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Eosinophil as a Target for Pathophysiological Factors and Pharmacological Compounds

ACADEMIC DISSERTATION
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I Ilmarinen P, Hasala H, Sareila O, Moilanen E and Kankaanranta H (2009): Bacterial DNA delays human eosinophil apoptosis. Pulmonary Pharmacology & Therapeutics 22: 167-176.

II Kankaanranta H, Ilmarinen P, Zhang X, Nissinen E and Moilanen E (2006): Anti-eosinophilic activity of orazipone. Molecular Pharmacology 69: 1861-1870.

III Ilmarinen-Salo P, Moilanen E and Kankaanranta H (2010): Nitric oxide induces apoptosis in GM-CSF-treated eosinophils via caspase 6-dependent lamin and DNA fragmentation. Pulmonary Pharmacology & Therapeutics 23: 365-371.

IV Ilmarinen-Salo P, Moilanen E, Kinnula V and Kankaanranta H (2012): Nitric oxide-induced eosinophil apoptosis is dependent on mitochondrial permeability transition (mPT), JNK and oxidative stress: apoptosis is preceded but not mediated by early mPT-dependent JNK activation. Respiratory Research 13: 73.

V Ilmarinen P, James A, Moilanen E, Pulkkinen V, Daham K, Saarelainen S, Laitinen T, Dahlén SE, Kere J, Dahlén B, Kankaanranta H. Enhanced expression of neuropeptide S receptor 1 (NPSR1) in eosinophils from severe asthmatics and subjects with total IgE above 100 IU/ml. Submitted for publication.

ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
AHR	airway hyperresponsiveness
ANT	adenine nucleotide translocator
AP-1	activator protein 1
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
BID	BH3-interacting domain death agonist
cAMP	3',5'-cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	3',5'-cyclic guanosine monophophate
CpG	cytosine linked to guanine by a phosphate bond
CytC	cytochrome c
EDN	eosinophil-derived neurotoxin
EPO	eosinophil peroxidase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GINA	Global Initiative for Asthma
GM-CSF	granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
IAP	inhibitor of apoptosis
IETD-CHO	Ile-Glu-Thr-Asp-aldehyde, caspase-8 inhibitor
IFN	interferon
Ig	immunoglobulin

IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IMS	intermembrane space
JNK	c-Jun N-terminal kinase
LT	leukotriene
MAPK	mitogen-activated protein kinase
MMP	mitochondrial membrane permeabilization
MOMP	mitochondrial outer membrane permeabilization
mPT	mitochondrial permeability transition
MyD88	myeloid differentiation primary response gene 88
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kB
NO	nitric oxide
NOS	nitric oxide synthase
NPS	neuropeptide S
NPSR1	neuropeptide S receptor 1
ODN	oligodeoxynucleotide
OR-2370	3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione; analogue of orazipone
OVA	ovalbumin
PARP	poly (adenosine diphosphate-ribose) polymerase
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
PI	propidium iodide
PG	prostaglandin
PI3K	phosphatidylinositol-3 kinase
Q-Vd-OPh	N-(2-Quinolyl)-Val-Asp-(2,6-difluorophenoxy)methyl ketone, broad-range caspase inhibitor
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
SNAP	S-nitroso-N-acetyl-DL-penicillamine, NO donor
SOD	superoxide dismutase

TGF	transforming growth factor
TNF	tumor necrosis factor
Th	T helper
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin I
Z-Asp-CH_2-DCB	benzyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone, pan-caspase inhibitor
Z-VEID-FMK	benzyloxycarbonyl-Val-Glu(OMe)-Ile-Asp(OMe)-fluoromethylketone, caspase-6 inhibitor
Z-DQMD-FMK	benzyloxycarbonyl-Asp(OMe)-Gln-Met-Asp(OMe)-fluoromethylketone, caspase-3 inhibitor

ABSTRACT

Asthma is a chronic inflammatory disease of the airways characterized by the accumulation of eosinophils into the airways in most phenotypes. Eosinophils release factors that damage the surrounding cells, induce bronchoconstriction and participate in the maintenance and exacerbation of inflammation. Eosinophils play an important role especially in asthma exacerbations. The number of eosinophils in tissues is regulated by their release from bone marrow, migration into tissues and by their removal by apoptosis or programmed cell death. In the absence of any inflammatory survival-prolonging factors, eosinophils die via apoptosis within a few days but in the presence of factors such as interleukin (IL)-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) their life-span can be prolonged for up to 1-2 weeks. Pharmacological agents that induce eosinophil apoptosis are therefore regarded as a natural treatment option for asthma. The induction of eosinophil apoptosis is a critical mechanism of action of glucocorticoids, the most important anti-inflammatory drug used today to treat asthma. Orazipone is a novel candidate drug for the treatment of inflammatory diseases, such as asthma and it was investigated in the present study for its effects on eosinophil apoptosis.

Several pathophysiological components are present in inflamed lungs potentially affecting eosinophil longevity and activity. The airways of asthmatics contain a high load of bacteria and their numbers are even increased during bacterial respiratory tract infections. Bacterial DNA is recognized by host cells via Toll-like receptor 9 (TLR9) based on unmethylated cytidine-phospho-guanosine (CpG) motifs. In addition, high levels of nitric oxide (NO) are produced during airway inflammation and it possesses both pro- and anti-inflammatory effects. NO has been reported to regulate eosinophil apoptosis but its mechanism of action remains unclear. Neuropeptide S receptor 1 (NPSR1) was identified in a search for asthma susceptibility genes from Finnish patients. The NPSR1 locus has been associated with asthma, increased levels of allergy-related antibody immunoglobulin (Ig) E, allergic conditions and bronchial hyperresponsiveness and it is known to be expressed in human eosinophils although its functions remain unclear. The aim of this study was to examine effects of bacterial DNA, NO, neuropeptide S (NPS) and orazipone on eosinophil apoptosis and further, to attempt to clarify their mechanisms of action. Study of NPS was extended by determining the expression and function of its receptor NPSR1 in human eosinophils. This study employed primary human blood eosinophils.

It was demonstrated that unmethylated bacterial DNA and synthetic CpG oligodeoxynucleotides (ODNs) resembling bacterial DNA delayed eosinophil apoptosis by a mechanism involving TLR9, phosphatidylinositol-3 kinase (PI3K) and nuclear factor (NF)-κB. Vertebrate DNA had no effect but surprisingly, methylated bacterial DNA reduced eosinophil apoptosis suggesting that bacterial DNA contains a previously unknown immunostimulatory sequence in addition to unmethylated CpG motif. The results provide a possible mechanism for maintenance and exacerbation of eosinophilic inflammation by bacteria in the airways of asthmatics.

Novel mechanisms of action were discovered for NO in eosinophils. In the presence of the survival-prolonging cytokine GM-CSF, NO first induced an early flickering mitochondrial permeability transition (mPT) and mPT-dependent JNK activation. Those events were not directly mediating eosinophil apoptosis but they may represent a stress response intended to support cell survival. In extended experiments, treatment with NO led to eosinophil apoptosis mediated by reactive oxygen species, late mPT, JNK and executor caspases 3 and 6. Since there is a close correlation between airway eosinophilia and exhaled NO-levels in asthmatics, it can be proposed based on the present results that in asthmatic lungs, NO produces predominantly a stress response in eosinophils supporting cell survival, not apoptosis.

The candidate drug orazipone and its analogue OR-2370 were shown to induce eosinophil apoptosis in the presence of the survival-prolonging cytokine IL-5. Similarly to NO, OR-2370-induced apoptosis was mediated by caspases 3 and 6 and JNK. Based on these results, orazipone and OR-2370 can be considered as potential candidates for treatment of eosinophilic inflammatory disorders such as asthma.

NPSR1 expression was found to be increased in eosinophils derived from subjects with high total IgE (>100 IU/ml) and in patients with severe asthma when compared to eosinophils from subjects with lower total IgE (<100 IU/ml) or from patients with mild asthma or from healthy controls, respectively. Functional studies revealed that treatment with NPS, the natural agonist of NPSR1, elevated intracellular cAMP levels and increased fMLP-induced adhesion molecule CD11b expression. The latter effect was seen only in eosinophils from subjects with high IgE levels. These results indicate that NPSR1 may have a pathological role in individuals with severe asthma and/or enhanced IgE level.

This study identified possible pathophysiological mechanisms underlying the eosinophilic airway inflammation and a novel mechanism of action for an anti-inflammatory candidate drug. This information produced can be utilized in the development of drugs for asthma or other eosinophilic conditions.

TIIVISTELMÄ

Astma on hengitysteiden krooninen tulehdussairaus, johon useimmiten liittyy eosinofiilisten tulehdussolujen kerääntyminen keuhkoihin. Eosinofiilit vapauttavat tekijöitä, jotka vahingoittavat ympäröiviä soluja, aiheuttavat keuhkoputkien supistumista sekä ylläpitävät tai pahentavat keuhkojen tulehdusta. Eosinofiileillä onkin merkittävä tehtävä erityisesti astman pahenemisvaiheessa. Näiden solujen määrää kudoksissa säätelee niiden kypsyminen ja vapautuminen luuytimestä, kulkeutuminen kudoksiin sekä apoptoosi, ohjelmoitu solukuolema. Eosinofiilit ohjautuvat apoptoosiin muutamassa päivässä ilman selviytymistä lisäävien tulehdustekijöiden vaikutusta. Tulehdustekijät, kuten interleukiinit IL-5 ja IL-3 sekä GM-CSF, pidentävät eosinofiilien elinikää jopa kahteen viikkoon. Eosinofiilien apoptoosia aiheuttavat lääkkeet ovat hyödyllisiä astman hoidossa. Eosinofiilien apoptoosin lisääminen on yksi tärkeimpiä vaikutusmekanismeja glukokortikoideille, jotka ovat yleisimmin käytössä olevia tulehdusta vähentäviä astmalääkkeitä. Oratsiponi on uusi tulehdussairauksien, kuten astman hoitoon kehitteillä oleva lääkeaine ja tässä työssä tutkittiin oratsiponin vaikutuksia eosinofiilien apoptoosiin.

Tulehtuneissa keuhkoputkissa on lukuisia patofysiologisia tekijöitä, jotka voivat vaikuttaa eosinofiilien elinikään ja aktiivisuuteen. Astmaatikkojen hengitysteissä on runsaasti bakteereita, joiden määrä lisääntyy vielä hengitysteiden bakteeri-infektion aikana. Bakteerin DNA:ta tunnistaa isäntäsolujen Toll-like reseptori 9 (TLR9) ja tämä tunnistus perustuu DNA:n metyloimattomiin sytidiini-fosfo-guanosiini (CpG)-rakenteisiin. Keuhkoputkitulehduksen aikana tuotetaan myös paljon typpioksidia, jolla on sekä tulehdusta lisääviä että estäviä vaikutuksia. Typpioksidin on aikaisemmin raportoitu säätelevän eosinofiilien apoptoosia mutta vaikutusmekanismit ovat epäselviä. Neuropeptidi S reseptori 1 (NPSR1) löydettiin etsittäessä astman alttiusgeenejä suomalaisista potilaista. NPSR1:n geenilokuksella on havaittu olevan yhteys astmaan, allergiaan liittyvän vasta-aineen immunoglobuliini (Ig) E:n lisääntyneeseen tasoon (>100 IU/ml), allergisiin tiloihin sekä keuhkojen hyperreaktiivisuuteen. Eosinofiilien tiedetään ekspressoivan NPSR1:tä mutta reseptorin tehtävää niissä ei tunneta. Tämän tutkimuksen tarkoituksena oli selvittää bakteerien DNA:n, typpioksidin, neuropeptidi S:n (NPS) ja oratsiponin vaikutuksia eosinofiilien apoptoosiin sekä niihin liittyviä vaikutusmekanismeja. NPS:n merkitystä tutkittiin laajemmin selvittämällä myös sen reseptorin, NPSR1:n, ilmenemistä ja toimintaa eosinofiileissä. Tutkimuksessa käytettiin ihmisen verestä eristettyjä eosinofiilejä.

Tutkimuksessa osoitettiin, että metyloimaton bakteerien DNA sekä bakteerien DNA:ta muistuttavat synteettiset CpG oligodeoksinukleotidit (ODN) viivästyttävät eosinofiilien apoptoosia. Vaikutus välittyi TLR9:n, proteiinikinaasi PI3K:n sekä transkriptiotekijä NF-κB:n kautta. Myös metyloitu bakteerien DNA vähensi yllättäen eosinofiilien apoptoosia, vaikka selkärankaisen DNA:lla ei vastaavaa vaikutusta ollut havaittavissa. Tulos viittaa siihen, että bakteerien DNA:ssa on metyloimattomien CpG-jaksojen lisäksi muitakin toistaiseksi tuntemattomia, immuunipuolustusta stimuloivia rakenteita. Työssä löydettiin kokonaan uusi mekanismi, jonka välityksellä

astmaatikoiden hengitysteissä olevat bakteerit voivat ylläpitää tai pahentaa eosinofiilistä tulehdusta.

Lisäksi tutkimuksessa tunnistettiin uusia typpioksidin vaikutuksia selittäviä mekanismeja eosinofiileissä. Solun selviytymistä lisäävän sytokiinin, GM-CSF:n, läsnäollessa typpioksidi aiheutti varhaisen mitokondrion kalvon permeabiliteetin muutoksen (mPT), joka oli luonteeltaan kohtauksittainen/palautuva ja johti proteiinikinaasi JNK:n aktivoitumiseen. Nämä tapahtumat eivät välittäneet apoptoosia vaan liittyvät todennäköisesti solun stressivasteeseen tähdäten solun selviytymiseen. Pitkäkestoisemmissa kokeissa typpioksidi aiheutti eosinofiilien apoptoosin, joka välittyi reaktiivisten happiyhdisteiden, myöhäisen mPT:n, JNK:n sekä kaspaasien 3 ja 6 kautta. Koska astmaatikoiden hengitysteiden eosinofiilimäärän sekä uloshengityskaasun typpioksidipitoisuuden välillä on vahva korrelaatio, on mahdollista että typpioksidi aiheuttaa fysiologisissa olosuhteissa ainoastaan eosinofiilien selviytymiseen tähtäävän stressivasteen ilman apoptoosia.

Tutkimuksessa havaittiin, että potentiaalinen lääkeaine oratsiponi ja sen analogi OR-2370 lisäsivät eosinofiilien apoptoosia erityisesti eosinofiilien selviytymistä lisäävän sytokiinin, IL-5:n, läsnäollessa. Apoptoosi välittyi kaspaasien 3 ja 6 sekä JNK:n kautta samaan tapaan kuin typpioksidin aiheuttama apoptoosi. Tulosten perusteella oratsiponi ja OR-2370 soveltuisivat erinomaisesti eosinofiilisten tulehdussairauksien, kuten astman hoitoon.

Tulokset osoittivat myös, että NPSR1:n ilmentyminen on lisääntynyt sellaisten tutkimuspotilaiden eosinofiileissä, joiden kokonais-IgE oli korkea (>100 IU/ml) verrattuna tutkimuspotilaisiin, joiden kokonais-IgE oli matalampi (<100 IU/ml). NPSR1:tä ilmentyi enemmän myös vaikeaa astmaa sairastavien potilaiden eosinofiileissä verrattuna lievää astmaa sairastavien potilaiden tai terveiden henkilöiden eosinofiileihin. Reseptorin luonnollisen ligandin, NPS:n, havaittiin lisäävän solunsisäisen toisiolähetin, syklisen AMP:n, määrää eosinofiileissä. Lisäksi NPS nosti bakteeriperäisen peptidin, fMLP:n, stimuloimaa adheesiomolekyyli CD11b:n ilmentymistä. Tämä vaikutus ilmeni ainoastaan niiden tutkimuspotilaiden eosinofiileissä, joilla oli korkea IgE. NPSR1 voi liittyä vaikean allergian ja astman patogeneesiin eosinofiileissä ilmentyvien vaikutustensa kautta.

Tässä väitöstutkimuksessa tunnistettiin kokonaan uusia eosinofiilisen tulehduksen patofysiologisia mekanismeja sekä löydettiin uusi vaikutusmekanismi tutkimuksen alla olevalle tulehdusta estävälle lääkeaineelle. Tutkimuksesta saatua tietoa voidaan hyödyntää uusien astman tai muiden eosinofiilisten sairauksien hoitoon tarkoitettujen lääkeaineiden kehitystyössä.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways especially affecting people in westernized countries. Asthma is a more complex disease than previously thought, manifesting in several different inflammatory forms (phenotypes). Even though detailed characterization of these phenotypes is still ongoing, approximately half of patients with asthma seem to belong to a phenotype associated with accumulation of eosinophils, a type of inflammatory cell, into the lungs. This phenotype is better known as allergic asthma. (Wenzel 2006, Woodruff et al. 2009)

Eosinophils are white blood cells of the myeloid lineage that develop in the bone marrow, circulate in the bloodstream and migrate into tissues, such as the lungs in patients with asthma. In the airways of asthmatics, they release harmful products that contribute to the maintenance and amplification of inflammation and constriction of bronchus (Hogan et al. 2008) and in that respect, their removal is considered beneficial. Programmed cell death (apoptosis) is a physiological and non-inflammatory way to eliminate cells because apoptotic cells break down into apoptotic bodies that are ingested by phagocytes preventing the release of harmful cell content into the surroundings. Necrosis, in contrast, is an accidental form of cell death producing inflammation. In healthy persons and in the absence of inflammation, eosinophils are short-living cells which die by apoptosis within a few days. Eosinophils from patients with asthma show delayed apoptosis (Simon et al. 1997, Kankaanranta et al. 2000) and pharmacological compounds that accelerate eosinophil apoptosis could be useful for the resolution of eosinophilic inflammation.

The lungs of asthmatics contain many inflammatory factors. Nitric oxide is a gaseous molecule produced in high amounts during airway inflammation and the exhaled levels of NO in asthmatics correlate strongly with the level of airway eosinophilia (Jatakanon et al. 1998, Lehtimaki et al. 2001, Payne et al. 2001). According to recent findings, airways are not sterile and contain a high load of bacteria (Edwards et al. 2012) and especially during respiratory tract infection, bacterial and/or viral components are abundant. Compounds that resemble bacterial DNA are also the focus of interest

because of their potential in the treatment of allergic diseases such as asthma (Fonseca and Kline 2009).

Neuropeptide S receptor 1 (NPSR1) was found in a search for asthma susceptibility genes from Finnish patients. The gene locus was linked to asthma and high IgE levels (IgE is an antibody the levels of which are typically elevated in allergic conditions) (Laitinen et al. 2004) but its significance and function in asthma/allergy remain unclear.

The aim of the present study was to examine the regulation of human eosinophil viability by pathophysiological and pharmacological compounds. An additional aim was to clarify the expression and function of NPSR1 in human eosinophils.

REVIEW OF THE LITERATURE

1 Asthma

Since approximately 400 B.C., any condition related to breathing has been called asthma. The English physician John Floyer (1649-1734) was probably the first who defined asthma as a separate pulmonary airway disorder where the cause of breathing difficulties was bronchial constriction (Sakula 1984). At present, asthma is defined as follows: *"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night and in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment"* (Global Initiative for Asthma (GINA) 2011). As knowledge has increased, it has become apparent that asthma is a heterogenous disease with many overlapping phenotypes (Wenzel 2006, GINA 2011). The phenotypes of asthma may be categorized according to the type of inflammation (eosinophilic, neutrophilic, mixed inflammatory or paucigranulocytic), triggering factors (e.g. allergic vs. non-allergic, aspirin-induced, exercise-induced) or clinical and physiological features (e.g. level of severity, frequency of exacerbations, glucocorticoid responsiveness, age of onset) (Wenzel 2006, Green et al. 2007, Henderson et al. 2009). However, at present there is no standardized categorization method (GINA 2011).

Asthma was a relatively rare disease until the second half of the 20th century but subsequently its prevalence has increased steadily. Approximately 300 million people suffer from asthma with the prevalence being highest in westernized countries (Masoli et al. 2004) although recently global differences have declined. In the 13-14 year age group, the prevalence of current asthma symptoms has reduced in many western countries but increased in regions such as Latin America and Africa (Pearce et al. 2007,

GINA 2011). Globally, the prevalence of asthma has increased in children (Pearce et al. 2007). Asthma may account for up to 250.000 deaths each year in different countries. Furthermore, the direct and indirect medical costs of asthma are substantial (Masoli et al. 2004, GINA 2011). Currently, approximately 4.3 % of Finns are entitled to a special reimbursement of drugs to treat asthma (Kauppi et al. 2012). In Helsinki, the prevalence of asthma is 9.4 % (Pallasaho et al. 2011). Asthma is the most common chronic disorder in Finnish children. However, the National Asthma Programme (1994-) has succeeded in decreasing hospitalization days due to asthma (Kauppi et al. 2012) as well as in reducing annual total costs of asthma (Haahtela et al. 2006). These improvements are probably due to earlier detection, increase in treatment effectiveness and improved guide for self-management of exacerbations (Kauppi et al. 2012).

1.1 Pathogenesis of allergic asthma

Clustering analyses have revealed the existence of several phenotypes of asthma but the underlying pathogenesis of most phenotypes is poorly known (Wenzel 2006, Woodruff et al. 2009, Baines et al. 2011). Approximately 50 % of patients with asthma belong to a phenotype characterized by airway and blood eosinophilia and a high degree of T helper 2 (Th2)-mediated inflammation (Woodruff et al. 2009). This is the only phenotype with a pathogenesis understood to certain extent and for this reason, it will be the focus of the following discussion.

Several factors may influence Th2 immune deviation. Both genetic predisposition and environmental factors such as reduced exposure to microbes, urban pollutants, severe viral upper respiratory tract infections and nutritional defects may contribute to the defective development of the infant lung and Th cell polarization towards Th2 cells (Calder et al. 2006, Holgate 2010, Locksley 2010). According to the current view, Th2 polarization is initiated by an impaired airway epithelial barrier which may be induced by the proteolytic activity of many allergens. The damaged epithelium releases thymic stromal lymphopoietin (TSLP), interleukin (IL)-33 and IL-25. TSLP directs dendritic cells to express the OX40 ligand, which primes naïve Th cells into a state where IL-4 production and Th2 differentiation are enabled. IL-33 and IL-25 stimulate Th2 cytokine (IL-4, IL-5, IL-13) production by mast cells, basophils and natural helper cells stimulating the terminal differentiation of Th2 cells (Locksley 2010, Bartemes and Kita

2012, Holgate 2012b). Th2 differentiation is reinforced by a positive auto-regulatory loop ending up in silenced Th1 cytokine expression and dampened Th1 differentiation (Bowen et al. 2008). Th2 cytokines together with allergen and co-stimulatory molecules promote the immunoglobulin (Ig) class-switching of B cells to IgE synthesis (IL-4, IL-13) and contribute to the recruitment, maturation and activation of mast cells (IL-4, IL-9, IL-13), eosinophils [IL-3, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF)] and basophils (IL-3, IL-4). (Galli et al. 2008, Holgate 2012b)

Allergic inflammation is often classified into three phases: early-phase, late-phase and chronic inflammation. The early phase-reaction occurs within minutes and mainly involves mast cell-mediated functions. B cell- derived allergen-specific IgE binds to its high-affinity IgE-receptors (FcεRI) on the surface of mast cells. Allergen binding to IgE triggers mast cell degranulation and the release of histamine and other preformed mediators that induce bronchoconstriction as well as the secretion of mucus, vasodilatation and increased vascular permeability. Mast cells also contribute to the transition to the late-phase reaction by releasing chemokines and chemotactic mediators that recruit other inflammatory leukocytes. (Galli et al. 2008, Holgate 2012b)

The late phase-reaction typically peaks at 6-9 hours after allergen exposure and involves an influx of Th2 cells, eosinophils, basophils and some neutrophils into the inflamed airways. The released mediators, such as cysteinyl leukotrienes and IL-13 trigger a contraction of bronchial smooth muscle and evoke the production of mucus. Additionally, eosinophil degranulation products such as basic proteins and reactive oxygen species injure airway epithelial cells. (Galli et al. 2008)

Chronic inflammation evolves when repeated allergen exposure leads to the prominent accumulation of Th2 cells, B cells, eosinophils, neutrophils and basophils to the airways. The release of toxic proteins and inflammatory mediators by these cells induces repeated airway injury and the subsequent repair processes lead to a thickening of the airway wall, known as airway remodelling. During airway remodelling, airway smooth muscle mass and angiogenesis increase and extracellular matrix proteins are deposited since there is enhanced function and a greater number of fibroblasts. There is an increase in the production of inflammatory mediators and the number of mucus-producing goblet cells in epithelium and this may lead to severe narrowing of airway lumen that is filled with mucus resulting in impaired lung function. Transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and IL-13 are considered central in the development of airway remodelling. However, several of the

mechanisms underlying airway remodelling remain unresolved. (Lloyd and Robinson 2007, Galli et al. 2008)

1.2 Drug treatment for asthma

Currently, the treatment of persistent asthma is based on the long-term use of inhaled glucocorticoids to suppress inflammation. Short-acting β_2-adrenoceptor agonists are used when needed to rapidly relieve bronchoconstriction. If asthma control is not achieved by a medium dose of glucocorticoids, long-acting β_2-adrenoceptor agonists can be supplemented. In moderate to severe disease, leukotriene modifiers or theophylline may be required as add-on medications. Drugs currently used in the treatment of asthma and their mechanisms of action are summarized in Table 1 (GINA 2011).

1.3 Drug development for asthma

Novel drugs for asthma are needed because approximately 20-35 % of patients with asthma exhibit a poor or no response to the actions of glucocorticoids (Malmstrom et al. 1999, Szefler et al. 2002). Additionally, the present treatments are not curative and must be continued often for the entire lifetime raising concerns of the side-effects (Adcock et al. 2008).

Improving the existing therapies is one strategy for the development of anti-asthmatic drugs. New bronchodilators, such as ultra-long-acting β_2-agonists (ultra-LABAs) and long-acting muscarinic antagonists (LAMAs) are in clinical trials to achieve once-daily dosage with a fast onset of action in patients with asthma and chronic obstructive pulmonary disease (COPD). In addition, combinations of LABA/ultra-LABA and LAMA as well as combinations of ultra-LABA and glucocorticoid and even triple combinations of ultra-LABA, LAMA and glucocorticoid are under development. Dual-acting muscarinic antagonist-β_2-agonists (MABAs), where these therapeutic actions are present in a single molecule, have been designed to achieve a fixed ratio of these effects in each region of the lung and a single pharmacokinetic profile (Cazzola et al. 2012). The aim in the development of dissociated glucocorticoids

Table 1. *Mechanisms of action of the drugs used to treat asthma.*

Drug class	Drug name	Function	Mechanism of action
Glucocorticoids -inhaled -systemic	 - Budesonide, Beclomethasone, Fluticasone, Mometasone Ciclesonide (prodrug) - Prednisolone	Anti-inflammatory	Suppress inflammatory gene transcription by transrepression, by increasing mRNA degradation and activating HDAC2. Transactivation resulting in increased production of anti-inflammatory cytokines. Apoptosis in many immune cell types. Vasoconstriction. (Alangari 2010)
β_2-adrenoceptor agonists - short-acting - long-acting - ultra-long acting	 - Fenoterol, Salbutamol, Terbutaline - Salmeterol, Formoterol - Indacaterol	Bronchodilator	Increase in cAMP leading to activation of PKA and phosphorylation of myosin light chain kinase resulting in bronchial smooth muscle relaxation. Bronchodilation also via opening of K+ channels and hyperpolarization. (Anderson 2006)
Leukotriene modifiers - Cysteine leukotriene 1 receptor antagonists - 5-lipoxygenase inhibitors	 - Montelukast, Zafirlukast Pranlukast - Zileuton	Bronchodilator and anti-inflammatory	Inhibit bronchoconstriction, mucus production and vascular permeability induced by leukotrienes. (Montuschi and Peters-Golden 2010)
Methylxanthines	Theophylline, Aminophylline	Bronchodilator and anti-inflammatory	Unknown. Thought to relax bronchial smooth muscle and inhibit immune cell function by inhibiting PDE (cAMP↑) and adenosine receptors. Activate HDAC2 resulting in suppression of inflammatory gene transcription. (Barnes 2013)
Anti-IgE	Omalizumab	Anti-inflammatory	Inhibits function of IgE by binding to circulating IgE: Prevents release of mediators involved in allergic cascade (histamine, leukotrienes, pro-inflammatory cytokines). (Holgate et al. 2009)
Anticholinergics	Ipratropium bromide, Tiotropium bromide	Bronchodilator	Inhibit muscarinic receptors non-selectively resulting in relaxation of acetylcholine-mediated bronchoconstriction and reduced secretion of mucus. (Gosens et al. 2006)

HDAC=histone deacetylase, cAMP=cyclic adenosine monophosphate, PKA=protein kinase A, PDE=phosphodiesterase, IgE=immunoglobulin E.

and non-steroidal glucocorticoid receptor modulators is to reduce the harmful effects of glucocorticoids. The goal is to design a glucocorticoid that would not induce gene activation (transactivation) that is thought to account for most of the adverse effects but would still efficiently repress the transcription factors NF-κB and AP-1 (transrepression) that are thought to mediate most of the beneficial effects (De Bosscher et al. 2010). However, this model has proved to be an oversimplification and only few compounds may succeed in clinical trials (Newton and Holden 2007, De Bosscher et al. 2010).

Antibodies against Th2-cytokines have been tested in several clinical trials but many of them, such as anti-IL-4, have not shown sufficient efficacy (Holgate 2012a, Maes et al. 2012). The characterization of asthma phenotype and selecting the correct subgroup of patients is of pivotal importance when designing these studies. The best example of this is the anti-IL-5 antibody, mepolizumab, which proved to be effective only in patients with eosinophilic asthma and recurrent exacerbations even when treated with high-dose glucocorticoids (Haldar et al. 2009, Nair et al. 2009, Pavord et al. 2012). Animal models have also hinted that IL-15, IL-17A, IL-25, IL-33, IL-31, IL-21 and TSLP might be interesting future drug targets (Holgate 2012a, Pelaia et al. 2012).

Other promising approaches include D-type prostanoid receptor (DP) 2 antagonists that inhibit the actions of prostaglandin D_2, chemokine receptor inhibitors (e.g. CCR3 and CCR4 as targets) and compounds that inhibit dendritic cells that drive Th2 differentiation such as OX40 ligand antagonists (Schuligoi et al. 2010, Vijayanand et al. 2010, Nguyen and Casale 2011, Wegmann 2011). Many kinases and transcription factors [p38, c-Jun N-terminal kinase (JNK), phosphatidylinositol-3 kinase (PI3K), nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT) 6, trans-acting T-cell-specific transcription factor (GATA-3)] are centrally involved in driving the lung inflammation and thus inhibition of these molecules is one drug development strategy (Bennett 2006, Duan and Wong 2006, Barnes 2008, Ohga et al. 2008, Edwards et al. 2009, Walker et al. 2009, Marwick et al. 2010). Finally, agonists of Toll-like receptors 7 and 9 are under active investigation (Fonseca and Kline 2009, Van et al. 2011).

In the future, the focus may be on the development of drugs for specific phenotypes of asthma. Because of the redundant inflammatory pathways, targeting a single mediator may not be an optimal approach and combination of biological drugs could be useful {1040 Pelaia,G. 2012;}. Developing drugs targeting eosinophil apoptosis could be a viable strategy for therapy of the phenotype of asthma in which there is a predominance of eosinophilic inflammation.

2 Eosinophil

Eosinophils were discovered by the German physician Paul Ehrlich in 1879. During the development of a histological staining method, he found blood cells with a bi-lobed nucleus and granules that were strongly stained with eosin and named them eosinophils (Ehrlich 1879). Together with neutrophils and basophils, eosinophils form the granulocyte subgroup of leukocytes. Eosinophils only account for approximately 3 % of blood leukocytes in healthy individuals, whereas the majority of leukocytes (~60 %) are neutrophils (Giembycz and Lindsay 1999, Siekmeier et al. 2001). Granulocytes are cells specialised to kill pathogens by both phagocytosis and the secretion of toxic mediators. The evolutionary function of eosinophils is thought to be the innate immune response against parasitic helminths (Klion and Nutman 2004) but they are critically involved also in the pathogenesis of allergic, gastrointestinal and hypereosinophilic disorders and in tumor immunity (Ellyard et al. 2007, Trivedi and Lloyd 2007, Zuo and Rothenberg 2007, Gleich and Leiferman 2009).

2.1 Eosinophil lifecycle

Eosinophils develop from $CD34^+$ haemotopoietic stem cells in the bone marrow. Their development is directed by transcription factors globin transcription factor 1 (GATA-1), PU.1 and CCAAT/enhancer-binding protein (C/EBP) as well as cytokines IL-3, IL-5 and GM-CSF (Campbell et al. 1987, Saeland et al. 1989, McNagny and Graf 2002). Of these transcription factors and cytokines, GATA-1 and IL-5 are the most specific for the eosinophil lineage development (Campbell et al. 1987, Yu et al. 2002). IL-5 and eotaxin govern the migration of eosinophils from bone marrow into the peripheral circulation (Collins et al. 1995, Palframan et al. 1998) where the half-life of eosinophils is 18 h (Steinbach et al. 1979). However, eosinophils are mainly tissue cells. In healthy individuals, they tend to migrate into gastrointestinal tract, thymus, spleen, mammary gland and uterus under the direction of eotaxin (Matthews et al. 1998, Gouon-Evans et al. 2000, Humbles et al. 2002). In the blood circulation, eosinophils live only 1-2 days but in tissues their longevity may be enhanced for up to 1-2 weeks according to *in vitro* observations (Rothenberg et al. 1987, Giembycz and Lindsay 1999).

In *in vitro* experiments, blood eosinophils die by apoptosis in the absence of survival-prolonging cytokines (Stern et al. 1992, Kankaanranta et al. 2000, Zhang et al. 2000). In addition to apoptosis, the life-span of eosinophils in airway tissue may be terminated via cytolysis due to necrosis, secondary necrosis or degranulation (Persson and Erjefalt 1997). Alternatively, eosinophils may migrate into airway lumen, undergo apoptosis and be engulfed by luminal macrophages or be eliminated with secretions and exudates (Uller et al. 2006b).

2.2 Eosinophil functions

The recruitment of eosinophils into inflammatory sites is mediated by several cytokines (mainly IL-4, IL-5, IL-13, GM-CSF), chemoattractant molecules (eotaxins, RANTES, complement components C3a and C5a), adhesion molecules [integrins α4β1 (VLA-4) and αMβ2 (CD11b), adhesion receptors intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and selectins] and by other molecules such as acidic mammalian chitinases (Moser et al. 1992, Venge et al. 1996, Venge et al. 1996, Horie et al. 1997, Zhu et al. 2004, Pope et al. 2005, DiScipio and Schraufstatter 2007, DiScipio and Schraufstatter 2007, Barthel et al. 2008). Of these cytokines, IL-5 and eotaxin seem to be the most important factors regulating eosinophil trafficking and activation (Wardlaw 1999).

Eosinophils store a wide array of mediators in their granules and are able to release these agents rapidly in response to various inflammatory stimuli. The most important secretagogue in inflamed airways is still unclear but a complex that mimics antigen cross-linked to secretory IgA (sIgA) antibody has been shown to be the most efficient *in vitro* (Abu-Ghazaleh et al. 1989, Kita et al. 1991a). Crystalloid granules are the largest of eosinophil granules and store all four highly basic eosinophil granule proteins and most of the preformed cytokines (Giembycz and Lindsay 1999, Hogan et al. 2008). Granule protein major basic protein (MBP) resides in the crystalloid core of the granule whereas eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) are packaged into the granule matrix (Lewis et al. 1978, Egesten et al. 1986, Peters et al. 1986). When released, these granule proteins are toxic not only to parasites but also to mammalian cells such as airway epithelial cells (MBP,

ECP, EPO) and have also antibacterial (MBP, ECP, EPO) and antiviral (EDN, ECP) properties.

In addition, eosinophils synthesize and store relatively small amounts of at least 35 different cytokines, chemokines and growth factors, which they are able to rapidly release (Giembycz and Lindsay 1999, Hogan et al. 2008). When activated, eosinophils can also release eicosanoids, especially leukotriene (LT) C_4, which evokes bronchoconstriction, elevates vascular permeability and increases mucus production (Back et al. 2011). Lipid bodies are the source of eicosanoids; they contain arachidonic acid and the enzymes required for eicosanoid synthesis (Melo et al. 2011). The primary granules of eosinophils contain Charcot-Leyden crystal protein which possesses lysophospholipase activity and small granules which store arylsulphatase B and acid phosphatase (Giembycz and Lindsay 1999).

Production of superoxide is another important mechanism for killing of pathogens by eosinophils. Eosinophils contain high amounts of membrane-bound NADPH oxidase that is responsible for superoxide generation (Someya et al. 1997).

2.3 Eosinophils in asthma

An elevated number of eosinophils is found in the bronchial biopsy, bronchoalveolar lavage (BAL) fluid, sputum and peripheral blood of approximately 50 % of patients with asthma (Bousquet et al. 1990, Rytila et al. 2000, Wenzel 2006, Woodruff et al. 2009). Eosinophils were traditionally regarded as end-stage effector cells in asthma. They have the potential to release products that participate in the maintenance and exacerbation of airway inflammation. The eosinophil-derived granule proteins damage airway epithelial cells, the lipid mediators induce bronchoconstriction, mucus hypersecretion and vascular permeability and the reactive oxygen species damage the airway epithelium (Hisamatsu et al. 1990, Hulsmann et al. 1994, Back et al. 2011) (Figure 1).

There is recent evidence suggesting that eosinophils also have an important immunoregulatory role and are able to promote Th2 polarization. These cells have been shown to induce Th cell activation, proliferation and production of IL-4, IL-5 and IL-13 through functioning as antigen-presenting cells (APCs) (Shi et al. 2004, Wang et al.

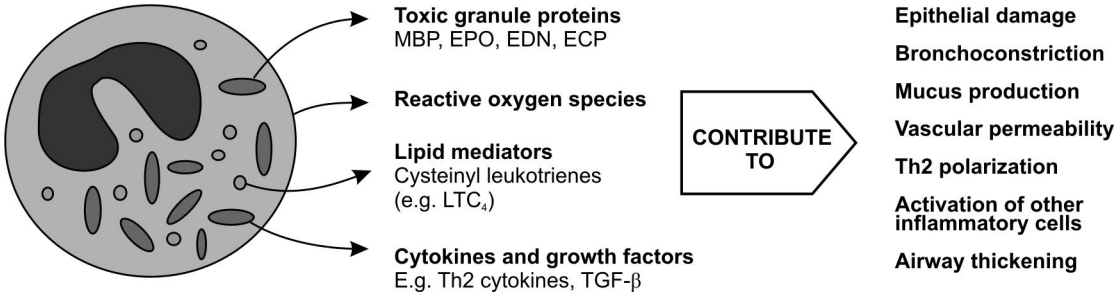

Figure 1. *Eosinophil products and their effects in the airways of asthmatics. MBP=major basic protein, EPO=eosinophil peroxidase, EDN=eosinophil-derived neurotoxin, ECP=eosinophil cationic protein, LTC_4=leukotriene C_4, Th2=T helper 2 cell, TGF-β=transforming growth factor-β.*

2007). Additionally, eosinophils produce cytokines (e.g. IL-4 and IL-25) and other molecules [e.g. indoleamine 2,3 dioxygenase (IDO)] that drive Th2 polarization (Chen et al. 2004, Odemuyiwa et al. 2004, Wang et al. 2007) (Figure 1). Indeed, ovalbumin (OVA)-sensitized/challenged eosinophil-deficient PHIL mice have reduced levels of Th2-cytokines in the airways and have poor recruitment of Th cells to the lungs (Jacobsen et al. 2008). In addition to inducing toxic effects, eosinophil granule proteins have been shown to activate both mast cells and dendritic cells (Zheutlin et al. 1984, Piliponsky et al. 2001, Yang et al. 2003, Yang et al. 2004).

Recent data from clinical studies with anti-IL-5 antibody and eosinophil-deficient mice suggests that eosinophils are important in asthma exacerbations and airway remodelling but not in airway hyperresponsiveness (AHR). Anti-IL-5 treatment had no clinical benefit in patients with mild asthma (Leckie et al. 2000, Flood-Page et al. 2003b) but led to a reduced exacerbation rate in patients with severe eosinophilic asthma. The patients were also able to reduce their glucocorticoid dose in response to anti-IL-5 treatment (Haldar et al. 2009, Nair et al. 2009). A recent meta-analysis concluded that adjustment of glucocorticoid dose according to eosinophil counts could be an effective way of reducing exacerbations (Petsky et al. 2012). Furthermore, many studies have detected a positive correlation between eosinophil number or ECP level and asthma severity (Synek et al. 1996, Louis et al. 2000). In contrast, clinical studies with anti-IL-5 suggest that eosinophils do not have any role in AHR. Additionally, eosinophil-deficient (delta double (Δdbl)-GATA) mice showed no improvement in AHR (Humbles et al. 2004, Haldar et al. 2009, Nair et al. 2009), even though in eosinophil-deficient PHIL mice, AHR did not develop after OVA challenge (Lee et al. 2004).

Eosinophils may be involved in mediating airway remodelling. Anti-IL-5 treatment in patients with mild asthma reduced levels of extracellular matrix proteins in bronchial mucosa, TGF-β1 mRNA in airway eosinophils and TGF-β1 protein in BAL fluid (Flood-Page et al. 2003a). In agreement, IL-5 receptor-deficient mice showed an attenuated formation of peribronchiolar and subepithelial fibrosis and decreased TGF-β1 levels after repeated allergen challenge (Tanaka et al. 2004). Furthermore, eosinophil-deficient Δdbl GATA mice could be protected from peribronchiolar collagen deposition and increases in airway smooth muscle mass but, however, no difference was found in their TGF-β1 levels (Humbles et al. 2004). In summary, eosinophils are important cells in the pathogenesis of asthma and are important target cells for anti-asthmatic drugs.

3 Programmed cell death

Programmed cell death is important in both the development and in the adult life of the organism. It is involved in the formation and deletion of structures, control of cell number, elimination of abnormal and damaged cells and in several pathological situations (Fuchs and Steller 2011). For decades, apoptosis was considered as the only form of programmed cell death. The morphological features of apoptosis such as cell shrinkage, chromatin condensation, DNA fragmentation, maintenance of membrane integrity, formation of apoptotic bodies and their engulfment by phagocytes were first described by Kerr and co-workers (Kerr et al. 1972). Necrosis is an accidental form of cell death that occurs in response to cell injury typically in the absence of ATP. It is characterized by swelling and cell rupture, and it results in the release of cellular contents into the surrounding tissue and this evokes local inflammation (Kerr et al. 1972). Necrosis has been traditionally regarded as an uncontrolled form of cell death. Recently, use of new biochemical techniques has revealed existence of many forms of programmed cell death including a form of regulated necrosis and thus had led to the development of a new functional classification system of cell death (Galluzzi et al. 2012). The new classification system is shown in Table 2. Apoptosis is the best understood form of programmed cell death. It can be executed via an extrinsic, receptor-mediated pathway or an intrinsic, mitochondrion-centered pathway.

Table 2. *Functional classification system of cell death. Modified from (Galluzzi et al. 2012).*

Cell death mode	Main biochemical features
Extrinsic apoptosis by death receptors	Death receptor signalling (Fas/TNFR1) Activation of caspase-8/caspase-3 cascade (type I cells) Activation of caspase-8, BID cleavage, MOMP, caspase-3 (type II cells)
Extrinsic apoptosis by dependence receptors	Ligand deprivation-induced dependence receptor signalling PP2A activation, DAPK1 activation Activation of caspase-9/caspase-3 cascade (direct or MOMP-dependent)
Intrinsic apoptosis, caspase-dependent	MOMP (activation of Bcl-2 members Bak/Bax or mPT) Irreversible $\Delta\psi_m$ dissipation, release of IMS proteins into cytosol (e.g. CytC) Activation of caspase-9/caspase-3 cascade
Intrinsic apoptosis caspase-independent	MOMP (activation of Bcl-2 members Bak/Bax or mPT) Irreversible $\Delta\psi_m$ dissipation Release of IMS proteins into cytosol (e.g. AIF, ENDOG)
Necroptosis (a specific case of regulated necrosis)	Death receptor signalling Caspase inhibition RIP1 and/or RIP3 activation
Autophagic cell death	Mainly a protective response induced by stress in dying cells. Lipidation of MAP1LC3, degradation of SQSTM1. Blocked by inhibitors of autophagy.
Mitotic catastrophe	Induced during abnormal mitosis. Activation of caspase-2 and TP53 or TP73, mitotic arrest.
Anoikis	Cells with lack of adhesion shows deficient β1-integrin attachment, downregulation of EGFR expression, inhibition of ERK1 signalling, overexpression of BIM.
Entosis	A non-phagocytic cell engulfs another similar type of cell and shows activation of Rho and ROCK1. For example, this type of death occurs in tumors.
Parthanatos	Cell death dependent on early PARP1-mediated accumulation of PAR, depletion of ATP and NADH, $\Delta\psi_m$ dissipation and AIF translocation to nucleus. A particular case of regulated necrosis?
Pyroptosis	Occurs at least in macrophages infected by certain bacteria. Activation of caspases-1 and -7, secretion of pyrogenic mediators IL-1β and IL-18.
Netosis	Restricted to granulocytes and often involves release of neutrophil extracellular traps (NETs). Activation of NADPH oxidase and ROS generation, dependent on autophagic machinery. Inhibition of caspases.
Cornification	Restricted to keratinocytes. Activation of caspase-14 and transglutaminases, which are involved in the generation of stratum corneum.

TNFR=tumor necrosis factor receptor, BID=BH3-interacting domain death agonist, MOMP=mitochondrial outer membrane permeabilization, PP2A=protein phosphatase 2A, DAPK1=death-associated protein kinase 1, Bcl-2=B-cell lymphoma 2, Bak= Bcl-2 antagonist/killer, Bax=Bcl-2-associated X protein, mPT=mitochondrial permeability transition, IMS=intermembrane space, CytC=Cytochrome c, AIF=apoptosis-inducing factor, ENDOG=endonuclease G, RIP1=receptor interacting protein kinase 1, MAP1LC3=microtubule-associated protein 1 light chain 3, SQSTM1=sequestesome 1,TP=tumor protein, ERK=extracellular-regulated kinase, EGFR=epidermal growth factor receptor, ROCK1=Rho-associated protein kinase 1, PARP1=poly(ADP-ribose) polymerase 1, PAR=poly(ADP-ribose), ATP=adenosine triphosphate, NADH=reduced form of nicotinamide adenine dinucleotide, ROS=reactive oxygen species.

