggest a survival-promoting role for IL-12 whereas IL-18 appears to have a pro-apoptotic function in these lymphocytes. In addition, blocking the β1 receptor resulted in an increased IL-6 production, a rather confusing finding given that the administration of antibodies neutralising IL-12 or IL-6 increases apoptosis of lamina propria T-cells and reduces the severity of the colitis in the gut of mice with Th1 induced colitis. Although IL-6 is a pleiotropic cytokine with mainly pro-inflammatory properties (32, 33), IL-6 can also exhibit anti-inflammatory effects under certain conditions (127). Moreover, IL-6 has also been shown to promote Th2 differentiation (128) and blocking the β1 sub-unit and thereby neutralising IL-12 would reduce the Th1 response and perhaps, by an increased IL-6 production, skew the inflammation into a Th2 response. Another possibility is that IL-12, in some situations, may have anti-inflammatory properties, which have been shown in mice lacking IL-12. In these studies, a more severe inflammation was observed in IL-12 knock out mice suffering from EAE compared to wild type mice suggesting a potential regulatory role for the IL-12/ INF γ signalling pathway(83, 129, 130). Blocking the β1 receptor could, theoretically, also neutralise IL-23 since β1 also associates with the IL-23 receptor sub-unit to form the IL-23 receptor complex. If so, a third potential scenario could involve the complementary effects of IL-6 and IL-23 in the generation of IL-17 producing T-cells demonstrating the complexity and redundancy of the immune system as blocking one inflammatory factor will up-regulate another.

We also show that in vitro treatment of BAL fluid lymphocytes from sarcoidosis patients with infliximab increased the amount of apoptotic lymphocytes compared to in vitro treatment of these cells with etanercept, another anti TNF-α drug. In contrast , no such difference in apoptosis induction between these two TNF-α neutralising drugs was observed in the 6 control patients which were diagnosed with other, non-sarcoid lung diseases. An explanation for this discrepancy could be that different cell types have an altered sensitivity to these two compounds. Sarcoidosis patients had a higher proportion CD4+ T-cells in their lungs compared to the control group, suggesting a possible higher sensitivity of this particular subtype of T-cells to infliximab-induced apoptosis compared to CD8+T-cells. The observed superior effectiveness of infliximab, compared to etanercept in inducing apoptosis in BAL fluid lymphocytes from sarcoidosis patients might be one factor related to the difference in therapy efficiency between these two drugs, in the treatment of patients with severe sarcoidosis.

5 Conclusions
I. In this work, we showed that low doses of TBT induced a dose-dependent activation of the caspase-3, followed by a typical apoptotic morphology, in resting PBL. Morphological changes, such as membrane blebbing and nuclear fragmentation, succeeded caspase activation. Moreover, a time-dependent induction of caspase-3 activity in the absence of an apoptotic morphology was observed when peripheral blood T lymphocytes were activated with CD3 mAbs. A progressive induction of the anti-apoptotic protein HSP27 accompanied the caspase-3 activity. Taken together, these data suggest that caspase-3 might have an additional role in the activation process of T lymphocytes.

II. Resistance to mitochondrial (TBT)-induced apoptosis developed after 3 days of anti-CD3-stimulation (when caspase-3 activity was high) in a minor lymphocyte population, expressing a CD45RO+ phenotype. A concurrent expression of the anti-apoptotic protein Bcl-xL accompanied the caspase-3 activity and the development of the apoptosis-resistant phenotype. Co-culturing with DEX blocked both the CD3-induced caspase-3 activity and the proliferation. Additionally, both the HLA-DR expression and the development of CD45RO+ T-cells were suppressed by DEX treatment. In conclusion, CD3-stimulated caspase-3 activity might be an important component in the proliferation process, and DEX blocks this process.