3.1 Apoptosis: extrinsic pathway

Extrinsic apoptosis can be initiated by ligation of Fas/CD95, tumor necrosis factor α (TNFα) or TNF related apoptosis inducing ligand (TRAIL) to their death receptors. Alternatively, the extrinsic pathway may be initiated by dependence receptors in the absence of their ligands (for mechanism see Table 2). Dependence receptors are not

structurally related but possess common functional properties: they trigger activation of survival pathway in the presence of ligand and activation of apoptotic pathway in the absence of ligand. (Galluzzi et al. 2012)

Death receptors are assembled as trimers and their ligation leads to recruitment of several proteins, such as pro-caspase-8, to the cytoplasmic death domain (DD). This multiprotein complex is called death-inducing signalling complex (DISC) and it regulates the activation of initiator caspase-8 (Figure 2). (Kroemer et al. 2007, Tait and Green 2010)

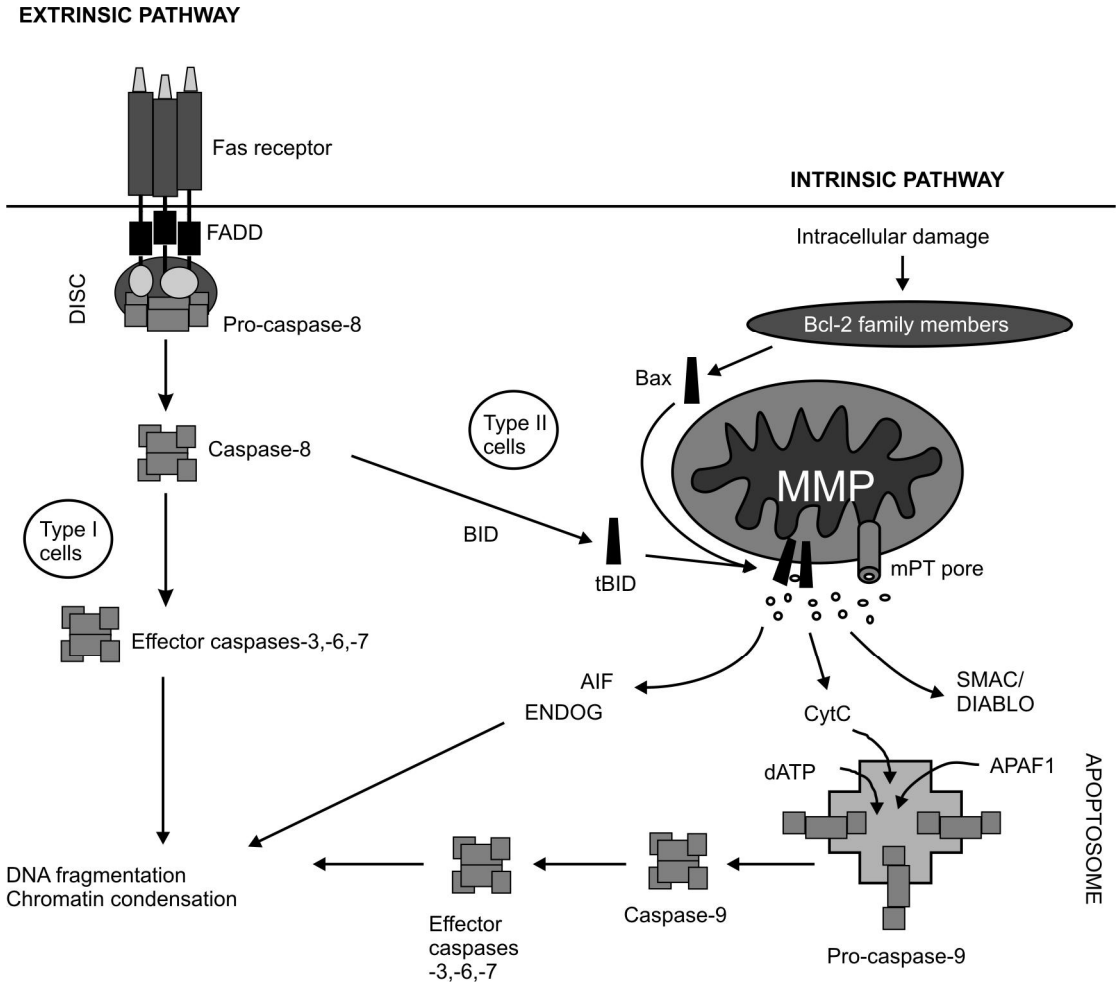

Figure 2. *Pathways of extrinsic and intrinsic apoptosis. Extrinsic apoptosis is initiated by ligation of death receptor Fas leading to activation of caspases. Sometimes mitochondrial route is required for caspase activation also in extrinsic apoptosis. Intracellular stress conditions initiate the intrinsic pathway of apoptosis, where Bcl-2 family members and mitochondrial membrane permeabilization play major roles. See text for details. SMAC-DIABLO facilitates activation of caspases by degrading inhibitors of apoptosis (IAPs). If caspases are inhibited, apoptosis is executed by AIF and ENDOG, which mediate DNA fragmentation and chromatin condensation. Abbreviations: FADD=Fas-associated protein with death domain, DISC=death-inducing signalling complex, tBID=truncated BID, mPT=mitochondrial permeability transition, AIF=apoptosis-inducing factor, ENDOG=endonuclease G, CytC=cytochrome c, Smac/Diablo= second mitochondria-derived activator of caspases/direct IAP binding protein with low pI, dATP= 2'-deoxy adenosine triphosphate, APAF1=apoptotic protease activating factor 1.*

Cells are divided into type I and II cells based on their need for an additional mitochondrial circuit for activation of effector caspases and the execution of apoptosis. In type I cells, initiator caspase-8 directly activates the effector caspases that execute apoptosis. In type II cells, caspase-8 cleaves BID [B-cell lymphoma (Bcl) 2 homology 3 (BH3)-interacting domain death agonist] into truncated BID (tBID), a fragment capable of permeabilizing the mitochondrial membrane leading to a release of intermembrane space (IMS) proteins such as cytochrome c (CytC) into the cytosol, activation of initiator caspase-9 and apoptosis as discussed in more detail in section 3.2. (Kroemer et al. 2007, Tait and Green 2010)

3.2 Apoptosis: intrinsic pathway

The intrinsic pathway can be initiated by several intracellular stress conditions such as DNA damage, oxidative stress and cytosolic Ca^{2+} overload. Typically both pro- and anti-apoptotic mechanisms are involved because the cells are struggling to survive in a stressful situation. (Galluzzi et al. 2012)

Members of Bcl-2 family are critical in monitoring intracellular damage. The Bcl-2 family consists of a group of anti-apoptotic proteins and two groups of pro-apoptotic proteins discriminated by their Bcl-2 homology (BH)-domains (BH1, BH2, BH3, BH4). Anti-apoptotic proteins contain all four BH-domains and pro-apoptotic proteins are divided into two groups based on whether they contain three BH domains or only one BH3 domain (Tait and Green 2010, Shamas-Din et al. 2011). In healthy cells, pro-apoptotic BH3 proteins are held inactive largely by anti-apoptotic Bcl-2 members. In response to an intracellular death signal, BH3-only proteins are released from these controlling mechanisms to mediate activation of pore-forming Bax, which results in mitochondrial outer membrane permeabilization (MOMP) and cell death (Tait and Green 2010). The members and functions of Bcl-2 protein families are described in more detail in Table 3.

Mitochondrial membrane permeabilization (MMP) is a central event in intrinsic apoptosis and it is considered as the point of no return (Kroemer et al. 2007, Galluzzi et al. 2012). In addition to the pore-forming activity of pro-apoptotic Bcl-2 family members, MMP can also be mediated via mitochondrial permeability transition (mPT),

Table 3. *Bcl-2 family of proteins and their role in intrinsic apoptosis.*

Bcl-2 protein family	Family members	Function
Anti-apoptotic (4 BH-domains)	Bcl-2, Bcl-X_L, Bcl-W, A1, Mcl-1	Maintain pro-apoptotic activator proteins inactive.
Pro-apoptotic ("BH3 only") - sensitizers - activators	BID, Bad, Bim, Bik, Bmf, Hrk, Noxa, Puma	Sensitizers release pro-apoptotic activator proteins from anti-apoptotic Bcl-2 proteins to promote apoptosis. Activators stimulate Bax to move to the mitochondrial outer membrane to form pores together with tBID or Bak.
Pro-apoptotic (3 BH-domains)	Bak, Bax, Bok	Form pores to mitochondrial outer membrane leading to mitochondrial outer membrane permeabilization.

Bcl-X_L=Bcl-extra large, Mcl-1=myeloid cell leukaemia-1, Bad= Bcl-2-associated death promoter, Bmf=Bcl-2-modifying factor, Hrk=harakiri, Puma= p53 upregulated modulator of apoptosis, Bak=Bcl-2 antagonist/killer, Bok= Bcl-2-related ovarian killer, tBID=truncated BID.

which will be discussed in the next section. (Kroemer et al. 2007, Rasola and Bernardi 2011). MMP results in loss of mitochondrial membrane potential ($\Delta\Psi_m$), disrupted mitochondrial ATP synthesis and the release of pro-apoptotic proteins from the IMS into the cytosol. One of the proteins released is CytC, which stimulates the formation of the apoptosome, a platform that activates initiator caspase-9 (Kroemer et al. 2007, Galluzzi et al. 2012) (Figure 2).

3.2.1 Mitochondrial permeability transition

Mitochondrial permeability transition (mPT) is one mechanism involved in the mitochondrial membrane permeabilization. During mPT, there is increased permeability of the inner mitochondrial membrane for solutes and molecules up to 1.5 kDa. A channel sensitive to Ca^{2+}, oxidants and pro-apoptotic Bcl-2 family members is responsible for this phenomenon (Kroemer et al. 2007, Rasola and Bernardi 2011)(Figure 3). Mitochondrial permeability transition results in mitochondrial matrix swelling, most likely due to the influx of ions that are accompanied by water. The mitochondrial outer membrane is ruptured as a result of this matrix swelling and apoptosis-inducing proteins are released into the cytosol (Kaasik et al. 2007).

The mPT channel is thought to be a multiprotein complex but its molecular structure is still unknown. Current evidence indicates that mitochondrial phosphate carrier PiC may form the core part of the pore (Leung and Halestrap 2008, Leung et al. 2008). Cyclophilin D and adenine nucleotide translocator (ANT) seem to play important regulatory roles since mice deficient for ANT or Cyclophilin D required 2-3-fold higher

Ca^{2+} concentration for mPT (Kokoszka et al. 2004, Basso et al. 2005). It has also been postulated that different proteins could participate in the formation of mPT channel depending on the cell type or specific trigger. Mitochondrial permeability transition can be inhibited by ligands of ANT (bongkrekic acid), Cyclophilin D (cyclosporin A) and some anti-apoptotic members of the Bcl-2 family (Halestrap et al. 1997, Marzo et al. 1998, Halestrap and Brenner 2003).

Mitochondrial permeability transition may function in two different modes. In addition to the irreversible sustained opening of the mPT channel occurring during cell death, the channel may also fluctuate between open and closed states (flicker) (Ichas et al. 1997, Petronilli et al. 2001). This mode does not lead to a permanent loss of mitochondrial membrane potential ($\Delta\Psi_m$) and cell death in contrast to the sustained mPT (Ichas et al. 1997, Petronilli et al. 2001). Flickering mPT occurs in healthy intact cells, in cells under minor stress and in cells prior to apoptosis (Petronilli et al. 2001, Saotome et al. 2009, Ma et al. 2011, Ma et al. 2011). Flickering mPT is believed to function as a fast calcium release mechanism, thereby participating in calcium-mediated signal transduction (Bernardi and Petronilli 1996, Barsukova et al. 2011).

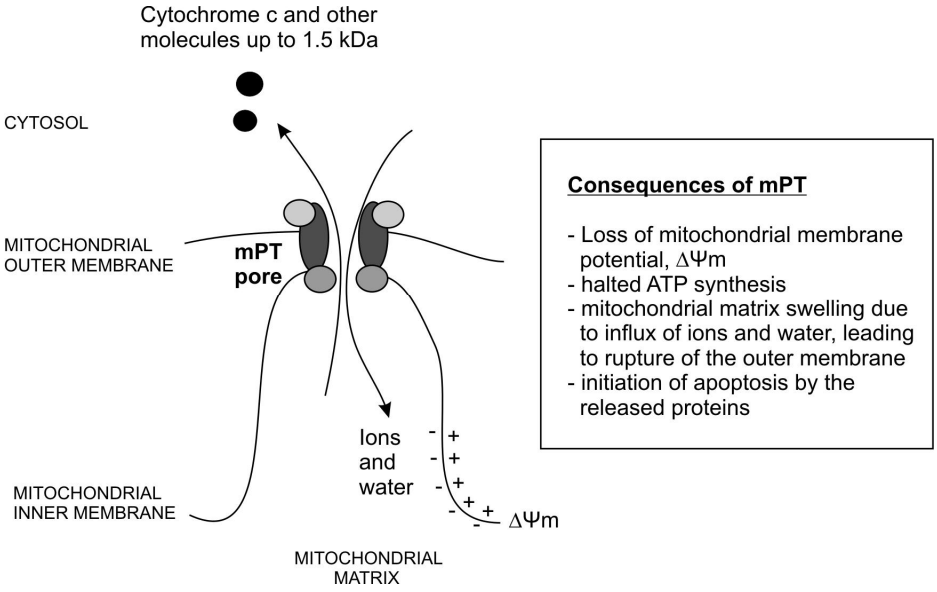

Figure 3. *Mitochondrial permeability transition (mPT) and its consequences. Mitochondrial permeability transition may be induced e.g. by increased matrix Ca^{2+}, Bcl-2 family members or oxidants.*

3.3 Caspases and calpains

Caspases are cysteine-dependent aspartate-specific proteases involved in the execution phase of apoptosis and processing of proinflammatory cytokines. Caspases have been traditionally classified into apoptotic (caspases 2, 3, 6, 7, 8, 9, 10) and proinflammatory caspases (caspases 1, 4, 5). Apoptotic caspases are further divided into initiators (caspases 8, 9, 10) and effectors (caspases 3, 6, 7) (Pop and Salvesen 2009). Caspase 2 displays features of both initiator and effector caspases (Troy and Shelanski 2003).

Initiator caspases are synthesized as inactive proenzymes containing an N-terminal prodomain followed by a large and a small subunit connected by linkers (Figure 4). Initiator caspases require dimerization for activation. This is enabled by platforms such as DISC or the apoptosome that are formed in response to apoptotic signals (Fuentes-Prior and Salvesen 2004, Pop and Salvesen 2009). Effector caspases are present as inactive dimers and require cleavage of the linker domain that separates the small and large fragments in order to form the cysteine-containing catalytic site and become activated (Figure 4). This is achieved by initiator caspases or other effector caspases. When activated, caspases cleave after a tetrapeptide sequence P_4-P_3-P_2-P_1 where P_1 is Asp and the three-dimensional structure of P_4-P_3-P_2 is optimal enough to fit the catalytic site. Additionally, the residue following Asp must be small and uncharged. For example, the optimal tetrapeptide sequences for caspases 3 and 6 are DEVD and VEH/ID, respectively, which occur in their natural substrates such as poly (ADP-ribose) polymerase (PARP) and lamin A, respectively (Thornberry et al. 1997, Crawford and

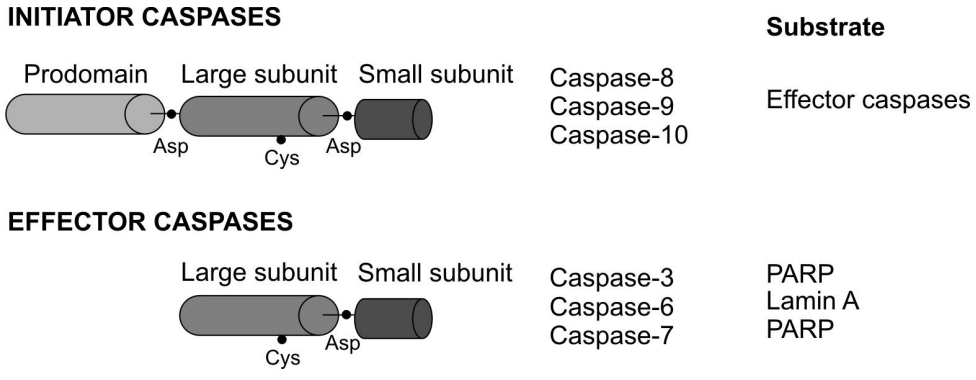

Figure 4. *Structure of initiator and effector caspases and examples of their substrates. Linkers are cleaved at Asp residue to activate caspases. Catalytic site containing Cys residue is also shown. Cys=cysteine residue, Asp=aspartate residue.*

Wells 2011). The numbers of proteins that have been reported as substrates of caspases is now approaching 1000 but the amount of biologically relevant substrates is unknown. Caspase-mediated cleavage of cellular substrates finally results in morphological signs of apoptosis such as chromatin condensation and DNA fragmentation. Members of IAP family act as inhibitors of caspases. IAPs may directly inhibit the catalytic site of caspases or increase their ubiquitination directing caspases to proteasomal degradation (Pop and Salvesen 2009, Crawford and Wells 2011).

Calpains are calcium-activated (papain-like) neutral proteases that are involved in the execution of both apoptosis and necrosis and there are at least 14 isoforms of calpains. Similarly to caspases, calpains are cysteine proteases but in contrast to caspases they require no particular amino acid in the substrate peptide sequence. Calpains are activated by increased intracellular calcium and their substrates include X-linked IAP (XIAP), Bcl-X_L, Bid and pro-caspases 3, 7, 8 and 9. When compared to caspases, much less is known of the role of calpains as mediators of cell death. (Harwood et al. 2005, Storr et al. 2011)

3.4 Regulation of eosinophil apoptosis

Eosinophils die spontaneously by apoptosis in the absence of any survival-prolonging cytokines in few days (Kankaanranta et al. 2005). However, the lifespan and rate of apoptosis may be modulated by survival-prolonging or apoptosis-inducing factors.

3.4.1 Survival-prolonging cytokines IL-5, IL-3 and GM-CSF

Eosinophil survival is markedly enhanced by IL-5, IL-3 and GM-CSF of which IL-5 is the most potent (Tai et al. 1991). GM-CSF seems to be the main eosinophil-survival prolonging cytokine in asthmatic airways, even though an elevated number of cells positive for mRNA of all of the three cytokines has been found in BAL from patients with asthma (Robinson et al. 1992, Adachi et al. 1995, Park et al. 1998). Prolongation of cell survival is one of the key functions of these cytokines supporting other functions.

Receptors for IL-5, IL-3 and GM-CSF have a similar β_c subunit but a distinctive α subunit, resulting in both overlapping and distinguishable effects (Giembycz and Lindsay 1999). Recently, it was demonstrated that the IL-5-IL-5R complex is

endocytosed after ligand binding in eosinophils and this is required for both signal amplification and signal termination (Lei and Martinez-Moczygemba 2008). After ligand binding, JAK2 phosphorylates cytoplasmic tyrosine residues in the β_c receptor subunit and these phosphorylation sites serve as binding sites for several proteins involved in intracellular signal transduction (Guthridge et al. 1998). IL-5 has been shown to activate lyn/syk-ras-raf1- mitogen activated protein kinase (MAPK) kinase (MAPKK)-extracellular signal-regulated kinases (ERK) 1/2, JAK2-STAT1 and PI3K-Akt pathways in eosinophils (Pazdrak et al. 1995a, Pazdrak et al. 1995b, Coffer et al. 1998) and at least lyn/syk, raf1, JAK2 and PI3K are important for IL-5-induced eosinophil survival (Yousefi et al. 1996, Pazdrak et al. 1998, Rosas et al. 2006). Treatment with IL-5 resulted in inhibition of Bax translocation to mitochondria and the subsequent release of cytochrome c and caspase activation (Dewson et al. 2001). The mechanism of Bax inhibition by GM-CSF was revealed recently. GM-CSF induced ERK-mediated phosphorylation of Thr167 in Bax thereby enabling an interaction of Bax with prolyl isomerase Pin1. Pin1 was proved critical in preventing the pro-apoptotic function of Bax (Shen et al. 2009). Interestingly, interaction between ICAM-1 and GM-CSF receptor was shown to be required for GM-CSF-induced eosinophil survival at later stages (Pazdrak et al. 2008).

3.4.2 Kinases MAPK and PI3K

MAPK are serine/threonine kinases mainly activated by proinflammatory cytokines, growth factors and environmental stress. MAPK family consists of JNK 1-3, ERK 1/2, 3, 5 and 7, and p38 family members. A series of phosphorylation cascades involving MAPK kinase kinases (MAPKKK) and MAPK kinases (MAPKK) leads to activation of MAPK. MAPK phosphorylate transcription factors resulting in transcription of genes involved in apoptosis, survival, proliferation and differentiation. Additionally, MAPK affect the function of numerous other proteins via phosphorylation. MAPK are inactivated by phosphoprotein phosphatases such as MAPK phosphatases (MKP). (Raman et al. 2007, Wancket et al. 2012)

JNK regulates apoptosis with several mechanisms. JNK phosphorylates transcription factors Jun, Fos and activating transcription factor 2 (ATF2), which cluster into the activator protein 1 (AP-1) complex and AP-1 induces transcription of apoptosis-related

proteins such as FasL and TRAIL-receptor 1 (Eichhorst et al. 2000, Guan et al. 2002). JNK also phosphorylates over 50 proteins including both pro- and anti-apoptotic mitochondrial Bcl-2 family proteins Bcl-2, Bcl-X_L, Mcl-1, Bad, Bim and Bax (Schroeter et al. 2003, Bogoyevitch and Kobe 2006, Raman et al. 2007) and stimulates mPT (Schroeter et al. 2003, Bogoyevitch and Kobe 2006, Hanawa et al. 2008, Lin et al. 2009). Reports exist also on the involvement of JNK in the pathway leading to caspase activation (Lu et al. 2006, Choi et al. 2009) and on JNK activation induced by caspase-mediated cleavage of Mst1 during apoptosis (Ura et al. 2007). By acting in these pathways, JNK has been shown to mediate apoptosis, apoptosis-related chromating condensation or DNA fragmentation (Ura et al. 2007, Choi et al. 2009). In eosinophils, JNK has been shown to mediate spontaneous apoptosis as well as apoptosis induced by nitric oxide and some drugs (Zhang et al. 2003, Hasala et al. 2007a, Hasala et al. 2007b, Kankaanranta et al. 2007). ERK1/2 may be involved in GM-CSF-induced eosinophil survival (Shen et al. 2009). Basal activity of MAPK p38 has been demonstrated to be important for eosinophil survival in the absence but not in the presence of cytokines (Kankaanranta et al. 1999).

Members of PI3K family are activated in response to cytokines, chemokines, growth factors and pathogenic components and the regulation of cell survival is one of their various functions. PI3Ks are divided into three classes on the basis of structural properties and substrate specificity. Class I members include a p110 catalytic subunit (α, β, δ or γ) and are activated by tyrosine kinase receptors or by the βγ subunit of GPCRs. Class II PI3Ks include three members with a C2 domain but their exact substrates and functions are unclear (Fougerat et al. 2009). The only member of class III PI3K is important for endocytosis, vesicular trafficking and Toll-like receptor signalling (Kuo et al. 2006). The function of PI3K is to catalyze the production of cell membrane phosphoinositides such as PtdIns(3,4,5)P_3 (Fougerat et al. 2009). Phosphoinositides enable phosphorylation of Akt, which is an important downstream target of PI3K. Forkhead transcription factor FOXO3a, glycogen synthase kinase 3 (GSK3) and components of cell death machinery such as caspase-9 and Bad act as substrates for Akt and their phosphorylation by Akt has been shown to suppress apoptosis (Datta et al. 1999). PI3K has been found to mediate survival induced by IL-5 and migration in eosinophils (Pinho et al. 2005, Rosas et al. 2006). In addition, eosinophil survival induced by beta-adrenergic agonists involves PI3K (Machida et al. 2005).

3.4.3 Transcription factor NF-κB

The NF-κB pathway is activated by stress and numerous endogenous and exogenous stimuli and it modulates the expression of an array of genes involved in the immune response. The pathway leading to activation of NF-κB is shown in Figure 5. The family of NF-κB consists of five members: p50, p52, p65 (RelA), c-Rel, and RelB. These proteins homo- or heterodimerize to form complexes that activate (p50-p65) or repress (p50-p50) transcription (Hayden and Ghosh 2008, Solt and May 2008). NF-κB dimers bind to κB sites within target gene promoters and regulate transcription of proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules and receptors (Barnes and Karin 1997, Hayden and Ghosh 2008).

In eosinophils, several cytokines (e.g. TNF-α, IL-15, leptin, TSLP), allergen (house dust mite extract) and some pathogenic components have been shown to stimulate NF-κB and induce NF-κB-dependent protein expression (Temkin and Levi-Schaffer 2001, Hoontrakoon et al. 2002, Coward et al. 2004, Wong et al. 2007a, Wong et al. 2007b, Wong et al. 2010). Constitutive NF-κB activity is critical for eosinophil survival and inhibition of NF-κB results in apoptosis (Ward et al. 1999, Fujihara et al. 2005). At least the productions of IL-8, GM-CSF and TNF-α are induced via the transcriptional activity of NF-κB (Temkin and Levi-Schaffer 2001, Coward et al. 2004). In that respect, NF-κB-mediated production of cytokines such as GM-CSF may act as an autocrine way of prolonging eosinophil survival (Temkin and Levi-Schaffer 2001, Coward et al. 2004).

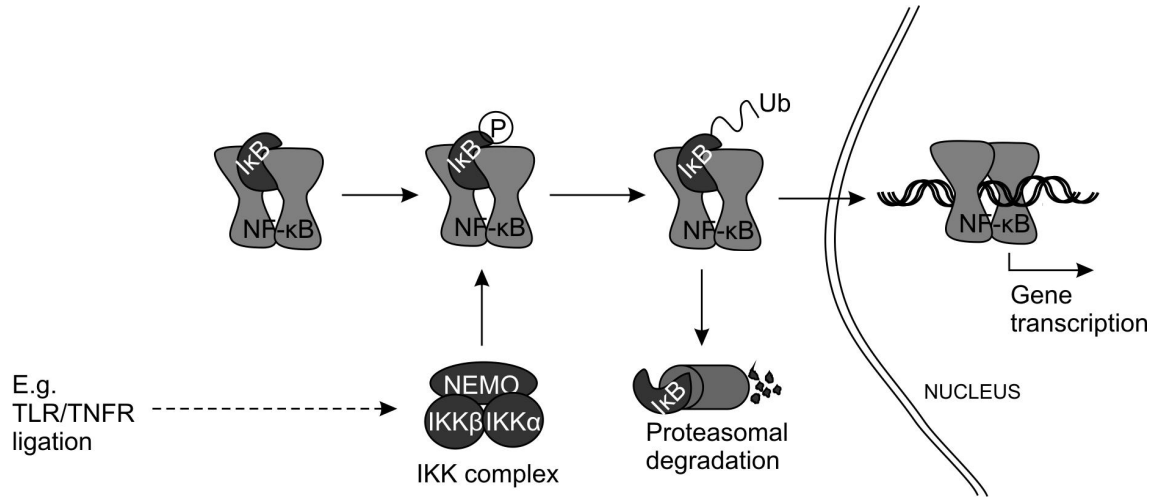

Figure 5. *Activation of NF-κB pathway. IKK complex mediates phosphorylation of IκB. In the unphosphorylated state, IκB prevents NF-κB dimers from nuclear translocation and DNA binding. Phosphorylation results in ubiquitination and proteasomal degradation of IκB and release of NF-κB, which is then transferred to the nucleus to modulate transcription of genes. TNFR=tumor necrosis factor α receptor, NEMO=NF-κB essential modulator, IκB=inhibitor of κB, IKK=IκB kinase, Ub=ubiquitin, P=phosphorylated.*

3.4.4 Caspases and calpains

Eosinophils express caspases-3, -6, -7, -8 and -9 (Zangrilli et al. 2000, Dewson et al. 2001). Some events associated with spontaneous apoptosis (e.g. phosphatidylserine surface expression, formation of apoptotic nuclei) but not all of them (e.g. loss of $\Delta\Psi_m$) are partly prevented by pan-caspase inhibitors (Dewson et al. 2001, Letuve et al. 2002). However, in Fas-induced apoptosis also early loss of $\Delta\Psi_m$ was reported to be prevented by a pan-caspase inhibitor as well as by inhibitors of caspases 3 and 8 (Letuve et al. 2001). Calpains have been shown to be involved in the cleavage of Bax in eosinophil apoptosis (Shen et al. 2009). Bax cleavage is a pro-apoptotic event leading to its mitochondrial targeting (Shen et al. 2009).

3.4.5 Mitochondria and Bcl-2 family proteins

In most cells, mitochondria function as "energy factories" producing ATP via enzymes in the electron transport chain maintaining $\Delta\Psi_m$. Eosinophils have a low number of mitochondria, approximately 24-36 per cell but they lack functional electron transport chain. Instead, eosinophil mitochondria maintain $\Delta\Psi_m$ via cytosolic ATP and its hydrolysis by F_1F_0-ATPase. However, eosinophil mitochondria are able to induce apoptosis. (Peachman et al. 2001)

Members of Bcl-2 family are critical in monitoring intracellular damage and the balance between anti-apoptotic and pro-apoptotic Bcl-2 members determines whether the cell undergoes intrinsic apoptosis. There is a controversy about whether there is anti-apoptotic Bcl-2 expression in human eosinophils; the expression seems to depend on the status of the patient and origin of eosinophils (blood, sputum or bronchial biopsy) (Dewson et al. 1999, Vignola et al. 1999). Lung eosinophils from patients with asthma and children with severe exacerbations have been shown to express higher levels of Bcl-2 than eosinophils from healthy individuals or children with mild-to-moderate exacerbations, respectively (Vignola et al. 1999, Maa et al. 2003, El-Gamal et al. 2004). In some but not all studies, IL-5 was shown to be capable of inducing Bcl-2 expression (Dewson et al. 1999, Zangrilli et al. 2000). The pro-apoptotic Bax is strongly expressed in eosinophils (Shen et al. 2009). Eosinophils also show high levels of pro-apoptotic Bid, which seems to be involved in both spontaneous and receptor-mediated apoptosis (Segal et al. 2007, Maret et al. 2009). Contradictory results exist on the expression of

anti-apoptotic Bcl-X_L in human eosinophils (Dibbert et al. 1998, Druilhe et al. 1998a, Zangrilli et al. 2000). Low levels of anti-apoptotic Mcl-1 have been detected in eosinophils (Druilhe et al. 1998a, Zangrilli et al. 2000). In summary, it seems that expression of Bcl-2 family proteins in eosinophils is balanced to pro-apoptotic members of the family, which is logical considering the usual rapid death of these cells.

3.5 Eosinophil apoptosis in the resolution of inflammation and as a target of anti-asthmatic drugs

Apoptosis of blood and nasal polyp tissue eosinophils was shown to be delayed in patients with asthma when compared to the situation in healthy individuals. The delayed eosinophil apoptosis in blood and tissue could only be partly prevented by anti-GM-CSF or anti-IL-5 antibodies, respectively, suggesting that these eosinophil survival-prolonging cytokines only partially explain the delay in apoptosis (Simon et al. 1997, Kankaanranta et al. 2000). It remains to be determined which factors in addition to GM-CSF, IL-3 and IL-5 are clinically relevant for the prolongation of eosinophil survival and the amplification and maintenance of eosinophilic inflammation.

In contrast to necrosis, apoptosis is a non-inflammatory form of cell death because the cell content is retained inside the cell and the apoptotic bodies formed are typically ingested by phagocytes. In this respect, eosinophil apoptosis is an important mechanism for resolution of eosinophilic airway inflammation (Woolley et al. 1996, Duncan et al. 2003). A sufficient amount of phagocytes in the airways is critical for a beneficial outcome of apoptosis because otherwise eosinophils undergo inflammation-aggravating secondary necrosis and piecemeal degranulation (Uller et al. 2005). The eosinophils which have undergone apoptosis are ingested by macrophages and at least in *in vitro* conditions, by the small airway epithelial cells (Stern et al. 1992, Woolley et al. 1996, Walsh et al. 1999).

Amplification of eosinophil apoptosis is one anti-inflammatory mechanism for glucocorticoids (Meagher et al. 1996, Zhang et al. 2000) and evidence for the clinical significance of eosinophil apoptosis has been obtained from steroid-treated asthmatics. An increased amount of apoptotic eosinophils has been found in sputum and bronchial submucosa from steroid-treated asthmatics when compared to steroid-untreated asthmatics (Woolley et al. 1996, Druilhe et al. 1998b). In addition, the ratio of apoptotic

eosinophils/total eosinophils inversely correlated with the severity of asthma (Vignola et al. 1999, Duncan et al. 2003). However, Uller and co-workers found that apoptotic eosinophils were rarely encountered in nasal biopsy of steroid-treated patients with allergic rhinitis (Uller et al. 2010) and in the airway tissue of steroid-treated allergen-challenged rodents (Uller et al. 2001, Uller et al. 2006a). According to the hypothesis of Uller and co-workers, eosinophils may migrate from airway tissue into airway lumen, possibly undergo apoptosis in the lumen and be eliminated through mucociliary transport or coughing (Uller et al. 2001, Erjefalt et al. 2004).

Glucocorticoids accelerate spontaneous eosinophil apoptosis and overcome the survival-increasing effect of low concentrations of IL-5, IL-3 or GM-CSF (Lamas et al. 1991, Wallen et al. 1991, Zhang et al. 2000). Glucocorticoid-induced apoptosis does not seem to differ markedly from spontaneous apoptosis. Glucocorticoid-specific events include the involvement of glucocorticoid receptor, increased ROS, early and late JNK activation and diminished production of X-linked inhibitor of apoptosis protein (XIAP) (Gardai et al. 2003).

Several other drugs used in the treatment of asthma are able to modulate eosinophil apoptosis. β_2-adrenoceptor agonists inhibit eosinophil apoptosis via a cAMP-dependent pathway (Kankaanranta et al. 2000, Kankaanranta et al. 2011). Cysteinyl leukotriene receptor antagonists and theophylline have been reported to accelerate apoptosis and to reverse GM-CSF-mediated eosinophil survival (Yasui et al. 1997, Lee et al. 2000).

4 Toll-like receptor 9

The immune system in mammals is divided into two parts: innate and acquired immunity. Innate immunity is the first-line defence against invading pathogens and it is mediated mainly by neutrophils, macrophages and dendritic cells. Acquired immunity is mediated by lymphocytes and involves the generation of an immunological memory and antigen-specificity. Cells of the innate immune system recognize pathogenic structures (pathogen-associated molecular patterns, PAMPs) via their pattern recognition receptors such as Toll-like receptors (TLRs). Recognition of PAMP via TLR leads to the production of proinflammatory cytokines via NF-κB and/or type I interferons via IRF3. The exclusive activation of the NF-κB pathway is dependent on MyD88 (myeloid

differentiation primary response gene 88). TRIF (Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β) mediates activation of both NF-κB and IRF3. In addition to production of interferons and cytokines, TLR activation leads to release of cytotoxic agents and promotes phagocytosis resulting in inflammation, pathogen eradication and activation of cells of the adaptive immune system. Ten members of TLRs have been identified in humans so far. TLRs 1, 2, 4, 5 and 6 recognize structures of bacteria, fungi and protozoa and reside primarily on the cell surface while TLRs 3, 7, 8 and 9 are expressed in endocytic compartments and recognize viral and bacterial nucleid acids (Figure 6). Expression and function of TLR 10 is unclear (Kawai and Akira 2010, Moresco et al. 2011). Eosinophils express many members of TLR family, including TLR9. TLR9 agonists are under active investigation because of their anti-inflammatory effects in animal models of allergic airway inflammation (Fonseca and Kline 2009), suggesting that they may have a role in modulating eosinophil apoptosis.

4.1 TLR9 agonists: bacterial DNA and CpG oligodeoxynucleotides

TLR9 is activated by single-stranded DNA containing unmethylated cytosine linked to guanine by a phosphate bond (CpG) (Hemmi et al. 2000). These motifs are frequently present in bacterial and viral DNA but are rare in mammalian DNA. For example, mouse genome contains 30-40-fold fewer unmethylated CpG dinucleotides when compared to the *Escherichia Coli (E. Coli)* genome (Stacey et al. 2003). The lack of any effect of mammalian DNA on TLR9 activation is also partially explained by its higher content of inhibitory, G-rich sequences when compared to bacterial DNA (Stacey et al. 2003). The CpG content of different bacterial DNAs varies markedly and correlates to the TLR9 activation potential of the DNA (Dalpke et al. 2006). *E. Coli* DNA contains intermediate frequency of CpG (7.27 %) while *Mycobacterium tuberculosis* contains a high frequency of CpG (12.74 %) (Dalpke et al. 2006).

Studies with synthetic CpG oligodeoxynucleotides (ODNs) have revealed that the nucleotides flanking CpG are important for potency. For example, the sequence GT**CG**TT with the phosphodiester backbone optimally activates human B-cells whereas GT**CG**AT may induce activity that is only approximately half of that induced by the optimal sequence. In addition to the number of CpG dinucleotides, the bases between

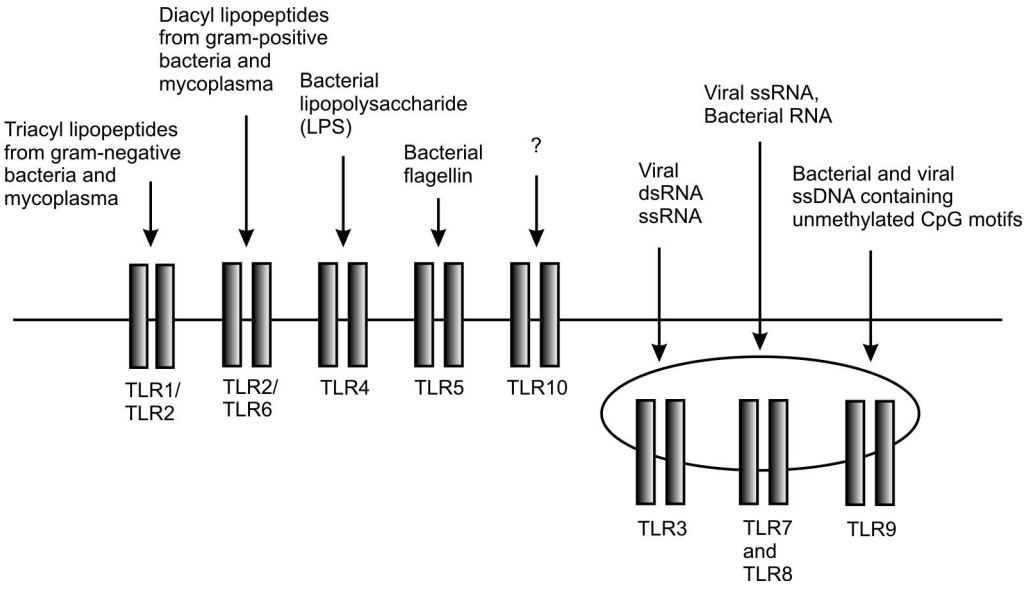

Figure 6. *Natural ligands of Toll-like receptors 1-10. ds=double-stranded, ss=single-stranded.*

them and backbone all affect the potency of the sequence (Hartmann and Krieg 2000, Hartmann et al. 2000).

The phosphorothioate backbone is used instead of the natural phosphodiester backbone to slow the degradation of the short synthetic ODNs normally catalyzed by exonucleases. In the phosphorothioate backbone, one of the non-bridging oxygens is replaced by sulphur at each linkage between nucleotides (Dias and Stein 2002). This modification increases cell retention but also enhances non-specific binding (Brown et al. 1994). A substantially lower concentration of CpG ODNs with phosphorothioate backbone is required to induce the maximal effect when compared to CpG ODNs with phosphodiester backbone. The backbone difference may also be reflected in different sequence and structure requirements for TLR9 activation (Hartmann and Krieg 2000).

Three types of synthetic CpG ODNs have been developed to induce a certain profile of immune activation. The main structural properties and immunostimulatory functions of class A, B and C CpG ODNs are shown in Table 4.

Table 4. *Properties of class A, B and C CpG oligodeoxynucleotides.*

ODN class	Structural properties Sequence example	Stimulatory functions	References
A	- PS-modified poly-G tails that forms quadruplex structures/hairpin-loops resulting in enhanced cellular uptake - Central palindrome with PO backbone and one or more CpG dinucleotides - For example gg**TGCAT**CG**ATGCA**Aggggg	- Induces high production of type I IFN by pDC	(Krug et al. 2001, Marshall et al. 2003)
B	- PS-modified, linear - contains several CpG dinucleotides - For example tgactgtgaa**cg**tt**cg**agatga	- Stimulates B cell functions	(Hartmann and Krieg 2000, Verthelyi et al. 2001)
C	- PS-modified - 1-2 TCG at or close to the 5' end - palindromic region of 10-12 bases containing at least two additional CpG spaced by 0-3 bases - For example tcgt**cg**aa**cg**tt**cg**agatgat	- Combines activities of A and B class CpG ODNs: stimulates B cell functions and type I IFN production by pDC	(Marshall et al. 2003)

In sequence examples, small letters and capitals indicate nucleotides with phosphorothioate and phosphodiester backbone, respectively. CpG dinucleotides are shown bolded and palindromic regions shaded with grey. PS=phosphorothioate, PO=phosphodiester, pDC=plasmacytoid dendritic cells.

4.2 Activation

TLR9 is an intracellular receptor residing in the endoplasmic reticulum (ER) in resting cells. TLR9 activation is preceded by endocytosis of DNA and the transfer of TLR9 from ER into DNA-containing structures such as early endosomes, and subsequently, into tubular lysosomal compartments (Latz et al. 2004). The binding of endocytosed DNA to TLR9 occurs only at acidic pH and the binding affinity is increased by reduction in pH, at least down to pH 5.5 (Rutz et al. 2004). This indicates that endosomal acidification (maturation of early endosomes into late endosomes) via function of vacuolar ATPase is beneficial for TLR9 activation (Lafourcade et al. 2008).

It seems that the CpG motif is an absolute requirement for TLR9 activation induced by phosphorothioate but not by phosphodiester DNA (Latz et al. 2007). Both CpG and non-CpG ODNs with phosphorothioate backbone bind to TLR9 with high affinity but only CpG ODNs induce a conformational change in the TLR9 homodimers enabling MyD88 recruitment and activation of the signalling cascade (Latz et al. 2007). In this respect, non-CpG ODNs act as inhibitors of TLR9. With respect to the phosphodiester DNA, the present evidence supports the view that it is the sugar group of DNA (2'-deoxyribose) that triggers TLR9 into a state of basal activity and although the activation can be enhanced by any bases, it is most potently stimulated by the unmethylated CpG

dinucleotide (Haas et al. 2008). Interestingly, a recent study suggests that the sequence-specific step is upstream of TLR9 leading to differential compartmentalization of unmethylated and methylated CpG DNAs with the phosphodiester backbone. According to this study, only unmethylated CpG DNA trigger a src-kinase (SRK)-mediated pathway that leads to their mobilization and co-localization into late endosomal compartments with TLR9 (de Jong et al. 2010). In addition, PI3K has been shown to mediate uptake and co-localization of CpG ODNs (Ishii et al. 2002). Interestingly, the distinct endosomal compartmentalization of different classes of CpG ODNs explains their divergent responses in plasmacytoid dendritic cells (pDC) (Guiducci et al. 2006).

4.3 Intracellular pathways and immunostimulatory effects induced by TLR9 agonists

Stimulation by CpG DNA results in a strong Th1 response. B-cells and plasmacytoid dendritic cells are considered as the main cell types expressing TLR9 and responding to CpG DNA. TLR9 signalling proceeds via MyD88, IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor (TRAF) 6 resulting in activation of MAPKs, PI3K, NF-κB and/or interferon regulatory factor (IRF) 7 leading to various immunostimulatory effects (Yi and Krieg 1998a, Hacker et al. 2000, Chuang et al. 2002, Gohda et al. 2004, Honda et al. 2005, Guiducci et al. 2008).

In plasmacytoid dendritic cells, CpG DNA induces the production of type I interferons, increased expression of co-stimulatory molecules, increased survival and maturation (Hartmann et al. 1999, Krug et al. 2001). In B-cells, CpG DNA stimulates production of IL-6, IL-10, TNF-α and IgM, expression of co-stimulatory molecules, increased survival, proliferation and differentiation from naïve and memory cells into plasma cells (Krieg et al. 1995, Yi et al. 1996, Yi et al. 1998, Hartmann and Krieg 2000, Jung et al. 2002).

Even though contradictory results exist, expression of TLR9 has been identified in human macrophages and monocytes and CpG DNA has been reported to promote the production of cytokines in these cells (Hornung et al. 2002, Mao et al. 2005, Kiemer et al. 2009). Bacterial DNA is significantly more potent in activating human macrophages than synthetic CpG ODNs (Kiemer et al. 2009). In neutrophils, bacterial DNA has been shown to inhibit apoptosis and to induce IL-8 production in a TLR9-dependent manner

(Jozsef et al. 2004, Jozsef et al. 2006). However, CpG- and TLR9-independent activation of neutrophils by bacterial DNA but not mammalian DNA has also been reported (Trevani et al. 2003). However, this activation was dependent on MyD88 (Alvarez et al. 2006). Eosinophils express TLR9 mRNA but class B CpG ODN (2006) were reported not to produce any effect on eosinophil survival or activation (Nagase et al. 2003). The effects of other classes of CpG ODNs and bacterial DNA on eosinophils have not yet been tested. There is evidence indicating that T-cell and natural killer (NK) cells are activated by CpG DNA only indirectly, in the presence of pDC (Sivori et al. 2004, Marshall et al. 2006).

CpG DNA increases the survival of several immune cell types. NF-κB, ERK, PI3K, up-regulation of cellular IAPs (cIAPs) as well as Bcl-2 and Bcl-X_L and/or down-regulation of active caspase-3 have been proposed to mediate the survival-prolonging effect of CpG DNA in these cells (Yi and Krieg 1998b, Park et al. 2002, Jozsef et al. 2004, O'Keeffe et al. 2005, Dil and Marshall 2009).

4.4 Therapeutic potential of CpG ODNs in allergic diseases

CpG ODNs have been proposed to have therapeutic potential in many diseases including allergy, cancer, infectious diseases as well as use as vaccine adjuvants (Vollmer and Krieg 2009). The interest in their potential use in the treatment of allergic diseases is based on the concept of inhibition of Th2 allergic immune response via an intense induction of Th1 response. Administration of CpG ODNs alone, together with antigen or physically linked to allergen have consistently decreased lung eosinophilia and bronchial hyperresponsiveness and increased the levels of Th1 marker IgG2a in acute and chronic murine models of allergic asthma (Kline et al. 2002, Santeliz et al. 2002, Jain et al. 2003). CpG ODN physically linked to allergen was the most effective (Tighe et al. 2000, Santeliz et al. 2002, Jain et al. 2003). CpG ODNs were effective in both preventing and reversing Th2-mediated lung inflammation, even though the effects of CpG ODNs on the levels of Th2 and Th1 cytokines have been inconsistent (Kline et al. 2002, Santeliz et al. 2002, Jain et al. 2003). Administration of CpG ODNs also prevented changes related to airway remodelling (Jain et al. 2002). Interestingly, CpG ODNs were effective also in IFN-γ and IL-12 double-knockout mice but only when 10 times higher doses were administered (Kline et al. 1999). These results indicate that Th1

cytokines IFN-γ and IL-12 may be involved but are not essential for the therapeutic effects of CpG ODNs.

In clinical trials, CpG ODN conjugated to ragweed-pollen allergen decreased symptoms in patients with allergic rhinitis, and this effect persisted throughout the subsequent ragweed season (Creticos et al. 2006). In another study, symptoms decreased or tended to decrease only during the second ragweed season (Tulic et al. 2004). However, in patients with allergic asthma, treatment with CpG ODN 1018 had no effect on allergen-induced decrease in FEV_1, sputum eosinophilia or Th2-related gene expression in sputum cells even though Th1-related gene expression was increased (Gauvreau et al. 2006). No adverse events were observed related to the study medication. The significance of the mechanisms of CpG ODNs for eosinophil apoptosis is unknown.

5 Nitric oxide

Nitric oxide (NO) is a gaseous molecule and a free radical with the formula •N=O. It is a signalling molecule involved in various biological processes, both physiological and pathophysiological. For example, NO is a vasodilator regulating blood pressure, a neurotransmitter and a toxic substance generated by phagocytes to kill bacteria. A high amount of NO is produced during inflammation resulting in both pro-inflammatory and regulatory effects. (Bogdan 2001, Korhonen et al. 2005, Kobayashi 2010)

NO is synthesized by nitric oxide synthases (NOS), which are composed of two NOS proteins and two calmodulins in their active form. NO is generated from L-arginine in the presence of NADPH, oxygen and co-factors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and iron protoporfyrin IX (haem). Three isoforms of NOS have been identified so far. Neuronal NOS (nNOS, NOS I) and endothelial NOS (eNOS, NOS III) are constitutively expressed and activated by agents that elevate the intracellular calcium concentration ($[Ca^{2+}]_i$) (Knowles and Moncada 1994, Alderton et al. 2001). These synthases are rapidly inactivated by decreased $[Ca^{2+}]_i$ and generate a low amount of NO. Inducible NOS (iNOS, NOS II) is the third NOS isoform. In contrast to nNOS and eNOS, expression of

iNOS is not constitutive but induced by microbial products (LPS) and proinflammatory cytokines (e.g. IL-1, TNF-α and IFN-γ) in many immune cells. Inducible NOS is functional in the presence of low $[Ca^{2+}]_i$ and once expressed it produces high amounts of NO for prolonged periods (Bogdan 2001, Korhonen et al. 2005, Kobayashi 2010).

5.1 Molecular effects of NO

As a hydrophobic small molecule, NO diffuses easily through lipid membranes into the cells. NO has both direct effects mediated by NO itself and indirect effects produced via reactive nitrogen species (RNS). RNS are often formed because NO reacts readily with other molecules due to its unpaired electron. Site and source of production, as well as the concentration of NO, determines whether direct or indirect effects are predominant (Davis et al. 2001, Korhonen et al. 2005).

Direct effects dominate in the presence of low concentrations of NO, as produced typically by eNOS and nNOS. NO reacts directly with transition metals, present in many enzymes leading to regulation of enzyme activity (Figure 7). The best known target of NO is the iron part of haem of the soluble guanylyl cyclase (sGC). NO stimulates production of cyclic GMP (cGMP) by sGC, which mediates the relaxing effect of NO on vascular smooth muscle (Korhonen et al. 2005, Bryan et al. 2009).

In the presence of a high NO concentration, as produced by iNOS, indirect effects induced by RNS predominate. Peroxynitrite ($ONOO^-$) is formed when NO reacts with superoxide ($O_2^{\bullet -}$) in the absence of adequate levels of superoxide scavenging agents

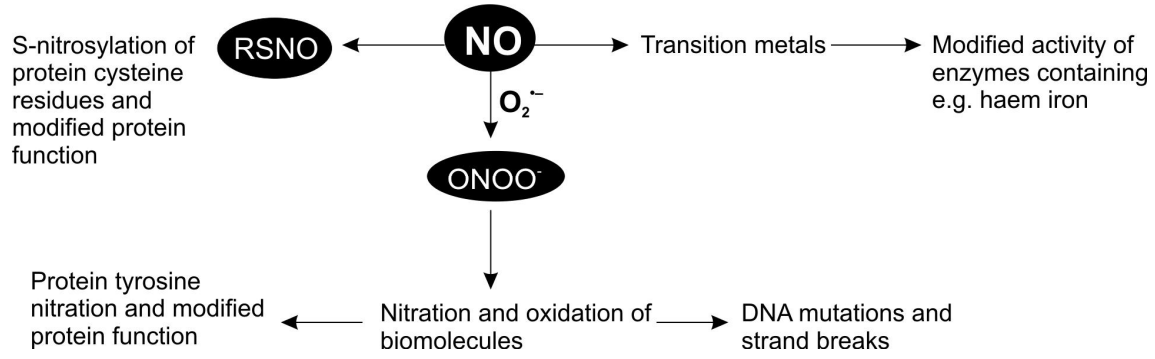

Figure 7. *Molecular effects of NO. RSNO=S-nitrosylated thiol.*

such as superoxide dismutases (SODs). $O_2^{\bullet-}$ is generated in certain immune cells such as eosinophils, neutrophils and macrophages via the activity of NADPH oxidase or xanthine oxidase, or alternatively as a side product of mitochondrial electron transport chain. Peroxynitrite is a highly reactive molecule that can nitrate and oxidize biomolecules leading to mutations and strand breaks in DNA, changes in the structure and function of lipid membranes and lipoproteins, and changes in protein activity (Figure 7). (Davis et al. 2001, Liaudet et al. 2009, Niki 2009)

RNS regulates protein function via nitration of tyrosine residues and S-nitrosylation of cysteine residues (Figure 7). Proteins dependent on tyrosine phosphorylation and many other proteins such as Akt, MAPK, protein kinase C (PKC) and NF-κB are regulated by tyrosine nitration (Liaudet et al. 2009). S-nitrosylation refers to the incorporation of NO into thiol group (RSH) leading to the formation of S-nitrosothiol (RSNO). Many transcription factors, kinases, ion channels and caspases are regulated by S-nitrosylation. In addition, the antioxidant glutathione can be S-nitrosylated and the S-nitrosoglutathione (GSNO) formed has been claimed to serve as a storage and transporter molecule for NO (Martinez and Andriantsitohaina 2009, Martinez-Ruiz et al. 2011).

5.2 Regulation of cell death by NO

Endogenous or exogenous NO can have both pro- and anti-apoptotic effects depending on the cell type, rate and amount of NO production and redox state of the cell. In simplistic terms, a high concentration of NO and a high oxidative state of the cell favour apoptotic or necrotic effects of NO whereas low levels of NO and an abundance of antioxidants support the anti-apoptotic action of NO. However, the cell type is also a critical determinant of the NO response. (Burney et al. 1997, Kim et al. 2001)

5.2.1 Anti-apoptotic mechanisms

NO increases survival in several cell types in the presence or absence of apoptotic stimuli (Genaro et al. 1995, Ciani et al. 2002b). The anti-apoptotic mechanisms of NO can be divided into cGMP-dependent and -independent mechanisms. NO-induced cGMP activates cGMP-dependent protein kinase (PKG), which directly phosphorylates

transcription factors such as cAMP response element-binding protein (CREB) ending up in regulation of expression of several genes, such as anti-apoptotic Bcl-2 (Walton and Dragunow 2000, Ciani et al. 2002a). Indeed, NO has been reported to up-regulate or retain levels of Bcl-2 and the mechanism may involve CREB (Genaro et al. 1995, Ciani et al. 2002a). Activation of PKG also leads to induction of the PI3K/Akt pathway and Akt-mediated phosphorylation of Bad (Ha et al. 2003). Modulation of these Bcl-2 family proteins results in a suppression of cytochrome c release and inhibition of mitochondrial apoptotic pathway. Cyclic GMP-independent anti-apoptotic mechanisms include S-nitrosylation of cysteine residues of caspases and Bcl-2, leading to inactivation of caspases and prevention of Bcl-2 degradation, respectively (Dimmeler et al. 1997, Azad et al. 2006). Increased levels of hsp70 and heme oxygenase-1 have also been reported to mediate the anti-apoptotic effect of NO in a cGMP-independent manner (Kim et al. 1995, Kim et al. 1997).

5.2.2 Pro-apoptotic mechanisms

Mitochondria occupy a central position in mediating the pro-apoptotic effects of NO. NO (or probably RNS) inhibits complexes I and II in the mitochondrial electron transport chain (Drapier and Hibbs 1988, Brown and Borutaite 2002). NO itself also competes with oxygen for binding to complex IV (cytochrome c oxidase) and inhibits this enzyme at low oxygen concentrations (Brown and Cooper 1994, Taylor and Moncada 2010). Because the function of the enzymes in the electron transport chain creates a proton gradient that is used to produce ATP, inhibition of these enzymes attenuates ATP production. Additionally, a few per cent of the oxygen consumed by the electron transport chain is converted to $O_2^{\bullet-}$ even in normal physiological situation but inhibition of these enzymes leads to increased $O_2^{\bullet-}$ leakage (Taylor and Moncada 2010). The subsequent formation of peroxynitrite leads to oxidation and nitration of DNA resulting in DNA strand breaks (Burney et al. 1997), upregulation of p53 (Messmer et al. 1996) and activation of PARP, an enzyme required in DNA repair (Heller et al. 1995). PARP-mediated DNA repair is energy-consuming and may lead to the depletion of ATP and cell death (Heller et al. 1995). Activation of p53 results also in up-regulation of pro-apoptotic Puma and Noxa and down-regulation of anti-apoptotic Bcl-2 and Bcl-X_L (Li and Wogan 2005). Furthermore, NO and peroxynitrite may activate

apoptosis by inducing mitochondrial permeability transition (Hortelano et al. 1997, Brown and Borutaite 2002). The mechanism seems to involve thiol oxidation of the mPT regulator ANT (Vieira et al. 2001, Piantadosi et al. 2002).

Another pro-apoptotic mechanism of NO is up-regulation of expression of apoptosis-related genes, such as Fas receptor and Fas ligand, DR5 and TRAIL (Fukuo et al. 1996, Chlichlia et al. 1998, Garban and Bonavida 1999, Huerta-Yepez et al. 2009). This up-regulation is mediated by S-nitrosylation of the transcriptional repressor Yin-Yang 1 (YY1), which leads to inhibition of its binding to Fas/DR5 promoter (Garban and Bonavida 2001, Hongo et al. 2005, Huerta-Yepez et al. 2009). Additionally, NO has been shown to promote apoptosis by inhibiting NF-κB activity via multiple mechanisms. NO S-nitrosylates p50 subunit of NF-κB and IKKβ leading to inhibition of DNA binding and suppressed activation of NF-κB, respectively (DelaTorre et al. 1997, Reynaert et al. 2004). NO has also been shown to increase the expression of the NF-κB-inhibitor, IκBα (Peng et al. 1995). The role of antioxidants, especially glutathione, is controversial in NO-induced apoptosis (Li and Wogan 2005).

5.3 NO, asthma and eosinophils

NO is produced in high amounts in the lungs of asthmatics and exhaled NO levels are strongly associated with eosinophilic airway inflammation, total IgE levels and skin-prick test scores (Jatakanon et al. 1998, Lehtimaki et al. 2001, Payne et al. 2001, Louhelainen et al. 2008). Asthmatics exhibit increased expression of iNOS in epithelial and inflammatory cells of the respiratory system and an increased concentration of exhaled NO when compared to healthy individuals (Kharitonov et al. 1994, Persson et al. 1994, Saleh et al. 1998, Maa et al. 2003). In addition to NO, these inflammatory cells produce superoxide and peroxidases and this mixture can evoke a high level of oxidative injury in the airway tissues of asthmatics (Louhelainen et al. 2008, Comhair and Erzurum 2010). However, the role of NO in asthma seems to be complex. NO has been shown to possess both enhancing and reducing properties with regard to lung eosinophilia.

The exhaled NO level correlates to BAL and sputum eosinophilia, eosinophil activation products and bronchial hyperresponsiveness (Jatakanon et al. 1998, Lehtimaki et al. 2001, Payne et al. 2001). Mice lacking iNOS showed reduced

accumulation of eosinophils in acute, but not in a chronic model of airway inflammation (Xiong et al. 1999, Naura et al. 2010). Consistently, inhibitors of NOS have reduced pulmonary eosinophilia in mice with acute lung inflammation (Feder et al. 1997). In patients with asthma, the exhaled NO was reduced by an inhaled glucocorticoid and an inhibitor of iNOS (Kharitonov et al. 1996, Yates et al. 1996). However, inhibitors of iNOS had no efficacy in reducing airway hyperreactivity or inflammatory cell numbers after allergen challenge in patients with asthma, even though iNOS inhibition reduced exhaled NO levels (Hansel et al. 2003, Singh et al. 2007).

NO have been shown to exert also inhibitory effects on pulmonary eosinophilia. In an allergic rat model, a form of NO-releasing prednisolone, was more potent than prednisolone alone in reducing pleural eosinophilia (Oliveira et al. 2008). In this rat model, the NO donor diethylenetriamine (DETA)-NONOate was also efficient in reducing eosinophil levels alone. Furthermore, in children with and without asthma, the apoptotic rate of sputum eosinophils was correlated to exhaled NO levels (Pontin et al. 2008). In addition, inhibition of NO production in eosinophils obtained from asthmatics resulted in increased Bcl-2 expression and decreased eosinophil apoptosis (Maa et al. 2003).

NO donors have been reported to have both apoptosis- and survival-promoting effects on eosinophils. NO donors (SNAP, SIN-1, S-nitroso-L-cysteine, NOC-18) were shown to induce eosinophil apoptosis or necrosis in the presence or absence of IL-5 (Beauvais and Joly 1999, Zhang et al. 2003). Our group demonstrated that the effect of NO donor SNAP in the presence of IL-5 could be reversed by the JNK inhibitor L-JNKI1 and pan-caspase inhibitor Z-Asp-CH_2-DCB but was independent of cGMP (Zhang et al. 2003). SNAP had no effect on Bcl-2 expression (Zhang et al. 2003). NO donors were reported to display reduced eosinophil apoptosis in the presence of heme-containing compounds due to the formation of nitrosyl-heme complex (Beauvais et al. 1995, Beauvais and Joly 1999). NO was anti-apoptotic also in the presence of Fas and both of these survival-prolonging effects were cGMP-dependent (Beauvais et al. 1995, Hebestreit et al. 1998). The role of NO in the regulation of eosinophil adhesion, chemotaxis or migration is controversial (Thomazzi et al. 2004, Pelaquini et al. 2011). In summary, NO has a complex role in asthma and in the regulation of eosinophil apoptosis and functions.

6 Neuropeptide S receptor 1

G-protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors. The G-protein-mediated signalling system consists of a receptor, a heterotrimeric G protein (α-subunit that hydrolyzes GTP and βγ-complex) and an effector, which may be an enzyme or an ion channel that is activated or inactivated by the G protein. GPCR-mediated system is versatile because many types of α, β and γ subunits exist, both the α-subunit and the βγ-complex are able to activate effectors and most GPCRs are able to activate several different G proteins. (Wettschureck and Offermanns 2005, Smrcka 2008)

The basic properties of heterotrimeric G proteins are defined by the α-subunit, which comprises of four families: $G\alpha_s$, $G\alpha_i/G\alpha_o$, $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$. Both $G\alpha_s$ and $G\alpha_i/G\alpha_o$ couple receptors to adenylyl cyclase but have opposite effects on the enzyme activity leading to increased or inhibited cAMP production, respectively. $G\alpha_q/G\alpha_{11}$ couples receptors to phospholipase C (PLC) terminating in the release of calcium from ER to cytosol and activation of protein kinase C. $G\alpha_{12}/G\alpha_{13}$ are typically stimulated by receptors coupling also to $G\alpha_q/G\alpha_{11}$ and are able to activate several signalling pathways (e.g. phospholipase A_2, JNK, RhoA). In granulocytes, GPCR-mediated pathways typically regulate adhesion, chemotaxis, superoxide production and degranulation. (Wettschureck and Offermanns 2005)

Neuropeptide S receptor 1 (NPSR1, also known as GPRA, GPR154, VRR1) is a GPCR that was simultaneously identified by two groups. It was identified as a G protein coupled receptor for asthma susceptibility (GPRA) (Laitinen et al. 2004) and as a vasopressin-related receptor 1 (VRR1) (Gupte et al. 2004). Neuropeptide S (NPS) was recognized as the endogenous ligand for the receptor (Gupte et al. 2004).

6.1 Genetic association study

Genetic susceptibility is recognized as a strong factor influencing asthma morbidity (Moffatt et al. 2010). In a genome-wide search for asthma susceptibility genes conducted in Finnish and French-Canadian families, a 133 kb risk segment was identified in chromosome region 7p14-15 (Laitinen et al. 2001). Thirteen SNPs formed

seven alternative combinations (haplotypes H1-H7) in the most conserved 77 kb area, all with frequencies over 2 % in the populations studied. Relative risk for serum total IgE above 100 IU/ml was 1.4 among homozygous H4 and H5 carriers in Finnish study population and relative risk for asthma was 2.5 among H2 carriers in the Canadian study population. NPSR1 was one of the two genes that were found in the 133 kb risk area.

One SNP was located in the third exon of the NPSR1 gene and this results in an altered amino acid (N107I) in the putative ligand binding area of the receptor (Laitinen et al. 2004). This mutation of NPSR1 was shown to lead to approximately 10-fold higher agonist potency but not to affect the ligand binding affinity (Reinscheid et al. 2005). Additionally, the mutation caused increased cell surface expression of the mutant NPSR1 receptor and resulted in an increased maximal efficacy (Bernier et al. 2006). Polymorphisms of the NPSR1 locus have been linked to susceptibility for asthma, high IgE, allergic conditions or bronchial hyperresponsiveness also in other populations (Laitinen et al. 2004, Kormann et al. 2005, Melen et al. 2005, Feng et al. 2006, Hersh et al. 2007, Malerba et al. 2007, Daley et al. 2009, Castro-Giner et al. 2010, Andiappan et al. 2011). In addition to asthma-related traits, polymorphism of the NPSR1 locus has also been linked to susceptibility to inflammatory bowel disease, schizophrenia and panic disorder (D'Amato et al. 2007, Domschke et al. 2011, Lennertz et al. 2011).

6.2 Expression

Two isoforms of NPSR1 (A and B) gene exist and they differ in the exons at the 3' end (Laitinen et al. 2004). These two isoforms were shown to regulate the same genes but the difference was quantitative: NPSR1-A induced stronger responses in intracellular secondary mediators and all other genes except CD69, a marker of Treg cells (Pietras et al. 2011). The expression of both NPSR1 and NPS has been consistently found in the central nervous system, especially hypothalamus and retina in both humans and rodents (Gupte et al. 2004, Xu et al. 2004, Allen et al. 2006, Xu et al. 2007). The expression in the lungs is rather conflicting. Originally, NPSR1 expression was found in bronchial epithelial and smooth muscle cells and it was demonstrated that the two isoforms were differentially expressed in asthmatic airways when compared to healthy airways (Laitinen et al. 2004). Additionally, NPSR1 mRNA was found to be up-regulated in the lungs in a mouse model of asthma (Laitinen et al. 2004). However, this result could not

be repeated in studies conducted by other groups (Allen et al. 2006, Zhu et al. 2011). Those studies did not find any significant expression of NPSR1 in human or rodent lungs (Xu et al. 2004, Allen et al. 2006, Zhu et al. 2011). Interestingly, peripheral blood leukocytes were shown to express NPSR1 and NPS (Xu et al. 2004, Pulkkinen et al. 2006). Monocytes and eosinophils expressed both isoforms of NPSR1 and Th cells expressed mainly isoform B (Pulkkinen et al. 2006). NPSR1-positive eosinophils and macrophages were found in the sputum of asthmatics and non-asthmatics with or without atopy (Pulkkinen et al. 2006).

6.3 Cell studies and knockout models

NPS was shown to increase both intracellular cAMP and Ca^{2+} levels suggesting that NPSR1 couples to both $G\alpha_s$ and $G\alpha_q$ (Gupte et al. 2004)(Figure 8). In a microarray analysis of HEK-293H cells transfected to overexpress NPSR1-A, NPS mostly altered the expression of genes related to cell proliferation, morphogenesis and the immune response. NPS was shown to dose-dependently increase mRNA expression of IL-8, MMP-10, inhibin beta A (INHBA), ephrin receptor A2 (EPHA2) and tenascin (Vendelin et al. 2006, Orsmark-Pietras et al. 2008). In this NPSR1-A-overexpressing cell line, NPS had an inhibitory effect on cell proliferation but did not induce apoptosis (Vendelin et al. 2006). NPS has been shown to affect the functions of immune cells. NPS was able to stimulate migration and phagocytosis of *E. Coli* by mouse macrophages (Pulkkinen et al. 2006). Additionally, NPS increased splenic lymphocyte proliferation, as well as the production of pro-inflammatory cytokines and phagocytosis of pulmonary alveolar macrophages in pigs (Yao et al. 2011). Furthermore, NPS was shown to stimulate chemotaxis of human monocytes via NPSR1 activation (Filaferro et al. 2012). The function of NPSR1 in eosinophils is completely unknown.

Two different NPSR1-deficient mice were developed to clarify the role of NPSR1 in lung inflammation. In both studies, a deficiency of functional NPSR1 resulted in unaltered development of airway inflammation in mice sensitized and challenged with OVA or *Aspergillus Fumigatus* (Allen et al. 2006, Zhu et al. 2011). However, intracerebroventricular administration of NPS led to an increase in the respiratory

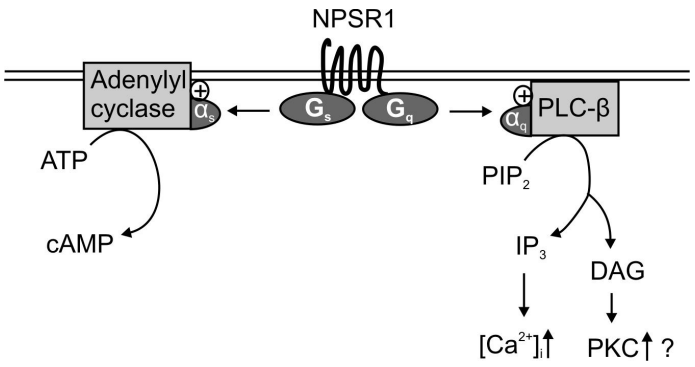

Figure 8. *Proposed model of intracellular pathways activated by NPSR1. G=G protein, PLC=phospholipase C, PIP_2= phosphatidylinositol 4,5-bisphosphate, IP_3=inositol 1,4,5-triphosphate, DAG=diacylglycerol, PKC=protein kinase C.*

frequency at baseline and in response to the bronchoconstricting agent, metacholine, in wild-type but not NPSR1-deficient mice (Zhu et al. 2011). In addition, NPS has been demonstrated to increase locomotor activity, stress-related corticosterone release and to have an anxiolytic effect in NPSR1-dependent manner (Zhu et al. 2010). Therefore, NPSR1 may regulate respiratory function through CNS and affect asthma also via a stress-related pathway. However, the role of NPSR1 in airway inflammation is obscure and the expression and function of NPSR1 in human immune cells such as eosinophils has not been adequately studied.

7 Orazipone

Orazipone [OR-1384; 3-({[4-methylsulfonyl]phenyl}methylene)-2,4-pentandione] and its derivatives OR-1958 [3-(3-chloro-4-methanesulfonyl-benzylidene)-pentane-2,4-dione] and OR-2370 [3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione] are anti-inflammatory compounds developed by the Finnish pharmaceutical company Orion Corporation (previously Orion Pharma)(Figure 9). Their mechanism of action is unique, since they probably form reversible conjugates with thiol groups (-SH) of proteins and glutathione (Nissinen et al. 1997). Orazipone and its derivatives have been reported to exert anti-inflammatory effects in animal models of colitis and asthma (Wrobleski et al. 1998, Ruotsalainen et al. 2000). Orazipone has decreased lung eosinophilia in PAF-induced airway inflammation in guinea pigs and has been able to reduce allergen-induced airway inflammation in rats (Ruotsalainen et al. 2000). At the cellular level,

orazipone has been shown to inhibit cytokine release from monocytes and T-cells and to reduce superoxide production in neutrophils (Nissinen et al. 1997). Additionally, orazipone and OR-1958 reduced histamine release and TNF-α production from mast cells (Vendelin et al. 2005a). In J774 macrophages, orazipone was shown to inhibit NF-κB and STAT1 activation and iNOS expression and these effects were mediated by the thiol-modulating properties of the compound (Sareila et al. 2008). The effects of orazipone on eosinophils at the cellular level are completely unknown.

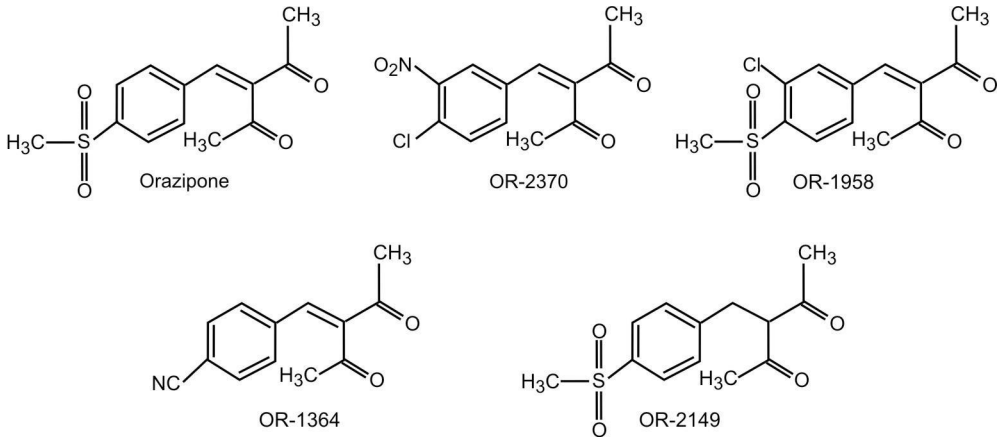

Figure 9. *Orazipone (OR-1384) and its derivatives OR-2370, OR-1958 and OR-1364. OR-2149 is a negative control compound lacking the thiol-binding property due to the presence of one reduced double-bond. (Reprinted with permission from: Kankaanranta et al. 2006, Mol Pharmacol. 69: 1861-70 © American Society for Pharmacology and Experimental Therapeutics)*

AIMS OF THE STUDY

Increased eosinophil survival and activation is important in the pathogenesis and maintenance of eosinophilic airway inflammation in asthma. The induction of eosinophil apoptosis is an important strategy to alleviate eosinophilic inflammation. The general aim of this study was to examine factors, pathophysiological components as well as drugs that regulate human eosinophil apoptosis and survival. This study was conducted to increase understanding of the pathophysiology of eosinophilic inflammation and to support the development of novel drugs.

The detailed aims of this study were:

1. to evaluate if agonists of Toll-like receptor 9 such as bacterial DNA regulate eosinophil survival and to characterize the mechanisms involved in this response (I).

2. to examine the effect of a candidate drug, orazipone, on eosinophil apoptosis and to elucidate the mechanism of action (II).

3. to investigate mechanisms involved in eosinophil apoptosis induced by exogenous NO in the presence of survival-prolonging stimulus GM-CSF, focusing especially on the role of mitochondria, JNK and caspases (III-IV).

4. to study NPSR1 expression in eosinophils and to test the hypothesis that expression is enhanced in patients with increased asthma severity and in subjects with elevated serum total IgE. One further aim was to evaluate the function of NPSR1 in eosinophils (V).

MATERIALS AND METHODS

1 Blood donors (I-V)

Blood donors were healthy subjects and patients with asthma or allergy. Before the blood donation, subjects provided a written informed consent to a study protocol approved by the ethics committee of Tampere University Hospital (Tampere, Finland). Characteristics of subjects with high and low IgE are shown in Table 5 and the characteristics of patients with asthma in Table 6 (V). The healthy volunteers shown in Table 6 were recruited by means of an advertisement and all asthmatics shown in Table 6 were recruited from the Lung and Allergy clinic in Karolinska University Hospital, Stockholm. The study was approved by the local ethics committee at Karolinska University Hospital in Stockholm Dnr 2006/728-31/2 and the participants all provided written informed consent. Asthmatic subjects (V) were grouped according to inhaled corticosteroid dose needed to control symptoms (i.e. mild asthma $\leq$800 µg budesonide equivalents, severe asthma $\geq$1200 µg budesonide equivalents + long-acting β_2 agonists and/or anti-leukotriene drugs). The inclusion criteria for the established aspirin-intolerant asthma (AIA) group were a positive aspirin challenge test within five years or an unequivocal history documented in hospital files.

2 Granulocyte isolation and culture (I-V)

Eosinophil and neutrophil isolation was conducted under sterile conditions. One hundred ml of venous blood was collected into 20 ml of solution containing acid citrate dextrose (ACD) anticoagulant. After sedimentation of red blood cells for 40-60 minutes in ACD and hydroxyethyl starch solution, the leukocyte-rich portion was collected and centrifuged, and the pellet layered onto Ficoll gradient solution. After centrifugation at

Table 5. *Characteristics of subjects with high and low IgE (V). Shown left are characteristics of all subjects and shown right are characteristics of those subjects participating in the study on NPSR1 expression.*

	All subjects		NPSR1 expression	
	IgE >100	**IgE<100**	**IgE >100**	**IgE<100**
No. subjects	21	23	7	6
Total serum IgE (IU/ml), mean (range)	240 (101-763)	26 (<5-88)	256 (142-526)	23 (5-71)
Healthy	2/21	17/23	0/7	6/6
Asthmatic	9/21	1/23	5/7	0/6
- Total serum IgE (IU/ml), mean (range)	249 (101-763)	13	190 (142-300)	-
- Use of ICS	4/9	1/1	3/5	-
- Dose of ICS (µg), mean (range)	1250 (400-2000)	800	1000 (400-1600)	-
- Use of long-acting β_2-agonist	2/9	0/1	1/5	-
- Use of leukotriene receptor antagonist	1/9	0/1	1/5	-
- Use of short-acting β_2-agonist when needed	5/9	0/1	2/5	-
- Use of regular oral antihistamine	3/9	0/1	3/5	-
- Use of regular nasal glucocorticoid	1/9	1/1	1/5	-
Allergic	10/21	5/23	2/7	0/6
- Total serum IgE (IU/ml), mean (range)	253 (116-526)	31 (17-45)	421 (316-526)	-
- Use of regular oral antihistamine	1/10	0/5	1/2	-
- Use of regular nasal glucocorticoid	1/10	2/5	0/2	-

ICS=inhaled corticosteroid dose (budesonide equivalent).

Table 6. *Characteristics of patients with asthma (V).*

	Healthy	**Mild$_1$**	**Severe$_2$**	**AIA$_3$**
No. subjects	10	10	10	8
Sex, male/female	3/7	1/9	4/6	4/4
Age, yr	34 ± 11	39 ± 15	48 ± 11	44 ± 12
Skin prick test, pos/neg	0/10	8/2	5/5	1/7
FEV_1, L	4.1 ± 0.9	3.0 ± 0.7	2.2 ± 0.8	3.1 ± 0.7
FEV_1, % predicted	118 ± 10	101 ± 15	73 ± 17	94 ± 17
FVC, L	4.9 ± 1.1	3.9 ± 0.8	3.5 ± 1.1	4.5 ± 1.1
FVC, % predicted	120 ± 12	111 ± 16	94 ± 15	112 ± 14
FENO, ppb	11.8 ± 4.5	15.8 ± 16.4	37.9 ± 35.1	26.5 ± 17.5
ICS dose, µg	0 ± 0	516 ± 212	2144 ± 774	440 ± 213
Blood lymphocytes ($x10^9$/l)	2.0 ± 0.4	2.1 ± 0.5	2.7 ± 0.8	2.2 ± 0.6
Blood monocytes ($x10^9$/l)	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1
Blood neutrophils ($x10^9$/l)	3.3 ± 1.3	3.6 ± 1.3	4.7 ± 2.1	4.1 ± 0.8
Blood eosinophils ($x10^9$/l)	0.1 ± 0.1	0.2 ± 0.1	0.8 ± 1.0	0.6 ± 0.2

Results are shown as mean ± SD.
$_1$ICS dose $\leq$ 800 µg budesonide equivalents
$_2$ ICS dose $\geq$1200 µg budesonide equivalents + long-acting β_2 agonists and/or anti-leukotriene drugs
$_3$A positive aspirin challenge test within five years or an unequivocal history documented in hospital files was the inclusion criterion for the group of aspirin intolerant asthma (AIA)
FEV_1=forced expiratory volume in 1 sec, FVC=forced vital capacity, FENO=fractional exhaled nitric oxide, ICS=inhaled corticosteroid dose (budesonide equivalent).

400 g for 30 min at room temperature (RT), the mononuclear cell layer was carefully removed and the granulocyte-rich pellet was washed and resuspended in Hank's balanced salt solution (HBSS). The remaining red blood cells were discarded by hypotonic lysis and granulocytes were washed in HBSS. In the neutrophil assays, the cells were resuspended at 2 x 10^6 cells/ml in Dutch modification of RPMI 1640 (containing 10 % fetal bovine serum (FBS), 50 units/ml penicillin, 50 µg/ml

streptomycin and 2 mM L-glutamine) and cultured at +37°C and 5 % CO_2 for the indicated times. Neutrophil purity was at least 99 %.

In the subsequent parts of the eosinophil separation, granulocytes were resuspended in RPMI 1640 containing 2 % FBS and 5 mM EDTA and anti-CD16-conjugated microbeads were added. After 40-60 minutes of incubation at +4°C, granulocytes were loaded onto a magnetic column where neutrophils with bound magnetic microbeads became attached whereas eosinophils passed through. Separated eosinophils were re-suspended in Dutch modification of RPMI 1640 containing antibiotics and L-glutamine. The eosinophil purity was at least 99 % and the contaminating cells consisted mostly of lymphocytes or neutrophils. Eosinophils were re-suspended at $1x10^6$ /ml and cultured in the presence or absence of test compounds at +37°C and 5 % CO_2 for the indicated times.

In some experiments (I), eosinophils were further purified by CD19- and CD304-negative selection to remove any possibly contaminating B-cells and plasmacytoid dendritic cells. Briefly, after the normal isolation process, the eosinophils were incubated with anti-CD19 and anti-CD304 microbeads in RPMI 1640 with 2 % FCS and 5 mM EDTA for 15 min at +4°C, loaded onto the magnetic column, re-suspended in Dutch modification of RPMI 1640 and counted. To ensure absence of contaminating B-cells (CD19+) and plasmacytoid dendritic cells (CD123+ CD303+), cells were labelled with FITC-conjugated anti-CD19 antibody or double-stained with FITC-conjugated anti-CD303 and R-phycoerythrin (PE) -conjugated anti-CD123 or the respective isotype controls (Table 7). After 20 min incubation in the dark at +4°C, excess antibody was removed by washing the cells with PBS buffer. B-cells and pDC were identified based on their low granularity and specific staining (CD19+ or CD123+ CD303+, respectively) with flow cytometric analysis by FACScan (Becton Dickinson, San Jose, CA, USA).

3 Cell death assays

3.1 DNA fragmentation assay (I-V)

DNA fragmentation conducted by endonucleases is a specific feature of cells undergoing apoptosis. DNA fragmentation was determined by flow cytometric analysis of permeabilized and propidium iodide (PI)-stained cells. After 40 hours of incubation eosinophils (1×10^5) were suspended into 300 µl of hypotonic solution containing 0.1 % sodium citrate, 0.1 % Triton-X and 50 µg/ml PI. Neutrophils were washed in PBS, fixed in 70 % ethanol and incubated at +4°C for at least 30 min. The neutrophil pellet was washed with PBS and resuspended in 50 µg/ml propidium iodide solution in PBS. After a time ranging from one hour to overnight incubation at +4°C protected from light, the samples were analysed by flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA) with excitation and emission wavelengths of 488 and 550 nm, respectively. The cells with reduced relative DNA content were considered apoptotic.

3.2 Annexin-V FITC / Propidium iodide counterstaining (I-IV)

The expression of phosphatidylserine residues on the outer leaflet of the cell membrane is a specific feature of apoptosis and it functions as a signal for engulfment by phagocytes. Phosphatidylserine levels on the cell surface can be detected by Annexin-V staining. Propidium iodide, permeable only to cells with a disrupted cell membrane, may be used to discriminate early (Annexin-V^+ propidium $iodide^-$) from late (Annexin-V^+ propidium $iodide^+$) apoptotic cells and to discriminate necrotic cells (Annexin-V^- propidium $iodide^+$). After the indicated time of incubation, eosinophils (1×10^5) were washed with phosphate buffered saline (PBS) and resuspended in 195 µl of binding buffer containing 10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, where 5 µl of Annexin-V FITC solution (containing 50 mM Tris, 100 mM NaCl, 1 % bovine serum albumin, 0.02 % sodium azide, pH 7.4) was added. After 10 min incubation at room temperature, the cells were washed with PBS and suspended in 190 µl of binding buffer. Propidium iodide at a final concentration of 10 µg/ml was added 1 min prior to

flow cytometric analysis. Cells positive for Annexin-V were considered apoptotic whereas Annexin-V^- propidium $iodide^+$ cells were considered necrotic.

3.3 Morphological analysis (I-V)

Analysis of eosinophil morphology was conducted by bright field microscope. After 40 hours of incubation, eosinophils were spun onto cytospin slides (25 g, 5 min) and fixed in methanol for 15 min. Subsequently, cells were stained with May-Grünwald-Giemsa. Cells with coalesced nucleus and condensed chromatin were classified as early apoptotic cells and "ghosts" as late apoptotic cells.

3.4 Caspase activity assays (II)

Caspase 3/7, 8 and 9 activity assays, based on cleavage of luminogenic caspase substrates, were carried out according to the instructions of the manufacturer (Promega, Madison, WI, USA). Cells were incubated with the test substances and transferred to white 96-well plates. An equal volume of Caspase Glo-reagent (3/7, 8 or 9) was added to the cells, mixed and incubated for 1 h at RT before measurement of luminescence.

3.5 Mitochondrial membrane potential (IV)

In living cells, the mitochondrial inner membrane is polarized with a negative charge inside. During cell death, the mitochondrial membrane potential ($\Delta\Psi_m$) collapses. The state of the mitochondrial membrane potential was determined by staining cells with the cationic dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Salvioli et al. 1997) (Table 7). In cells with intact $\Delta\Psi_m$, JC-1 accumulates into the negatively charged matrix side of the mitochondrial membrane. After JC-1 has reached a threshold concentration, it aggregates and emits red fluorescence. In cells with collapsed $\Delta\Psi_m$, JC-1 remains in its monomeric form in the cytoplasm emitting green fluorescence. After the indicated time of incubation, 2.5-5 x 10^5 eosinophils were stained with 10 µg/ml JC-1 for 15 min at +37°C, washed twice in medium and resuspended in PBS. Flow cytometric analysis was carried out. K^+ ionophore

valinomycin is a drug able to collapse $\Delta\Psi_m$ and was used as a positive control (Salvioli et al. 1997). The gates defining cells with intact and lost $\Delta\Psi_m$ were based on cells treated with GM-CSF (10 pM) and valinomycin (1 µM), respectively, in each experiment individually.

3.6 Mitochondrial permeability transition (IV)

Mitochondrial permeability transition was determined by the MitoProbe transition pore assay kit (Molecular Probes Inc., Eugene, OR, USA), a technique based on calcein acetoxymethyl ester (AM) and $CoCl_2$ (Petronilli et al. 1999). Calcein AM (Table 7), a non-fluorescent molecule, accumulates into cells including mitochondria and its cleavage by intracellular esterases liberates the fluorescent calcein that is not able to cross mitochondrial or cell membranes. $CoCl_2$ is a fluorescence quencher that accumulates into mitochondria only when the mPT pore is open. Therefore, a reduced calcein fluorescence is indicative of mPT. The calcein AM/$CoCl_2$-method requires the activity of intracellular esterases that are functional only in viable cells restricting use of this method. Ionomycin induces complete mPT and was used as a positive control. Cells (5×10^5) were labeled with 10 nM calcein AM, 400 µM $CoCl_2$ and/or 0.5 µM ionomycin for 15 min at +37°C, washed and re-suspended in PBS. Calcein fluorescence was determined by flow cytometric analysis. Calcein fluorescence is expressed as a percentage of its initial value.

3.7. Primary necrosis (I)

The cells were incubated in the presence and absence of study compounds for 60 min at +37°C with 5 % CO_2 and thereafter diluted in 250 µl of HBSS. Five minutes before flow cytometric analysis, PI was added at a final concentration of 20 µg/ml. PI is able to enter cells with ruptured cell membranes which is characteristic for necrosis. Cells positive for PI were considered as necrotic.

4 Eosinophil activation assays (V)

4.1 Eosinophil degranulation and mediator release assays

Eosinophils (3-5 x 10^5) were incubated in the presence or absence of test compounds for 30 min or 3 h at +37°C with 5 % CO_2 and then the culture media were collected and frozen at -80°C. Secretory IgA-coated beads used as stimulant for degranulation were prepared as previously described by Kita and co-workers (Kita et al. 1991a). ELISA for eosinophil-derived neurotoxin (EDN) (MBL International Corporation, Woburn, MA, USA), vasoactive intestinal peptide (VIP) (Cusabio Biotech Co., Ltd, Wuhan, China) or somatostatin (Uscn Life Science Inc., Wuhan, China) was carried out according to the manufacturer's instructions. All samples were assayed as duplicates.

For stimulation of peripheral blood leukocytes with NPS, cells were adjusted to a concentration of 1x10^6/ml in RPMI supplemented with L-glutamine and 0.1% BSA. One ml aliquots were incubated in 24-well plates for 6 h at +37°C with either 0-1 µM NPS, 1 µM A23187 (calcium ionophore) or 10 ng/ml LPS as well as the relevant vehicle controls. Samples were then centrifuged and supernatants collected and stored at -80°C until further analysis. Enzyme immunoassay kits for PGE_2, LTB_4 (Cayman chemical, Michigan, USA), matrix metalloproteinase-10 (MMP10) (R & D Systems, Minneapolis, MN, USA) and IL-8 (BIOrad, Hemel Hempstead, UK) were carried out according to the manufacturers' instructions.

4.2 Eosinophil peroxidase activity assay

Eosinophil peroxidase (EPO) activity was determined by oxidation of o-phenylenediamine (OPD) in the presence of hydrogen peroxide (Strath et al. 1985). Eosinophils were diluted at 1 x 10^6/ml in color-free RPMI 1640 containing FBS, antibiotics and L-glutamine. After incubation for 30 min or 3 h with the test compounds as duplicates (5 x 10^4 eosinophils in 50 µl/well), 100 µl of EPO substrate solution (0.4 mg/ml OPD, 50 mM Tris-HCl, 0.003 % hydrogen peroxide) was added to each well and incubated for 30 min at RT protected from light. Sulphuric acid (50 µl, 2 M) was added to terminate the reaction and the absorbance read with a microplate reader at 490 nm.

EPO activity in lysed eosinophils was linear at least up to an eosinophil concentration of 5×10^4 as determined by using a standard curve.

4.3 Superoxide generation

Superoxide dismutase (SOD)-inhibitable reduction of cytochrome c was used to determine generation of superoxide designating that corresponding samples differing only by the presence or absence of SOD were prepared to assess the specific formation of superoxide. Eosinophils were re-suspended at 0.5 million/ml in HBSS with 10 mM HEPES and 0.1 % gelatin. Cells were supplied with cytochrome c (100 µM) and SOD (0.6 µM) or solvent after which the test compounds or solvent were added and the cells transferred to 96-well plate, 200 µl/well as duplicates. Absorbance at 550 nm was read with a microplate reader immediately (0 min) and at 30 min intervals up to 3 h. Between the measurements cells were incubated at +37°C without CO_2. The amount of superoxide generated was calculated as nmol of cytochrome c reduced/million cells by using extinction coefficient of $2.1 \times 10^{-4}\ M^{-1}\ cm^{-1}$. Finally, values from the SOD samples were deducted from the values of corresponding samples without SOD.

4.4 CD11b surface expression

After incubation with the test substances, cells were washed with ice-cold PBS and re-suspended in 20 µl of PBS containing 0.5 % BSA. One µl of FITC-conjugated CD11b antibody or IgG2a isotype control (Table 7) was added and the cells incubated for 30 min at +4°C. The cells were washed and re-suspended in PBS and analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, California, USA).

5 Determination of intracellular cyclic AMP (V)

Eosinophils (3×10^5 at 37°C) were treated with test compound or solvent for 30 s and spinned down at 12.000 g for 10 seconds. Cells were lysed and intracellular cAMP determined with ELISA (Cell Biolabs, San Diego, CA, USA). Briefly, 210 µl of cell

lysis buffer (provided with the ELISA kit) was added to the eosinophil pellet. After incubation of 30 min at +4°C and centrifugation of 10 min at 12.000 g, supernatants (200 µl) were obtained and stored at -80°C. The samples were acetylated to improve the sensitivity of the cAMP determination and cyclic AMP levels were measured by ELISA according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA).

6 Methylation and denaturation of DNA (I)

In some experiments (I), *E. Coli* DNA was methylated at CpG dinucleotides by incubation with CpG methyltransferase M.SssI (2U/µg DNA) and 160 µM S-adenosylmethionine for 3 h at +37°C in NE buffer 2 (buffer supplied with the enzyme). Reaction was stopped by heating at +65°C for 20 min. Methylated and unmethylated DNA (for control) were purified from enzymes by phenol extraction and ethanol precipitation. Briefly, an equal volume of phenol was added to the sample. After centrifugation at 8.000 rpm for 2 min, the water and phenol phases were separated and the water phase containing DNA was transferred to another tube. DNA was incubated in 0.1 volume of sodium acetate (3 M, pH 5.5) and a double volume of 100 % ethanol over-night at -20°C. The mix was centrifuged at 10.000 rpm for 10 min at +4°C. Ethanol (100 %) was removed, after which 1 ml of 70 % ethanol was added. After centrifugation at 10.000 rpm for 5 min at +4°C, 70 % ethanol was removed carefully and the DNA was air-dried and dissolved in water.

To confirm a successful methylation process, we treated DNA with a restriction enzyme BstUI known to cleave at unmethylated CpG (10 unit BstUI/1 µg DNA in 50 µl NE buffer 2) at +60°C for 1 h. Methylated and unmethylated DNA samples were loaded onto 0.7 % agarose gel with 0.5 µg/ml ethidium bromide and electrophoresis conducted in Tris-base, acetic acid and EDTA (TAE) buffer. Methylated DNA was completely resistant to digestion by restriction endonuclease BstUI, indicating successful methylation. *E. Coli* and salmon sperm DNA were diluted in sterile water and always made single-stranded before use by heating at +95°C for 10 minutes and then rapidly cooling on ice.

7 NPSR1 expression by flow cytometry (V)

Previously characterized (Laitinen et al. 2004, Vendelin et al. 2005b, Pulkkinen et al. 2006) antibodies raised against intracellular epitopes of NPSR1-A and -B were used to investigate NPSR1 expression in human eosinophils with flow cytometry. Optimal antibody concentrations were identified by titration, and purified rabbit immunoglobulins at equivalent concentrations were used as negative controls. Flow cytometric analysis was performed on fixed, permeabilised blood leukocytes by FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, California, USA). Eosinophils were gated on the basis of their high autofluorescence and high granularity according to a method previously described (Carulli et al. 1998) and specificity was confirmed by staining for eosinophil granule protein. The results are shown as MFI values after subtraction of the relevant isotype control.

8 Protein extraction and western blotting (I-V)

Eosinophil pellets were washed with ice-cold PBS and lysed in ice-cold radioimmuno precipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8, 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.5 mM phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM N-octyl-β-D-glucopyranoside). After 15-30 min incubation at +4°C, lysates were centrifuged at 12.000 g for 5 minutes at +4°C. Supernatant was mixed 1:4 in sodium dodecyl sulfate (SDS) loading buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.025 % bromophenol blue and 5 % β-mercaptoethanol) and stored at -20°C until western blotting was carried out. Protein quantification was conducted by Bradford method, based on binding of Coomassie blue to protein (Bradford 1976).

After boiling of protein samples in SDS buffer for 10 min, equal amounts of protein (20-30 µg/lane) were loaded onto SDS-polyacrylamide gel (8 % for TLR9, 10 % for lamins and JNK, 12 % for caspase-6 and NPSR1). The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a buffer containing 25 mM Tris-base,

Table 7. *Commercial antibodies used in western blotting and flow cytometry.*

Antibody	Product	Supplier
Antibodies used in western blotting		
Anti-actin I-19 rabbit polyclonal	sc-1616R	Santa Cruz Biotechnology
Anti-actin C-11 rabbit polyclonal	sc-1615R	Santa Cruz Biotechnology
Anti-caspase 6 rabbit polyclonal	9762	Cell Signaling Technology
Anti-GAPDH goat polyclonal	sc-20357	Santa Cruz Biotechnology
Anti-JNK rabbit polyclonal	sc-571	Santa Cruz Biotechnology
Anti-pJNK (Thr183/Tyr185) rabbit polyclonal	9251	Cell Signaling Technology
Anti-pJNK (Thr183/Tyr185) mouse monoclonal	sc-6254	Santa Cruz Biotechnology
Anti-lamin A/C rabbit polyclonal	2032	Cell Signaling Technology
Anti-lamin B goat polyclonal	sc-6216	Santa Cruz Biotechnology
Anti-NPSR1 rabbit polyclonal	ab92425	Abcam
Anti-nucleolin (aka C23) rabbit polyclonal	sc-13057	Santa Cruz Biotechnology
Anti-TLR9 rabbit polyclonal	3739	ProSci Inc.
Secondary goat anti-rabbit, HRP-conjugated	sc-2004	Santa Cruz Biotechnology
Secondary donkey anti-goat, HRP-conjugated	sc-2020	Santa Cruz Biotechnology
Secondary sheep anti-mouse, HRP-conjugated	NXA 931	GE Healthcare Bio-sciences
Antibodies and other reagents used in flow cytometry		
Annexin-V FITC	BMS306FICE	Bender Medsystems
Anti-CD11b mouse IgG2a, FITC-conjugated	H12140F	EuroBioSciences
Anti-CD123 mouse IgG2a, PE-conjugated	554529	BD Biosciences Pharmingen
Anti-CD19 mouse IgG1κ, FITC-conjugated	555412	BD Biosciences Pharmingen
Anti-CD303 mouse IgG1κ, FITC-conjugated	130-090-510	Miltenyi Biotech
Anti-TLR9 rat IgG2a, PE-conjugated	12-9099	eBioscience
Calcein AM	M34153	Molecular Probes
JC-1	30001	Biotium
Mouse IgG1 isotype control, FITC-conjugated	130-092-213	Chemicon International
Mouse IgG1κ isotype control, FITC-conjugated	555748	eBioscience
Mouse IgG2a isotype control, PE-conjugated	CBL601P	Chemicon International
Mouse IgG2a isotype control, FITC-conjugated	C12386F	EuroBioSciences
Propidium iodide	P4170	Sigma-Aldrich
Rat IgG2a isotype control, PE-conjugated	12-4321	eBioscience

All antibodies used in flow cytometry were monoclonal. GAPDH=glyceraldehyde 3-phosphate dehydrogenase, HRP=horseradish peroxidase, PE=phycoerythrin, AM=acetoxymethyl ester, JC-1=5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide.

250 mM glycine and 0.1 % SDS and then, electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, UK, Ltd., Little Chalfont, Buckinhamshire, UK) with a semidry blotter. Membranes were blocked in Tris-buffered saline with 0.1 % Tween-20 (TBST) containing 5 % nonfat dry milk or bovine serum albumin (BSA) for 1 hour at RT. Primary antibody (Table 7) in blocking solution was added to the membrane and incubated overnight at +4°C. The membrane was washed three times with TBST for 5 min and incubated with horse radish peroxidase-(HRP)-conjugated secondary antibody (Table 7) in the blocking solution for 30 min at RT. After three 5 min washes with TBST, bound antibody was detected by using Super Signal West Pico, Dura or Femto chemiluminescent substrate (Pierce, Rockford, Ill, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, Calif, USA) or ImageQuant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Actin was used as a loading control. For detection of actin, when necessary, the membrane was stripped of previous primary and secondary antibodies. Membranes were incubated in stripping buffer (62.5 mM Tris-Hcl, pH 6.8, 2 % SDS, 0.68 % β-mercaptoethanol) for 20 min at +55°C and washed twice with TBST.