III. In this study, we demonstrated a decrease in the apoptotic sensitivity of BAL fluid lymphocytes recovered from asthmatic subjects following airway allergen inhalation. This increased apoptotic resistance appeared to be related to the mitochondrial apoptotic pathway and was associated with an increase in Bcl-2 expressing lymphocytes, indicating similar mechanisms as shown in paper 1 and 2. We also recorded a significantly higher baseline level of caspase-3 activity in BAL fluid lymphocytes from these asthmatic subjects compared to PBL from the same subjects. These data suggest that airway inflammation in asthma is associated with a reduced apoptosis susceptibility, which may lead to an enhanced survival of lymphocytes in the bronchial mucosa and consequently prolonged inflammation.
IV. In the fourth and last work, the results suggest a pro-survival role for IL-12 and/or IL-23 whereas IL-18 appears to have the opposite effect in the lungs of sarcoidosis patients. Interestingly, we also detected an increase in IL-6 production after blocking the IL-12/IL-23 receptor sub-unit, which might mirror a Th2 stimulating milieu that the absence of IL-12 would give. We did also show that infliximab but not etanercept *in vitro* increased significantly the proportion of apoptotic BAL fluid lymphocytes from sarcoidosis patients which might be related to the superior effect of infliximab to etanercept in the treatment of patients with severe sarcoidosis.
6. Summary and future perspectives

In the present study, we have shown that the activation state of the T-lymphocyte influences the sensitivity to apoptosis both in vitro by activating T-cells with anti-CD3 antibodies and in vivo in BAL fluid lymphocytes from patients with atopic asthma exposed to airborne antigen. Our data demonstrate a possible participation of caspase-3 in the early activation- and proliferation machinery of T-cells, and that DEX can inhibit this process. Moreover, IL-12 and IL-18 might be additional factors influencing apoptosis-sensitivity of activated T-cells participating in chronic inflammation.

Our future aims are to further investigate the mechanism behind the apoptosis resistance seen in lung lymphocytes from sarcoidosis patients. Particularly it would be very exciting to investigate the potential different actions of IL-23 and IL-12 in the lungs of patients with sarcoidosis and how IL-6 is related to the actions of these two cytokines. This is especially so in the light of new evidence suggesting a potential immuno-modulatory role of IL-12 and that IL-23 might be the master cytokine driving chronic inflammation.

Another challenging task is to investigate the mechanisms behind the altered therapeutic outcomes of different approaches to inhibit the actions of TNF-α i.e. etanercept and infliximab in granulomatous disease.

Moreover, questions remained to be answered concerning the exact role for caspase-3 in viable activated T-cells:

Which are the target substrates for caspase-3 in the activation process?

How is the cell protected from the well known deadly activities of this enzyme?

And finally:

What signals are needed to turn caspase-3 enzyme into pro-life instead of pro-death?

In summary, understanding the pro- and anti-apoptotic effects of inflammatory mediators such as cytokines as well as the activation phenotypes of the participating cells may aid the rational design of other therapeutic strategies to treat inflammatory diseases.
**Acknowledgements**

This is a surprise and for sure it would not have been real if you lot haven’t been around. I would especially thank

My supervisor, **Johan Grunewald** for introducing me to the endless expanding field of immunology, for encouragement and for teaching me the beauty of the T-cell. Now then, will it speed up, slow down or just implode.

My co-supervisor **Anders Eklund** for believing in me, for your enthusiasm, kindness and making me realise that there are no ways quite like the airways.

My other co-supervisor **Héléne Stridh** for introducing me into the lab and guiding me into the world of apoptosis.

**Jan Whalström** for being a living dictionary, the kindest man alive and critically reading my work as well as letting me know when religious right wing powers are high-jacking my brain. I really don’t now how to thank you

**Everybody** at lungforskningslab for making it such a nice and creative atmosphere.

**Benita Dahlberg** for cells, teaching me flow cytometry and for good laughs and **Lotta Müller-Suur** for antibodies, LPS and also good laughs.