9 RNA extraction and real-time polymerase chain reaction (V)

Eosinophils were homogenized by adding 1 ml TRIzol® reagent to a pellet of 5-8 x 10^6 cells. After 5 min incubation at RT for 5 min, the homogenate was stored at -80°C until extraction was continued. Two-hundred µl of high purity chloroform was added to the homogenate, vortexed vigorously for 15 s and allowed to stand at RT for 10 min. The sample was centrifuged at 12.000 g for 15 min at +4°C, after which three phases were distinguished: the lowest phenol-chloroform-phase and the interphase contained DNA while RNA resided in the upper water phase. The water phase was transferred to a sterile tube, chloroform added for a second time and the sample centrifuged again as described above. The sample was supplied with 0.5 ml of 2-propanol and let stand in RT for 5-10 min. RNA was precipitated to the bottom of the tube after centrifugation of 12.000 g for 8 min. Supernatant was removed and RNA washed twice by adding 1.5 ml of 75 % ethanol and centrifuging at 7.500 g for 5 min. After removal of the supernatant, the RNA precipitate was air dried for 5-10 min and finally dissolved in DEPC-water. RNA content was quantified spectrophotometrically. RNA (500 ng) was reverse-transcribed to cDNA by using Taqman Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). Parameters for the reverse transcription RT reaction were: incubation at +25ºC for 10 min, reverse transcription at +48ºC for 30 min and inactivation of reverse transcription at +95ºC for 5 min.

Complementary DNA was subjected to real-time polymerase chain reaction (PCR) with ABI PRISM 7500 Sequence Detection System applying SybrGreen chemistry (Applied Biosystems). PCR primers were CCCCCTCATCTACTGTGTCTTCA (forward) and TCTCTCCCGGAACGTCATTCT (reverse) for NPSR1-A, CCCCCTCATCTACTGTGTCTTCA (forward) and TCGTTGAGGGCAGAGCATTA (reverse) for NPSR1-B, CTGAACCATCCAGGCCAAAT (forward) and

GCCGTGTGGCAATCCAAT (reverse) for the house-keeping gene EEF1A1 (eukaryotic translation elongation factor 1 alpha 1). PCR reaction parameters were: incubation at +50ºC for 2 min, incubation at +95ºC for 10 min, 40 cycles of denaturation at +95ºC for 15 s, annealing and extension at +60ºC for 1 min. Each sample was determined in triplicate.

10 Statistics (I-V)

Results are presented as mean ± SEM and p-values below 0.05 were considered significant. Statistical significance of differences were analyzed by repeated measures one-way ANOVA with Dunnett's post test (I, III-V), Student-Newman-Keuls post test (II), t-test (I-V) or repeated measures two-way ANOVA with Bonferroni's post test (V). Association studies were performed by Spearman's correlation analysis and a rho-value above 0.3 was considered to indicate a positive correlation (V).

11 Reagents (I-V)

AEOL 10150 was a kind gift from Prof. James Crapo (University of Colorado, Denver, USA). Other reagents used in this study were obtained as follows: wortmannin, LY294002, PD169316, SP600125, negative control for SP600125, PD98059, SB202474, JNK inhibitor VIII, bongkrekic acid, apocynin, Z-DQMD-FMK (Caspase-3 inhibitor), Ac-IETD-CHO (Caspase-8 inhibitor), Z-VEID-FMK (Caspase-6 inhibitor), Ac-LEVD-CHO (Caspase-4 inhibitor), Ac-LEHD-CHO (Caspase-9 inhibitor) Q-VD-OPh, Calpeptin, May-Grünwald's eosine-methylene blue (Merck, Darmstadt, Germany), Z-Asp-CH_2-DCB (Peptide Institute, Inc., Osaka, Japan), JNK peptide inhibitor 1 and TAT control peptide (L-stereoisomers) (Alexis Corp. Läufelfingen, Switzerland), Caspase-Glo 3/7, 8 and 9 assay (Promega, Madison, VI, USA), orazipone, OR-2370, OR-1958, OR-1364, OR-2149 (Orion Corporation, Espoo, Finland) anti-CD16, anti-CD19 and anti-CD304 microbeads and the magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany), RPMI 1640 medium with Dutch modification, L-glutamine, penicilline-streptomycin (Life Technologies Ltd, Paisley,

UK), HBSS, RPMI 1640 (Lonza Walkersville Inc, Walkersville, MD, USA), human Annexin-V FITC kit (Bender Medsystems GmbH, Vienna, Austria), fetal bovine serum (Euroclone, Pero, Italy or Life Technologies Ltd, Paisley, UK), Giemsa staining solution (J.T. Baker, Deventer, Holland), MG-132 (Tocris Bioscience, Bristol, UK), *E. Coli K12* DNA, salmon sperm DNA, chloroquine (Invivogen, San Diego, CA, USA), CpG methyltransferase M.SssI and BstUI (New England Biolabs, Ipswich, MA, USA), human NPS (SFRNGVGTGMKKTSFQRAKS) (AnaSpec, San Jose, CA, USA or Sigma Genosys, Haverhill UK), cAMP ELISA kit (Cell Biolabs, San Diego, CA, USA), TRIzol® reagent (Molecular Research Center Inc,. Cincinnati, Ohio, USA), RayBio Human cytokine antibody array (RayBiotech, Inc., Norcross, GA, USA), rhGM-CSF, rhIFN-γ, rhTNF-α, enzyme immunoassay for MMP10 (R & D Systems, Minneapolis, MN, USA), enzyme immunoassay for IL-8 (BIOrad, Hemel Hempstead, UK), SNAP, leukotriene B_4, enzyme immunoassay kits for PGE_2 and LTB_4 (Cayman Chemical, Ann Arbor, MI, USA), N-acetyl-D,L-penicillamine (Alfa Aesar, Karlsruhe, Germany), monoclonal antibody CD95 (Fas) (Beckman Coulter, Brea, CA, USA), purified human secretory IgA (MP Biomedicals, Aurora, OH, USA), CNBr-activated Sepharose™ 4B, Ficoll-Paque PLUS (GE Healthcare Bio-sciences AB, Uppsala, Sweden), human EDN ELISA kit (MBL International Corporation, Woburn, MA, USA), human VIP ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, China), human somatostatin ELISA kit (Uscn Life Science Inc., Wuhan, China), JC-1 mitochondrial membrane potential detection kit (Biotium Inc., Hayward, CA, USA), MitoProbe transition pore assay kit (Molecular Probes Inc., Eugene, OR, USA). ODNs and all other reagents were from Sigma-Aldrich Co., St. Louis, MO, USA) and the ODN sequences used are shown in Table 8.

Table 8. *CpG and non-CpG oligodeoxynucleotides used in this study (I).*

ODN class	ODN name	CpG ODN Sequence	Non-CpG control sequence
A	D19	5'-ggTGCAT**CG**ATGCAGggggg-3'	5'-ggTGCATGCATGCAGggggg-3' (Dc)
B	1018	5'-tgactgtgaa**cg**tt**cg**agatga-3'	5'-tgactgtgaaccttagagatga-3' (1040)
B	2006	5'-t**cg**t**cg**ttttgt**cg**ttttgt**cg**tt-3'	
C	C274	5'-t**cg**t**cg**aa**cg**tt**cg**agatgat-3'	5'-tgcttgcaagcttgcaagca-3' (C661)
iODN	iODN	5'-ttagggttagggttagggttaggg-3'	

In sequences, small letters and capitals indicate nucleotides with phosphorothioate and phosphodiester backbone, respectively. CpG dinucleotides are shown bolded. ODNs were diluted in Tris-EDTA (10 mM Tris, pH 7.5-8.0, 1 mM EDTA) to prevent degradation of the short ODNs known to occur in acidic conditions. iODN=inhibitory ODN.

SUMMARY OF THE RESULTS

1 Regulation of eosinophil longevity (I, III-V)

Most of isolated eosinophils underwent apoptosis when cultured for 40 h in the absence of any survival-prolonging factors. As determined by DNA fragmentation assay, morphological examination and annexin-V staining, spontaneous apoptosis occurred in 53.87 ± 2.13 % (n=56), 55.53 ± 3.35 % (n=23) and 76.12 ± 2.98 % (n=12) of eosinophils after 40 h of incubation, respectively. After a shorter, 16-20 h incubation time, 25.25 ± 2.54 % (n=18) had undergone spontaneous apoptosis as assayed by annexin-V staining. Mitochondrial membrane potential was lost in 19.21 ± 3.92 % (n=5) and 49.99 ± 8.95 % (n=5) of cells after 20 h and 40 h of incubation, respectively.

Inhibition of NF-κB pathway by the pharmacological inhibitors BMS-345541, MG-132 or pyrrolidine dithiocarbamate (PDTC) increased spontaneous apoptosis as did also inhibition of p38 and PI3K by SB203580 and wortmannin, respectively (Figure 10A). This indicates the importance of these signalling pathways in the maintenance of eosinophil survival.

Spontaneous eosinophil apoptosis was found to be mediated by caspases 3 and 6 and calpains 1 and/or 2 because their inhibition reduced apoptosis markedly (Figure 10B). More careful examination with different apoptosis determination methods revealed that caspase-6 inhibitor Z-VEID-FMK totally reversed only apoptosis-related DNA fragmentation (Figure 10B) but not the morphological signs of apoptosis (apoptotic cells 62.20 ± 9.06 % vs. 75.70 ± 3.82 % in the absence and presence of caspase-6 inhibitor) or phosphatidylserine exposure (apoptotic cells 78.20 ± 5.45 % vs. 58.77 ± 7.12 % in the absence and presence of caspase-6 inhibitor). However, morphological examination also showed that the proportion of early apoptotic cells of total apoptotic cells was significantly increased from 38.04 ± 3.22 % to 75.99 ± 8.07 % by treatment with caspase-6 inhibitor indicating delayed or halted spontaneous apoptosis. Lamin A/C

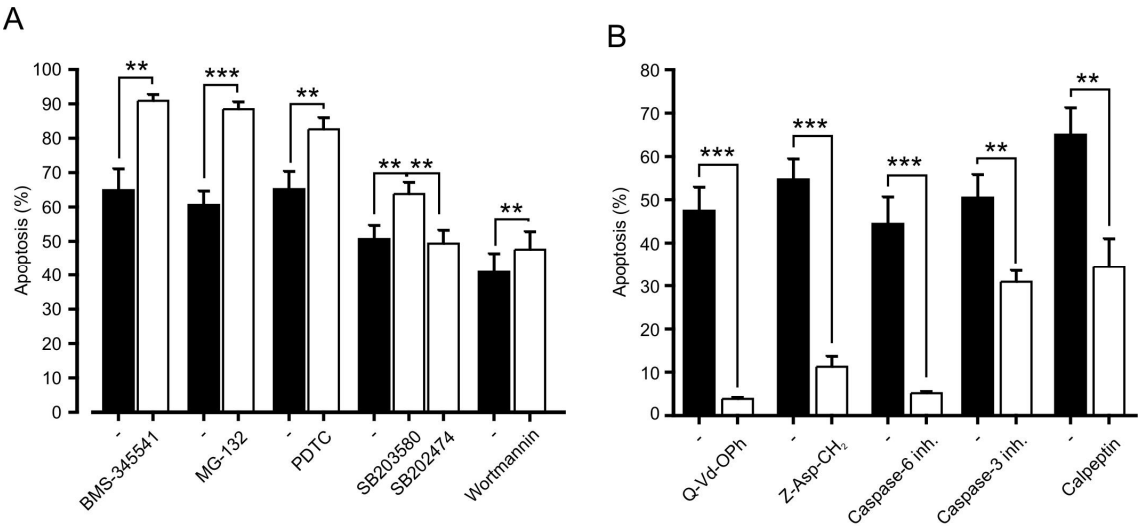

Figure 10. *Effects of inhibition of NF-κB, p38, PI3K (A), caspase and calpain (B) pathways on spontaneous eosinophil apoptosis. Eosinophils were treated with solvent or inhibitors of NF-κB pathway BMS-345541, MG-132 or pyrrolidine dithiocarbamate (PDTC, 10 µM) for 1 h, p38 inhibitor SB203580 or its negative control SB202474 (10 µM) for 30 min, PI3K inhibitor wortmannin (100 nM) for 20 min, pan-caspase inhibitors Q-Vd-OPh (20 µM), Z-Asp-CH_2 (100 µM), caspase-6 inhibitor Z-VEID-FMK (200 µM) or caspase-3 inhibitor Z-DQMD-FMK (200 µM) for 20 min or calpain inhibitor calpeptin (50 µM) for 60 min at RT. After 40 h of incubation apoptosis was determined by DNA fragmentation assay. Values are mean ± SEM of 5-13 individual experiments.*

and B are substrates of caspase-6. In confirmation of the activation of caspase-6, there was a 41.25 ± 13.75 % ($p<0.05$, n=4) decrease in caspase-6 proenzyme levels and increases of 60.66 ± 24.01 % ($p<0.05$, n=7) and 158.54 ± 67.76 % ($p<0.05$, n=5) in lamin A/C and lamin B fragment levels, respectively, in untreated cells when compared with GM-CSF-treated cells.

2 Effect of TLR9 agonists on eosinophil survival (I)

Because TLR9 agonists CpG oligodeoxynucleotides (ODNs) had shown anti-inflammatory efficacy in murine models of allergic asthma, it was studied whether synthetic CpG ODNs from classes A, B and C and native bacterial DNA could affect eosinophil longevity. Bacterial but not vertebrate DNA delayed eosinophil apoptosis (Figure 11A). Furthermore, class B and C CpG ODNs with phosphorothioate backbone inhibited eosinophil apoptosis, and the largest effect (18.1 ± 5.2 % decrease in apoptosis, $p<0.01$, n=7) was seen with class B CpG ODN 1018. Therefore, CpG ODN 1018 and its non-CpG ODN control 1040 were chosen for further studies. CpG ODN

1018 had no effect in the presence of the survival-prolonging GM-CSF ($p>0.05$, $n=6$) or apoptosis-inducing glucocorticoid budesonide ($p>0.05$, $n=6$).

A study conducted by Matsumoto and co-workers (Matsumoto et al. 2006) raised concerns about whether the survival-prolonging effect of CpG DNA was due to contaminating pDC or B-cells, known to release high amounts of interferons and/or cytokines in response to CpG DNA. The amount of these cell types as contaminating cells was very low in normally purified eosinophils (0.02 ± 0.01 % of CD19+ B-cells and 0.001 ± 0.000 % of CD123+ CD303+ pDC cells) and after extensive purification they were completely absent. Even in these highly purified eosinophils, CpG ODN 1018 reduced apoptosis by 12.80 ± 2.63 % ($p<0.01$, $n=6$).

Surprisingly, it was found that also non-CpG ODN 1040 inhibited apoptosis when apoptosis was determined by morphological examination (Figure 11A) and Annexin-V FITC staining ($p<0.01$, $n=6$). In that respect, it was of interest to study whether the effect of bacterial DNA was dependent on unmethylated CpG motifs. It was possible to successfully methylate CpG motifs of bacterial DNA (as determined by treatment with restriction endonuclease BstUI) but interestingly, the apoptosis-delaying effect of bacterial DNA was not abolished by methylation (Figure 11B). Altogether, these results

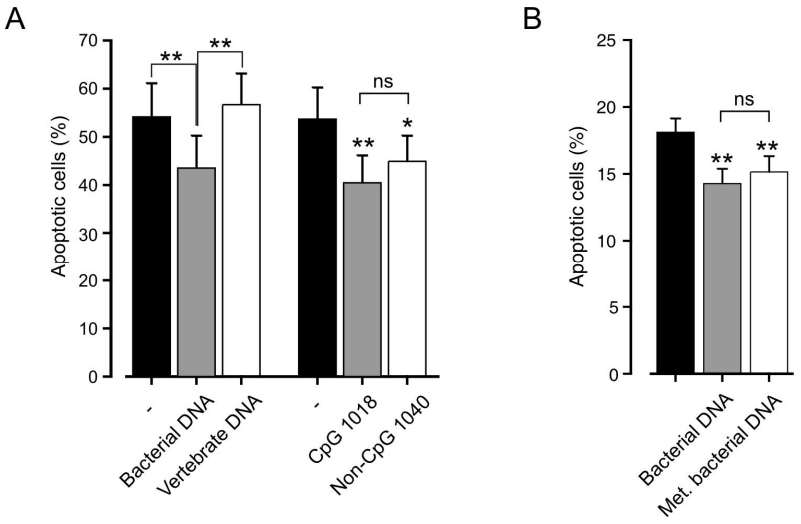

Figure 11. *Effects of bacterial DNA, CpG ODN 1018, non-CpG ODN 1040 (A) and CpG-methylated bacterial DNA (B) on eosinophil apoptosis. In A, eosinophils were treated with bacterial DNA (30 µg/ml), vertebrate DNA (30 µg/ml), CpG ODN 1018 (3 µM) or non-CpG ODN 1040 (3 µM) for 40 h and apoptosis was determined by morphological examination. In B, eosinophils were treated with unmethylated or methylated bacterial DNA (30 µg/ml) for 20 h and apoptosis assayed by Annexin-V FITC staining. Bacterial/vertebrate DNA was always added at the beginning and once after 16-20 h incubation. (Reprinted with permission from: Ilmarinen et al. 2009, Pul Pharmacol Ther. 22:167-76. © Elsevier Ltd., modified)*

indicate that the anti-apoptotic effect of CpG DNA is independent on CpG methylation and they suggest that, in addition to unmethylated CpG, bacterial DNA contains another unidentified immunostimulatory sequence that is absent from vertebrate DNA.

2.1 Mechanism of action

As determined by flow cytometry (Figure 12A) and western blotting (n=3), it was demonstrated that human eosinophils express TLR9 protein. Approximately similar expression levels were found in eosinophils from both atopic and healthy donors.

To study whether the anti-apoptotic effect of bacterial DNA, CpG ODN 1018 or non-CpG ODN 1040 was mediated via TLR9, inhibitors of the TLR9 pathway were used. These included inhibitors of endosomal acidification (bafilomycin A1 and chloroquine) and inhibitory ODN. Bafilomycin and inhibitory ODN prevented the anti-apoptotic effects of both bacterial DNA and CpG ODN 1018 (Figures 12B-C). Chloroquine prevented the effect of CpG ODN 1018 on apoptosis but bacterial DNA decreased apoptosis by 18.18 ± 2.28 % ($p<0.01$, n=6) and 13.84 ± 2.58 % ($p<0.05$, n=6) in the absence and presence of chloroquine, respectively. However, the anti-apoptotic effect of non-CpG ODN 1040 was not prevented by bafilomycin (Figure 12B) or chloroquine ($p<0.05$, n=6). These results suggest that TLR9 mediates the anti-apoptotic action of CpG ODNs and bacterial DNA but not that of non-CpG ODNs.

Finally, the roles of NF-κB and PI3K in mediating anti-apoptotic effect of CpG DNA were studied by using pharmacological inhibitors of those pathways. The anti-apoptotic action of CpG ODN 1018 was totally prevented by two inhibitors of the NF-κB pathway, BMS-345541 (Figure 12D) and PDTC ($p>0.05$, n=7). Additionally, MG-132, a proteasome inhibitor that inhibits NF-κB by preventing degradation of IκB, reduced the anti-apoptotic effect of CpG ODN 1018 from 16.50 ± 4.59 % ($p<0.01$, n=13) to 6.81 ± 2.67 % ($p<0.05$, n=13). Inhibition of PI3K by wortmannin completely abolished the anti-apoptotic effect of CpG ODN 1018 (Figure 12E). In summary, these results suggest that bacterial DNA and CpG ODNs inhibit eosinophil apoptosis via TLR9, PI3K and NF-κB.

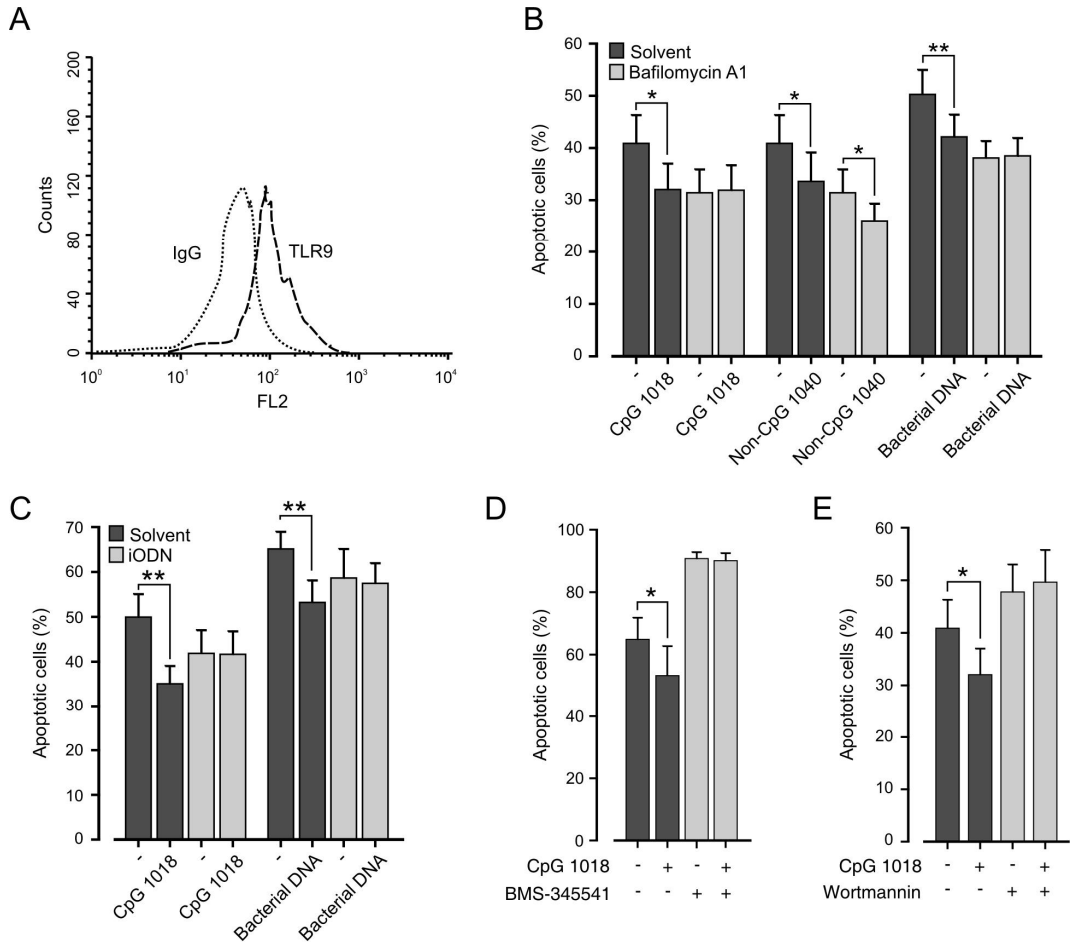

Figure 12. *TLR9 expression in eosinophils and effects of inhibitors of TLR9, NF-κB and PI3K pathways on CpG DNA-induced delayed eosinophil apoptosis. In A, shown is TLR9 expression in eosinophils as determined by flow cytometry. Typical experiments of three with similar results are displayed. In B-E, eosinophils were pretreated with bafilomycin A1 (100 nM) or wortmannin (100 nM) for 20 min, iODN (10 μM) for 30 min or BMS-345541 (10 μM) for 60 min and supplied with 3 μM ODNs, 30 μg/ml bacterial DNA or solvent. Bacterial DNA (30 μg/ml) was added once again after 16-20 h of incubation. After total incubation time of 40 h, apoptosis was determined by DNA fragmentation assay. The results are shown as mean ± SEM of 5-6 independent experiments. (Reprinted with permission from: Ilmarinen et al. 2009, Pul Pharmacol Ther. 22:167-76. © Elsevier Ltd., modified)*

3 Effect of orazipone and its analogues on eosinophil apoptosis (II)

Since oxygen radicals and thiol group-containing compounds such as the antioxidant glutathione are critical in modulating apoptosis in eosinophils (Wedi et al. 1999, Kankaanranta et al. 2002, Lee and Shin 2009), it was interesting to study the effects of a novel thiol-modulating anti-inflammatory compound orazipone on eosinophil apoptosis. It was found that orazipone and its structural analogs OR-2370 and OR-1958 induced

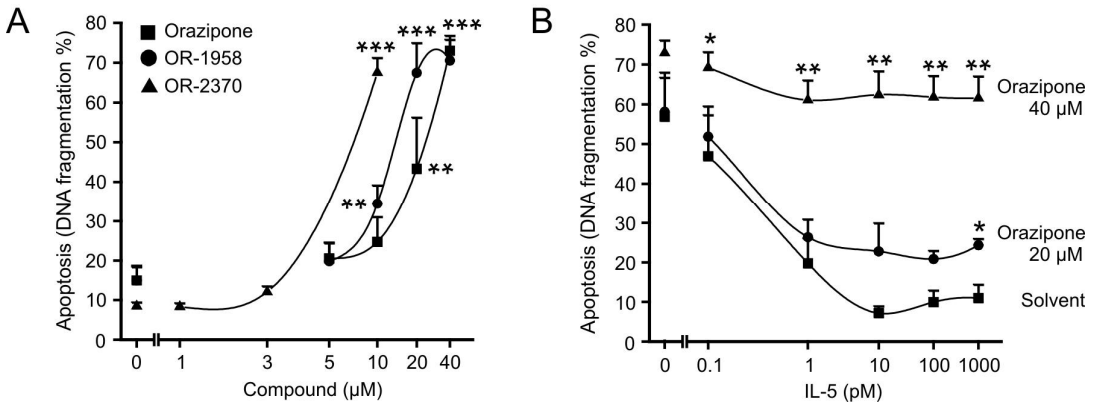

Figure 13. *Effect of orazipone (A-B) and its structural analogs (A) on eosinophil apoptosis in the presence of IL-5. Eosinophils were cultured for 40 h. In A, IL-5 concentration was 10 pM. Each data point represents mean ± SEM of 5-6 independent experiments. * indicates p<0.05, ** p<0.01, *** p<0.001 as compared with cells treated with similar concentration of IL-5. (Adapted from Kankaanranta et al. 2006, Mol Pharmacol. 69: 1861-70 © American Society for Pharmacology and Experimental Therapeutics)*

apoptosis in IL-5-treated eosinophils in a concentration-dependent manner (Figure 13A). The negative control compound OR-2149, which is not able to react with thiol-containing compounds due to its reduced double bond in the side chain, had no effect on eosinophil apoptosis in the presence of IL-5 ($p>0.05$, n=3). Orazipone retained its pro-apoptotic effects even when higher (10-100 pM) concentrations of IL-5 were used (Figure 13B), which is in contrast to glucocorticoids that are known to lose their pro-apoptotic effect when the IL-5 concentration is increased (Hagan et al. 1998, Zhang et al. 2000). Additionally, orazipone enhanced spontaneous apoptosis ($p<0.05$, n=6) and Fas-induced apoptosis ($p<0.05$, n=5) but did not induce primary necrosis ($p>0.05$, n=6). The pro-apoptotic effect of orazipone was not general because orazipone had no effect on neutrophil apoptosis, in the absence or presence of Fas or GM-CSF (Table 9).

Table 9. *Effect of orazipone on neutrophil apoptosis.*

	Apoptotic cells (%)
Spontaneous apoptosis	
Solvent control	68.00 ± 2.60
Orazipone 10 μM	63.12 ± 4.69
Orazipone 40 μM	67.62 ± 4.25
GM-CSF (70 pM)	
Solvent control	52.92 ± 5.41
Orazipone 10 μM	48.44 ± 5.22
Orazipone 40 μM	56.78 ± 5.23
Fas (CH-11 100 ng/ml)	
Solvent control	84.36 ± 2.96
Orazipone 10 μM	84.17 ± 3.09
Orazipone 40 μM	84.40 ± 4.41

After 16 h of incubation, apoptosis was determined by DNA fragmentation assay. Values are mean ± SEM of five independent experiments. There were no statistically significant differences when compared to the solvent control.

3.1 Mechanism of action

Since OR-2370 was the most potent of the tested compounds in increasing eosinophil apoptosis in the presence of IL-5, it was decided to clarify its mechanism of action. OR-2370 evoked a time-dependent early increase in pJNK levels in IL-5-treated eosinophils (Figure 14A). Additionally, a peptide inhibitor of JNK, L-JNKI1, prevented the effect of OR-2370 on DNA fragmentation in the presence of IL-5 (Figure 14B) but interestingly, did not reverse OR-2370-induced morphological signs of apoptosis or phosphatidylserine exposure ($p>0.05$, n=4-8). This indicates that JNK is involved in later stages of OR-2370-induced apoptosis, specifically in the DNA fragmentation pathway. Inhibition of ERK (PD98059), p38 (SB203580 and PD169316) or PI3K (LY294002 and wortmannin) did not prevent the pro-apoptotic effect of OR-2370 ($p>0.05$, n=4-7), indicating that they are not involved in mediating this effect.

OR-2370 increased activities of caspases 3/7, 8 and 9 when compared to IL-5-treated eosinophils (Figure 15A). Consistently, the apoptosis-increasing effect of OR-2370 in IL-5-treated eosinophils was totally prevented by a broad-range caspase-inhibitor Q-Vd-OPh (Figure 15B) and a pan-caspase inhibitor Z-Asp-CH_2-DCB ($p<0.001$, n=7). Of the specific inhibitors tested, the caspase-6 inhibitor Z-VEID-FMK totally abolished and

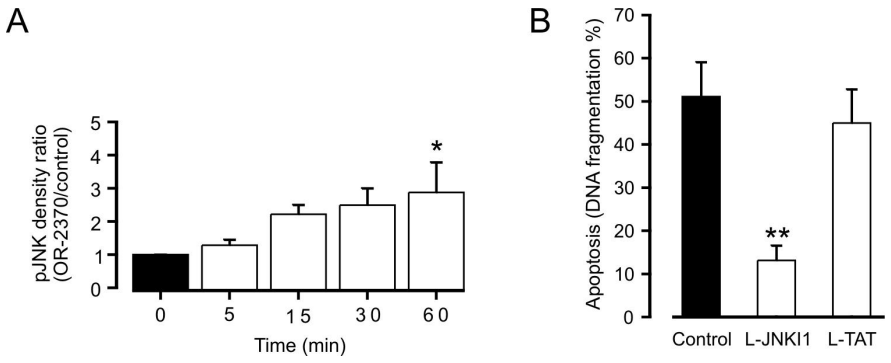

Figure 14. *Effect of OR-2370 on JNK phosphorylation (A) and effect of JNK inhibitor L-JNKI1 on OR-2370-induced apoptosis (B) in IL-5-treated eosinophils. In A, shown is density ratio of pJNK of cells treated with OR-2370 (10 µM) and IL-5 (10 pM) when compared with IL-5 (10 pM) -treated control cells. Shown are mean ± SEM of six repeats and * indicates $p<0.05$ when compared to 0 min cells. In B, the effects of L-JNKI1 (10 µM) and control peptide L-TAT (10 µM) are shown on apoptosis of eosinophils treated with IL-5 (10 pM) and OR-2370 (10 µM) for 40 h. Apoptosis in the presence of 10 pM IL-5 was 9.3 ± 1.0. Values are mean ± SEM of eight individual experiments. ** indicates $p<0.01$ when compared to L-TAT-treated cells. Reprinted with permission from: Kankaanranta et al. 2006, Mol Pharmacol. 69: 1861-70 © American Society for Pharmacology and Experimental Therapeutics, modified)*

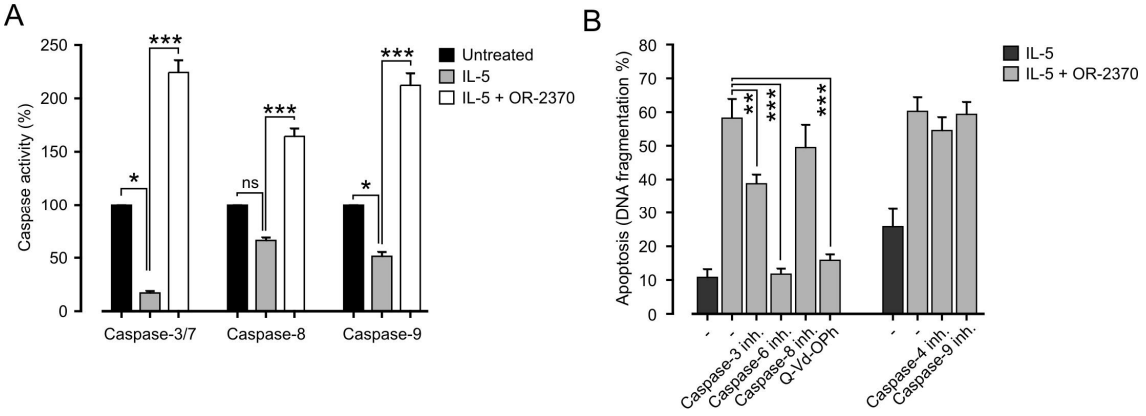

Figure 15. *Effect of OR-2370 on activities of caspases 3/7, 8 and 9 (A) and effect of caspase inhibitors on OR-2370-induced DNA fragmentation (B) in IL-5-treated eosinophils. In A, eosinophils were treated for 16 h in the presence or absence of 10 pM IL-5 and 10 µM OR-2370, after which caspase-activity was determined by the Caspase Glo assay. In B, eosinophils were pretreated with caspase-3 inhibitor Z-DQMD-FMK (200 µM), caspase-6 inhibitor Z-VEID-FMK (200 µM), caspase-8 inhibitor Ac-ITED-CHO (100 µM), caspase-4 inhibitor Ac-LEVD-CHO (100 µM) or caspase-9 inhibitor Ac-LEHD-CHO (100 µM) for 20 min and then supplied with 10 pM IL-5 and 10 µM OR-2370. Total incubation time was 40 h. Means ± SEM are shown of 4-5 individual experiments and * indicates p<0.05, ** p<0.01 and *** p<0.001. (Adapted from Kankaanranta et al. 2006, Mol Pharmacol. 69: 1861-70 © American Society for Pharmacology and Experimental Therapeutics)*

caspase-3 inhibitor Z-DQMD-FMK partially prevented the effect of OR-2370 on DNA breakdown (Figure 15B). OR-2370-induced DNA fragmentation was not mediated by caspases 4, 8 or 9 (Figure 15B) as determined by their inhibition, even though OR-2370 enhanced the activity of initiator caspases 8 and 9 (Figure 15A). The inhibitors of caspases 8 and 9 (Ac-ITED-CHO and Ac-LEHD-CHO, respectively) decreased activities of these caspases by 99 and 65 % (n=2), respectively. Thus, orazipone and its structural analogues possess anti-eosinophilic properties and OR-2370-induced DNA fragmentation in IL-5-treated cells was mediated via JNK and caspases 3 and 6.

4 Mechanism of NO-induced eosinophil apoptosis (III, IV)

NO is produced in high amounts in asthmatic lungs and asthmatics show increased concentration of exhaled NO when compared to healthy individuals (Kharitonov et al. 1994, Persson et al. 1994, Lehtimaki et al. 2001). The NO donor SNAP was previously shown to induce apoptosis of IL-5-treated eosinophils. The mechanism was proposed to involve JNK and caspases but it was decided to elucidate a more detailed mechanism for the pro-apoptotic effect of NO. It was found, as expected, that SNAP but not the negative control compound penicillamine induced eosinophil apoptosis in a

concentration-dependent manner also in the presence of GM-CSF (10 pM). The effect reached statistical significance at the 1 mM SNAP concentration ($p<0.01$, $n=7$). The time-response curve revealed that marked apoptosis occurred 40 h but not 24 h after addition of SNAP and GM-CSF (Figure 16A).

4.1 Role of mitochondria

Mitochondria and mitochondrial permeability transition have been previously demonstrated to be central in mediating pro-apoptotic effects of NO in murine macrophages and thymocytes (Hortelano et al. 1997, Borutaite et al. 2000, Brown and Borutaite 2002). We continued by clarifying whether mPT mediates pro-apoptotic effects of NO by using bongkrekic acid, an inhibitor of mPT. Bongkrekic acid, indeed, mostly prevented SNAP-induced apoptosis as determined by DNA fragmentation assay (Figure 16B), morphological analysis ($p<0.01$, $n=6$) and Annexin-V assay ($p<0.01$, $n=6$). Bongkrekic acid was much less effective in preventing spontaneous apoptosis (Figure 16B, 11.83 ± 3.08 % decrease in apoptosis by Annexin-V assay $p<0.05$, $n=6$, no effect in morphological analysis $p>0.05$, $n=6$).

The occurrence of partial mPT by SNAP was revealed at 1 h by using a technique based on calcein AM and $CoCl_2$ (Figure 16C). Treatment with bongkrekic acid was able to prevent this effect (Figure 16C). However, the early partial mPT was not significant for apoptosis because addition of bongkrekic acid at the 16 h time-point still efficiently prevented SNAP-induced apoptosis (Figure 16D). This result indicated that the early mPT may be transient/flickering and the threshold for apoptosis-inducing permanent mPT occurred at 16-40 h after addition of SNAP and GM-CSF. Further evidence for the flickering nature of early mPT was gained by showing that $\Delta\psi_m$ was not lost at the 20 h time-point (Figure 16E).

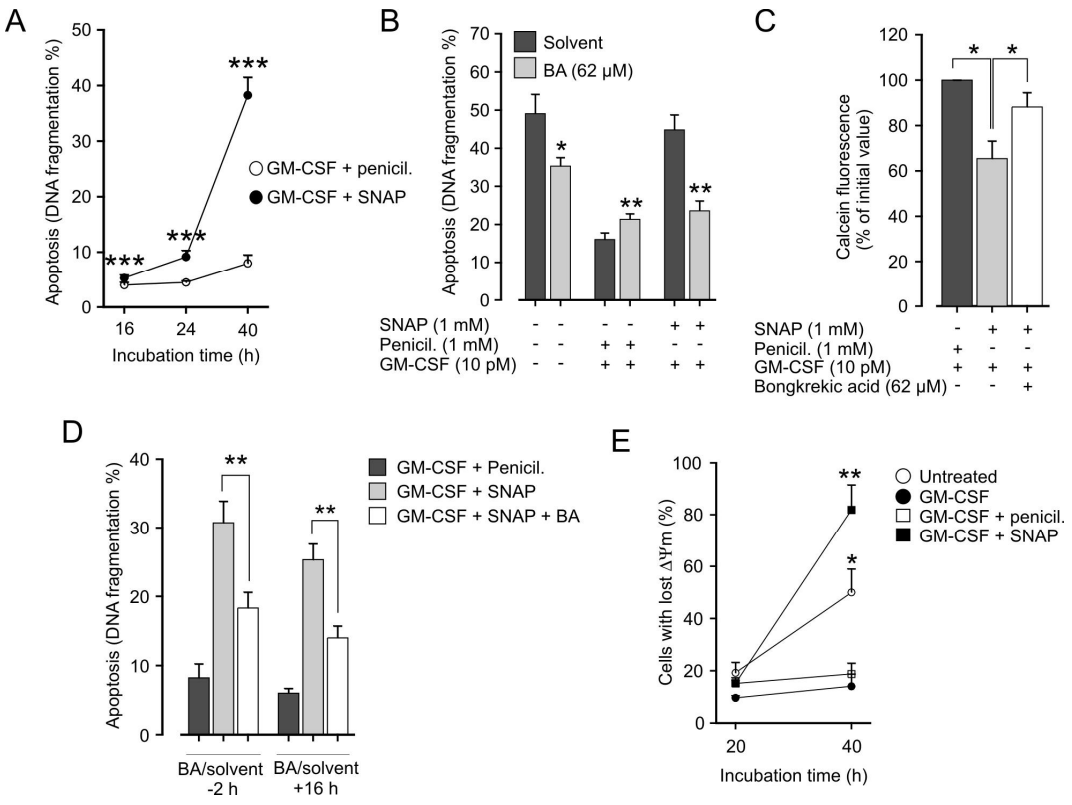

Figure 16. *Effect of SNAP on eosinophil apoptosis (A), effect of mPT inhibition on SNAP-induced apoptosis (B, D), effect of SNAP on mPT (C) and effect of SNAP on mitochondrial membrane potential (E) in the presence of GM-CSF. In B eosinophils were pretreated for 2 h with bongkrekic acid (BA) or solvent after which GM-CSF, SNAP and/or penicillamine was added and incubation continued for 40 h. In C, cells were pretreated with BA for 30 min before addition of GM-CSF, SNAP and/or penicillamine. Calcein AM/$CoCl_2$ staining was conducted after 1 h incubation. In D, bongkrekic acid was added either 2 h before or 16 h after GM-CSF, SNAP and/or penicillamine. Total incubation time was 40 h. In E, the state of mitochondrial membrane potential ($\Delta\psi_m$) was determined by JC-1 staining after indicated incubation times. When not indicated, the concentrations used were GM-CSF 10 pM, SNAP 1 mM, penicillamine 1 mM and bongkrekeic acid 62 µM. Shown are mean ± SEM of five to six independent experiments.*

4.2 Role of JNK

The experiments were continued by evaluating the role of JNK in SNAP-induced apoptosis. A strong increase in phospho-JNK (pJNK) levels was detected at 2 h and smaller increases at 1 h, 20 h and 30 h time-points (Figure 17A). Phospho-JNK levels were stable in untreated cells (Figure 17A). The SNAP-induced peak in pJNK at 2 h was completely prevented by bongkrekic acid (Figure 17B) indicating that JNK activation is a consequence of the early mPT.

Results from western blotting showed both early and late activation of JNK. Previously, early and transient activation has been observed to function as a stress

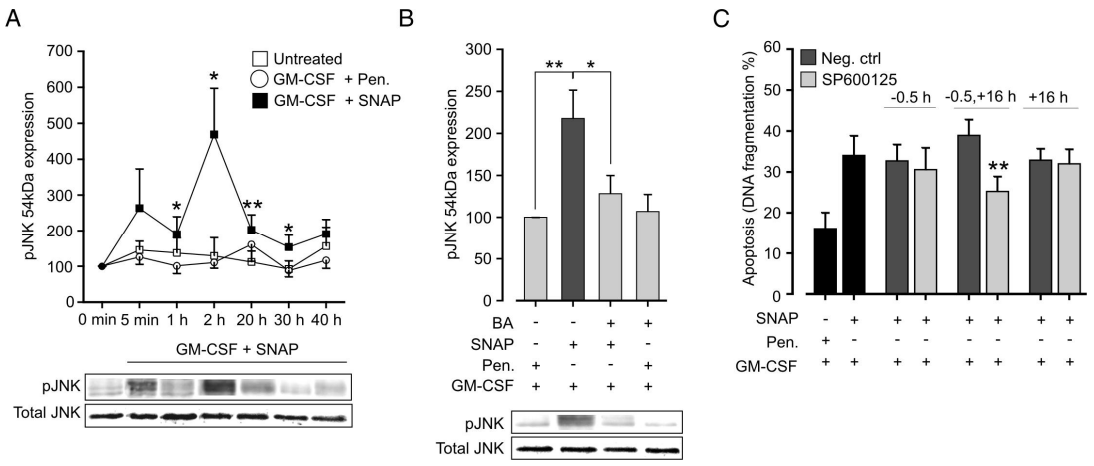

Figure 17. *Effect of SNAP on pJNK levels (A), role of mPT in early JNK activation (B) and effect of JNK inhibition on SNAP-induced apoptosis (C). In A, eosinophils were lysed for western blotting after the indicated treatments and incubation times. pJNK and total JNK levels were normalized against the 0 min GM-CSF (10 pM) sample. Total JNK was used as the loading control and 54 kDa pJNK and total JNK are shown. * indicates p<0.05 and ** p<0.01 compared with eosinophils stimulated with GM-CSF and penicillamine for the corresponding time, n=7. Untreated cells were compared to 0 min GM-CSF sample, n=5. In B, eosinophils were preincubated with bongkrekic acid for 2 h before adding SNAP, penicillamine (pen) and/or GM-CSF. Incubation was continued for 2 h before cell lysis and western blotting. Means ± SEM are shown, n=6. In C, time-points shown above the columns refer to the addition times of SP600125 (10 µM) or its negative control (10 µM). Time zero represents the time of adding GM-CSF (10 pM) and SNAP (1 mM) or penicillamine (1 mM). Total incubation time was 40 h. Values are mean ± SEM, n=5 and ** indicates p<0.01 versus respective control.*

response while delayed and sustained JNK activation mediated apoptosis (Sanchez-Perez et al. 1998, Ventura et al. 2006). To test this hypothesis, the JNK inhibitor SP600125 was added at different time-points to examine the significance of its early or late activation on apoptosis. Addition of SP600125 at two different time-points (at the beginning and at 16 h) partly prevented SNAP-induced apoptosis but its addition at either time-point alone was not sufficient to prevent apoptosis (Figure 17C). This result suggests that the later phase of JNK activation is involved in mediating SNAP-induced apoptosis but it may be initiated before the 16 h time-point.

JNK has mediated mPT or loss of $\Delta\psi_m$ in previous studies with other cell types (Hanawa et al. 2008, Lin et al. 2009). Treatment with two different JNK inhibitors did not prevent SNAP-induced loss of $\Delta\psi_m$ at 40 h ($p>0.05$, n=4-5) suggesting that JNK has some other role than mediating mPT in SNAP-induced apoptosis.

4.3 Role of ROS

A SOD mimetic, AEOL 10150, was used to assess whether generation of O_2^- and the formation of peroxynitrite is involved in SNAP-induced apoptosis. AEOL partly prevented SNAP-induced apoptosis (Figure 18). The addition of AEOL 10150 at 16 h time-point was no longer effective, indicating importance of early formation of ROS (Figure 18). According to these results, SNAP specifically induced the generation of O_2^- because spontaneous apoptosis was not prevented by AEOL. It was also found that NADPH oxidase was not the source of O_2^- because its inhibition by diphenylene iodonium (DPI) or apocynin had no effect on SNAP-induced apoptosis ($p>0.05$, n=6-8).

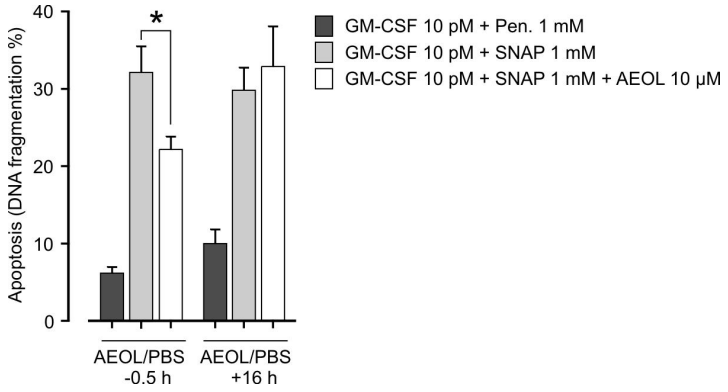

Figure 18. *Effect of SOD mimetic AEOL 10150 on SNAP-induced apoptosis. AEOL was added either 30 min before or 16 h after GM-CSF, SNAP and/or penicillamine. Total incubation time was 40 h. Values are mean ± SEM, n=5 and * indicates $p<0.05$.*

4.4 Role of caspases

Caspase inhibitors were used to study the role of caspases in SNAP-mediated apoptosis. SNAP-induced DNA fragmentation was totally abolished by a caspase-6 inhibitor Z-VEID-FMK, partially prevented by a caspase-3 inhibitor Z-DQMD-FMK but not affected by a caspase-8 inhibitor IETD-CHO (Figure 19A). Caspase-6 inhibition partly prevented SNAP-induced phosphatidylserine expression as determined by Annexin-V staining (41.44 ± 6.88 % decrease in Annexin$^+$ cells, $p<0.01$, n=6) but it did not prevent the SNAP-induced morphological signs of apoptosis ($p>0.05$, n=5). However,

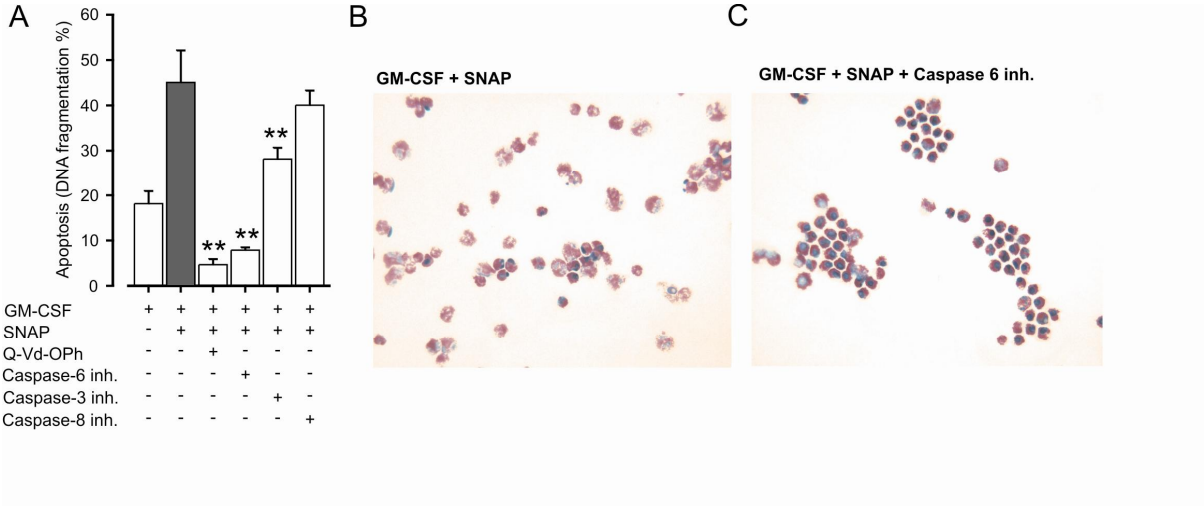

Figure 19. *Effects of caspase inhibitors on SNAP-induced apoptosis. Eosinophils were pretreated with 20 µM Q-Vd-OPh, 200 µM Z-VEID-FMK (caspase-6 inhibitor), 200 µM Z-DQMD-FMK (caspase-3 inhibitor) or 100 µM IETD-CHO (caspase-8 inhibitor) or solvent for 20 min after which 1 mM SNAP and 10 pM GM-CSF was added. Total incubation time was 40 h. In A, the results are shown as mean ± SEM of 6 independent experiments. In B and C, representative figures are shown from morphological analysis. (Reprinted with permission from: Ilmarinen-Salo et al. 2010, Pulm Pharmacol Ther. 23:365-371 © Elsevier Ltd., modified).*

morphological examination revealed that the inhibition of caspase-6 increased the proportion of early apoptotit cells (as identified by cell shrinkage and chromatin condensation) and decreased the proportion of late apoptotic cells (“ghosts”) of the total number of apoptotic cells (Figure 19B-C). This indicates that apoptosis can be delayed or halted to the early apoptotic phase when DNA fragmentation is inhibited in eosinophils.

Caspase-6 activation was confirmed by examining the caspase-6 proenzyme level and degradation of its substrates, lamin A/C and B (Orth et al. 1996, Takahashi et al. 1996). SNAP clearly decreased level of caspase-6 proenzyme indicating cleavage into active fragments (Figure 20A). Furthermore, SNAP increased lamin A/C (Figure 20B) and B fragment levels (3.2 fold increase, $p<0.05$, $n=5$) when compared to cells treated with GM-CSF and penicillamine and both of these effects could be totally prevented by the caspase-6 inhibitor (Figure 20B and lamin B, $p<0.01$, $n=5$). Calpeptin, an inhibitor of calpains 1 and 2, had no effect on SNAP-induced DNA fragmentation ($p>0.05$, $n=6$).

In summary, it was demonstrated that NO could induce apoptosis in GM-CSF-treated eosinophils via early ROS production, late JNK activation, late mPT and caspases 3 and 6. In addition, we found that before initiation of apoptosis NO induced early partial mPT that led to early and strong JNK activation.

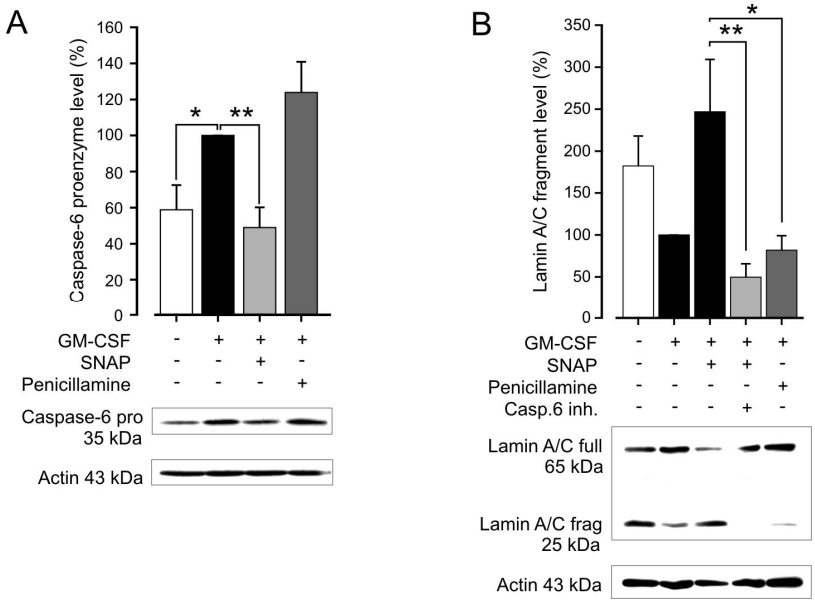

Figure 20. *Effect of SNAP on caspase-6 activity. In A, eosinophils were incubated with 10 pM GM-CSF, 1 mM SNAP and/or 1 mM penicillamine for 40 h and lysed for western blotting. In B, eosinophils were pretreated with 200 µM caspase-6 inhibitor Z-VEID-FMK for 20 min before addition of 10 pM GM-CSF, 1 mM SNAP and/or 1 mM penicillamine. After total incubation time of 40 h, eosinophils were lysed for western blotting. Actin was used as loading control. The results are shown as mean ± SEM of 4-5 repeats and representative blots. * indicates p<0.05 and **p<0.01. (Reprinted with permission from: Ilmarinen-Salo et al. 2010, Pulm Pharmacol Ther. 23:365-371 © Elsevier Ltd., modified).*

5 Expression and function of NPSR1 in eosinophils (V)

As NPSR1 was identified as a gene linked to asthma and high IgE (e.g. total IgE above 100 IU/ml) the aim of this study was to compare expression of NPSR1 in eosinophils derived from subjects with high and low serum IgE and in eosinophils derived from healthy controls and from patients with varying severities of asthma. By western blotting analysis, eosinophils from individuals with total IgE above 100 IU/ml were found to express a higher level of NPSR1 protein when compared to eosinophils from subjects with total IgE below 100 IU/ml (Figure 21A-B). Normalization of NPSR1 against β-actin (Figure 21A-B), nucleolin and GAPDH produced parallel results. A tendency for positive correlation existed between NPSR1/β-actin expression and total serum IgE levels, as analyzed by Spearman's correlation analysis (rho=0.5447, p=0.0542). However, none of the tested inflammatory mediators (GM-CSF, TNF-α,

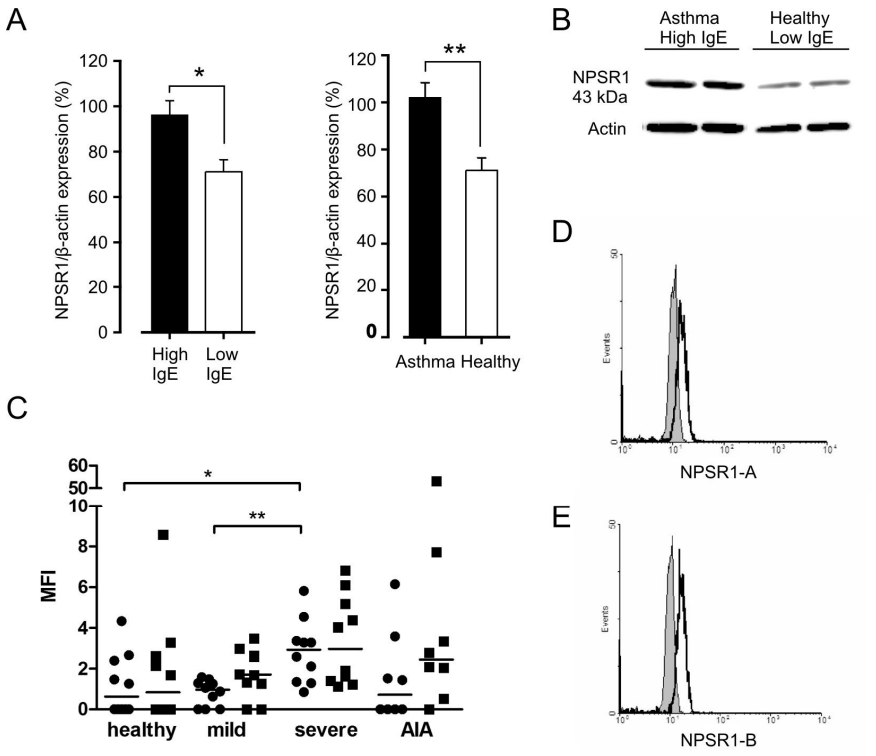

Figure 21. *NPSR1 expression in eosinophils from subjects with total IgE above and below 100 U/ml and in eosinophils from patients with different severities of asthma. In A, density of the first band was set as 100 %. Values are mean ± SEM of 5-7 repeats. In B, freshly purified eosinophils were lysed for western blotting. As an example, bands of NPSR1 and actin (43 kDa) from eosinophils derived from two different high IgE asthmatics (total n=5) and two different low IgE healthy (total n=6) are shown. In C, expression of NPSR1-A (●) and NSPR1-B (■) is displayed as measured by flow cytometry in healthy controls, and patients with mild (ICS dose ≤ 800 µg budesonide equivalent), severe (ICS dose ≥ 1200 µg budesonide equivalent + long-acting β_2 agonists and/or anti-leuk otriene drugs) and aspirin-intolerant asthma (AIA). *p<0.05, **p<0.01. D and E illustrate representative graphs from flow cytometric analysis from a subject with severe asthma.*

IFN-γ, TNF-α + IFN-γ, LPS, LTB_4 or fMLP) elevated NPSR1 levels in eosinophils from high or low IgE subjects ($p>0.05$, n=6).

Additionally, severe asthmatics showed a higher expression of NPSR1-A, but not NPSR1-B, in their eosinophils when compared to healthy controls or mild asthmatics as determined by flow cytometric analysis (Figure 21C-E). A positive correlation was also found between the percentage of eosinophils positive for NPSR1-A and a daily dose of inhaled corticosteroids (Spearman's correlation analysis rho=0.445, p=0.005, df=36).

5.1 Functional studies

The next stage was to evaluate whether the natural NPSR1 agonist, NPS, had any effects on eosinophil functions, such as integrin CD11b surface expression or release of mediators, granule proteins or superoxide. The function of NPSR1 was evaluated in

eosinophils obtained from subjects with IgE>100 IU/ml as patients with severe asthma are treated with multiple drugs (e.g. high dose inhaled glucocorticoids and long-acting β_2-agonists) known to affect eosinophil longevity (Kankaanranta et al. 2000, Zhang et al. 2000).

GM-CSF and fMLP were used either separately or combined to stimulate CD11b surface expression and all of these treatments enhanced CD11b levels in eosinophils from subjects with high IgE (fMLP data in Figure 22A, $p<0.01$, n=8). However, only the combination of fMLP and GM-CSF increased CD11b levels in eosinophils from subjects with low IgE ($p<0.01$, n=6). NPS further increased CD11b levels in fMLP-treated eosinophils from high IgE subjects but had no effect in eosinophils from low IgE subjects (Figure 22A-B). NPS produced no effect in the presence of GM-CSF in eosinophils from high or low IgE individuals ($p>0.05$, n=6-8). A tendency for positive correlation between serum IgE level and the magnitude of the effect of NPS on fMLP-stimulated CD11b was also found (Fig. 22C).

Furthermore, NPS showed a tendency to increase fMLP-stimulated EDN release in eosinophils from subjects with high IgE (Figure 23A). NPS tended to increase also superoxide production and EPO release induced by immobilized secretory IgA in eosinophils from high IgE donors (Figure 23B-C). In these eosinophils, NPS increased the EPO production induced by immobilized secretory IgA at a supra-high 50 µM

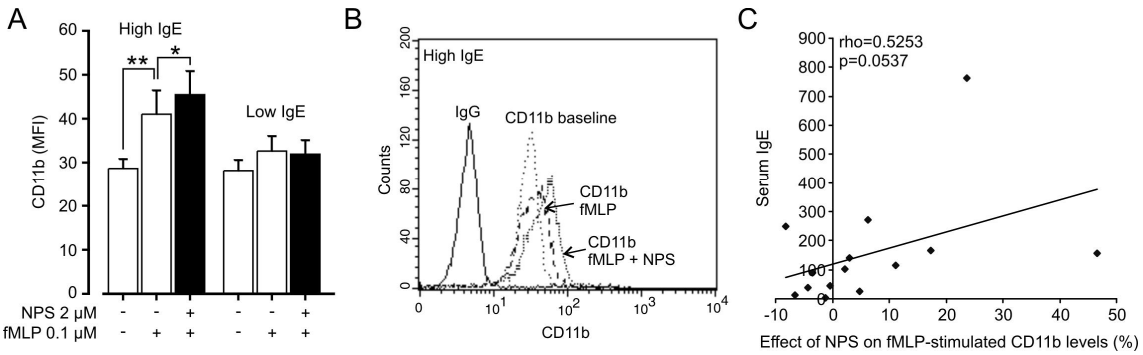

Figure 22. *Effect of NPS on fMLP-induced CD11b expression in eosinophils from high or low IgE donors. Eosinophils were treated with 2 µM NPS for 19 h after which 0.1 µM fMLP or solvent was added and incubation continued for additional 10 min. CD11b levels were determined by flow cytometry. A shows mean ± SEM of 6 (low IgE) or 8 (high IgE) individual experiments and *p<0.05, **p<0.01. B shows a representative overlaid plot of the effect of NPS on fMLP-induced CD11b expression in eosinophils from a high IgE donor. The correlation between serum IgE and the magnitude of the effect of NPS on fMLP-stimulated CD11b levels is shown in C.*

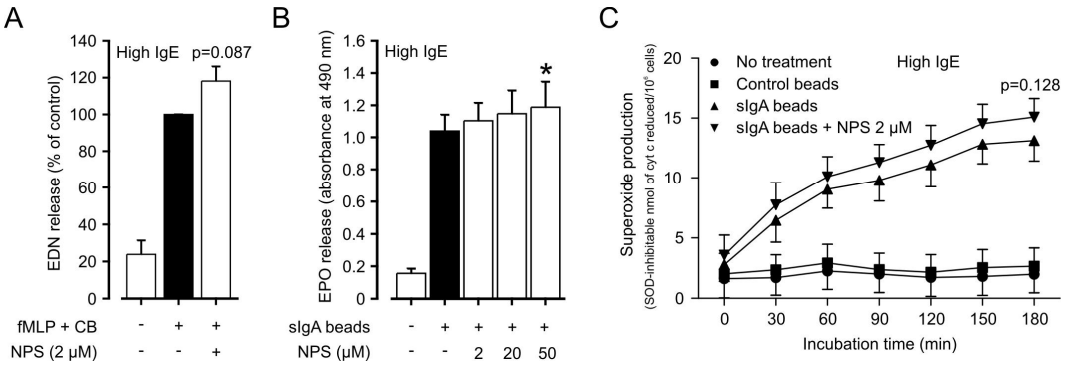

Figure 23. *Effect of NPS on stimulated release of granule proteins (EPO and EDN) and superoxide. In A and B, eosinophils were preincubated with solvent or NPS (A-B) and in the presence or absence of 5 µg/ml cytochalasin B (CB) (A) for 15 min, after which solvent, 0.1 µM fMLP (A) or 0.5 mg/ml sIgA-coupled beads (B) were added and the incubation continued for 3 h at +37°C. In C, eosinophils were preincubated with 100 µM cytochrome c with or without 0.6 µM SOD and 2 µM NPS for 15 min. Cells were supplied with solvent, immobilized sIgA (0.5 mg/ml) or control beads and transferred to 96-well plate for detection of absorbance at 550 nm. The results are shown as mean ± SEM of 5-6 individual experiments and * represents p<0.05 as compared with the respective control.*

concentration in a statistically significant manner. No effects were observed on superoxide production or on the release of granule proteins EDN or EPO by NPS treatment alone ($p>0.05$, n=4-7). Additionally, it was examined whether NPS could stimulate blood leukocytes from healthy controls or severe asthmatics to release PGE_2, IL-8, MMP-10 or LTB_4 but NPS was ineffective ($p>0.05$, n=10). NPS (1 µM) had no effect on eosinophil longevity, in the presence or absence of GM-CSF, Fas or budesonide ($p>0.05$, n=5-7).

To test whether NPS could affect intracellular signalling in eosinophils, the effect of NPS on intracellular cAMP levels was examined. NPS induced a rapid increase in cAMP from 114.37 ± 21.01 fmol/ml to 179.81 ± 45.23 fmol/ml ($p<0.05$, n=8) at 30 seconds at 20 µM concentration. The effect was smaller when compared to the elevation in the cAMP level induced by histamine (from 114.37 ± 21.01 fmol/ml to 449.66 ± 159.11 fmol/ml, $p<0.01$, n=8).

In summary, eosinophils from subjects with high IgE level and patients with severe asthma showed increased expression of NPSR1. NPSR1 may be involved in regulating eosinophil adhesion as NPS could increase fMLP-induced CD11b surface expression in eosinophils collected from subjects with high IgE. However, clarifying the effects of NPS on eosinophil functions will require further studies.

6 Summary of the mechanisms regulating eosinophil apoptosis and survival

In all, it was studied whether TLR9 agonists, NO, orazipone, its analogue OR-2370 or NPS affects eosinophil longevity. NO and the analogues of orazipone induced apoptosis, TLR9 agonists increased survival whereas NPS had no effect on eosinophil longevity. Many similarities exist in the mechanisms of eosinophil apoptosis and survival irrespective of the apoptosis- or survival-inducing stimulus. NF-κB and PI3K are important for both baseline survival of eosinophils and TLR9-agonist induced survival.

According to the results of these studies, DNA fragmentation during eosinophil apoptosis irrespective of the stimulus seems to be generally mediated by caspases 3 and 6. The role of calpains shows stimulus-dependency, since they are involved in spontaneous apoptosis but not in NO-induced apoptosis. JNK was activated only in the presence of inducers of apoptosis such as NO and OR-2370. The importance of the mitochondrial permeability transition is emphasized in the presence of NO but some degree of mPT also seems to mediate spontaneous apoptosis. Superoxides have a critical role in mediating NO-induced but not spontaneous eosinophil apoptosis. A summary of the mechanisms of apoptosis revealed in this thesis is shown in Table 10.

Table 10. *Summary of the mechanisms involved in the regulation of spontaneous apoptosis and apoptosis induced by OR-2370 and nitric oxide.*

	Casp3	Casp4	Casp6	Casp8	Casp9	Calp	JNK	mPT	ROS
Spontaneous	++	ND	+++	–	ND	++	–	+	–
OR-2370 (IL-5)	++	–	+++	–	–	ND	+++	ND	ND
NO (GM-CSF)	++	ND	+++	–	ND	–	++	+++	++

Abbreviations: +++ = apoptosis is completely dependent, ++ = apoptosis is partially (appr. 50 %) dependent, + = minor role in apoptosis, – = no role in apoptosis, ND = not determined, casp = caspase, calp = calpain.

DISCUSSION

1 Methodology

One major strengtht of this study is that primary human eosinophils were utilized to study eosinophil apoptosis and functions. Results with human eosinophils are more applicable to human pathophysiology when compared to murine eosinophils that do not establish asthmatic airway inflammation in nature and may function differentially. The use of a malignant eosinophilic cell line is not an option in the study of apoptosis because cell death pathways are transformed when the cells become immortalized. Human tissue eosinophils would produce results most applicable to human pathophysiology but because of the ethical aspects regarding bronchoscopy and the difficulties in obtaining sputum eosinophils, eosinophils from peripheral blood were used. Differences have been reported to exist in the function of blood and tissue eosinophils and this should be kept in mind when interpreting the results. For example, some inflammatory stimulants have been found to induce a stronger response in tissue eosinophils when compared to blood eosinophils (Sedgwick et al. 1992).

The shortage of eosinophils and their short lifespan were issues that complicated this study. The number of eosinophils obtained in 100 ml blood from a healthy person typically does not exceed 6 million, although 6-30 million cells can often be obtained from subjects with allergy or asthma. Therefore the shortage of cells often restricts carrying out long time-series with many different treatments, especially with methods such as western blotting that require close to one million cells per sample. The short life-span of eosinophils excludes use of methods such as transfection and RNA interference.

Many things have to be considered when using primary human eosinophils. Asthmatics require regular medication to control symptoms and were allowed to take their medication when participating in this study. Glucocorticoids have been shown to modulate eosinophil apoptosis (Meagher et al. 1996, Zhang et al. 2000) and to affect

cytokine production (Miyamasu et al. 1998). Even though drugs present in plasma were mainly washed away during the isolation process, their effects may be long-lasting and influence the eosinophil function assessed during the experiments. As an example of this, apoptosis of blood eosinophils was shown to be delayed in steroid-naïve patients with asthma but normalized in steroid-treated patients with asthma (Kankaanranta et al. 2000). These issues were born in mind when interpreting the results.

Eosinophils of asthmatic and allergic subjects are derived from an environment with increased levels of inflammatory mediators and also these mediators may have long-lasting effects on eosinophil functions and thus influence the experimental results. For example, it was noted that some inflammatory mediators increased surface expression of integrin CD11b only in eosinophils derived from patients with total serum IgE above 100 IU/ml (V). NPSR1 levels were also enhanced only in eosinophils from these subjects. This probably reflects the background influence of donor-derived inflammatory mediators. Another possible explanatory factor is genetic background. The donor background has to be considered when choosing donors for particular experiments and again, when interpreting the results.

Eosinophils were isolated to a purity of above 99 %. However, some contaminating cells always remain in eosinophil suspensions, mostly lymphocytes or neutrophils. In particular, contaminating lymphocytes may produce cytokines or other mediators that may in some situations have significant contribution to the effect produced by the test compound on eosinophils. This possibility was excluded by performing extensive eosinophil purification when studying the effect of CpG DNA on eosinophil survival because plasmacytoid dendritic cells and B-lymphocytes are extremely strongly activated by CpG DNA (Yi et al. 1996, Hartmann et al. 1999, Krug et al. 2001). Contaminating cells did not most likely play any significant role in the other studies of this thesis.

A diverse array of methods was applied to determine apoptosis in this thesis. Because all methods have their drawbacks, the use of more than one method is crucial if one wishes to obtain reliable results. The DNA fragmentation assay, where cellular DNA content is determined by propidium iodide staining of permeabilized cells, was used as the primary method for determination of apoptosis but this method does not discriminate necrosis. Instead, necrosis can be distinguished from apoptosis by Annexin-V FITC/propidium iodide counterstaining. Annexin-V staining is generally considered as a sensitive and reliable method (Walsh et al. 1998), even though some

apoptotic cells have been reported to lack phosphatidylserine exposure (Qu et al. 2007). Subjectivity is the greatest disadvantage of morphological analysis but this method can distinguish the early from late stages of apoptosis and some visible functional aspects of the cell (e.g. degranulation). In study III, morphological analysis was utilized to examine the role of caspase-6 and the consequences of caspase-6 inhibition in eosinophil apoptosis. Generally, the main effects were investigated by all three methods to ensure reliability but some mechanistic studies were conducted only by DNA fragmentation assay because of limited time and resources. The results from all three apoptosis determination methods correlated generally well. Annexin-V staining revealed the highest percentage of apoptosis and phosphatidylserine expression seemed to precede other manifestations of apoptosis, which is consistent with a report from a previous study (Vermes et al. 1995).

Several methods were used to determine caspase activity. The luminescence assay is a rapid and sensitive method with which to determine caspase activity but it measures caspase activity only at the exact time-point studied. Detection of fragmented caspase substrate levels by western blotting represents a more cumulative method and it reveals also deceased caspase activity. The use of a cumulative method may be of critical importance in some situations. For example, nitric oxide is known to inactivate caspases via S-nitrosylation (Dimmeler et al. 1997) indicating that caspases may be active or inactive depending on the time-point studied. For this reason, cumulative methods were preferred in the study of the effects of NO.

Many methods were established in our laboratory during this thesis. The state of mitochondrial membrane potential was measured by the cationic dye JC-1. JC-1 staining has been previously conducted in eosinophils (Gardai et al. 2003) and is considered as a reliable and accurate method when one wishes to measure whether mitochondrial membrane is polarized (viable cells) or depolarized (apoptotic cells) (Perry et al. 2011). The reliability was enhanced by the use of clear positive and negative controls in each experiment separately. The results obtained by JC-1 staining and other apoptosis determination methods were closely similar, supporting reliability of this method.

Mitochondrial permeability transition was measured by the MitoProbe transition pore assay kit, a technique based on calcein/$CoCl_2$ staining and flow cytometric analysis. This method has not been previously used in eosinophils. The positive control ionomycin and inhibitor of mPT functioned as expected indicating reliability. Since

transient mPT has been shown to function as a calcium release mechanism, the determination of intracellular calcium levels would have provided further support for the result. It was not possible to determine mPT at late time-points because the calcein AM/$CoCl_2$ method can only be used in cells with an intact plasma membrane and functional intracellular esterases. It seems that cells that are on an inevitable path towards apoptosis may lose their intracellular esterase activity or plasma membrane integrity quite rapidly because the amount of eosinophils with negative calcein staining in the absence of $CoCl_2$ was increased at later time-points (16-40 h).

Many methods were exploited to examine eosinophil activation. EPO activity, EDN release, superoxide generation and CD11b levels were studied by standard methods, all widely used in eosinophil studies. These eosinophil functions are typically, but not always, closely related. Therefore, the use of several methods reduces the possibility for flaws due to method limitations or false interpretations. For example, the size of the effect of NPS on fMLP-induced CD11b expression can be argued as relatively small, raising the question of whether it is a true effect but the tendencies seen in degranulation and superoxide assays support the reliability of the CD11b effect.

Standard methods, western blotting and flow cytometry, were used to study protein expression levels. In the western blotting, protein concentrations were determined in advance to enable loading of similar protein amount into each well. Furthermore, when protein levels were compared, the protein of interest was always proportioned to one or more loading controls. Specific antibodies were used and in some cases, the specificity was confirmed with a blocking peptide. In flow cytometry, isotype controls were used always when unfamiliar antibodies were utilized in order to assess the level of unspecific binding.

Specific inhibitors were used as pharmacological tools to examine mechanisms of actions of the compounds of interest because as discussed above, the short life span of eosinophils excludes the use of many other methods such as siRNA. Pharmacological inhibitors may exert unspecific effects, which has to be kept in mind when interpreting the results. For example, small molecule inhibitors of caspases 3, 8 and 9 (Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK) have been reported to inhibit also non-target caspases at high concentrations (Berger et al. 2006). Additional methods such as caspase activity assays were used in these studies to further support the role of specific caspases. Thus, since all single research tools have limitations, it was decided to attempt

to exploit variety of apoptosis and activation determination methods and several inhibitors for each protein under investigation to increase reliability.

2 General mechanisms of eosinophil survival and apoptosis

Many similarities exist in the mechanisms of eosinophil apoptosis and survival, independently of the presence or absence of the stimulus. This study confirmed the previous observations that NF-κB and PI3K are factors mediating eosinophil survival (Ward et al. 1999, Fujihara et al. 2005, Pinho et al. 2005, Rosas et al. 2006). Constitutive NF-κB activity seems to be important for eosinophil survival independent of the stimulus (Ward et al. 1999, Fujihara et al. 2005). PI3K has been shown to mediate survival-prolonging effects of IL-5 and β_2 agonists (Machida et al. 2005, Rosas et al. 2006) but the results of this study suggest that it plays a role also in baseline survival in the absence of survival-prolonging agents.

These results suggest that activation of caspases 3 and 6 is a general feature of eosinophil apoptosis. Previously, the involvement of these caspases, especially caspase-3, has been found in spontaneous eosinophil apoptosis and apoptosis induced by various stimuli (Zangrilli et al. 2000, Dewson et al. 2001, Letuve et al. 2001, Hasala et al. 2008). However, the specific function of caspase 6 has not been previously studied in eosinophils. The combination of the results gained by DNA fragmentation assay, morphological analysis, Annexin-V staining and western blotting suggest that lamin degradation and DNA fragmentation are caspase 6-dependent events and their inhibition can delay or terminate apoptosis at the level of chromatin condensation but does not prevent apoptosis. Consistently, in other cell types, caspase 6 has been shown to play a role in lamin degradation, DNA fragmentation and/or chromatin condensation (Rao et al. 1996, Thornberry et al. 1997, Allsopp et al. 2000, Ruchaud et al. 2002). Caspases have been previously shown to be involved in mediating phosphatidylserine externalization (Verhoven et al. 1999, Min et al. 2004) but the results of this study extended the previous knowledge by showing that phosphatidylserine externalization was partly dependent on caspase-6.

It was demonstrated that calcium-dependent cysteine proteases, calpains, are involved in DNA fragmentation during spontaneous eosinophil apoptosis but not during NO-mediated apoptosis. This indicates that the mechanisms involved in eosinophil apoptosis differ at the level of proteases depending on the stimulus. During spontaneous eosinophil apoptosis, calpains have been previously shown to play a role in mediating the cleavage of Bax into the pro-apoptotic, mitochondrial targeting form (Shen et al. 2009).

3 Bacterial DNA and delayed eosinophil apoptosis

Bacterial DNA is a pathogenic component that induces an intense Th1 response leading potentially to inhibition of Th2-type allergic immune response. Based on this concept, CpG oligodeoxynucleotides that resemble bacterial DNA are a focus of interest for the treatment of allergic diseases (Fonseca and Kline 2009). Respiratory tract infections, on the other hand, may exacerbate asthma, or according to the most recent findings, even initiate asthma (Jackson et al. 2011, Edwards et al. 2012). In this light, it was interesting to study the effects of TLR9 agonists, unmethylated CpG ODNs and bacterial CpG DNA, on eosinophil longevity. It was demonstrated that CpG ODNs and bacterial DNA but not vertebrate DNA delayed eosinophil apoptosis and the effect was mediated via TLR9, PI3K and NF-κB. Shortly after this study, survival-prolonging effect of CpG ODNs on eosinophils was confirmed by Mansson and co-workers (Mansson and Cardell 2009). One previous study reported a lack of effect (Nagase et al. 2003). Eosinophil activation has been reported to be enhanced (Mansson and Cardell 2009) or not to be affected by CpG ODNs (Nagase et al. 2003, Matsumoto et al. 2006, Wong et al. 2007a). This inconsistency may be a consequence of the different backgrounds of eosinophil donors (Mansson and Cardell 2009). Indeed, CpG ODNs enhanced eosinophil activation more efficiently in eosinophils derived from allergic than healthy subjects (Mansson and Cardell 2009) but the difference did not arise from divergent levels of TLR9, in agreement with the present results.

It was found that bacterial DNA and CpG ODNs reduced eosinophil apoptosis in a TLR9-dependent manner but the anti-apoptotic effect of non-CpG ODNs was not mediated by TLR9. Consistently, TLR9 activation has been reported to strictly require

CpG when the backbone is phosphorothioate-modified (Latz et al. 2007). The anti-apoptotic action of non-CpG ODNs may be attributable to unspecific effects induced by phosphorothioate backbone, e.g. induction of Jak2 (Too 1998). Non-CpG controls were not employed in other eosinophil studies (Nagase et al. 2003, Wong et al. 2007a, Mansson and Cardell 2009) indicating a strength for this study. It was demonstrated that methylated bacterial DNA reduced eosinophil apoptosis but it is not clear if that effect is TLR9-dependent. However, it can be proposed that this effect is independent of TLR9 because methylated and unmethylated CpG DNA have been reported to be differentially compartmentalized and only unmethylated CpG DNA was able to co-localize with TLR9 (de Jong et al. 2010). Consistent with these results, neutrophils were reported to be activated by unmethylated and methylated bacterial DNA but not by vertebrate DNA (Trevani et al. 2003) and there is convincing evidence for TLR9-independent recognition of bacterial DNA (Wang and Krieg 2003, Yasuda et al. 2005, Stetson and Medzhitov 2006). One possible receptor for methylated bacterial DNA has been recently identified. The complement receptor 2 (CR2) was shown to bind methylated bacterial DNA (in the absence of complement C3) with much higher affinity than mammalian DNA and activation of CR2 has been reported to result in activation of NF-κB (Asokan et al. 2013). Therefore, it seems that many receptors exist to recognize different structures of foreign DNA and recognition of bacterial DNA is not solely dependent on unmethylated CpG and TLR9.

It was discovered that CpG DNA-induced eosinophil survival was dependent on PI3K and NF-κB, consistently with the results in other cell types (Park et al. 2002, Jozsef et al. 2004, O'Keeffe et al. 2005). Production of IL-8 by CpG ODNs has also been reported further supporting a role for NF-κB (Mansson and Cardell 2009).

There is increasing evidence to support the idea that microbes play a role in the development and pathogenesis of asthma (Edwards et al. 2012). In contrast to traditional views, the lower airways are not sterile and contain a mean of 2.000 bacterial genomes per cm^2 surface (Hilty et al. 2010). This is comparable to the numbers of bacteria in the upper parts of small intestine. In a study involving healthy volunteers and patients with asthma and COPD, the highest counts of bacterial genomes ($50.000/cm^2$) were found in the bronchi of an asthmatic subject. Additionally, the bronchi of asthmatics contain significantly more pathogenic proteobacteria, such as *Haemophilus* species, than airways of healthy controls (Hilty et al. 2010). Bacterial DNA reduced apoptosis at a concentration between 10 and 30 µg/ml but in order to determine the

biological significance, it is critical to elucidate whether this concentration can be reached in natural conditions. A 20 μg/ml concentration of bacterial DNA has been detected in the sputum of patients with cystic fibrosis (Schwartz et al. 1997) suggesting that the results may have biological relevance. Bacterial respiratory tract infections have been associated with asthma exacerbations (Cunningham et al. 1998, Wark et al. 2002) and our results provide one possible mechanism for asthma exacerbation induced by bacterial respiratory tract infection. The present study also provides a possible mechanism on how bacterial DNA might increase eosinophilic inflammation and contribute to the development and maintenance of asthma.

4 Orazipone and nitric oxide: mechanisms of eosinophil apoptosis

In this study, the drug candidate orazipone and NO-donor SNAP were both found to induce eosinophil apoptosis in the presence of survival-prolonging cytokine GM-CSF or IL-5. The signalling pathways these compounds stimulated in eosinophils had many similarities such as early JNK activation and activation of caspases 3 and 6 suggesting that there may be further similarities.

Oxidative stress has previously been shown to promote eosinophil apoptosis (Wedi et al. 1999, Kankaanranta et al. 2002, Lee and Shin 2009). Antioxidants, such as glutathione and N-acetyl-cysteine (NAC), protect cells against oxidative stress and they have been shown to inhibit spontaneous eosinophil apoptosis and apoptosis induced by oxidative stress and Fas (Wedi et al. 1999). It was demonstrated in this study that NO-induced apoptosis in GM-CSF-treated eosinophils was dependent on ROS. The mechanism of orazipone-induced eosinophil apoptosis was shown to involve thiol-modulating property of the drug because the negative control compound OR-2149, which is not able to modulate thiols due to the reduced double bond, had no effect. The antioxidant glutathione is one important thiol-containing compound with which orazipone is likely to form adducts with (Sareila et al. 2008). Glutathione is considered to be the most important antioxidant defence in mitochondria (Orrenius et al. 2007). Inactivation of glutathione results in increased oxidative stress and it would be interesting to study whether orazipone, similarly to NO, actually induces eosinophil

apoptosis mediated by oxidative stress. SNAP or orazipone were not toxic as demonstrated by the low amount of Annexin-V^- propidium $iodide^+$ eosinophils.

Mitochondrial permeability transition (mPT) typically plays a critical role in the apoptosis induced by oxidative stress (Corry 2002, Orrenius et al. 2007), and therefore its involvement in NO-induced apoptosis was evaluated. It was observed that NO could induce early partial mPT. However, instead of being a critical mediator of NO-induced apoptosis, this partial mPT led to strong JNK activation. It was concluded that the early mPT was transient or flickering because it was partial and $\Delta\psi_m$ was not lost at the 20 h time-point. There is limited but consistent evidence indicating that flickering mPT is a means to discharge mitochondrial matrix calcium overload, thereby preventing the initiation of intrinsic apoptosis (Bernardi and von Stockum 2012). There is also evidence of flickering mPT-mediated release of superoxide or ROS (Zorov et al. 2006, Ma et al. 2011). Since ROS is a known inducer of JNK, this may be the mechanism for the strong JNK activation demonstrated at 2 h. Interestingly, glucocorticoids have been shown to enhance ROS production in eosinophils and ROS was shown to mediate glucocorticoid-induced eosinophil apoptosis. ROS is believed to have a critical role also in glucocorticoid-induced T-cell apoptosis (Zamzami et al. 1995a, Zamzami et al. 1995b). Dexamethasone induced an early JNK activation that was dependent on early production of ROS (Gardai et al. 2003). Consistently, orazipone induced rapid activation of JNK at 1 h. In summary, this study may have identified a general mechanism to explain early JNK activation induced by drugs enhancing oxidative stress in eosinophils. Substances that induce oxidative stress in these cells (NO, glucocorticoids, possibly orazipone) may induce flickering mPT, leading to release of ROS and activation of JNK. The previous knowledge was also extended by showing that the early JNK activation is not directly related to induction of apoptosis in eosinophils subjected to oxidative stress. Indeed, in other cell types early JNK activation has been described to represent a stress response resulting in cell survival signalling (Sanchez-Perez et al. 1998, Ventura et al. 2006). The contribution of JNK to cell survival may be mediated via JunD (Lamb et al. 2003).

NO induced also a later and lower level JNK activation that was shown to mediate apoptosis. According to investigations of others, this type of delayed and sustained activation of JNK is indeed typically related to apoptosis (Sanchez-Perez et al. 1998, Ventura et al. 2006). Dexamethasone also stimulated both early (40 min) and late (16 h) JNK activation (Gardai et al. 2003). It would have been interesting to study the effects

of orazipone on pJNK levels at later time-points. Drugs producing oxidative stress seem to induce both early and late activation of JNK, of which the early activation may be a stress response and the late activation mediates apoptosis.

JNK has been previously shown to mediate apoptosis through several pathways: AP-1-mediated transcription of FasL and TRAIL-receptor 1 (Eichhorst et al. 2000, Guan et al. 2002), phosphorylation of Bcl-2 family protein members (Schroeter et al. 2003, Bogoyevitch and Kobe 2006, Raman et al. 2007), mPT induction (Hanawa et al. 2008, Lin et al. 2009) and phosphorylation of histone H2AX required for DNA fragmentation (Lu et al. 2006). According to these results in eosinophils, JNK inhibition prevented only DNA fragmentation, not the other apoptotic events studied. Similarly, inhibition of caspase 6 mainly inhibited DNA fragmentation. This suggests that functions of JNK and caspases are closely connected to mediate DNA fragmentation. According to the results of a previous study, JNK-induced phosphorylation of histone H2AX was required for DNA breakdown during apoptosis and JNK was also required for the activation of caspase 3. It was concluded that JNK seems to regulate caspase-mediated fragmentation of DNA (Lu et al. 2006).

Treatment with NO ultimately induced eosinophil apoptosis that was dependent on late mPT. The results do not explain whether flickering mPT is continuous leading eventually to the threshold for permanent mPT or whether the flickering mPT ceases and the permanent mPT is a distinct late event. The accumulation of oxidative damage may be a critical determinant that stimulates the occurrence of permanent mPT and the subsequent apoptosis and this is supported by the finding that oxidative stress produced before 16 h was critical for NO-induced apoptosis.

Inhibitors of initiator caspases (8 or 9) did not show any capability to prevent orazipone-induced apoptosis, even though these caspases, especially caspase 9, were activated by orazipone. The inhibitors used in the present study inhibited the activities of caspases 8 and 9 by 99 % and 65 %, respectively. It is not clear if the residual 35 % of caspase 9 activity would be sufficient to activate effector caspases. Thereby, the possibility does exist that this result is a consequence of an inefficient inhibitor. Effector caspases 3 and 6 were involved in both NO- and orazipone-induced apoptosis and their role was discussed in the section 2.

These present results produced significant novel information on the mechanisms of eosinophil apoptosis induced by NO and oxidative stress. Oxidative stress may be a surprisingly common mediator of eosinophil apoptosis induced by various factors such

as glucocorticoids (Gardai et al. 2003). Consequently, the mechanisms revealed by this study may be rather general. Based on the results of this study, a hypothetical model of the effects of low or high level of oxidative stress on eosinophils is shown in Figure 24. The interesting question is whether these mechanisms are active also in physiological situations. It is difficult to assess the physiological level of NO in the lungs because NO is a free radical and reacts readily with proximal molecules. However, it is clear that oxidative and nitrosative stress is abundant in the lungs of asthmatics this being reflected in the increased levels of exhaled NO (Lehtimaki et al. 2001), excessive oxidative protein products in the lungs (nitrotyrosine, 3-bromotyrosine, chlorotyrosine) and reduced levels of antioxidants (Comhair and Erzurum 2010). A close correlation exists between airway eosinophilia and exhaled NO-levels (Jatakanon et al. 1998, Payne et al. 2001, Malerba et al. 2008) although airway epithelial cells seem to be the major source of NO instead of eosinophils (Guo et al. 1995, Guo et al. 1997, MacPherson et al. 2001). Based on these results one could speculate that NO may induce a protective response in eosinophils in a physiological situation, involving early flickering mPT and JNK activation. These events might occur also at lower NO concentration than used in this study. However, NO may also induce eosinophil apoptosis in some clinical situations as supported by a study involving children with and without asthma. In those children, apoptotic rate of sputum eosinophils was correlated to exhaled NO (Pontin et al. 2008). Taken together, the role of NO in the regulation of eosinophils functions

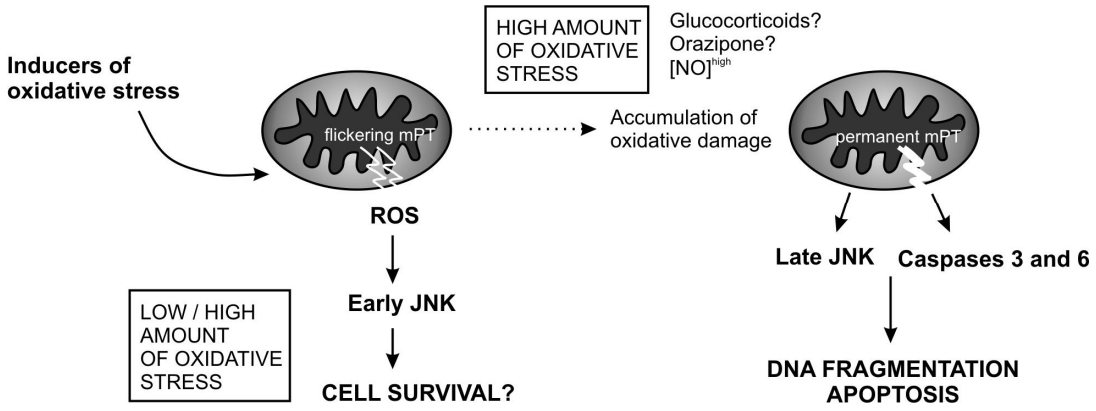

Figure 24. *Hypothetical model of consequences of oxidative stress in eosinophils. Low level of oxidative stress may result in eosinophil survival while high level of oxidative stress induces eosinophil apoptosis. Levels of NO produced during airway inflammation might support eosinophil survival. Oxidative stress may mediate eosinophil apoptosis induced by various factors/drugs, for example glucocorticoids.*

seems to be complex and the end response may depend on the local concentration of NO and the local environment. This study is one step forward in attempts to explain the conflicting effects of NO on eosinophils.

5 NPSR1 expression and function in eosinophils

This study detected higher expression of NPSR1 in blood eosinophils from subjects with high IgE (IgE>100 U/ml) when compared to subjects with low IgE (IgE<100 U/ml). Additionally, it was noted that eosinophils from patients with severe asthma expressed higher levels of NPSR1 than eosinophils from patients with less severe disease or from healthy subjects. Consistent with these results, there is evidence that higher NPSR1 expression is involved in inflammatory conditions (Laitinen et al. 2004, D'Amato et al. 2007). Additionally, IFNγ and TNFα, and LPS enhanced NSPR1 mRNA levels in human mononuclear cells (Bruce et al. 2009, Sundman et al. 2010). The reason for the enhanced NPSR1 expression in eosinophils from patients with high IgE remains unclear because none of the tested mediators (GM-CSF, TNF-α, IFN-γ, TNF-α + IFN-γ, LPS, LTB_4 or fMLP) increased NPSR1 protein levels *in vitro*. However, in future studies it would be interesting to clarify whether some Th2 cytokines such as IL-4 or IL-33 can enhance the expression of NPSR1. Another interesting topic for further studies would be the effect of the amino acid change N107I on NPSR1 expression or function in eosinophils.

According to these results, it seems clear that higher NPSR1 expression is related to allergic/atopic asthma characterized by high total IgE, and to severe asthma. According to the skin-prick tests, 50 % of the patients with severe asthma and 80 % of the patients with mild asthma were atopic, raising the question of the contribution of atopy to the increased NPSR1 levels detected in eosinophils from severe asthmatics. However, because concomitant total IgE levels were not known, this remains speculative. Furthermore, based on this study one can not state that NPSR1 level is increased in non-asthmatic allergic patients with high IgE because of the small number of those patients examined in the expression study. However, this is an interesting topic for future study.

NPS has been previously shown to increase both intracellular cAMP and Ca^{2+} suggesting activation of both G_s- and G_q-pathways in HEK293 cells overexpressing

NPSR1 (Pietras et al. 2011). Consistently, raised levels of cAMP were detected in NPS (20 μM)-treated human eosinophils. Enhancers of cAMP such as β_2-agonists and PGE_2 typically decrease the rate of eosinophil apoptosis and inhibit eosinophil functions such as degranulation (Kita et al. 1991b, Giembycz and Lindsay 1999, Peacock et al. 1999, Chang et al. 2000, Parkkonen et al. 2008, Kankaanranta et al. 2011) but NPS did not induce any such effects with the exception of decreased CD11b levels in eosinophils from low IgE donors. The trend in eosinophils was that NPS produces some increase in activation (increased adhesion molecule expression, superoxide production and degranulation) in eosinophils derived from subjects with high IgE. In previous studies, agents elevating intracellular Ca^{2+} such as PAF, fMLP and Ca^{2+} ionophore have typically enhanced eosinophil activation (Sedgwick et al. 1988, Kroegel et al. 1989, Sedgwick et al. 1992, Giembycz and Lindsay 1999, Tan and Lim 2008) suggesting that intracellular Ca^{2+} levels may mediate the stimulatory effects of NPS on eosinophil activation. It would have been interesting to examine the effect of NPS on intracellular Ca^{2+} content in eosinophils. A possible subject for future study is also the potential chemotactic effect of NPS on eosinophils, which has been recently described in human blood monocytes (Filaferro et al. 2012).

Adhesion molecules such as CD11b are critical for recruitment of eosinophils from blood circulation to the inflamed tissues, such as asthmatic airways. It was found that NPS increased CD11b levels in fMLP- but not GM-CSF-stimulated eosinophils from donors with high IgE. This effect may be explained by synergistic effect of NPS and fMLP on intracellular Ca^{2+} via their GPCRs. Many mechanisms have been described to explain the synergistic action of GPCRs (Werry et al. 2003). Eosinophils derived from subjects with high IgE exhibit increased levels of NPSR1 but may also more often express the NPSR1 variant that displays enhanced function (Laitinen et al. 2004, Reinscheid et al. 2005, Bernier et al. 2006) explaining why this effect is observed only in eosinophils from these donors.

Even though NPSR1 was initially reported to be up-regulated in a mouse model of airway inflammation and expression of its isoforms differed in bronchial biopsies of patients with asthma and healthy controls (Laitinen et al. 2004), results from two different mice lacking functional NPSR1 have indicated that NPSR1 may not have a crucial role in the regulation of allergic airway inflammation (Allen et al. 2006, Zhu et al. 2011). A general view has evolved that NPSR1 mainly mediates the functions of the central nervous system (Xu et al. 2004, Zhu et al. 2010) and may contribute to asthma,

e.g. by affecting respiratory functions (Zhu et al. 2011). However, recent evidence of the effects of NPS on immune cell functions (Table 11) and the present study offer grounds to reassess the role of NPSR1 in human allergic asthma and indicates that further work should be conducted in this area.

Table 11. *Current evidence of the regulation of immune cell functions by NPS*

Immune cell type	Effect of NPS on cell function	Reference
Primary human monocytes	Increases chemotaxis	(Filaferro et al. 2012)
Mouse macrophage cell line RAW 264.7	Stimulates phagocytosis of *E.Coli* and migration	(Pulkkinen et al. 2006)
Pig splenic lymphocytes	Increases proliferation	(Yao et al. 2011)
Pig pulmonary alveolar macrophages	Increases production of proinflammatory cytokines and phagocytosis	(Yao et al. 2011)
Primary human eosinophils from subjects with high IgE	Increases adhesion molecule CD11b expression induced by fMLP	(V)

6 Eosinophil as a target for pathophysiological factors and pharmacological compounds

Eosinophils are abundantly present in most phenotypes of asthma and they contribute to the maintenance and exacerbation of the disease (Wenzel 2006, Hogan et al. 2008). Their removal via induction of apoptosis confers clinical benefits and thus the promotion of eosinophil apoptosis is considered as one important mechanism of action of glucocorticoids (Woolley et al. 1996, Druilhe et al. 1998b). Eosinophils are surrounded by various pathophysiological factors including nitric oxide, pathogenic components and possibly NPS and it is important to elucidate the effects of those agents on eosinophils.