For three years I have been sitting next chair to **Cecilia Kemi** I guess she got a reason and she picks up the phone and calls the company who made the heating plate alone.

My roommate burned her hand she says, you are making incredible useless heating plates

Give us a good one or go to jail. Keep up the spirit

I am going to miss sitting next chair to Cecilia Kemi.

**Lukas Didon** and **Charlotta Dagnell** for introducing me into the brownish, bluish and pink world of immunohistochemistry. Green never really made it. **Farah Idali** and **Maria Wikén** for teaching me CBA and being very nice persons. For the record, you are all very nice. **Ernesto Silva** for trying to show me a second dimension and **Mikael Mikko** for giving me a telephone, for icecream with yellow raspberries and for a very refreshing giggle. **Staffan Nyström** for nice chats. **Caroline Olgart Höglund** for good discussions, wild sense of humour and for in the
middle of the night on a stormy ocean far away from home: I have never heard about a boat splitting in two halves so that is left to happen. (I am in a hurry/panic so excuse me for my english). Bettina Levänen for being a very fun and nice person. Åsa Wheelock for interesting discussions, Magnus Nord for microscope help and for, the hardest bit is to convince your self. Ulrika Zagai thanks for teaching a totally unteacheble person butterfly swim, our lesson ended very naturally, when there was no water left in the pool and if you haven’t been, this thesis would still be in my broken computer. Karin Fredriksson for interesting horse and science discussions and all former colleges at lungforskningslab Kia Katchar, Fariba Sabounchi Schütt, Jenny Barton, Anders Plank and Tove Berg

Margitha Dahl, Gunnel de Forest och Helene Blomqvist for good chats, BAL cells and helping me understand the secrets of FEV1, PD20 and RAST. Magnus Sköld for challenging questions and comments. Magnus Löfdahl och Pär Gyllfors and all the nurses and doctors at the lung clinic.

Eva Marie Karlsson for taking care of everything.

My family my mother Monica Müller Ljungberg for helping me with everything, including feeding me, Joni and Elita as well as for being the best mother and Gösta Ljungberg for your incredible kindness and for putting everything together. My father Jan Müller also for helping me with everything, driving me and my horse around Sweden and just being a great dad and Lisbeth Müller for taking such a good care of Elita. I am very greatful for all support and understanding I have got from you this year. I promise to get a grip now.

My sister Camilla Müller, I love you I would not have survived without you. Gustav Nord for a great sense of humour and the coolest tattoo. Tyra Müller Nord my beloved sister-daughter you the sweetest and you know, when you are a grown up you can have as many dogs as you like.

My lovely sister Julia Ljungberg, thanks for all help with Elita, she loves you.

My brother Gustav Ljungberg for all god stories and songs and your daughter princess Linnea.
My sister Sara Djärv for an understanding personality and a tremendous voice. Håkan Djärv for: if a new I were going to move a whole car line I would have taken two cups of coffee.

Anna Bolmström for a long and beautiful friendship and your family, Johan Lepistö and Arvind, Vikram and Niil for always welcoming me to your house. Charlotte Albrektsson and Sofia Ponzio for being such a good friends. Nadja Svensson for the wildest discussions and thoughts. Camilla Dahlqvist for being a very good friend, you always know without saying. Mårten Kivi I hope Holland is treating you nice. I miss you a lot. Nilla Hallgren it was really a pleasant surprise to hear from you and Uppsala is, after all, not the end of the world.

My beautiful daughter Joni Müller af Uhr you are my everything, I love you. I promise not to be such a boring mother (not as often anyway) anymore.
This work was supported by the Swedish Medical Research Council, the Swedish Heart Lung Foundation, the King Oscar II Jubilee Foundation, Åke Wiberg Foundation and Karolinska Institutet.
References


92. Kay AB. The role of T lymphocytes in asthma. Chem Immunol Allergy 2006;91:59-75.