Asthma is characterized by a high bacterial load (Hilty et al. 2010, Edwards et al. 2012) and increased levels of exhaled NO (Kharitonov et al. 1994, Persson et al. 1994, Saleh et al. 1998, Lehtimaki et al. 2001, Maa et al. 2003). Here it was found that bacterial DNA increases eosinophil longevity and this represents a putative mechanism for maintenance or exacerbation of eosinophilic inflammation in the asthmatic airways by bacteria. NO together with inflammatory cell-derived superoxide and peroxidases leads to continuous oxidative stress in the airways (Comhair and Erzurum 2010). This study revealed a possible mechanism to explain how NO could induce a protective

effect in eosinophils via inducing rapid flickering mPT and early JNK activation. The protective effect of NO on eosinophils is supported by clinical observations (Jatakanon et al. 1998, Payne et al. 2001, Malerba et al. 2008). In this study it was also demonstrated that treatment of eosinophils with high concentration of NO *in vitro* resulted in apoptosis mediated by ROS, JNK and caspases. Even though it remains unclear whether bronchial NO induces eosinophil apoptosis in physiological situations, apoptosis mediated by oxidative stress may be a surprisingly general and may well explain the pro-apoptotic actions of some therapeutic drugs and other compounds. For example, mechanisms of apoptosis induced by NO and glucocorticoids seem to involve oxidative stress and display many similarities (Gardai et al. 2003). Therefore, these results may also be applied to better understand the mechanism of action of glucocorticoids in eosinophils. According to the results of this study, subjects with severe asthma or high IgE are characterized by increased levels of NPSR1 in eosinophils. More studies will be needed to reveal the precise role of NPSR1 in eosinophils, other inflammatory cells and asthma/allergy in general. It remains to be decided whether NPSR1 is a potential drug target for the treatment of allergic asthma.

This thesis produced a substantial amount of information that may be utilized in drug development. A significant proportion, 20-35 %, of patients with asthma respond poorly to glucocorticoids (Malmstrom et al. 1999, Szefler et al. 2002) indicating that novel anti-inflammatory drugs are needed to treat asthma. Orazipone and CpG ODNs have both exhibited high potency to reduce eosinophilia in animal models of allergic airway inflammation (Ruotsalainen et al. 2000, Kline et al. 2002, Santeliz et al. 2002, Jain et al. 2003) even though CpG ODNs were ineffective when administered to patients with allergic asthma despite of induction of Th1-related genes (Gauvreau et al. 2006). The suitability of using nitric oxide donors to treat asthma is also under investigation, these compounds may possess antibronchoconstrictive and anti-inflammatory effects (Redington 2006, Larsson et al. 2007, Oliveira et al. 2008, Turner et al. 2010, Foster et al. 2011). NO-releasing drugs have shown contradictory effects on eosinophilia in animal models of asthma/allergy (Oliveira et al. 2008, Foster et al. 2011). On the basis of the present results, one could speculate that the effect may depend on the concentration of NO. However, inhibitors of iNOS had no effect in patients with asthma (Hansel et al. 2003, Singh et al. 2007). The present data reveals induction of eosinophil apoptosis as a novel mechanism to explain the efficacy of orazipone in reducing airway eosinophilia. Orazipone may be even more potent than glucocorticoids in reducing

eosinophilia because the pro-apoptotic effect of orazipone was not abolished by elevated concentration of IL-5, in contrast to the pro-apoptotic action of glucocorticoids (Hagan et al. 1998). Orazipone breaks down in the blood but would be suitable as an inhalation formulation to treat asthma (Aho et al. 2001). Our results, however, do not explain the anti-eosinophilic efficacy of CpG ODNs in animal models of asthma but may partly explain their inefficiency in the treatment of patients with asthma. It remains to be determined whether pro-apoptotic effect of NO on eosinophils is of importance in explaining the action of NO-releasing drugs *in vivo*. The effects of NO are complex during airway inflammation, and for this reason the therapeutic potential of NO donors or iNOS inhibitors in human asthma may be limited.

In summary, this thesis produced information on the possible pathophysiological mechanisms underlying eosinophilic airway inflammation. In addition, findings of this thesis may be applied in the development of drugs targeting eosinophils to treat asthma and other eosinophilic disorders.

SUMMARY AND CONCLUSIONS

The aim of this study was to examine the regulation of human eosinophil viability by pathophysiological and pharmacological compounds such as bacterial DNA, nitric oxide, NPS and the novel candidate drug, orazipone. Study of NPS was extended by determining the expression and function of its receptor NPSR1 in human eosinophils. All experiments were conducted in primary human blood eosinophils.

The major findings and conclusions were:

1. Bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG motifs delayed human eosinophil apoptosis via TLR9, PI3K and NF-κB. In addition, methylated bacterial DNA but not vertebrate DNA delayed apoptosis suggesting that bacterial DNA may contain additional immunostimulatory features.

2. A novel candidate drug orazipone and its analogue OR-2370 induced eosinophil apoptosis in the presence of survival-prolonging cytokine IL-5. It was determined that caspases 3 and 6 and JNK mediated OR-2370-induced DNA fragmentation and apoptosis.

3. Nitric oxide induced early flickering mPT and mPT-dependent JNK activation in GM-CSF-treated eosinophils; this phenomenon did not mediate apoptosis but may represent a stress response. Extended exposure to NO finally led to eosinophil apoptosis mediated by ROS, late mPT, JNK and caspases 3 and 6.

4. NPSR1 expression was increased in eosinophils from subjects with high total IgE (>100 IU/ml) and from patients with severe asthma when compared to eosinophils from subjects with lower total IgE (<100 IU/ml) or healthy subjects and patients with mild asthma, respectively. NPS increased fMLP-induced

adhesion molecule CD11b expression in eosinophils from patients with high IgE but not from those with low IgE. NPS also elevated intracellular cAMP levels.

This study provides a possible mechanism to explain maintenance and exacerbations of eosinophilic lung inflammation in the presence of bacteria in the respiratory tract. The results also support multiple functions of NO in the regulation of eosinophil apoptosis possibly depending on the NO concentration and the cellular environment. According to this study, NPSR1 may have a pathophysiological role in patients with severe asthma and/or elevated IgE levels. Finally, the results indicate that orazipone and its analogues might have beneficial effects in the treatment of eosinophilic inflammatory disorders such as asthma.

In conclusion, this thesis extended the previous knowledge on the pathophysiological mechanisms underlying eosinophilic airway inflammation and produced information that can be utilized in the development of drugs targeting eosinophils to treat asthma and other eosinophilic disorders.

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Bacterial DNA delays human eosinophil apoptosis

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ABSTRACT

Oligodeoxynucleotide (ODN) sequences containing unmethylated cytidine phosphate guanosine (CpG) motifs prevalent in bacterial DNA attenuate allergic lung inflammation in experimental models of asthma but failed to inhibit eosinophilia and improve lung function in patients with asthma. Bacterial respiratory tract infections exacerbate asthma in humans. Increased eosinophil survival is a critical factor leading to persistent eosinophilic airway inflammation. Apoptosis is regarded as a key mechanism in the resolution of eosinophilic inflammation. The aim of this study was to investigate the effects of bacterial DNA and CpG ODNs on human eosinophil apoptosis *in vitro* and to elucidate the signalling pathway.

Eosinophils were isolated from human peripheral blood by CD16- or CD16-, CD19- and CD304-negative selection. Apoptosis was determined by flow cytometric analysis of relative DNA content, Annexin-V staining and/or morphological analysis. Toll-like receptor 9 (TLR9) expression was studied by using western blotting and intracellular flow cytometry.

Bacterial DNA and phosphorothioate-modified CpG ODNs, but not vertebrate DNA, were found to delay spontaneous eosinophil apoptosis. The effect of CpG ODNs was dependent on endosomal acidification and reversed by inhibitory ODN, which suggests involvement of TLR9 pathway. Furthermore, we demonstrated TLR9 expression in eosinophils derived from both atopic and healthy donors. Non-CpG ODNs had occasionally parallel but less profound effect on eosinophil apoptosis, which was not dependent on endosomal acidification. The anti-apoptotic effect of CpG ODNs was dependent on phosphatidylinositol 3-kinase (PI3K) and nuclear factor-κB (NF-κB) but not mitogen-activated protein kinases (MAPKs) as determined by inhibitor studies. Although our results suggest CpG-dependent involvement of TLR9 in the action of phosphorothioate-modified ODNs, we interestingly found that the anti-apoptotic action of native bacterial DNA in eosinophils is not dependent on unmethylated CpG motifs. This suggests that bacterial DNA contains a currently unknown recognition structure lacking from vertebrate DNA. Bacterial DNA-mediated suppression of eosinophil apoptosis is a novel mechanism for exacerbation of eosinophilic lung inflammation associated with bacterial respiratory tract infection.

1. Introduction

Asthma is a chronic inflammatory disease, where eosinophilic granulocytes are numerous in the lungs. Release of eosinophil products such as toxic granule proteins, cysteinyl leukotrienes, pro-inflammatory cytokines and reactive oxygen species leads to epithelial cell damage, mucosal damage, bronchoconstriction and increased mucus secretion and vascular permeability [1]. Additionally eosinophils have recently been demonstrated to have an essential role in airway remodelling and a significant regulatory role in T-helper cells 2 (TH_2)-cytokine production [2,3]. Increased eosinophil survival is a critical factor leading to persistent eosinophilic airway inflammation. Apoptotic cell death is considered as an important removal mechanism of eosinophils from the lungs but in patients with asthma eosinophil apoptosis is delayed [4].

Pathogenic components modulate allergic inflammation in several ways. According to the hygiene hypothesis, infections may prevent development of allergic disease. On the other hand, respiratory tract infections seem to exacerbate established asthma and contribute to asthma chronicity [5,6]. These infections, even though originally defined of viral origin, often involve mixed bacterial co-infection and several types of bacterial infections have been associated with acute asthma exacerbations or chronic stable

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asthma [6,7]. Eosinophils have been suggested to play an important role in asthma exacerbations [8,9].

Bacterial DNA is characterized by unmethylated cytidine phosphate guanosine (CpG) dinucleotides. In vertebrate DNA, unmethylated CpG dinucleotides are uncommon. DNA containing unmethylated CpG dinucleotides is a pathogen-associated molecular structure recognized by Toll-like receptor 9 (TLR9), a receptor of innate immunity [10]. In mice, bacterial DNA has been previously found to exert both pro-inflammatory and anti-inflammatory effects. Bacterial DNA was reported to induce inflammation in the lower respiratory tract of mice [11]. On the other hand, synthetic CpG oligodeoxynucleotides (ODNs) are currently under intense investigation due to their anti-inflammatory effects in mouse models of asthma [12,13]. Stimulation by CpG ODNs activates TH_1-type innate immune response, which is thought to inhibit TH_2-type allergic immune response. Synthetic CpG ODNs with different sequences and backbones activate distinct cell types, which has led to their categorization into classes A, B and C. Class A CpG ODNs induce high secretion of type I interferons (IFNs) by plasmacytoid dendritic cells (pDC), whereas class B CpG ODNs induce interleukin (IL)-6 production and proliferation of B-cells. Class C CpG ODNs were developed to have immunostimulatory activity that is combination of the activities induced by class A and B CpG ODNs [14].

Only scarce information exists of TLR9 function in human eosinophils [15,16]. To our knowledge, no information exists of the effects of native bacterial DNA on eosinophils. Similarly, it is not known whether the effect of CpG DNA on eosinophils involves TLR9 or how the signalling is mediated. To evaluate the role of eosinophils in the modulatory action of CpG DNA in inflammation, we aimed to study the effects of bacterial DNA and synthetic CpG ODNs on human eosinophil apoptosis. Additionally, we aimed to establish the signalling pathway mediating the effect.

2. Materials and methods

2.1. Oligodeoxynucleotides and DNA

ODNs were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. We used ODNs with the following sequences (phosphorothioate bases are shown with small letters and phosphodiester bases with capitals): Class A CpG ODN D19 5′-ggTGCATCGATGCA Gggggg-3′, non-CpG ODN Dc (control for D19) 5′-ggTGCATGCATG CAGggggg-3′, Class B CpG ODN 1018 5′-tgactgtgaacgttcgagatga-3′, non-CpG ODN 1040 (control for 1018) 5′-tgactgtgaaccttagagatga-3′, Class B CpG ODN 2006 5′-tcgtcgttttgtcgttttgtcgtt-3′, Class C CpG ODN C274 5′-tcgtcgaacgttcgagatgat-3′, non-CpG ODN C661 (control for C274) 5′-tgcttgcaagcttgcaagca-3′ and inhibitory ODN 5′-ttagggtt agggttagggttaggg-3′. ODNs were diluted in Tris–EDTA (10 mM Tris, pH 7.5–8.0, 1 mM EDTA) to prevent degradation of the short ODNs known to occur in acidic conditions. *Escherichia coli* (*E. coli*) *K12* DNA and salmon sperm DNA were purchased from Invivogen, San Diego, CA, USA. They were diluted in nuclease- and endotoxin-free sterile water according to the manufacturer's instructions. *E. coli* and salmon sperm DNA were made single-stranded before use by heating at 95 °C for 10 min, after which they were rapidly cooled on ice. In each experiment, *E. coli* DNA and salmon sperm DNA were added in the beginning and once after 16–18 h of culture. For some experiments, *E. coli* DNA was treated in NE buffer 2 with CpG methyltransferase (2 U/μg DNA) and 160 μM *S*-adenosylmethionine for 3 h at 37 °C. Methylated and unmethylated DNA (for control) was purified by phenol extraction and ethanol precipitation and dissolved in sterile water. To confirm successful methylation process, we treated DNA with restriction enzyme BstUI followed by agarose gel electrophoresis.

2.2. Other materials

Other reagents were obtained as follows: Anti-TLR9 antibody and its blocking peptide (ProSci Inc., Poway, CA, USA), horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phycoerythrin (PE)-conjugated anti-TLR9 antibody, PE-conjugated rat IgG2a isotype control, fixation buffer and permeabilization buffer (eBioscience, San Diego, CA, USA), CpG methyltransferase M.SssI and BstUI (New England Biolabs, Ipswich, MA, USA), bafilomycin A1 from *Streptomyces griseus*, BMS-345541, pyrrolidine dithiocarbamate (PDTC), SB203580, budesonide, dimethyl sulfoxide (DMSO) and propidium iodide (PI) (Sigma–Aldrich Co., St. Louis, MO, USA), wortmannin, SP600125, negative control for SP600125, PD98059, SB202474 (Merck Biosciences Darmstadt, Germany), anti-CD16, anti-CD19 and anti-CD304 microbeads, fluorescein isothiocyanate (FITC)-conjugated anti-CD303 antibody and the magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-CD123 antibody, FITC-conjugated anti-CD19 antibody, $IgG_1\kappa$ isotype control (BD Biosciences Pharmingen, San Jose, CA, USA), PE-conjugated IgG2a, FITC-conjugated IgG1 (Chemicon International Inc., Temecula, CA, USA), MG-132 (Tocris Bioscience, Bristol, UK), chloroquine (Invivogen, San Diego, CA, USA). Other reagents were obtained as described elsewhere [4,17,18]. Stock solutions of wortmannin, bafilomycin A1, mitogen-activated protein kinase (MAPK) inhibitors and their negative controls, BMS-345541 and MG-132 were prepared in DMSO. Final DMSO concentration in the cells was 0.3%. Budesonide stock was prepared in ethanol and the final ethanol concentration in the cells was 0.2%. Similar concentration of the solvent was added to the control cultures.

2.3. Human eosinophil isolation and culture

The blood samples (100 ml) were taken from eosinophilic donors, mostly from patients with asthma and/or allergy. All donors gave written informed consent to a study protocol approved by the Ethical Committee of Tampere University Hospital. Eosinophils were isolated by immunomagnetic CD16-negative selection under sterile conditions as previously described [4,17,19]. Purity of eosinophils after the isolation process was at least 99%. For some experiments, eosinophils were further purified by CD19- and CD304-negative selection to remove possible contaminating B-cells and plasmacytoid dendritic cells. Cells were resuspended at 10^6/ml and cultured in Dutch modification of RPMI 1640 containing 10% fetal bovine serum, antibiotics and L-glutamine at 37 °C with 5% CO_2 in 96-well plates. If not otherwise stated, eosinophils isolated by CD16-negative selection were used in the experiments.

2.4. Apoptosis assays

Relative DNA fragmentation assay and flow cytometric analysis of PI-stained cells were performed as previously described [4,17,19]. Cells with reduced DNA content were considered as apoptotic. For morphological analysis eosinophils were spun onto cytospin slides (25 g, 5 min), fixed in methanol for 15 min and stained with May-Grünwald–Giemsa. Shrunken cells with nuclear coalescence and chromatin condensation were considered apoptotic. Annexin-V binding assay was performed as previously described [18,19]. The cells displaying positive Annexin-V FITC labelling (FITC+/PI− and FITC+/PI+) were regarded as apoptotic.

2.5. Determination of the amount of contaminating CD19+ and CD123+ CD303+ cells in eosinophil suspensions

The amount of contaminating B-cells (CD19+) and pDC (CD123+ CD303+) after eosinophil isolation was assessed by

immunofluorescence and flow cytometric analysis of 20,000 cells as described by Matsumoto et al. [20]. Briefly, the cells were incubated for 20 min at +4 °C in PBS buffer containing 0.5% BSA and 2 mM EDTA, Fc receptor blocking reagent and fluorophore-conjugated monoclonal antibody or corresponding IgG control at concentration recommended by the manufacturer. Cells were washed with PBS buffer and analyzed by flow cytometer.

2.6. TLR9 expression

TLR9 expression was determined by western blotting and intracellular flow cytometry. For western blotting, eosinophils were lysed in ice-cold radioimmuno precipitation assay (RIPA)-buffer, after which protein was mixed in sodium dodecyl sulfate (SDS)-containing loading buffer and loaded onto 8% SDS-polyacrylamide electophoresis gel. After electrophoresis, proteins were electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, UK, Ltd., Little Chalfont, Buckinghamshire, UK) and blocked for 1 h in Tris-buffered saline with tween (TBST) containing 5% bovine serum albumin (BSA). Membrane was incubated overnight at +4 °C in the blocking solution with 1 μg/ml anti-TLR9 or blocked anti-TLR9. Blocking of anti-TLR9 was conducted by incubating the antibody with 1 μg/ml blocking peptide at +37 °C for 30 min. For flow cytometric analysis of TLR9 expression, eosinophils were fixed, permeabilized and stained with PE-conjugated TLR9 antibody or IgG control (1 μg/million cells) according to the manufacturer's instructions.

2.7. Statistics

Results are expressed as mean ± standard error of mean (SEM). Apoptosis is expressed as percentage of apoptotic cells (number of apoptotic cells/total number of cells × 100). Statistical significance was calculated by paired *t*-test or by repeated measures analysis of variance with Dunnett's post-test. Differences were considered significant when $p < 0.05$.

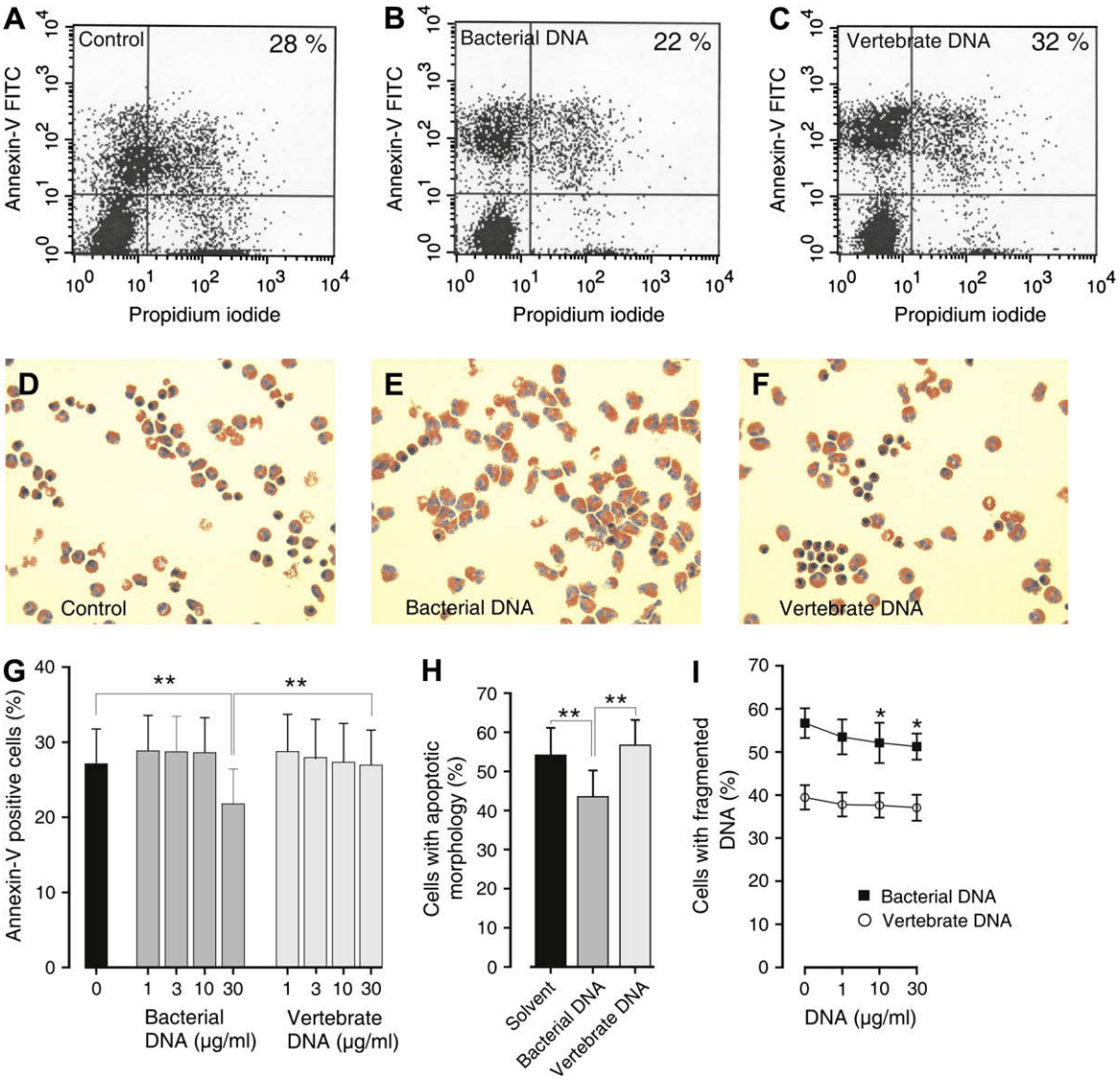

Fig. 1. Effect of bacterial DNA on human eosinophil apoptosis. Eosinophils were incubated in the absence or presence of bacterial or vertebrate DNA for 20 h (A–C, G) or for 40 h (D–F, H–I) after which apoptosis was determined by Annexin-V FITC binding assay (A–C, G), morphological analysis (D–F, H) or relative DNA fragmentation assay (I). In A–C and D–F shown are representative graphs of 6–8 experiments. Percentage of Annexin-V positive cells (FITC+/PI− and FITC+/PI+) is shown in the upper right corners of A–C. Where not stated, DNA concentration was 30 μg/ml. In G–I shown are mean ± SEM of cells isolated from six to eight donors. Asterisk * indicates $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Effect of bacterial DNA on spontaneous eosinophil apoptosis

Human eosinophils undergo spontaneous apoptosis in the culture in the absence of life-supporting cytokines. An early sign of apoptosis, translocation of phosphatidylserine from the inner to the outer leaflet of the lipid bilayer in the cell membrane, was inhibited by bacterial DNA (30 μg/ml) but not by vertebrate DNA (30 μg/ml) as determined by Annexin-V binding assay (Fig. 1A–C, G). Similarly, treatment with bacterial DNA reduced the percentage of eosinophils with apoptotic morphology as compared to treatment with solvent or vertebrate DNA when measured after 40 h of incubation (Fig. 1D–F, H). In addition, treatment with bacterial DNA (30 μg/ml) led to reduction in the number of eosinophils with decreased relative DNA content indicative of reduction in the amount of fragmented DNA (Fig. 1I). In contrast, vertebrate DNA had no effect on DNA fragmentation (Fig. 1I).

3.2. Effects of synthetic class A, B and C CpG ODNs on spontaneous eosinophil apoptosis

Bacterial but not vertebrate DNA inhibited human eosinophil apoptosis. To determine whether this is due to the CpG structure more prevalent in bacterial DNA, we tested the effects of class A, B and C CpG ODNs on eosinophil apoptosis. As determined by DNA fragmentation assay, class B CpG ODN 1018 inhibited apoptosis in a concentration-dependent manner, while non-CpG ODN 1040 had no effect (Fig. 2A–C, G, H). Similarly, treatment with 3 μM 1018 resulted in a reduction in the number of eosinophils with apoptotic morphology (Fig. 2D–F, I). Also the number of cells expressing phosphatidylserine on the outer leaflet of the cell (Annexin-V positive) was reduced by $19 \pm 3\%$ ($p < 0.01$, $n = 6$). Interestingly, however, treatment with non-CpG ODN 1040 reduced the amount of eosinophils with apoptotic morphology (Fig. 2I) and bound Annexin-V ($15 \pm 2\%$ decrease in apoptosis, $p < 0.01$, $n = 6$), although no statistically significant effect occurred in DNA fragmentation assay. Treatment with another class B CpG ODN 2006 or class C CpG

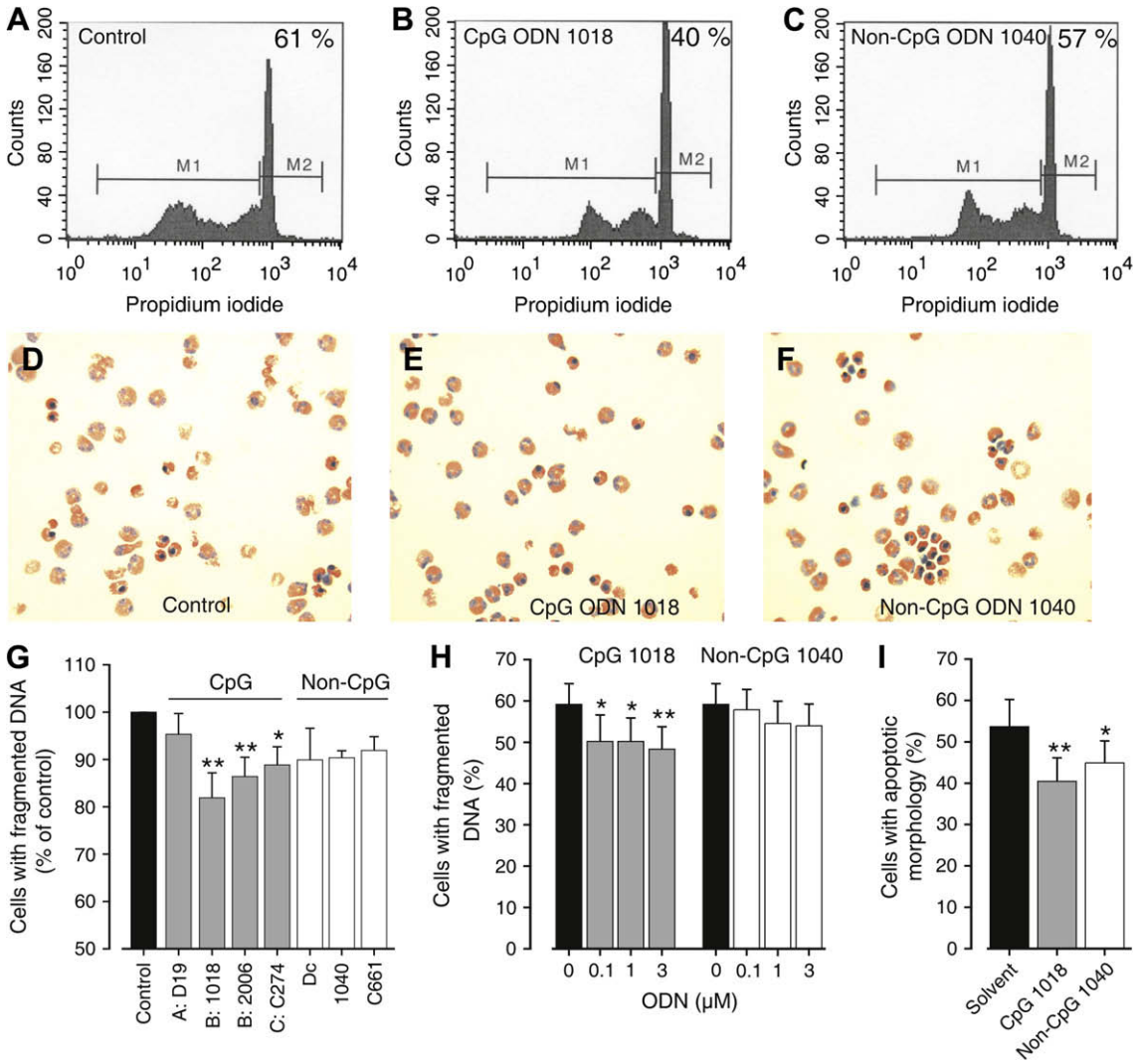

Fig. 2. Effects of class A, B and C CpG ODNs on eosinophil apoptosis. Typical graphs from relative DNA fragmentation assay are shown in A–C and typical cell morphologies in D–F after eosinophils were treated with or without class B CpG ODN 1018 (3 μM) or respective non-CpG ODN 1040 (3 μM) for 40 h. Proportion of cells with decreased DNA content (gate M1) is shown in the upper right corner of A–C. Data in G–I represents mean ± SEM of 6–7 similarly performed experiments, where apoptosis was assessed by relative DNA fragmentation assay (G–H) or morphological analysis (I). Letters preceding CpG ODN codes refers to the CpG ODN classes. When not mentioned, ODN concentration was 3 μM. Asterisk * indicates $p < 0.05$ and ** $p < 0.01$ as compared with the respective solvent control.

ODN C274 led to statistically significant reduction in apoptosis (Fig. 2G), whereas non-CpG ODN C661 had no effect. However, class A CpG ODN D19 or the corresponding non-CpG ODN Dc did not affect apoptosis rate of eosinophils (Fig. 2G). The most potent apoptosis-inhibiting CpG ODN 1018, and its corresponding non-CpG ODN 1040 were chosen for further studies.

3.3. Effect of CpG DNA on primary eosinophil necrosis

Inhibition of apoptosis may not only lead to increased cell survival but to induction of other forms of cell death such as cytolysis or primary necrosis of cells. In fact, cytolysis of eosinophils has been described in asthmatic airways [21]. Therefore we tested the effect of CpG ODN 1018 and non-CpG ODN 1040 on primary eosinophil necrosis by PI-staining after 1 h and 2 h incubation. PI can only enter cells with ruptured plasma membrane, which is a typical feature of a necrotic cell. Thereby, necrotic cells are typically positively stained by PI after a short incubation. At 1 h timepoint, $1.6 \pm 0.1\%$ of untreated, $1.7 \pm 0.2\%$ of 1018-treated and $1.7 \pm 0.1\%$ of 1040-treated cells showed PI-positive staining ($p > 0.05$, $n = 6$). At 2 h timepoint, percentages of PI-positive cells were $1.6 \pm 0.1\%$, $1.9 \pm 0.1\%$ and $1.7 \pm 0.2\%$ in the absence and presence of 1018 and 1040, respectively ($p > 0.05$, $n = 6$). These results suggest that CpG ODN 1018 or non-CpG ODN 1040 do not induce primary necrosis of eosinophils.

3.4. Role of contaminating cells in the anti-apoptotic effect of CpG DNA on eosinophils

There has been a debate over whether the effects of CpG DNA on some cell types are direct or indirect [20,22]. A small amount of contaminating pDC and B-lymphocytes may lead to false interpretations, as they produce high quantities of interferons and/or cytokines in response to CpG DNA. To evaluate this possibility we further purified our eosinophil preparations by using CD16-, CD19- and CD304-negative selection. On average, in anti-CD16 isolated eosinophils we found $0.020 \pm 0.010\%$ CD19+ cells and $0.001 \pm 0.000\%$ CD123+ CD303+ cells as assessed by flow cytometric analysis ($n = 6$). After extensive purification flow cytometric analysis revealed complete absence of CD19+ and CD123+ CD303+ cells ($n = 6$). In these highly purified eosinophil populations, CpG ODN 1018 reduced the proportion of apoptotic cells by $13 \pm 3\%$ ($p < 0.01$, $n = 6$) as determined by relative DNA fragmentation assay. However, the inhibiting effect of CpG ODN 1018 was somewhat larger in cells simultaneously prepared using CD16-negative selection only ($22 \pm 5\%$, $p < 0.01$, $n = 6$). Thus, CpG ODNs affect eosinophils directly in the absence of pDC and B-cells. However, in the presence of pDC and B-cells the effect of CpG ODNs may be more pronounced.

3.5. Toll-like receptor 9 expression in human eosinophils

To see whether eosinophils express TLR9 protein and to determine whether the expression is dependent on hypersensitivity, we conducted western blotting and flow cytometric analysis with eosinophils isolated from both healthy and atopic individuals. In western blotting, peripheral blood mononuclear cells served as a positive control. A band corresponding to the size of TLR9 was detected in eosinophil lysates derived from both healthy (data not shown) and atopic donors (Fig. 3A), and the band was not seen if the antibody was first incubated with the specific blocking peptide (Fig. 3A). Expression of TLR9 in eosinophils derived from both healthy and atopic individuals was confirmed by flow cytometric analysis (Fig. 3B and C).

3.6. Effect of CpG methylation on the anti-apoptotic action of bacterial DNA

Methylation of the cytosines in CpG motifs is believed to mask the ability of CpG DNA to activate TLR9. We conducted experiments to see whether the effect of bacterial DNA on eosinophil apoptosis is

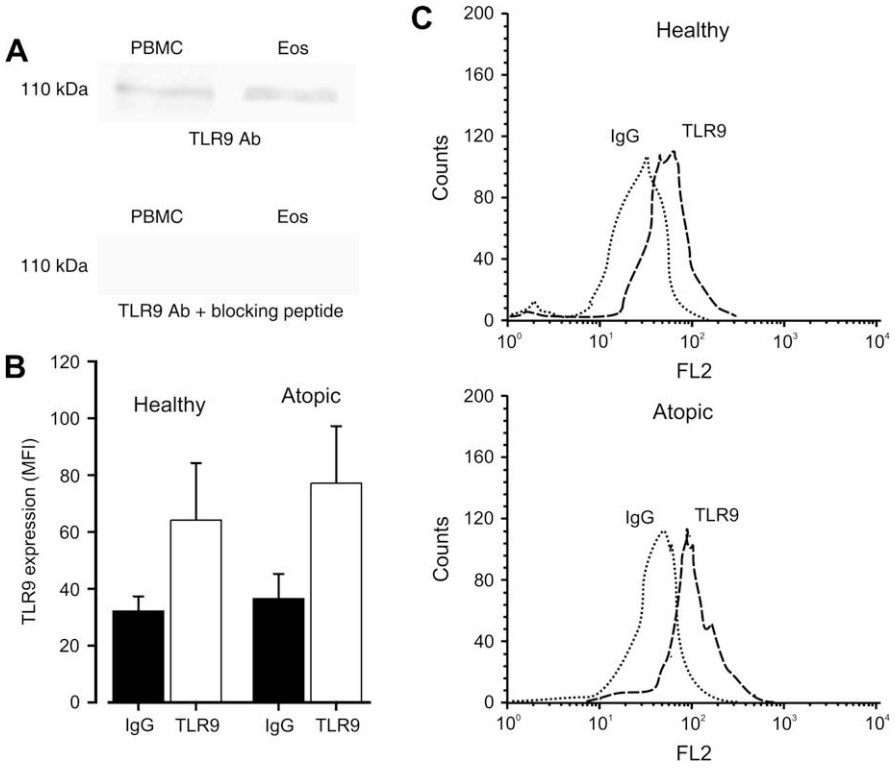

Fig. 3. TLR9 expression in human eosinophils. Expression was determined by western blotting (A) and intracellular flow cytometry (B–C) in eosinophils derived from atopic and healthy donors. In A and C shown is one typical experiment of three with similar results. In B, TLR9 expression determined by flow cytometric analysis is shown as mean ± SEM.

abrogated due to CpG methylation, which would support a role for TLR9. Surprisingly, CpG methylation of bacterial DNA did not result in loss of the anti-apoptotic effect (Fig. 4A). Methylated DNA was completely resistant to digestion by restriction endonuclease BstUI (Fig. 4B), indicating successful methylation. These results suggest that the apoptosis-delaying effect of bacterial DNA is not dependent on unmethylated CpG motifs.

3.7. Effect of inhibitors of endosomal acidification and inhibitory ODN on the anti-apoptotic action of CpG ODNs, non-CpG ODNs and bacterial DNA

To explore the role of TLR9 in the effects of CpG ODNs, non-CpG ODNs and bacterial DNA on eosinophils, we employed inhibitors of TLR9-activation pathway. Low endosomal pH is a prerequisite for the activation of intracellularly localized TLR9. Bafilomycin A1 and chloroquine are inhibitors of endosomal acidification with different mechanisms of action. Inhibitory oligodeoxynucleotides are a new, recently identified class of oligodeoxynucleotides, which have been reported to specifically inhibit TLR9 activation induced by CpG DNA [23]. Bafilomycin A1 totally abrogated the effects of 1018 (Fig. 5A) and bacterial DNA (Fig. 5D) on eosinophil apoptosis. Chloroquine also reduced the anti-apoptotic effect of 1018 in a concentration-dependent manner (Fig. 5B) but bacterial DNA induced statistically significant anti-apoptotic effect even in the presence of chloroquine (Fig. 5E). However, preincubation with inhibitory ODN completely abolished the effect of both 1018 (Fig. 5C) and bacterial DNA (Fig. 5F). Bafilomycin (100 nM), chloroquine (10 μM) and inhibitory ODN (10 μM) attenuated spontaneous apoptosis by $23 \pm 4\%$, $10 \pm 2\%$ and $17 \pm 6\%$, respectively. Overall, these results indicate that the anti-apoptotic effect of phosphorothioate-modified CpG ODN 1018 is dependent on intracellular TLR9. Evidence was also obtained for TLR9-dependent action of bacterial DNA.

To see, whether the occasionally occurring effects of non-CpG ODN 1040 were dependent on endosomal acidification, we studied the effect of 1040 on eosinophil apoptosis in the presence or absence of bafilomycin A1 and chloroquine. We included only those experiments where 1040 inhibited apoptosis as determined by DNA fragmentation assay. Treatment with 100 nM bafilomycin A1 or 10 μM chloroquine did not affect the anti-apoptotic effect of 1040 (data not shown). The result suggests that the effects of non-CpG on eosinophil apoptosis are not mediated by intracellularly localized TLR9.

3.8. Role of PI3K, NF-κB and MAPKs in the anti-apoptotic effect of CpG ODNs

PI3K, NF-κB and MAPKs have been previously described as mediators of TLR9 signalling pathway [10,24]. To see the involvement of these mediators in CpG DNA-activated pathway leading to delayed eosinophil apoptosis, we conducted experiments with pharmacological inhibitors of PI3K (wortmannin), NF-κB pathway (PDTC, MG-132 and BMS-345541) and MAPKs (SB203580, SP600125 and PD98059). Wortmannin (100 nM), PDTC, BMS-345541 and MG-132 (10 μM) suppressed the effect of CpG ODN 1018 on apoptosis (Fig. 6A–D), even though 1018 retained statistically significant anti-apoptotic effect in the presence of MG-132 (Fig. 6C). Wortmannin (100 nM) slightly enhanced spontaneous apoptosis by 1.2-fold ($p < 0.01$, $n = 6$) whereas 1.3–1.5-fold increase in spontaneous apoptosis was found after treatment with 10 μM PDTC, BMS-345541 or MG-132 (in each case $p < 0.01$, $n = 6$–13), as previously reported for inhibitors of NF-κB [25]. SB203580, SP600125 or PD98059 (10 μM) did not inhibit the anti-apoptotic effect of CpG ODN 1018 on eosinophils in a statistically significant manner ($n = 5$–7, data not shown).

3.9. Effect of CpG DNA on GM-CSF-induced eosinophil survival

Granulocyte macrophage-colony stimulating factor (GM-CSF) is an important eosinophil survival-increasing cytokine. Asthmatic patients have elevated levels of GM-CSF in their bronchoalveolar lavage (BAL) fluid [26], which may be a significant cause leading to eosinophilia. We studied next whether CpG ODN 1018 and bacterial DNA increase eosinophil survival in the presence of 10 pM GM-CSF. When apoptosis was measured by DNA fragmentation assay, GM-CSF-treatment decreased eosinophil apoptosis significantly after 40 h of incubation (Table 1). Bacterial DNA or CpG ODN 1018 had no further apoptosis-inhibiting effect in the presence of GM-CSF (Table 1).

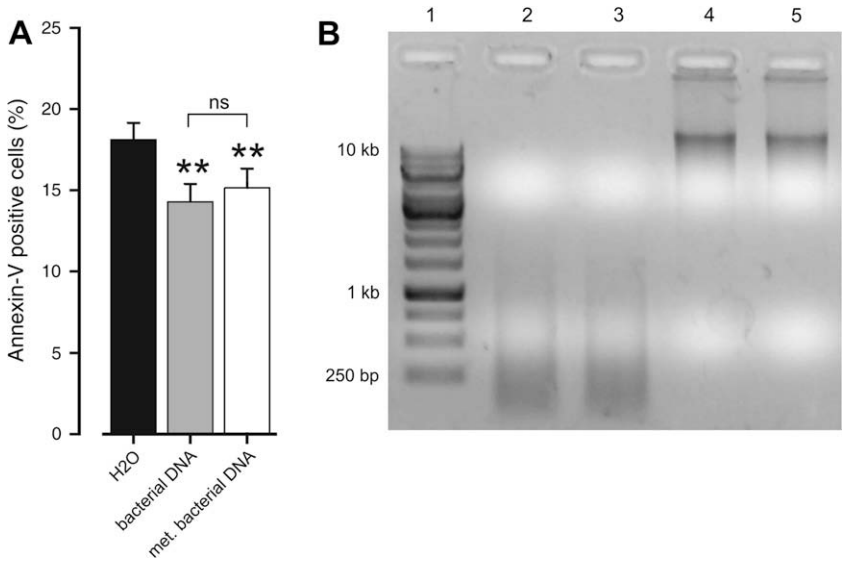

Fig. 4. Effect of CpG methylation on the anti-apoptotic action of bacterial DNA. Eosinophils were incubated in the absence or presence of 30 μg/ml unmethylated or methylated bacterial DNA for 20 h after which apoptosis was determined by Annexin-V FITC binding assay (A). Asterisk ** indicates $p < 0.01$ as compared with the respective solvent control. Agarose gel electrophoresis of unmethylated and methylated bacterial DNA treated with restriction enzyme BstUI for 3 h (B). Lane 1: marker, lane 2: unmethylated DNA with 1 U BstUI/μg DNA, lane 3: unmethylated DNA with 10 U BstUI/μg DNA, lane 4: methylated DNA with 1 U BstUI/μg DNA, lane 5: methylated DNA with 10 U BstUI/μg DNA. Each lane contained 400 ng DNA.

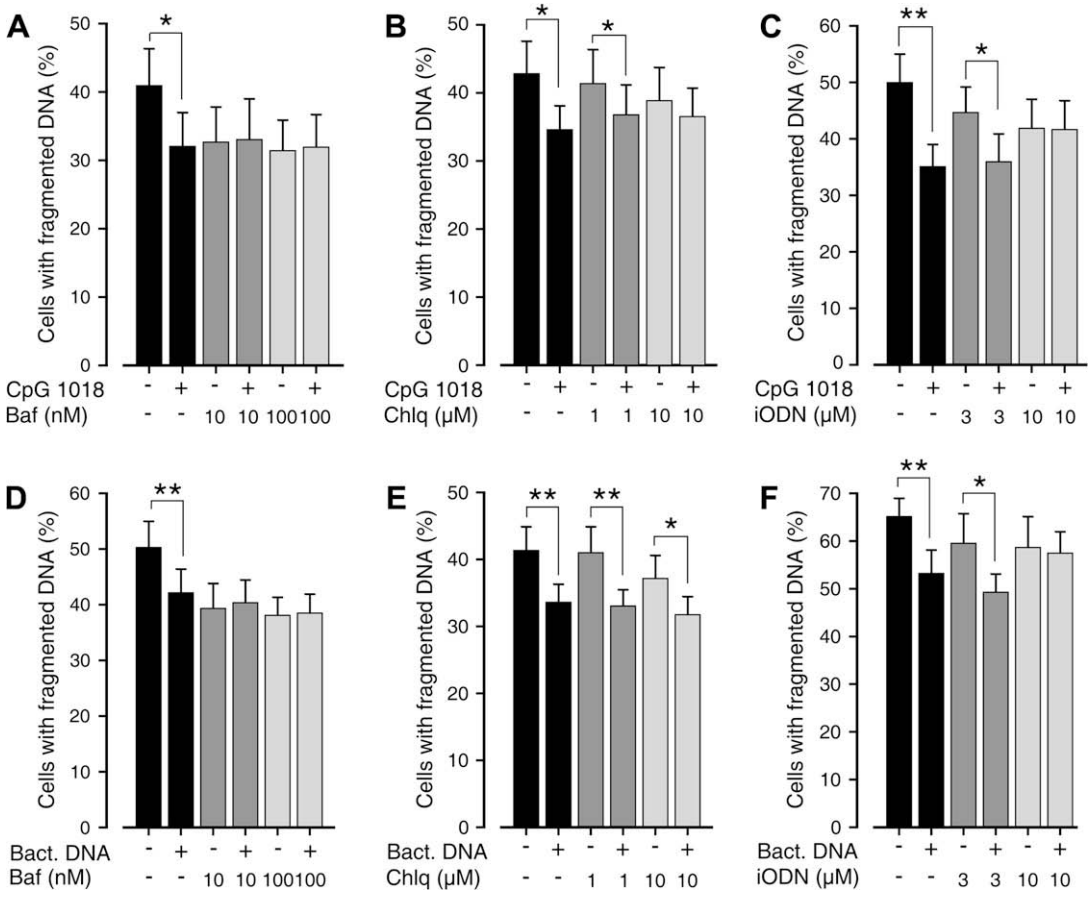

Fig. 5. Effects of inhibitors of endosomal acidification and inhibitory ODN on the anti-apoptotic action of CpG ODN 1018 and bacterial DNA. Eosinophils were pretreated with solvent or indicated concentrations of bafilomycin (A, D) or chloroquine (B, E) for 20 min or with inhibitory ODN (C, F) for 30 min, after which solvent, CpG ODN 1018 (3 μM) or bacterial DNA (30 μg/ml) was added. Apoptosis was measured by DNA fragmentation assay after 40 h of culture. Experiments were repeated 5–8 times with eosinophils derived from different individuals. Data is shown as mean ± SEM. Asterisk * indicates $p < 0.05$ and $^{**}p < 0.01$.

3.10. Effect of CpG DNA on eosinophil apoptosis in the presence of a glucocorticoid

Glucocorticoids reduce the number of eosinophils in the airways of patients with asthma [27]. They enhance spontaneous eosinophil apoptosis [17,18] and partially reverse cytokine-induced eosinophil survival [28]. We used glucocorticoid budesonide to examine whether CpG DNA is able to reverse glucocorticoid-induced apoptosis of eosinophils. As determined by DNA fragmentation assay after 40 h of incubation budesonide increased apoptosis in

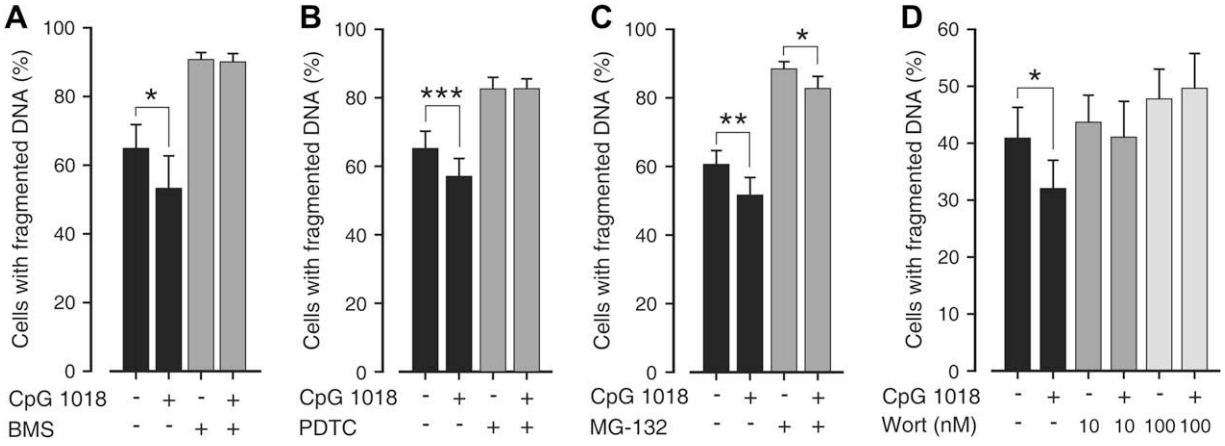

Fig. 6. Effects of inhibitors of NF-κB pathway (A–C) and PI3K (D) on the anti-apoptotic action of CpG ODN 1018. Eosinophils were incubated with solvent or CpG ODN 1018 (3 μM) for 40 h after 1 h pre-treatment with solvent or BMS-345541 (A), PDTC (B) or MG-132 (C) or after 20 min pre-treatment with solvent or wortmannin (D). Apoptosis was determined by relative DNA fragmentation assay. Mean ± SEM of 6–13 experiments with eosinophils from different donors is represented. Asterisk * indicates $p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 1
Effects of bacterial DNA and CpG ODN 1018 on GM-CSF-induced eosinophil survival.

	Apoptotic cells (%) Mean ± SEM
Untreated	57 ± 4
GM-CSF 10 pM	9 ± 2***
GM-CSF 10 pM + Bacterial DNA 30 μg/ml	9 ± 1
Untreated	41 ± 5
GM-CSF 10 pM	9 ± 1**
GM-CSF 10 pM + CpG ODN 1018 3 μM	10 ± 1
GM-CSF 10 pM + Non-CpG ODN 1040 3 μM	10 ± 1

$N = 5–6$, $p < 0.01$**, $p < 0.001$*** as compared to untreated cells.

Table 2
Effects of bacterial DNA and CpG ODN 1018 on budesonide-induced apoptosis of human eosinophils.

	Apoptotic cells (%) Mean ± SEM
Untreated	58 ± 4
Budesonide 1 μM	70 ± 3*
Budesonide 1 μM + Bacterial DNA 30 μg/ml	63 ± 2
Untreated	59 ± 7
Budesonide 1 μM	76 ± 7**
Budesonide 1 μM + CpG ODN 1018 3 μM	72 ± 9
Budesonide 1 μM + Non-CpG ODN 1040 3 μM	75 ± 6

$N = 5–6$, $p < 0.05$*, $p < 0.01$** versus untreated eosinophils.

a statistically significant manner as previously described (Table 2) [17]. Bacterial DNA and CpG ODN 1018 had no effect in the presence of budesonide (Table 2).

4. Discussion

In the present study we showed that bacterial DNA as well as synthetic class B and C CpG ODNs but not vertebrate DNA delay spontaneous apoptosis of human peripheral blood eosinophils *in vitro*. Additionally, we found TLR9 expression in eosinophils derived from both atopic and healthy individuals. Our results suggest a mechanism dependent on TLR9, PI3K and NF-κB to explain the effects of phosphorothioate-modified CpG ODNs on eosinophil apoptosis. We also showed that unmethylated CpG dinucleotides are not essential for the anti-apoptotic effect of native bacterial DNA in eosinophils suggesting presence of a novel immunostimulatory pattern in bacterial DNA lacking from vertebrate DNA.

Bacterial DNA and CpG ODN were found to reduce spontaneous eosinophil apoptosis maximally by approximately 20%. The size of this effect is smaller than that of the survival-prolonging cytokines IL-3, IL-5 or GM-CSF, but is largely similar to the well characterized effects of β_2-agonists and other cyclic AMP elevating agents on human eosinophils [4,29,30].

In the current study, CpG ODN 1018 reduced apoptosis of extensively purified eosinophils, although to a somewhat lesser extent than that of normally purified eosinophils. This result excludes the possibility that the effect is dependent on contaminating B-cells and pDC, which are known to be highly activated by CpG ODNs [20] but suggests that the presence of these contaminating cells may further augment the apoptosis-delaying effect of CpG ODNs on eosinophils. Direct response of eosinophils to CpG ODNs is supported by our finding that eosinophils express TLR9 protein as shown by western blotting and flow cytometric analysis. This is in concordance with a recent report [16]. In addition, human eosinophils have been previously demonstrated to express TLR9 mRNA [15]. Furthermore, treatment with CpG ODNs has been found to resist apoptosis in other TLR9-expressing cells such as pDC [31], B-cells [32] and neutrophils [33]. Interestingly, survival of neutrophils was prolonged with a 10–20-fold lower concentration of bacterial DNA [33] as compared to that found in eosinophils (present study), which may reflect more essential role of neutrophils in the first-line host defence against bacteria as compared to eosinophils.

We found TLR9 expression in eosinophils derived from both healthy and allergic donors indicating that TLR9 expression is not induced by hypersensitivity. It is thought that TLR9 is an intracellular receptor requiring acidic endosomal pH for its activation [34]. Bafilomycin A1, a highly specific inhibitor of vacuolar type H^+-ATPase, abolished the effect of CpG ODNs and bacterial DNA on eosinophil apoptosis completely. In contrast chloroquine, which acts as a weak base and accumulates inside endosomes neutralizing their pH [35], reversed most of the effect of synthetic CpG ODNs but not that of bacterial DNA. One possibility is that this inconsistency is due to interaction of DNA with chloroquine [36], which may be influenced by backbone differences. The result may also indicate differences in endosomic processing of bacterial DNA and phosphorothioate-modified CpG ODNs. Inhibitory ODNs have been reported to inhibit TLR9 activation induced by CpG ODNs very recently. Inhibitory ODNs were shown to bind TLR9 with similar affinity to CpG DNA. However, unlike stimulatory CpG DNA, inhibitory ODN did not induce conformational change in TLR9 required for its activation [23]. Preincubation of eosinophils with inhibitory ODNs completely reversed the effect of CpG ODNs and bacterial DNA on apoptosis. Altogether, these results combined with the observation of TLR9 expression in eosinophils suggest that intracellularly localized TLR9 mediates the anti-apoptotic action of synthetic CpG ODNs in human eosinophils. Evidence was obtained also for the involvement of TLR9 in the suppression of eosinophil apoptosis by bacterial DNA but further studies are needed to strengthen this finding.

In this study, treatment with non-CpG ODN 1040 had inhibitory effect on spontaneous apoptosis of eosinophils in some occasions. In contrast to the actions of CpG ODNs, the effect of 1040 was not dependent on endosomal acidification, which is critical for the activation of intracellular TLR9 [34]. Phosphorothioate backbone, where one of the non-bridging oxygens at each phosphodiester linkage is replaced by a sulphur atom, has been previously reported to exert sequence-independent activity on several cellular events [37,38]. Stimulation with phosphorothioate backbone has led to the activation of Jak2 [37], which is a critical component in the survival-promoting effects of IL-5, IL-3 and GM-CSF on eosinophils [39]. It is tempting to speculate that the weak inhibitory effects of non-CpG ODNs are due to TLR9-independent activation of Jak2.

Several previous studies have shown that bacterial DNA stimulates leukocyte populations via a mechanism dependent on unmethylated CpG dinucleotides and TLR9 [33,40,41]. However, recently many groups have demonstrated TLR9 activation independently from unmethylated CpG motifs [42,43]. Also CpG- and TLR9-independent immunostimulatory effects induced by bacterial but not vertebrate DNA have been demonstrated [44]. This suggests that the recognition of bacterial DNA by the host cell may not be as simple as previously thought. Unmethylated CpG motifs may not be the only TLR9-activating pattern in bacterial DNA or also other sensors distinguishing bacterial DNA from host DNA may exist. Our results support the view that immune cells discriminate bacterial DNA from vertebrate DNA also via another molecular pattern than unmethylated CpG motifs. The results with the inhibitors of TLR9-activation pathway suggest involvement of TLR9 in the recognition process of bacterial DNA but we cannot completely rule out other, currently unknown receptors or cytosolic sensors. In previous studies, sequences activating TLR9 in a CpG-independent manner have contained phosphorothioate backbone and 5′-TC dinucleotide in a thymidine-rich background or modified nucleotides with a bicyclic heterobase in the place of C in CpG [42,43,45]. CpG-methylated DNA with phosphodiester backbone has also been

shown to interact with TLR9, however, the interaction was weaker and led to reduced NF-κB activity when compared to unmethylated CpG DNA [34]. Interestingly, Hartmann et al. demonstrated that certain active CpG sequences with phosphodiester backbone lose their activity when phosphorothioate modified [46]. This suggests that backbone differences lead to different sequence and structure requirements for TLR9 activation. Stiffer phosphorothioate backbone with limited conformations available may have stricter sequence requirements for TLR9 activation as compared to phosphodiester DNA. Altogether, sequence requirements for TLR9 activation seem not to be limited to unmethylated CpG and may be critically influenced by the backbone.

PI3K has been previously described to play an important role in eosinophil survival *in vivo* [47]. In this study, the effect of CpG ODNs on eosinophil apoptosis was dependent on member(s) of PI3K family, since it was completely reversed by PI3K inhibitor wortmannin. The finding is consistent with the results of a previous study, where regulation of vesicular trafficking concerning TLR9 and CpG DNA was suggested to include PI3K [24]. In dendritic cells, CpG DNA was reported to induce phosphorylation of Akt, which is a known downstream target of PI3K [48].

Activation of transcription factor NF-κB is a critical survival-prolonging mechanism in eosinophils and many other immune cell types [25]. Stimulation of TLR9 has been described to result in activation of NF-κB [10]. Consistent with this, PDTC and BMS-345541, inhibitors of NF-κB and IκB kinase, respectively, blocked completely the anti-apoptotic effect of CpG ODNs on eosinophils. Also proteasome inhibitor MG-132 which inhibits degradation of IκB, an inhibitory subunit of NF-κB, suppressed the anti-apoptotic effect of CpG ODNs. Taken together, our data suggests a cascade where activation of TLR9 by CpG ODNs leads to activation of PI3K – NF-κB pathway resulting in prolonged survival of human eosinophils. MAPKs seem not to be involved in the action of CpG ODNs in eosinophils. Interestingly, NF-κB and PI3K have been demonstrated to act in concert in several studies. The following mechanisms have been suggested: 1) PI3K-activated Akt enhances degradation of IκB and nuclear translocation of NF-κB by inducing IκB kinase phosphorylation and activation [49], 2) PI3K-Akt axis is not involved in the nuclear translocation of NF-κB but induces Ser^{536} phosphorylation of the p65 subunit of NF-κB thus increasing its transcriptional activity [50]. Whether these mechanisms are involved in the action of CpG ODNs in eosinophils remains to be determined.

CpG ODNs have exerted anti-inflammatory effects in murine models of asthma and allergy [12,13] but has shown contradictory results in the first clinical trials [51,52]. The present data suggests that treatment with CpG ODNs may lead to exacerbation of eosinophilic inflammation and that particular carefulness should be followed when administrating CpG ODNs to humans.

Pro-inflammatory action of bacterial DNA has been described in mice. Administration of bacterial DNA resulted in significant accumulation of polymorphonuclear leukocytes in the lower respiratory tract suggesting that bacterial DNA may play a pathogenic role in inflammatory lung diseases [11]. In humans, evidence exists of the role of bacterial infection in the exacerbation of asthma and atopic dermatitis [6,53]. Accumulation of appropriate amounts of bacterial DNA in the infection site is essential for the biological significance of the current results. In the study of Schwartz et al. up to 20 μg/ml bacterial DNA was found from sputum of patients with cystic fibrosis [11]. The anti-apoptotic effect of bacterial DNA on eosinophils occurred between concentrations of 10 μg/ml and 30 μg/ml, suggesting biological relevance. According to the present findings direct contact between bacterial DNA and eosinophils leads to prolonged eosinophil lifespan which presents a novel mechanism of asthma exacerbation during bacterial respiratory tract infection.

In summary, the current results suggest that phosphorothioate-modified CpG ODNs inhibit human eosinophil apoptosis through a pathway involving TLR9, PI3K and NF-κB. We also showed that bacterial but not vertebrate DNA suppresses eosinophil apoptosis but the effect is independent of unmethylated CpG motifs. This indicates existence of a novel, bacterial DNA-specific recognition pattern lacking from vertebrate DNA. The present study revealed an additional pro-inflammatory action of bacterial DNA *in vitro*, which may have clinical relevance in contributing exacerbation of eosinophilic lung disease such as asthma.

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Antieosinophilic Activity of Orazipone[S]

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ABSTRACT

Orazipone is a novel sulfhydryl-reactive compound that has been previously shown to reduce lung eosinophilia in guinea pigs and rats and to inhibit degranulation in mast cells and cytokine production in monocytes and T-cells. However, the effects of orazipone on granulocyte longevity are unknown. Orazipone and its derivative 3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione (OR-2370) reversed interleukin-5-afforded survival of human eosinophils by inducing apoptosis. In contrast, orazipone did not affect granulocyte macrophage-colony-stimulating factor–induced survival of human neutrophils. The effect of orazipone on eosinophil apoptosis is different from that of glucocorticoids in that even high con-centrations of interleukin-5 were not able to overcome the effect of orazipone. Orazipone further enhanced spontaneous apoptosis as well as that induced by CD95 ligation without inducing primary necrosis. OR-2370-induced DNA fragmentation was shown to be dependent on caspases 3 and 6 and c-jun-N-terminal kinase, whereas extracellular regulated kinase, p38 mitogen-activated protein kinase, and phosphatidylinositol 3-kinase as well as caspases 4, 8, and 9 seem not to mediate its actions. Our results suggest that orazipone and its derivative OR-2370 possess strong antieosinophilic activity and thus may have anti-inflammatory efficacy in the treatment of asthma and/or allergic conditions.

Eosinophils are thought to play a critical role in allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis (Giembycz and Lindsay, 1999; Gleich, 2000). In asthmatic patients, activation of eosinophils in the airways is believed to cause epithelial tissue injury, contraction of airway smooth muscle and increased bronchial responsiveness. Apoptosis or programmed cell death is regarded as an important feature in the resolution of asthmatic inflammation (Kankaanranta et al., 2005). Apoptosis is characterized by specific biochemical and morphological changes, including cell shrinkage, surface blebbing, DNA fragmentation and loss of nucleoli (Kankaanranta et al., 2005), so that the apoptotic cell is phagocytosed intact without release of its contents. In vitro, eosinophil apoptosis is inhibited by cytokines, such as interleukin-3, interleukin-5, and granulocyte macrophage–colony-stimulating factor (GM-CSF) (Giembycz and Lindsay, 1999; Kankaanranta et al., 2005). Eosinophil apoptosis is up-regulated by Fas (CD95/APO-1), a 45-kDa transmembrane protein belonging to the tumor necrosis factor receptor family (Kankaanranta et al., 2005). Eosinophil apoptosis is delayed in patients with asthma or inhalant allergy (Wedi et al., 1997; Kankaanranta et al., 2000a). Furthermore, the number of eosinophils in asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils (Vignola et al., 1999). Thus, pharmacological induction of eosinophil apoptosis is considered an in-teresting possibility to treat eosinophilic inflammatory conditions such as asthma and/or allergic diseases.

Orazipone and its derivatives OR-1958 and OR-2370 (Fig.

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ABBREVIATIONS: GM-CSF, granulocyte macrophage–colony-stimulating factor; JNK, c-jun-N-terminal kinase; DMSO, dimethyl sulfoxide; Z-, *N*-benzyloxycarbonyl-; FMK, fluoromethyl ketone; Ac-, *N*-acetyl-; CHO, aldehyde; MAPK, mitogen-activated protein kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD098059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole; PI3K, phosphatidylinositol 3-kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4 pyridyl)-1*H*-imidazole; DCB, [(2,6-dichlorobenzoyl)oxy]methane; L-JNKI1, GRKKRRQRRR-PP-RPKRPTTLNLFPQVPRSQD-amide; L-TAT, RKKRRQRRR-amide, negative control for L-JNKI1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; IL, interleukin; OR-2370, 3-(4-chloro-3-nitro-benzylidene)-pentane-2,4-dione; OR-1958, 3-(3-chloro-4-methanesulfonyl-benzylidene)-pentane-2,4-dione; OR-2149, 3-(4-methanesulfonyl-benzyl)-pentane-2,4-dione.

1) are novel anti-inflammatory compounds that exert their effects by forming reversible conjugates with the thiol groups of proteins and glutathione (Wrobleski et al., 1998). Their effect on thiol groups is readily reversible and makes them unique among the other thiol-modulating compounds. Orazipone (OR-1384) has been shown previously to suppress the release of interleukin-1β, interleukin-8, and tumor necrosis factor-α from human blood monocytes and to suppress oxygen free radical production in polymorphonuclear leukocytes (Nissinen et al., 1997, 1998; Serkkola and Nissinen, 1999). Recently, orazipone and its derivative OR-1958 were shown to inhibit histamine release and tumor necrosis factor-α production in rat and human mast cells (Vendelin et al., 2005). Orazipone and OR-1958 have been shown to reverse the platelet-activating factor–induced pulmonary eosinophilia in a dose-dependent manner in guinea pigs (Aho et al., 2001). Furthermore, orazipone was shown to prevent lung eosinophilia in ovalbumin-sensitized rats after repeated administration with efficacy equal to that of budesonide (Ruotsalainen et al., 2000). However, at the cellular level, the effects of orazipone on eosinophils are not known. In addition to its efficacy in experimentally induced pulmonary inflammation, orazipone has been shown to have anti-inflammatory effects in models of experimental colitis (Wrobleski et al., 1998). The exact intracellular mechanism of the anti-inflammatory action of orazipone remains unknown but may be related to the modulation of intracellular signaling system by inhibition of the function of thiol-containing proteins.

Given the critical role of thiol groups and oxygen radicals in eosinophil apoptosis (Wedi et al., 1999; De Souza et al., 2002; Kankaanranta et al., 2002; Gardai et al., 2003), our aim was to test the possible anti-inflammatory effects of orazipone on human eosinophils. The present study describes the ability of orazipone and its derivative OR-2370 to induce apoptosis in human eosinophils and to reverse interleukin-5–afforded eosinophil survival as well as evaluates their possible mechanisms of action.

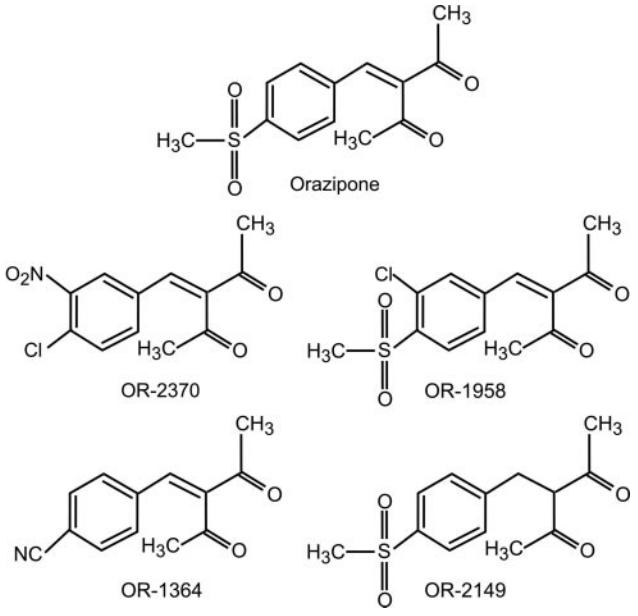

Fig. 1. The chemical structures of orazipone and OR-2370.

Materials and Methods

Granulocyte Purification. Blood (50–100 ml) for eosinophil experiments was obtained from persons with eosinophilia. However, patients with hypereosinophilic syndrome were excluded because of the possibly different signaling in the myeloproliferative variant hypereosinophilic syndrome expressing FIP1L1-PDGFRA-fusion kinase (Schwartz 2003). For neutrophil experiments, blood was obtained from healthy volunteers. Eosinophils and neutrophils were isolated to >99% purity under sterile conditions as reported previously (Kankaanranta et al., 1999, 2000a,b; Zhang et al., 2000, 2002). The cells were resuspended at 10^6 cells/ml and cultured in RPMI 1640 medium, 10% fetal calf serum, and antibiotics. Subjects gave informed consent to a study protocol approved by the ethical committee of Tampere University Hospital.

Determination of Granulocyte Apoptosis. Unless otherwise stated, eosinophil and neutrophil apoptosis was determined by relative DNA fragmentation method and flow cytometry as described previously (Kankaanranta et al., 1999, 2000a,b; Zhang et al., 2000, 2002). The cells showing decreased relative DNA content were considered to be apoptotic, as described previously (Kankaanranta et al., 2000b). Annexin-V binding and morphological analysis was performed as previously reported (Kankaanranta et al., 2000b; Zhang et al., 2002). Oligonucleosomal DNA fragmentation in eosinophils was analyzed by agarose gel DNA electrophoresis as described previously (Kankaanranta et al., 1999, 2000b).

Caspase Activity Assay. Caspase 3/7, 8, and 9 activities in human eosinophils were measured with the use of Caspase-Glo 3/7, 8, and 9 assays according to the manufacturer's instructions. In brief, eosinophils (10^6 cells/ml) were cultured in RPMI 1640 medium, 10% fetal calf serum, and antibiotics in the presence or absence of interleukin-5 (10 pM) and OR-2370 (10 μM) for 16 h. Equal volumes of Caspase-Glo 3/7, 8, or 9 substrates were added, and the sample was incubated for 1 h at room temperature before measurement of luminescence.

Immunoblot Analysis. Eosinophils were suspended at 10^6 cells/ml and cultured at 37°C. At the time points indicated in Fig. 6, cells were centrifuged at 12,000*g* for 10 min. The cell pellet was lysed by boiling for 5 min in 30 μl of 6× Laemmli buffer, centrifuged at 12,000*g* and debris was carefully removed. Samples were then stored at −20°C until immunoblot analysis. For immunoblot analysis, each protein sample was loaded on 10% SDS-polyacrylamide gel and electrophoresed for 2 h at 100 V. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) with semidry blotter, blocked using 5% nonfat dry milk in 20 mM Tris-base, pH 7.6, 150 mM NaCl, and 0.1% Tween 20. Proteins were labeled using specific antibody and subsequently detected using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL) Western blotting detection agents and detected by using Fluorchem 8800 equipment and software (Alpha Innotec, San Leandro, CA). Quantification of relevant bands was performed by densitometry. The activated c-jun-N-terminal kinase (JNK) was identified and quantified by Western blot analysis using specific antibody recognizing the dual phosphorylated (i.e., activated) form of JNK. Control time curves with the solvent (0.5% DMSO) were prepared to see the change in JNK activation in similar conditions in the absence of OR compounds. The increase in activation of JNK by OR-2370 is expressed as the phospho-JNK activity in OR-2370–treated cells compared with the simultaneously prepared control cells with the solvent.

Materials. Reagents were obtained as follows: Caspase-Glo 3/7, 8, and 9 assays (Promega), L-JNKI1 (JNK peptide inhibitor 1, L-stereoisomer), and L-TAT control peptide (Alexis Corp., Läufelfingen, Switzerland), Z-Asp-CH_2-DCB (Peptide Institute, Inc., Osaka, Japan), orazipone, OR-2370, OR-1958, OR-1364, and OR-2149 (Fig. 1) (OrionPharma Ltd., Espoo, Finland), phosphospecific JNK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), horseradish peroxide-linked anti-rabbit IgG (GE Healthcare) and Z-D(OMe)QM-

D(OMe)-FMK, Z-VEID-FMK, Ac-LEVD-CHO, Ac-IETD-CHO, Ac-LEHD-CHO, Q-VD-OPh, LY294002, PD169316, and wortmannin (Merck, Darmstadt, Germany). Unless otherwise stated, the reagents were obtained as described previously (Kankaanranta et al., 1999, 2000a,b, 2002; Zhang et al., 2000, 2002, 2003). The incubation time is 40 h unless otherwise stated. L-JNK1, L-TAT, PD098059, SB203580, LY294002, PD169316, and wortmannin and caspase inhibitors were added 20 min before OR-2370. Stock solutions of orazipone and OR-compounds, PD098059, SB203580, LY294002, PD169316, wortmannin and caspase inhibitors were prepared in DMSO. The final concentration of DMSO in the culture was 0.5 to 0.75% (1.25% in Caspase 4 and 9 inhibitor experiments). A similar concentration of DMSO was added to the control incubations.

Statistical Analysis. Results are expressed as means ± S.E.M. Apoptosis is expressed as apoptotic index. Apoptotic index is the number of measured apoptotic cells divided by the total number of measured cells. Statistical significance was calculated by analysis of variance for repeated measures supported by Student-Newman-Keuls test by using Instat software (GraphPad Software, San Diego, CA). Differences are considered significant if $P < 0.05$.

Results

Effects of Orazipone on Interleukin-5–Afforded Eosinophil Survival. Interleukin-5 inhibited human eosinophil apoptosis in a concentration-dependent manner, and maximal inhibition of apoptosis was obtained at 10 pM interleukin-5 (apoptotic indexes, 0.57 ± 0.09 and 0.07 ± 0.02 in the absence and presence of interleukin-5, respectively; $n = 5$, $P < 0.001$). Orazipone increased the number of apoptotic eosinophils in the presence of interleukin-5 (Fig. 2A). This increase in the number of apoptotic cells was confirmed by increased phosphatidylserine expression on the outer leaflet of cell membrane of interleukin-5–treated cells. The proportion of Annexin-V–positive cells in the absence and presence of orazipone (40 μM) was 0.07 ± 0.02 and 0.64 ± 0.10, respectively; $n = 6$, $P < 0.001$ (Fig. 2, B and C). Furthermore, an increase in the number of eosinophils showing the typical features of apoptosis (such as nuclear coalescence, chromatin condensation, and cell shrinkage) was found with orazipone (apoptotic index, 0.02 ± 0.01 and 0.54 ± 0.12 in the absence and presence of 40 μM orazipone, respectively; $n = 6$, $P < 0.001$; Fig. 2, D and E). To further confirm the ability of orazipone to induce eosinophil apoptosis, DNA breakdown, the typical hallmark of apoptosis was analyzed. Orazipone (40 μM) reversed the interleukin-5–afforded inhibition of DNA breakdown, and a typical "ladder" pattern was found, indicating the occurrence of apoptotic cell death (Fig. 2F). Two structural sulfhydryl-reactive analogs of orazipone had a similar effect, whereas nonsulfhydryl-reactive analog OR-2149 did not reverse the effect of interleukin-5 on DNA breakdown in eosinophils (Fig. 2F).

Glucocorticoids are known to partially reverse the survival-prolonging action of interleukin-5 on eosinophils. However, this effect of glucocorticoids is abolished when interleukin-5 is used at higher concentrations (Hagan et al., 1998; Zhang et al., 2000, 2002; Druilhe et al., 2003). For example, budesonide (1 μM) partly reversed cytokine-afforded survival in the presence of low (1 pM) but not in the presence of higher (10–100 pM) concentrations of interleukin-5 (Fig. 3A). To

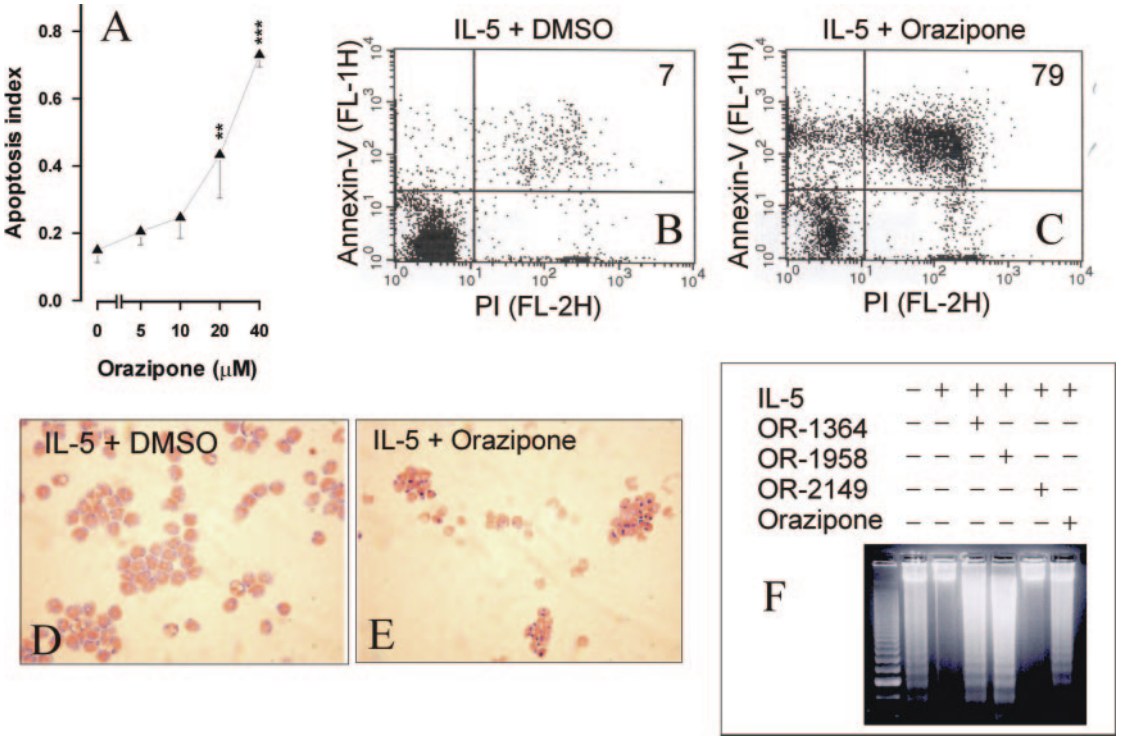

Fig. 2. The effect of orazipone (A) on apoptosis in 10 pM IL-5–treated human eosinophils during culture for 40 h. Each data point represents the mean ± S.E.M. of six independent determinations using eosinophils from different donors. **, $P < 0.01$; ***, $P < 0.001$ compared with respective solvent control. Representative graphs from Annexin V-FITC (FL-1H) and uptake of propidium iodide (PI: FL-2H) are shown in B and C. B and C, top right, total number of early apoptotic eosinophils (annexin V-FITC^{+ve} and PI^{-ve}) and late apoptotic eosinophils (annexin V-FITC^{+ve} and PI^{+ve}). D, morphology of eosinophils cultured for 22 h in the presence of 10 pM IL-5. E, typical morphology of apoptotic and late apoptotic eosinophils cultured in the presence of 10 pM IL-5 and 40 μM orazipone. F, the effect of 10 pM IL-5 and 40 μM OR-1364, OR-1958, orazipone, and the negative control compound OR-2149 on DNA fragmentation in eosinophils cultured for 22 h. A representative of six (B–E) and three (F) similar experiments using eosinophils from different donors is shown.

evaluate whether the effect of orazipone is similar to glucocorticoids, its effects were studied in the presence of different concentrations of interleukin-5. Interestingly, orazipone (20–40 μM) reversed the effect of interleukin-5 on eosinophil apoptosis even in the presence of high concentrations of interleukin-5 (Fig. 3B). Thus, the effect of orazipone on eosinophil apoptosis seems to be different from that of glucocorticoids so that even high concentrations of the survival-prolonging cytokine interleukin-5 are unable to reduce its effects.

Effect of Orazipone on Fas-Induced Eosinophil Apoptosis. Relatively few compounds exist that are able to reverse the effect of interleukin-5 on eosinophil survival (Kankaanranta et al., 2005). One of those is nitric oxide, which has been shown to reverse the effect of interleukin-5 by inducing apoptosis (Zhang et al., 2003). However, nitric oxide can also reverse the apoptosis inducing effect of Fas in eosinophils (Hebestreit et al., 1998). This prompted us to evaluate whether orazipone has effects on Fas-induced apoptosis. Orazipone (5–40 μM), further enhanced the apoptosis-inducing effect of Fas-ligation in human eosinophils (Supplemental Data file 1).

Effect of Orazipone on Spontaneous Eosinophil Apoptosis. Glucocorticoids are able to enhance apoptosis of cytokine-deprived eosinophils at clinically relevant drug concentrations (Zhang et al., 2000, 2002). Similar to glucocorticoids, orazipone (40 μM) was able to enhance apoptosis of cytokine-deprived eosinophils 1.2- to 3-fold as assessed by relative DNA fragmentation assay, morphological analysis, or Annexin-V binding assay (n = 6, Supplemental Data file 2).

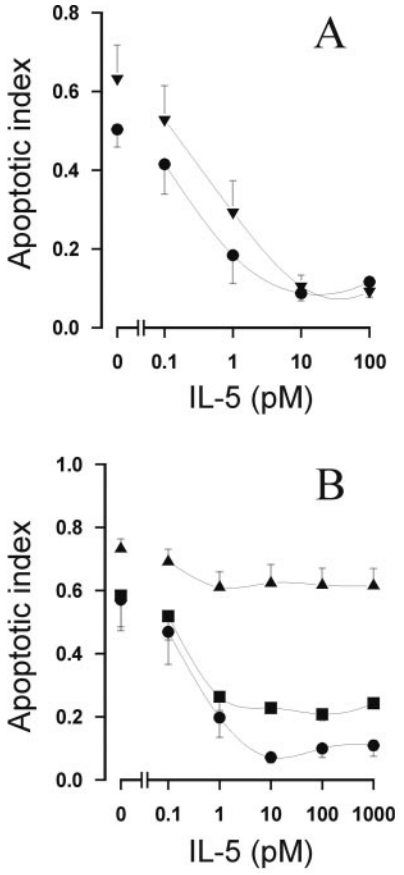

Fig. 3. The effect of budesonide (▼, 1 μM) (A) and orazipone (■, 20 μM; ▲, 40 μM) (B) on apoptosis in eosinophils cultured for 40 h in the presence of different concentrations of IL-5. ●, the concentration-curve of IL-5 in the presence of solvent control. Each data point represents the mean ± S.E.M. of four to five independent determinations using eosinophils from different donors. When not visible, error bars are within the symbol size.

Effect of Orazipone on Primary Eosinophil Necrosis. An important feature for a drug possessing anti-eosinophilic activity is that it should not induce primary necrosis that could lead to the release of eosinophil contents to the surrounding tissue. To evaluate this possibility, the effects of orazipone on primary eosinophil necrosis were evaluated by using counterstaining with Annexin-V and propidium iodide, where positive staining with propidium iodide indicates a rupture of the plasma membrane and the absence of staining with Annexin-V indicates that the cell has not undergone apoptosis. Thus, cells showing positive staining with propidium iodide but not with Annexin-V can be considered to have the typical feature of primary necrosis (i.e., the plasma membrane breakdown). In the absence of interleukin-5, the percentages of propidium iodide$^+$/Annexin-V$^-$ cells were 5 ± 2 and 4 ± 1% in the absence and presence of 40 μM orazipone, respectively (n = 6, $P > 0.05$), and in the presence of interleukin-5 (10 pM), the corresponding percentages were 5 ± 3 and 5 ± 1% (n = 6, $P > 0.05$). Thus, it can be concluded that orazipone does not induce primary necrosis in eosinophils.

Effect of Orazipone on Apoptosis in Human Neutrophils. To exclude a general toxic effect by orazipone on all cell types, the effects of orazipone on apoptosis and cytokine-afforded survival of human neutrophils were studied. Orazipone (5–40 μM) did not affect spontaneous neutrophil apoptosis (Table 1). GM-CSF (70 pM) inhibited human neutrophil apoptosis during culture for 16 h (Table 1). Orazipone (5–40 μM) did not reverse GM-CSF–afforded survival of human neutrophils (Table 1). Ligation of Fas-enhanced neutrophil apoptosis (Table 1). Orazipone did not affect Fas-induced apoptosis in human neutrophils (Table 1). The lack of effect of orazipone (40 μM) on spontaneous apoptosis and GM-CSF–afforded neutrophil survival were confirmed by Annexin-V binding assay. In the absence of GM-CSF, the

TABLE 1
Lack of effect of orazipone on spontaneous or Fas-induced neutrophil apoptosis or GM-CSF-afforded neutrophil survival
Data is expressed as mean ± S.E.M., n = 5. Ligation of Fas was induced by CH-11 monoclonal antibody (100 ng/ml). There were no statistically significant differences between any of the concentrations of orazipone versus solvent control.

	Apoptotic Index
Spontaneous apoptosis	
Solvent control	0.68 ± 0.03
Orazipone 5 μM	0.61 ± 0.05
Orazipone 10 μM	0.63 ± 0.05
Orazipone 20 μM	0.66 ± 0.03
Orazipone 40 μM	0.68 ± 0.04
Fas	
Solvent control	0.84 ± 0.03
Orazipone 5 μM	0.86 ± 0.03
Orazipone 10 μM	0.84 ± 0.05
Orazipone 20 μM	0.80 ± 0.05
Orazipone 40 μM	0.84 ± 0.04
GM-CSF (70 pM)	
Solvent control	0.53 ± 0.05
Orazipone 5 μM	0.55 ± 0.04
Orazipone 10 μM	0.48 ± 0.05
Orazipone 20 μM	0.54 ± 0.04
Orazipone 40 μM	0.57 ± 0.05

apoptotic indexes were 0.78 ± 0.01 and 0.70 ± 0.08 and in its presence 0.73 ± 0.03 and 0.71 ± 0.03 in the absence and presence of orazipone; $n = 4$, $P > 0.05$. Similar to that described in eosinophils, there was no significant induction of primary necrosis in neutrophils (in the absence of GM-CSF, 1 ± 1 and 3 ± 1% necrotic cells in the absence and presence of 40 μM orazipone, respectively, and in the presence of GM-CSF 2 ± 1 and 2 ± 1% necrotic cells in the absence and presence of 40 μM orazipone, respectively).

Effects of Analogs of Orazipone on Eosinophil Apoptosis. Data published previously suggest that structural analogs of orazipone may have improved anti-inflammatory efficacy compared with orazipone (Vendelin et al., 2005). For comparison, structurally related analogs of orazipone were studied. Both OR-1958 and OR-2370 reversed interleukin-5–afforded human eosinophil survival in a concentration-dependent manner by inducing apoptosis (Fig. 4). OR-1958 and another structurally related sulfhydryl-reactive analog OR-1364 reversed interleukin-5 inhibited DNA breakdown similarly to orazipone, whereas a nonsulfhydryl-reactive analog OR-2149 (Nissinen et al., 1997) did not reverse the effect of interleukin-5 on DNA breakdown in eosinophils (Fig. 2F). The ability of OR-2370 to induce apoptosis in interleukin-5-treated human eosinophils was confirmed by showing the increase in the number of Annexin-V positive eosinophils (apoptosis index, 0.09 ± 0.02 and 0.55 ± 0.15 in the absence and presence of 10 μM OR-2370, respectively; $n = 6$, $P < 0.001$). Because OR-2370 was found to be even more potent in inducing eosinophil apoptosis than orazipone and OR-1958, OR-2370 was used in further studies to evaluate the mechanisms of orazipone-induced apoptosis in eosinophils.

Role of Caspases in OR-2370–Induced Apoptosis. A pan-caspase inhibitor, Z-Asp-CH_2-DCB (20–200 μM) significantly reversed 10 μM OR-2370–induced apoptosis in interleukin-5–treated eosinophils during 40 h of incubation (Table 2). To further evaluate the role of caspases in OR-2370–induced apoptosis, the activities of caspase 3/7, 8, and 9 were measured. During culture for 16 h active caspases 3/7, 8, and 9 were detected. Caspase 3/7 and 9 activities were significantly reduced by interleukin-5 (Fig. 5). OR-2370 significantly increased the activity of caspases 3/7, 8, and 9 in the presence of interleukin-5 (Fig. 5). A broad-range inhibitor of caspases 1, 3, 8, 9, 10, and 12, Q-VD-OPh almost completely reversed OR-2370–induced apoptosis (Table 2). Q-VD-OPh (20 μM) also inhibited caspase 3/7, 8, and 9 activities in human eosinophils in the presence of OR-2370 by 75 to 99% ($n = 2$, data not shown). A more specific inhibitor of caspase 3 [Z-D(OMe)QMD(OMe)-FMK] partly reversed OR-2370–induced apoptosis (Table 2). Inhibitor of caspase 6 (Z-VEID-FMK) also reversed OR-2370–induced apoptosis in eosinophils (Table 2). To evaluate the role of other potential caspases, inhibitors for caspase 4 (Ac-LEVD-CHO), 8 (Ac-ITED-CHO), and 9 (Ac-LEHD-CHO) were investigated. Inhibitors of caspases 4, 8, and 9 did not reverse OR-2370–induced apoptosis (Table 2), although Ac-ITED-CHO and Ac-LEHD-CHO inhibited caspase 8 and 9 activities in human eosinophils by 99 and 65%, respectively ($n = 2$; data not shown). This suggests that even though caspases 8 and 9 are activated by OR-2370 in eosinophils, they do not mediate OR-2370-induced DNA breakdown.

Role of Mitogen-Activated Protein and Phosphatidylinositol 3-Kinases in OR-2370–Induced Apoptosis in Eosinophils. When interleukin-5–treated eosinophils were incubated at 37°C in the presence of OR-2370 (10 μM), a time-dependent increase in JNK activity was detected using Western blotting with an anti-pJNK antibody that recognizes the dual phosphorylated (i.e., activated) form of JNK (Fig. 6, A and B). To evaluate the functional role of JNK activation in OR-2370–induced apoptosis in interleukin-5–treated cells, a novel cell-permeable inhibitor peptide specific for JNK, L-JNKI1 (Bonny et al., 2001), was used. L-JNKI1 (10 μM), but not the negative control peptide L-TAT, almost completely reversed 10 μM OR-2370–induced DNA breakdown in interleukin-5–treated eosinophils (Figs. 6C and 7, A–C). To determine whether JNK activation is central to the OR-2370–induced apoptosis, the effect of L-JNKI1 (10 μM) on OR-2370–induced apoptosis was analyzed by using the morphological analysis and measurement of phosphatidylserine appearance on the outer cell membrane using Annexin-V binding assay. Interestingly, L-JNKI1 did not reduce the number of cells showing the typical early signs of apoptosis, such as apoptotic morphology or phosphatidylserine

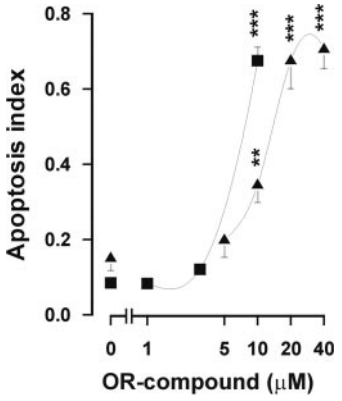

Fig. 4. The effect of OR-1958 (▲) and OR-2370 (■) on apoptosis in eosinophils cultured for 40 h in the presence of IL-5 (10 pM). Each data point represents the mean ± S.E.M. of six independent determinations using eosinophils from different donors. **, $P < 0.01$; *** $P < 0.001$ compared with respective solvent control.

TABLE 2

The effect of caspase inhibition on OR-2370 (10 μM)-induced apoptosis in interleukin-5-treated eosinophils

Data is expressed as mean ± S.E.M., $n = 4$–7. The corresponding apoptotic index in the presence of 10 pM interleukin-5 but in the absence of OR-2370 was 0.11 to 0.25.

	Apoptotic Index
Control	0.46 ± 0.03
Z-Asp-CH_2-DCB	
20 μM	0.34 ± 0.03**
200 μM	0.06 ± 0.01***
Control	0.58 ± 0.06
Q-VD-OPh	
20 μM	0.16 ± 0.02***
Z-D(OMe)QMD(OMe)-FMK	
200 μM	0.39 ± 0.03**
Z-VEID-FMK	
200 μM	0.12 ± 0.02***
Ac-ITED-CHO	
100 μM	0.49 ± 0.07
Control	0.60 ± 0.04
Ac-LEVD-CHO	
100 μM	0.55 ± 0.04
Ac-LEHD-CHO	
100 μM	0.59 ± 0.04

**, $P < 0.01$; ***, $P < 0.001$ compared with the respective solvent control in the absence of caspase inhibitors.

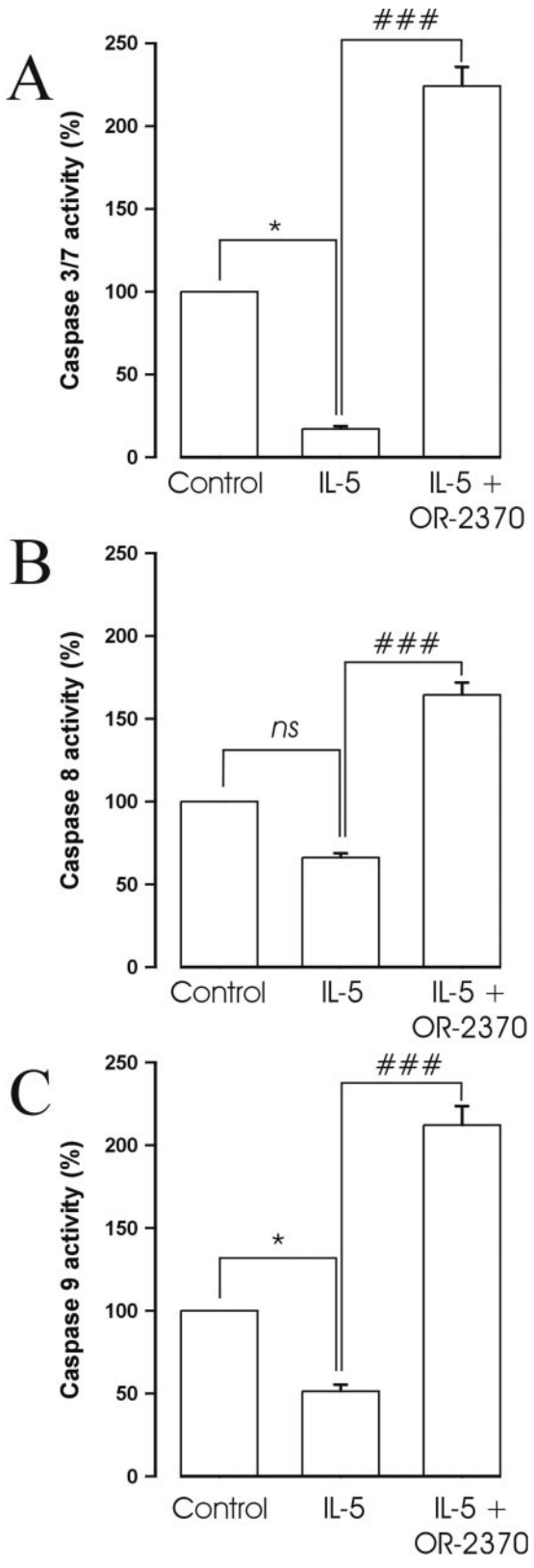

Fig. 5. The effect of 10 μM OR-2370 on caspase 3/7 (A), caspase 8 (B), and caspase 9 (C) activation in human eosinophils cultured for 16 h in the presence of 10 pM IL-5. Caspase activity was measured with Caspase-Glo assay system. Each data point represents the mean ± S.E.M. of four independent determinations using eosinophils from different donors. *, $P < 0.05$ compared with respective solvent control in the absence of interleukin-5; ###, $P < 0.001$ compared with the respective solvent control in the presence of interleukin-5.

expression on the outer cell membrane. By using morphological criteria for apoptosis in cells treated with 10 μM OR-2370 and 10 pM interleukin-5, the apoptotic indexes were 0.64 ± 0.19 and 0.74 ± 0.13 in the presence of 10 μM L-TAT and 10 μM L-JNKI1, respectively; $n = 5$, $P > 0.05$) after culture for 20 h (Fig. 7, G–I). Likewise, the proportion of Annexin-V–positive cells was not reduced by L-JNKI1 compared with cells treated with L-TAT (apoptotic indexes, 0.49 ± 0.16 and 0.61 ± 0.14 in the presence of 10 μM L-TAT and 10 μM L-JNKI1, respectively; $n = 5$; Fig. 7, D–F). These results suggest that JNK activation is not an early event

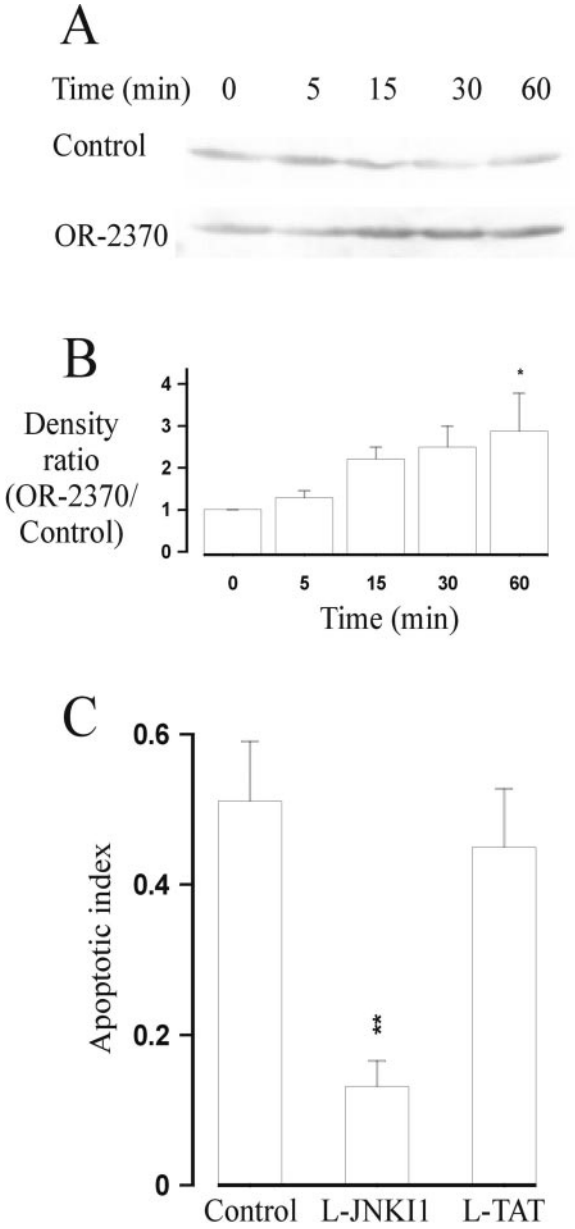

Fig. 6. A, the effect of 10 μM OR-2370 on phosphorylation of JNK. The upper lane shows JNK phosphorylation in the simultaneously prepared control cells from the same donor treated with solvent control (0.5% DMSO). A typical experiment of six similar is shown. B, density ratio of JNK phosphorylation in OR-2370-treated cells compared with the simultaneously prepared control cells. Mean + SEM, $n = 6$. *, $P < 0.05$ compared with 0 min timepoint. C, the effect of L-JNKI1 (10 μM) and the negative control peptide L-TAT (10 μM) on apoptotic index in 10 μM OR-2370 and 10 pM IL-5 treated cells. Mean ± S.E.M., $n = 8$. ***, $P < 0.01$ compared with the negative control peptide L-TAT.

mediating orazipone-induced apoptosis; rather, it mediates orazipone-induced DNA fragmentation.

Extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) have been proposed to be involved in the regulation of eosinophil apoptosis (Kankaanranta et al., 1999; Hall et al., 2001). Thus, to evaluate the role of these kinases in OR-2370–induced eosinophil apoptosis, we used a pharmacological approach to inhibit the activity of ERKs with the use of the MAPK kinase inhibitor PD098059 and the activity of p38 MAPK by SB203580 and PD169316. However, PD098059, SB203580, and PD169316 did not affect 10 μM OR-2370–induced apoptosis in interleukin-5–treated human eosinophils (Table 3).

To evaluate whether activation of phosphatidylinositol 3-kinase (PI3K) could mediate OR-2370–induced apoptosis, pharmacological inhibitors of PI3K were employed. However, neither LY294002 nor wortmannin reversed OR-2370–induced eosinophil apoptosis (Table 3).

Discussion

In the present study, we showed that the thiol-modulating compounds orazipone and OR-2370 induced apoptosis in interleukin-5–treated human eosinophils and were able to enhance spontaneous eosinophil apoptosis without inducing primary necrotic cell death. In contrast to eosinophils, orazipone does not induce apoptosis in human neutrophils. The mechanism of action of orazipone seems to involve caspases 3 and 6 as well as JNK-mediated DNA breakdown.

Induction of eosinophil apoptosis is currently considered

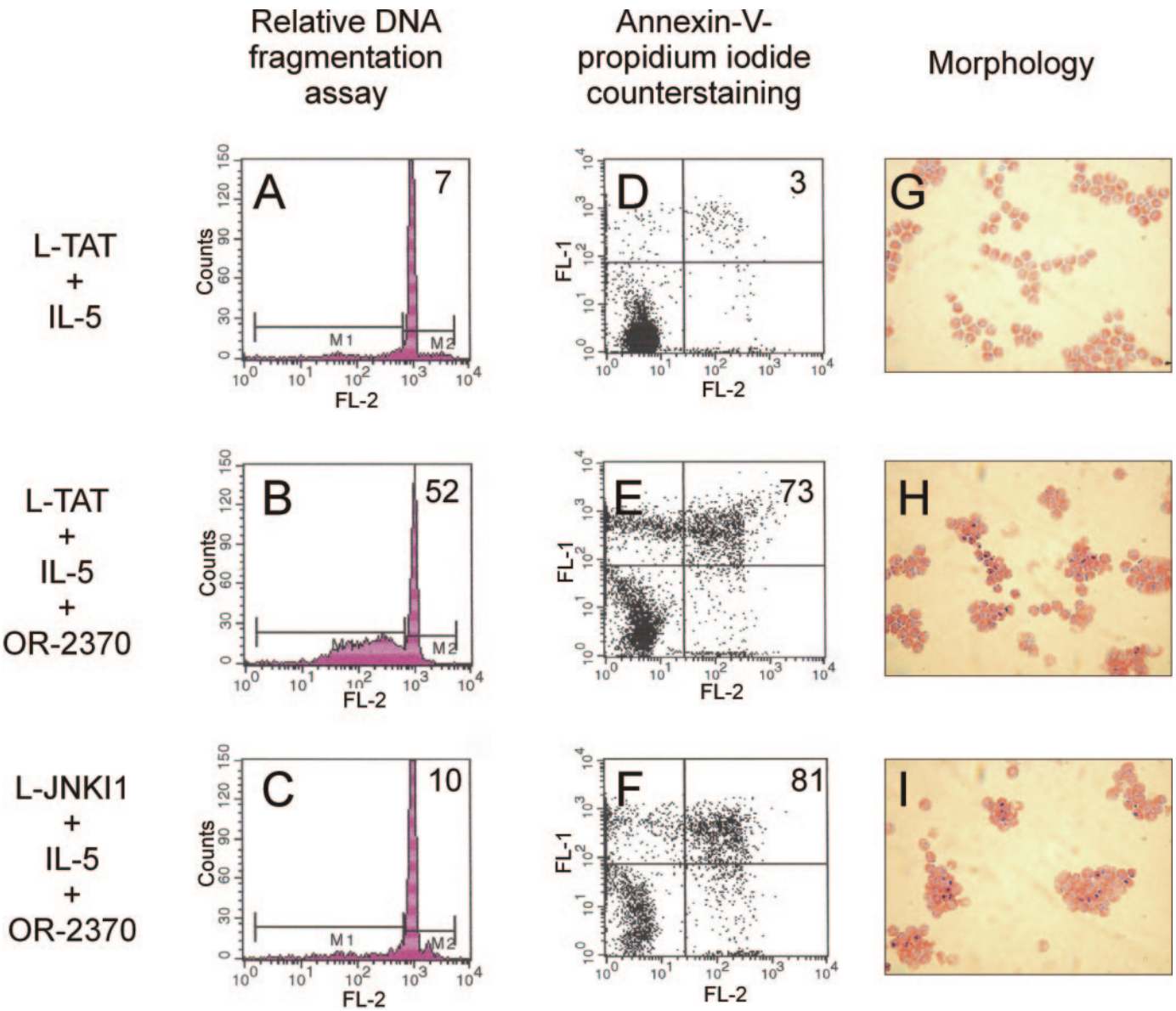

Fig. 7. The effect of 10 μM L-JNKI1 (C, F, and I) on 10 μM OR-2370-induced apoptosis in 10 pM interleukin-5 treated eosinophils compared with the negative control peptide 10 μM L-TAT (B, E, and H). A solvent control incubated in the presence of 10 μM L-TAT and 10 pM interleukin-5 but in the absence of OR-2370 is shown in (A, D, and G). Representative graphs from relative DNA fragmentation assay of propidium iodide-stained eosinophils (A–C), Annexin V-FITC (FL-1) and uptake of propidium iodide (PI; FL-2) (D–F), and morphological analysis of eosinophils (G–I) are shown. D–F, top right, total number of early apoptotic eosinophils (annexin V-FITC^{+ve} and PI^{-ve}) and late apoptotic eosinophils (annexin V-FITC^{+ve} and PI^{+ve}). Representative graphs of experiments with similar results using eosinophils from four to eight different donors are shown.

one of the key mechanisms of the antiasthmatic effectiveness of glucocorticoids (Druilhe et al., 2003; Walker et al., 2003; Walsh et al., 2003; Kankaanranta et al., 2005). Glucocorticoids are able to enhance spontaneous eosinophil apoptosis at clinically relevant drug concentrations (Zhang et al., 2000, 2002). In the present study, we showed that orazipone and OR-2370 were able to enhance constitutive apoptosis of cytokine-deprived eosinophils. More importantly, they were able to fully reverse the interleukin-5–afforded eosinophil survival by inducing apoptosis. Glucocorticoids have been reported to partly reverse interleukin-5-afforded survival, but the effect of steroids falls off as the concentration of interleukin-5 increases (Adachi et al., 1996; Druilhe et al., 2003). In contrast to glucocorticoids, the effect of orazipone was not reversed by higher concentrations of interleukin-5, suggesting that the mechanism of action orazipone is different from that of glucocorticoids and that orazipone may have a better antieosinophilic activity compared with current glucocorticoids.

The exact mechanism of action of orazipone and its derivatives OR-1958 and OR-2370 remains unknown, but they probably exert their effects by forming reversible conjugates with the thiol groups of proteins and glutathione (Vendelin et al., 2005). The ability of orazipone and OR-1958 to inhibit cytokine production is dependent on their ability to react with glutathione in mononuclear cells or activated neutrophils. The effect of orazipone and OR-1958 has been shown to be reversible, and this has been tested by incubating compounds with glutathione and measuring the formation of compound-glutathione adduct and its dissociation back to the parent compound and glutathione (Nissinen et al., 1997). If the double bond in the side chain is reduced, the compounds do not react with glutathione, and they lose their ability to inhibit the activation of these cells (Nissinen et al., 1997). In the present study, we used also OR-2149, which has a reduced double bond in the side chain as a negative control compound. OR-2149 did not induce DNA breakdown or apoptosis in eosinophils. This suggests that the effect of orazipone is related to its ability to modulate thiols.

TABLE 3

The effect of inhibition of ERK (PD098059), p38 MAPK (SB203580, PD169316) and PI3K (LY294002, wortmannin) on 10 μM OR-2370-induced apoptosis in interleukin-5-treated human eosinophils

Data is expressed as mean ± S.E.M., n = 4–7. The corresponding apoptotic index in the presence of 10 pM interleukin-5 but in the absence of OR-2370 was 0.12 to 0.15.

	Apoptotic Index
PD098059	
0	0.52 ± 0.06
1 μM	0.48 ± 0.05
10 μM	0.49 ± 0.05
SB203580	
0	0.38 ± 0.08
1 μM	0.41 ± 0.10
10 μM	0.43 ± 0.10
PD169316	
0	0.59 ± 0.08
0.1 μM	0.61 ± 0.09
1 μM	0.58 ± 0.11
LY294002	
0	0.59 ± 0.08
10 μM	0.66 ± 0.05
50 μM	0.72 ± 0.05
Wortmannin	
0	0.59 ± 0.08
10 nM	0.59 ± 0.07
100 nM	0.61 ± 0.06

Thiol antioxidants such as *N*-acetylcysteine and glutathione have been shown to inhibit spontaneous and sodium arsenite- and Fas-induced apoptosis in human eosinophils (Wedi et al., 1999). Furthermore, we have shown that oxidative stress, especially H_2O_2, enhances spontaneous apoptosis and reverses interleukin-5–afforded eosinophil survival by inducing apoptosis (Kankaanranta et al., 2002). Recently, oxidant-induced mitochondrial injury was reported to be pivotal for eosinophil apoptosis and glucocorticoids were shown to enhance it in a JNK-mediated manner that is in turn inhibited by the survival-prolonging cytokine GM-CSF (Gardai et al., 2003). These data support the idea that eosinophil survival is regulated by thiol-sensitive redox regulation. The present results on the effects of orazipone on eosinophil apoptosis could be explained by formation of reversible conjugates with thiols, thereby preventing their effect on survival. Another possibility for the mechanism of action of orazipone is that it specifically saves some critical thiol-groups from modulation by interleukin-5, which leads to inhibition of interleukin-5-afforded survival.

Regulation of caspase activity is believed to be central during apoptosis. The presence of caspases 3, 6, 7, 8, and 9 and their processing during spontaneous or nitric oxide-induced apoptosis in eosinophils has been described previously (Zangrilli et al., 2000; Dewson et al., 2001; Zhang et al., 2003) and spontaneous eosinophil death can be blocked by broad specificity caspase inhibitors such as Z-Asp-CH_2-DCB or Z-VAD-FMK (Dewson et al., 2001; De Souza et al., 2002). However, the detailed caspase cascades mediating apoptosis in eosinophils remain unknown (Daigle and Simon, 2001). The effect of OR-2370 could be reversed by the broad specificity caspase inhibitors Z-Asp-CH_2-DCB and Q-VD-OPh, suggesting the mediator role of caspases in OR-2370–induced apoptosis. Furthermore, OR-2370–induced apoptosis was reduced by inhibitors of caspase 3 and 6, suggesting their involvement. It is noteworthy that specific inhibitors for caspases 8 or 9 were not able to reverse orazipone-induced apoptosis in IL-5–treated eosinophils even though these inhibitors efficiently suppressed caspase activities in these cells. These results suggest that the effects of orazipone on eosinophils are not mediated via caspase 8 or 9 pathways, but use a caspase pathway involving caspases 3 and 6.

The role of MAPKs and PI3K in the regulation of human eosinophil apoptosis has gained attention (Kankaanranta et al., 1999; Miike et al., 1999; Hall et al., 2001; Gardai et al., 2003; Zhang et al., 2003). There exists some controversy regarding whether ERK pathway is involved in the survival-prolonging action of cytokines (Kankaanranta et al., 1999; Miike et al., 1999; Hall et al., 2001), whereas p38 MAPK seems to be involved in spontaneous eosinophil survival (Kankaanranta et al., 1999). By using pharmacological inhibitors, we were able to exclude ERK, p38 MAPK, and PI3K as targets of OR-2370. Recently, JNK has been proposed to be involved in eosinophil apoptosis induced by dexamethasone (Gardai et al., 2003) and NO (Zhang et al., 2003). OR-2370 enhanced activation of JNK as evidenced by Western blot analysis, showing an increase in the amount of phosphorylated JNK. Inhibition of JNK activity by a specific inhibitor, L-JNKI1, reversed the effect of OR-2370 when apoptosis was measured using the relative DNA fragmentation assay, suggesting that JNK

mediates orazipone-induced apoptosis. However, when the effects of L-JNKI1 on OR-2370-induced apoptosis were analyzed using morphological features of apoptosis and the expression of phosphatidylserine on the outer leaflet of the cell membrane (Annexin-V binding assay), L-JNKI1 was not able to reverse the effect of OR-2370. These results suggest that JNK activity is enhanced in human eosinophils in response to orazipone and mediates orazipone-induced DNA breakdown, but JNK activation is not involved in the early signaling of orazipone-induced apoptosis. The role of JNK in the regulation of apoptosis in other cell types, mainly of malignant nature, has been widely studied, and it has been found to have both pro- and antiapoptotic effects (Lin and Dibling, 2002; Manning and Davis, 2003). The exact relationship between JNK activation and DNA fragmentation/apoptosis in eosinophils remains to be established.

Orazipone and its derivative OR-1958 have been shown to inhibit platelet-activating factor-induced lung eosinophilia in guinea pigs (Aho et al., 2001) and to prevent lung eosinophilia in ovalbumin-sensitized rats after repeated administration with efficacy equal to that of budesonide (Ruotsalainen et al., 2000). Orazipone has been shown to inhibit IL-1β, IL-2, tumor necrosis factor-α, and IL-8 secretion from monocytes or lymphocytes as well as superoxide release and degranulation in neutrophils and histamine release from mast cells (Wrobleski et al., 1998; Vendelin et al., 2005). In the present study, we found that orazipone induced apoptosis in IL-5–treated human eosinophils in vitro. Whether induction of eosinophil apoptosis explains the ability of orazipone to inhibit lung eosinophilia and to what extent its inhibitory effects on cytokine production and other inflammatory cells contribute to its effects remain currently unknown.

In the present study, we show for the first time that orazipone induces apoptosis in human eosinophils. To exclude the possibility that orazipone induces nonspecific toxicity toward all human cells, we studied the effect of orazipone on spontaneous and Fas-induced apoptosis and GM-CSF–afforded survival of human neutrophils. Orazipone, at concentrations that induced apoptosis in eosinophils, did not affect neutrophil apoptosis at all. Whether orazipone induces apoptosis in any cell type other than eosinophils remains to be evaluated. Orazipone has been successfully administered to healthy volunteers and patients with asthma as an inhalation preparation in two phase I-Ib trials (E. Moilanen, unpublished observations). However, the effects on asthmatic inflammation in vivo in humans remain unresolved.

Taken together, our results suggest that thiol-modulating compounds orazipone and OR-2370 have antieosinophilic activity and are potent candidates for the treatment of eosinophilic inflammatory conditions.

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Nitric oxide induces apoptosis in GM-CSF-treated eosinophils via caspase-6-dependent lamin and DNA fragmentation

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ABSTRACT

Asthma is characterized by accumulation of eosinophils in the lungs and delayed apoptosis may be one mechanism leading to eosinophilia. Nitric oxide (NO), present in inflamed lungs, has been shown to possess both anti- and proeosinophilic properties. We previously showed that NO induces apoptosis in the presence of survival prolonging cytokine IL-5 in human eosinophils. In the present study, we examined the intracellular mechanisms of NO-induced apoptosis in granulocyte macrophage-colony stimulating factor (GM-CSF)-treated eosinophils concentrating on the role of caspases and calpains. Eosinophils were isolated from human blood and apoptosis was determined by relative DNA fragmentation assay, morphological analysis and/or Annexin-V FITC assay. We showed that NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) induced apoptosis in GM-CSF-treated eosinophils. SNAP-induced DNA fragmentation was totally prevented by an inhibitor of caspase-6 (Z-VEID-FMK). Decreased levels of caspase-6 proenzyme and increased amounts of cleaved lamin A/C in SNAP-treated cells indicated activation of caspase-6. Furthermore, SNAP-induced lamin A/C and B fragmentation was totally abolished by an inhibitor of caspase-6. According to our results, caspase-6 mediates lamin and DNA fragmentation also in spontaneously dying eosinophils. Inhibitor of calpains prevented most of DNA fragmentation related to spontaneous apoptosis but had no effect in eosinophils undergoing NO-induced apoptosis. In the present study we showed that caspase-6 is essential for the executive phase involving lamin and DNA fragmentation in both NO-induced and spontaneous eosinophil apoptosis. However, differences in the involvement of calpains suggest that the intracellular signalling in NO-induced apoptosis has specific features at the level of proteases. This study demonstrates new mechanisms for NO-induced and spontaneous apoptosis in human eosinophils.

1. Introduction

Asthma is characterized by accumulation of eosinophils in the lungs and prolonged eosinophil viability [1]. Eosinophil activation leads to release of harmful substances from granules resulting in increased mucus production, mucosal and epithelial cell damage and bronchoconstriction [2]. Other functions of eosinophils include regulation of Th2 cytokine production and participation in the airway remodelling [3,4]. Apoptosis is a useful non-inflammatory removal mechanism of eosinophils from the lungs and is characterized by cell shrinkage, chromatin condensation, nuclear coalescence, lamin fragmentation, DNA fragmentation and mitochondrial changes.

Nitric oxide is a gaseous molecule produced by constitutive and inducible nitric oxide synthases (NOS) in the human body. Nitric oxide has several physiological functions in the human body, such as regulation of blood pressure and neurotransmission but it also participates in the pathophysiology of several diseases [5]. Inducible nitric oxide synthase (iNOS) in alveolar macrophages and bronchial epithelial cells produce high amounts of NO in inflamed lungs. In asthma, role of nitric oxide is ambiguous. Patients with asthma show elevated levels of NO in the exhaled air, which correlates to clinical symptoms of asthma, sputum eosinophilia and eosinophil activation markers [6,7]. However, NO-releasing glucocorticoid was shown to be more potent in inhibiting eosinophilic inflammation in a rat-model of asthma than the glucocorticoid alone [8]. In addition, NO-releasing compound DETA-NONOate was as potent as a glucocorticoid in inhibiting eosinophil accumulation [8].

In vitro, NO was shown to have both pro- and antiapoptotic effects on eosinophils [9,10]. We demonstrated previously that NO-releasing compound S-nitroso-N-acetyl-D,L-penicillamine (SNAP) induces apoptosis in IL-5-treated eosinophils. Apoptosis is typically

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executed by caspases, cysteine aspartate-specific proteases, which are activated by proteolytic cleavage of the inactive proenzyme by other caspases, proteases or auto-activation. Proapoptotic effect of SNAP on eosinophils was abolished by a pan-caspase inhibitor suggesting a significant role for caspases in mediating the effect of NO [9]. However, the involvement of specific caspases remains unresolved. Calpains are calcium-activated neutral proteases that reside in the cytosol as inactive proenzymes until intracellular calcium influx leads to their activation. Several cell functions have been shown to involve calpains [11]. Interestingly, calpain activation has been demonstrated during apoptotic cell death [12] and as a consequence of oxidative stress [13]. Calpains were recently shown to participate in eosinophil apoptosis [14] but very little is known about calpain function in eosinophils. This study was conducted to examine the specific roles of effector caspases and calpains in NO-induced apoptosis in GM-CSF-treated cells.

2. Materials and methods

2.1. Materials

Materials were purchased as follows: SNAP (Cayman Chemical, Ann Arbor, MI, USA), N-acetyl-D,L-penicillamine (Alfa Aesar, Karlsruhe, Germany), Z-DQMD-FMK (Caspase-3 inhibitor), IETD-CHO (Caspase-8 inhibitor), Z-VEID-FMK (Caspase-6 inhibitor), Q-VD-OPh, Calpeptin (Merck, Darmstadt, Germany), lamin A/C and caspase-6 antibody (Cell Signaling Technology Inc., Danvers, MA, USA), lamin B and actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Other reagents were purchased as described elsewhere [1,15]. Stock solutions of SNAP, penicillamine, caspase and calpain inhibitors were prepared in DMSO. Final DMSO concentration in the cells was 0.5–1%. Similar concentration of the solvent was added to the control cultures.

2.2. Human eosinophil purification and culture

Eosinophils were isolated from blood sample of voluntary eosinophilic donors under sterile conditions. All donors gave written informed consent to a study protocol approved by the ethical committee of Tampere University Hospital. One hundred ml of venous blood was collected into acid citrate dextrose (ACD) anticoagulant. After sedimentation of red blood cells, the supernatant containing leukocytes and remaining red blood cells was removed and washed. The pellet was mixed into HBSS after which it was layered onto Ficoll gradient solution and centrifuged at 700*g* for 30 min to separate mononuclear cells. The remaining red blood cells were discarded by hypotonic lysis. Eosinophils were separated from neutrophils by negative selection using immunomagnetic anti-CD16 microbeads. After 40–60 min of incubation at 4 °C, granulocytes were loaded onto a magnetic separation column. After final wash, eosinophils were resuspended at 1×10^6/ml in Dutch modification of RPMI 1640 containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. Eosinophil purity was determined by morphological analysis of Kimura-stained cells and was at least 99%. Cells were resuspended at 10^6/ml and cultured in Dutch modification of RPMI 1640 containing 10% fetal bovine serum, antibiotics and L-glutamine and incubated at 37 °C with 5% CO_2 in 96-well plates for the indicated times.

2.3. Apoptosis assays

DNA fragmentation was determined by flow cytometric analysis of propidium iodide (PI)-stained cells. After 40 h of incubation eosinophils (1×10^5) were suspended in 300 μl hypotonic solution containing 0.1% sodium citrate, 0.1% Triton-X and 50 μg/ml PI. After 1–24 h incubation at 4 °C the samples were analysed by flow cytometer (FACScan, Becton Dickinson, San Jose, CA, USA) [16]. Cells with hypodiploid amount of DNA were considered as apoptotic. For morphological analysis eosinophils were spun onto cytospin slides (25 g, 5 min), fixed in methanol for 15 min and stained with May-Grünwald-Giemsa. Shrunken eosinophils with coalesced nuclei and condensed chromatin were regarded as early apoptotic while cells with "ghost"-like structure were considered as late apoptotic. Annexin-V binding assay was conducted as previously described [16]. The cells displaying positive Annexin-V FITC labelling (Anx+/PI− and Anx+/PI+) were considered as apoptotic.

2.4. Western blotting

Eosinophils were washed with ice-cold PBS and lysed in ice-cold RIPA (radioimmuno precipitation assay)-buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodiumdeoxycholate, 0.1% SDS) containing phenylmethylsulfonylfluoride, sodiumorthovanadate, leupeptin, aprotinin, sodium fluoride, sodium pyrophosphate and N-octyl-β-D-glucopyranoside. After 15–30 min incubation at 4 °C, lysate was centrifuged at 12,000*g* for 5 min. Supernatant was mixed in SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol) and boiled for 10 min. Protein (20–30 μg) was loaded onto 10% SDS-polyacrylamide electophoresis gel. After 2 h of electrophoresis (120 V) in a buffer containing 25 mM Tris-base, 250 mM glycine and 0.1% SDS, proteins were electrically transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, UK, Ltd., Little Chalfont, Buckinhamshire, UK) with a semidry blotter. After transfer, membrane was blocked in TBST containing 5% nonfat dry milk or bovine serum albumin (BSA) for 1 h at RT. Membrane was incubated over-night at 4 °C in the blocking solution with primary antibody. The membrane was washed three times with TBST for 5 min, incubated with HRP-conjugated secondary antibody in the blocking solution for 30 min at RT and washed again three times with TBST for 5 min. Bound antibody was detected by using Super Signal West Pico, Dura or Femto chemiluminescent substrate (Pierce, Rockford, Ill, USA) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA).

2.5. Statistics

Results are shown as mean ± standard error of mean (SEM). Apoptosis is expressed as percentage of apoptotic cells (number of apoptotic cells/total number of cells × 100). Statistical significance was calculated by repeated measures analysis of variance with Dunnett's post-test by using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. SNAP induces apoptosis in GM-CSF-treated human eosinophils

Treatment with GM-CSF rescued eosinophils from undergoing spontaneous apoptosis (Fig. 1). SNAP, a donor of nitric oxide, induced apoptosis of GM-CSF-treated cells as determined by DNA fragmentation assay after 40 h of incubation (Fig. 1). Similarly, when determined by Annexin-V assay and morphological analysis at 40 h, only 17.3 ± 2.5% and 10.1 ± 0.9% (mean ± SEM) of cells were apoptotic after treatment with GM-CSF but SNAP (1 mM) induced apoptosis in 84.3 ± 1.4% (mean ± SEM, $p < 0.0001$, $n = 5$) and 86.4 ± 1.6% (mean ± SEM, $p < 0.0001$, $n = 5$) of GM-CSF-treated eosinophils, respectively (Annexin-V data not shown, morphological data

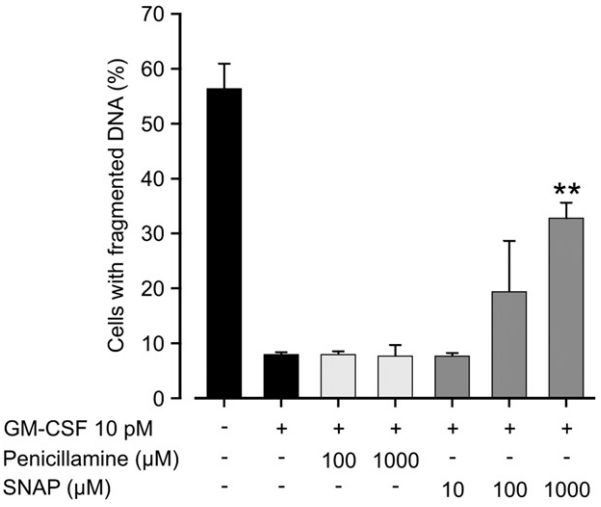

Fig. 1. SNAP induces apoptosis in GM-CSF-treated eosinophils. Eosinophils were treated with indicated concentrations of SNAP, penicillamine and/or GM-CSF for 40 h. Apoptosis was measured by DNA fragmentation assay. Shown are mean ± SEM of six independent experiments and ** indicates $p < 0.01$ when compared with GM-CSF-treated cells.

also shown in Fig. 2I). Negative control of SNAP, N-acetyl-D,L-penicillamine (penicillamine), had no effect on DNA fragmentation (Fig. 1) or morphological signs of apoptosis in the presence of GM-CSF (mean ± SEM 10.1 ± 0.9% and 10.1 ± 1.1% of cells with apoptotic morphology after treatment with GM-CSF and GM-CSF + penicillamine, respectively, $p > 0.05$, $n = 5$) (Fig. 2I) but slightly decreased phosphatidylserine (PS) exposure in Annexin-V staining assay (mean ± SEM 17.3 ± 2.5% and 11.7 ± 2% Annexin-positive cells after treatment with GM-CSF and GM-CSF + penicillamine, respectively, $p < 0.01$, $n = 5$)(data not shown) indicating decreased apoptosis. This suggests that the effect of SNAP was mediated by the released nitric oxide.

3.2. Caspase-6 mediates DNA fragmentation in SNAP-induced apoptosis

To evaluate the involvement of caspases in SNAP-induced apoptosis, we employed general and specific caspase-inhibitors. A broad spectrum caspase-inhibitor Q-Vd-OPh (20 μM) totally reversed the effect of SNAP on DNA fragmentation in the presence of GM-CSF after 40 h of incubation (Fig. 2A) indicating major role for caspases in the DNA fragmentation process. A specific caspase-6 inhibitor (Z-VEID-FMK, 200 μM) had a similar effect on SNAP-induced DNA fragmentation at 40 h as Q-Vd-OPh (Fig. 2A–C) suggesting that DNA fragmentation is principally caspase-6-dependent. Similarly, DNA fragmentation related to spontaneous apoptosis was totally abolished by 200 μM caspase-6 inhibitor (mean ± SEM 44.5 ± 6.2% and 5.2 ± 0.4% of cells with fragmented DNA in the absence and presence of caspase-6 inhibitor, respectively, $p < 0.001$, $n = 7$). Caspase-3 inhibitor (Z-DQMD-FMK, 200 μM) also partly prevented DNA fragmentation at 40 h in SNAP-treated (Fig. 2A and D) and untreated cells (mean ± SEM 50.5 ± 5.3% and 30.9 ± 2.6% of cells with fragmented DNA in the absence and presence of caspase-3 inhibitor, respectively, $p < 0.01$, $n = 6$) but caspase-8 inhibitor (IETD-CHO, 100 μM) had no effect in the presence (Fig. 2A) or absence of GM-CSF and SNAP (mean ± SEM 53.0 ± 7.4% and 69.9 ± 6.3% of cells with fragmented DNA in the absence and presence of caspase-8 inhibitor, respectively, $p > 0.05$, $n = 6$).

To more specifically determine the role of caspase-6 in mediating NO-induced apoptosis in GM-CSF-treated eosinophils, we studied the effect of caspase-6 inhibitor (200 μM) on PS exposure and morphology in the presence and absence of SNAP, penicillamine and GM-CSF at 40 h time-point. Caspase-6 inhibitor partly prevented the effect of SNAP on PS exposure as determined by Annexin-V FITC staining (Fig. 2E–G). Similarly, PS exposure during spontaneous apoptosis was partly prevented by inhibiting caspase-6 (Fig. 2E). In morphological analysis inhibition of caspase-6 did not prevent SNAP-induced or spontaneous eosinophil apoptosis (Fig. 2I) but clearly altered the ratio of early apoptotic and late apoptotic cells by increasing the relative amount of early apoptotic cells. In the presence of GM-CSF and SNAP 30.09 ± 4.82% (mean ± SEM) of all apoptotic cells were early apoptotic but treatment with caspase-6 inhibitor increased the proportion of early apoptotic cells to 87.75 ± 4.10% (mean ± SEM) (Fig. 2J–K). Caspase-6 inhibition had no effect on apoptosis in the presence of GM-CSF and penicillamine in Annexin-V staining assay or morphological analysis (Fig. 2E, H, I and L).

3.3. Caspase-6 is activated during SNAP-induced apoptosis

To date, caspase-6 is the only protease that has been shown to cleave lamin A/C during apoptosis [17,18]. Lamin B cleavage has been shown to be conducted by at least caspases-3 and -6 [17,19–21]. To confirm caspase-6 activation in eosinophils, we examined caspase-6 proenzyme levels and lamin A/C and B cleavage by western blotting. Significant changes in caspase-6 proenzyme levels were observed at 40 h but not at 16 h time-point (mean ± SEM of caspase-6 proenzyme levels 88.3 ± 22.3% and 98.4 ± 24.5% of GM-CSF-control after 16 h treatment with GM-CSF + SNAP and GM-CSF + penicillamine, respectively, $n = 5$, $p > 0.05$). After 40 h of incubation, caspase-6 proenzyme levels were significantly reduced in SNAP-treated cells, indicating that caspase-6 proenzyme had been cleaved into an active form (Fig. 3A). Similar reduction in caspase-6 proenzyme levels occurred in untreated eosinophils (Fig. 3A). Furthermore, increased amounts of caspase-6 activation products, cleaved lamin A/C and B fragments, were observed in SNAP-treated eosinophils after 40 h of incubation (Fig. 3B and C). Amounts of cleaved lamin B were enhanced also in untreated cells (Fig. 3C). SNAP-induced lamin A/C and B degradation was completely reversed by 200 μM caspase-6 inhibitor (Fig. 3B and C).

3.4. Calpains mediate spontaneous but not SNAP-induced DNA fragmentation

Next we tested whether calpains are involved in SNAP-induced or spontaneous eosinophil apoptosis by using calpeptin, an inhibitor of calpains 1 and 2. Interestingly, pre-treatment with 50 μM calpeptin partly reversed DNA fragmentation at 40 h in spontaneously dying eosinophils (Fig. 4). However, calpeptin had no effect on SNAP-induced DNA fragmentation in the presence of GM-CSF (Fig. 4).

4. Discussion

In the present study, we explored the involvement of caspases 3, 6 and 8 and calpains 1 and 2 in apoptosis induced by NO in GM-CSF-treated human eosinophils. We demonstrated that caspase-6 has a major role in lamin and DNA fragmentation during NO-induced as well as spontaneous apoptosis. Calpains 1 and 2, however, were not involved in NO-induced apoptosis even though DNA fragmentation in spontaneously dying eosinophils was partly reversed by inhibition of calpains 1 and 2.

Apoptosis is typically executed by caspases, cysteine aspartate-specific proteases. Caspases are activated by proteolytic cleavage

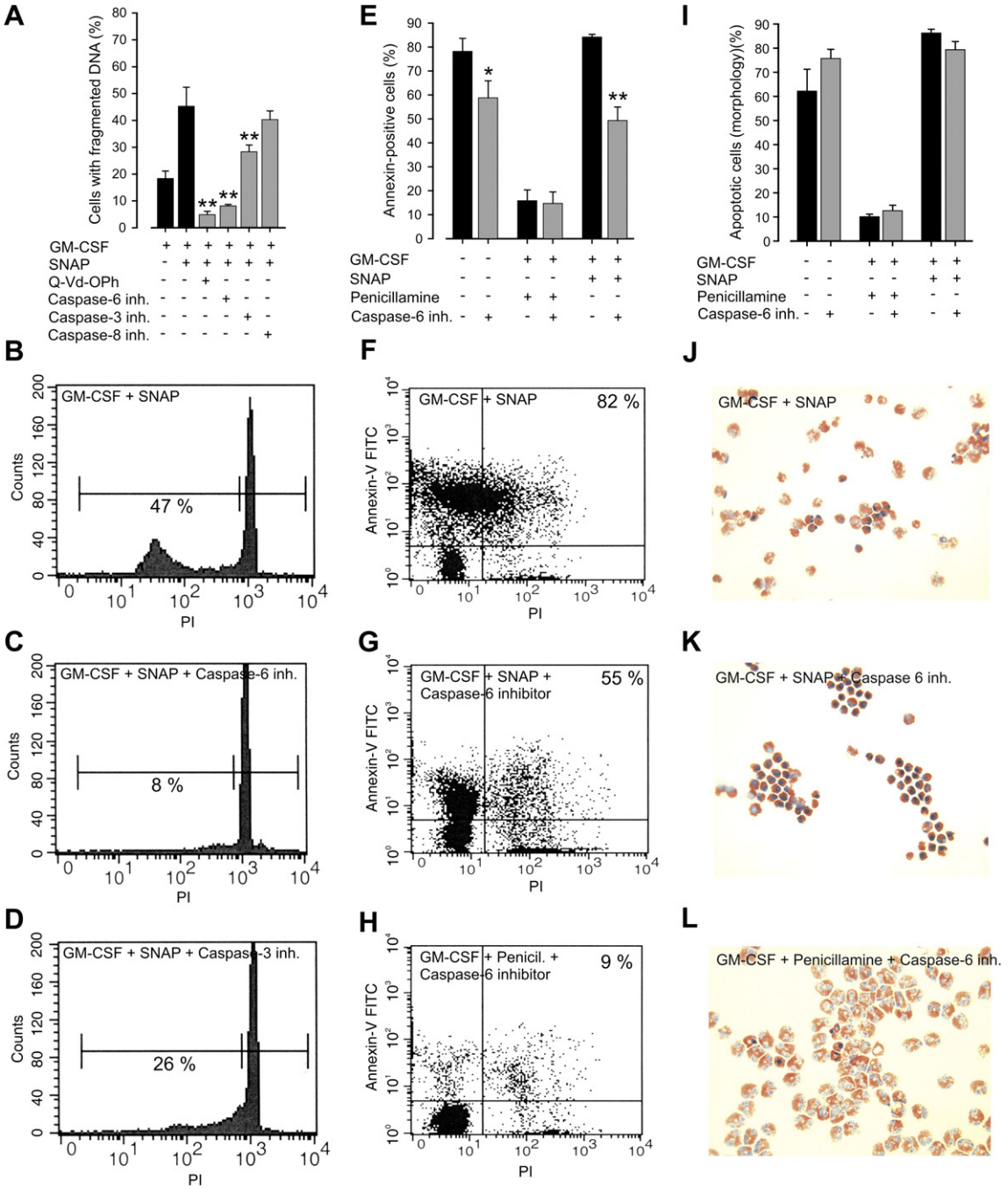

Fig. 2. Role of caspases in SNAP-induced apoptosis. Eosinophils were pretreated with solvent or caspase-6 inhibitor (Z-VEID-FMK, 200 μM) (A–C, E–L), caspase-3 inhibitor (Z-DQMD-FMK, 200 μM) (A, D), caspase-8 inhibitor (IETD-CHO, 100 μM) (A) or broad spectrum caspase inhibitor (Q-Vd-OPh, 20 μM) (A) for 20 min before addition of SNAP (1 mM), penicillamine (1 mM) and/or GM-CSF (10 pM). After 40 h of incubation apoptosis was determined by DNA fragmentation assay (A–D), Annexin-V FITC staining (E–H) or morphological analysis (I–L). In B–D shown are representative graphs from DNA fragmentation assay, in F–H from Annexin-V FITC staining and in J–L from morphological analysis. Values are mean ± SEM of five to six independent experiments and $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared to the respective control (black bar).

of their inactive proenzymes. Initiator caspases 2, 8, 9 and 10 are activated by upstream molecules whereas activation of effector caspases 3, 6 and 7 is triggered by caspases functioning upstream. Effector caspases act by cleaving downstream cellular substrates thereby conducting the execution phase of apoptosis. Previously, expression and activation of caspases 3, 6, 7, 8 and 9 was detected during spontaneous apoptosis of human eosinophils [22,23]. In our previous study, NO-induced apoptosis in IL-5-treated cells was abolished by a pan-caspase inhibitor but we could not identify the caspases mediating NO-induced apoptosis [9]. Here we demonstrate that DNA fragmentation in NO-induced apoptosis in GM-CSF-treated eosinophils is dependent on caspase-6. Decreased levels of caspase-6 proenzyme and increased formation of the known caspase-6 degradation products cleaved lamin A/C and B [17,18] in SNAP-treated cells demonstrate activation of caspase-6. Additionally, we found that SNAP-induced lamin A/C and B fragmentation was totally abolished by caspase-6 inhibitor. According to our results, caspase-6 mediates lamin and DNA fragmentation also in spontaneously dying eosinophils. Similarly, a significant role for caspase-6 in histamine- and orazipone-induced apoptosis

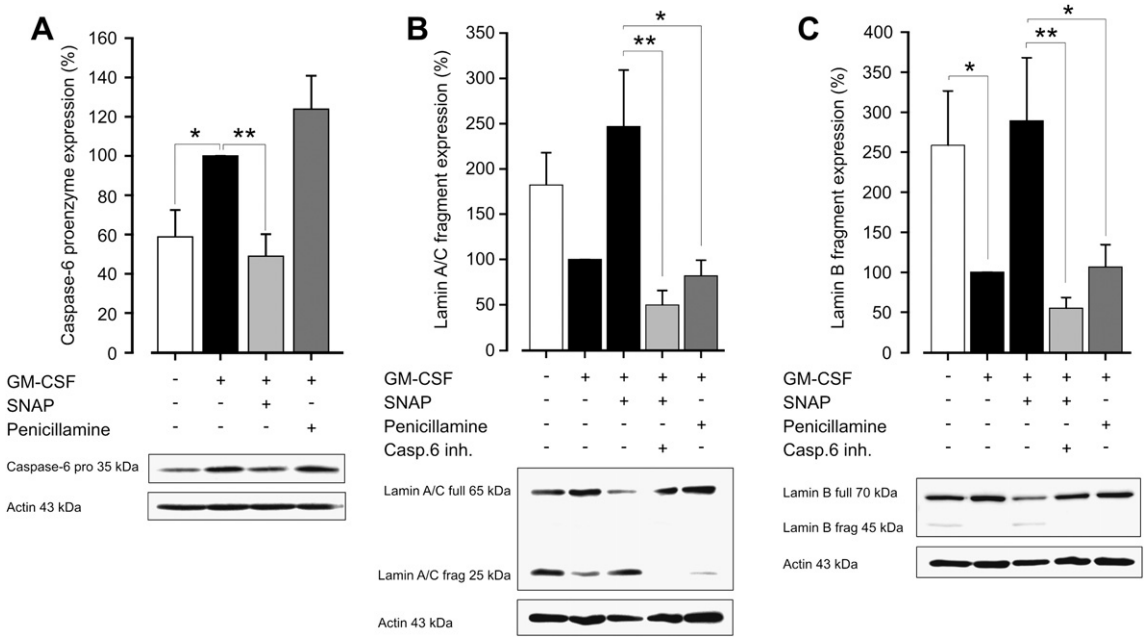

Fig. 3. SNAP induces caspase-6 activation and caspase-6-mediated lamin A/C and B fragmentation. Eosinophils were incubated in the absence or presence of solvent, GM-CSF (10 pM), SNAP (1 mM) or penicillamine (1 mM) for 40 h with or without pre-treatment with caspase-6 inhibitor Z-VEID-FMK (200 μM) for 20 min. Cells were lysed and lamin A/C, lamin B, caspase-6 and actin (as loading control) levels were measured by western blotting. Results are shown as mean ± SEM and * indicates $p < 0.05$ and **$p < 0.01$ as compared with the respective control. Shown are representative blots of 4–5 independent experiments.

has been previously reported [24,25]. This suggests that caspase-6 activation is a general feature of eosinophil apoptosis independently from the proapoptotic stimulus.

We showed that DNA fragmentation during eosinophil apoptosis was strictly dependent on caspase-6. Participation of caspase-6 in apoptotic DNA fragmentation has been contradictory according to previous studies in other cell types. In some cell types DNA fragmentation has been abolished or delayed by inhibition of caspase-6 or by expression of uncleavable mutant lamins in accordance with our results [26,27]. However, an experiment with isolated cell nuclei suggested that caspase-6 has a significant role in chromatin condensation but not in DNA fragmentation [21]. In this study, examination of cell morphology revealed that caspase-6 inhibition does not totally prevent eosinophil apoptosis, only delays or halts it, which is expected when function of a specific effector caspase is quenched. Most of the cells treated with GM-CSF and SNAP appeared as late apoptotic ghosts after 40 h of incubation. However, caspase-6 inhibition resulted in shrunken eosinophils with condensed chromatin thus resembling features of early apoptosis. According to the result we conclude that lack of function of caspase-6 and the following inhibition of lamin and DNA fragmentation either delays chromatin condensation or halts apoptosis at the level of chromatin condensation thereby preventing progression of apoptosis and formation of apoptotic bodies. Consistently, treatment with caspase-6 inhibitor resulted in dramatic inhibition of late membrane blebbing in apoptotic HeLa cells [28] and absence of apoptotic bodies in isolated nuclei with induced apoptosis [29]. Results also show that chromatin condensation can occur independently from DNA fragmentation in eosinophils, which is in concordance with the results with other cell types or non-cell extracts [30,31].

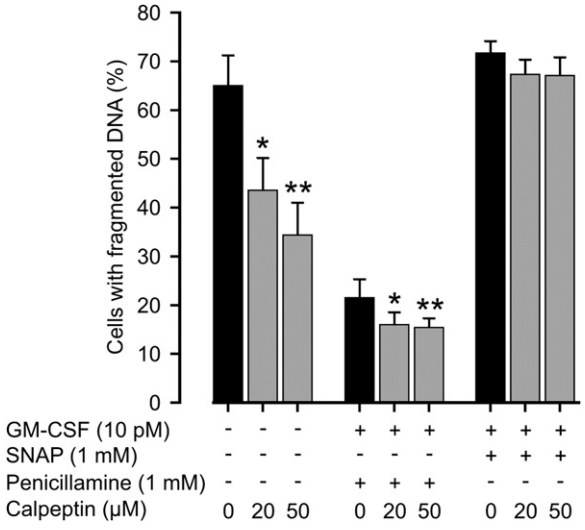

Fig. 4. Calpains are involved in spontaneous but not SNAP-induced eosinophil apoptosis. Eosinophils were treated with solvent or indicated concentrations of calpeptin for 60 min prior to adding solvent, GM-CSF, SNAP or penicillamine. Apoptosis was determined by DNA fragmentation assay after 40 h of incubation. Values are mean ± SEM of six independent experiments. * indicates $p < 0.05$ and **$p < 0.01$ as compared with the respective control (black bar).

Activation of caspase-3 has been shown during spontaneous eosinophil apoptosis and apoptosis induced by several different stimulants [22,32,33]. We previously demonstrated caspase-3 activation in eosinophils treated with NO and IL-5 but could not inhibit apoptosis-related DNA fragmentation by the used caspase-3 inhibitor (Ac-DMQD-CHO). However, the caspase-3 inhibitor used in the present study (Z-DQMD-FMK) partly prevented the effect of NO on eosinophil DNA fragmentation in the presence of GM-CSF. The reason for this inconsistency is unknown but may involve differences in cellular uptake or unspecific inhibition of caspase-6 by the caspase-3 inhibitor in question. Altogether, the involvement of caspase-3 in eosinophil DNA fragmentation during apoptosis remains unclear.

Recognition of PS on the cell surface is one of the mechanisms directing phagocytosis of apoptotic cells by macrophages [34]. PS

exposure has been shown to be dependent or independent of caspases depending on the stimulus. For example, PS exposure in PMA-induced but not in glucocorticoid-induced apoptosis was caspase-dependent in T lymphocytes [35]. PS exposure induced by a helminth parasite was shown to be caspase-dependent in human eosinophils [36]. However, no information exists about the specific caspases involved in PS exposure. We demonstrate here partial dependence of NO-induced PS exposure on caspase-6 function. Similarly, PS exposure in spontaneously dying cells was partly dependent on the function of caspase-6. This suggests similar mechanisms of PS exposure during spontaneous and NO-induced apoptosis.

Calpains are involved in a variety of calcium-regulated cellular functions, such as signal transduction, cell cycle, gene expression and apoptosis [11]. Our results indicate involvement of calpains in DNA fragmentation in spontaneously dying but not in eosinophils undergoing NO-induced apoptosis. The mechanism for calpain-mediated DNA fragmentation during spontaneous eosinophil apoptosis may be any of the following: Calpains have been shown to function downstream of caspases [12], mediate release of AIF from mitochondria [37] and to mediate cleavage of Bax to an active form [14,38]. Especially, calpain-mediated cleavage of proapoptotic Bax has been shown to be central for spontaneous eosinophil apoptosis [14], which is consistent with our data. In this study, NO-induced DNA fragmentation in GM-CSF-treated eosinophils was not found to be mediated by calpains. This difference may arise from NO-induced S-nitrosylation of calpains and the following inactivation [39,40]. Despite of the similarities in the involvement of caspases in both NO-induced and spontaneous apoptosis, this result shows that these two apoptotic pathways have differences at the level of calcium-activated neutral proteases.

In summary, we showed that caspase-6 is central for the executive phase involving lamin and DNA fragmentation in both NO-induced and spontaneous eosinophil apoptosis. However, calpains mediate DNA fragmentation only in spontaneously dying eosinophils but not in eosinophils undergoing NO-induced apoptosis providing evidence that NO-induced intracellular apoptotic signalling has specific features at the level of proteases. This study demonstrates novel mechanisms for NO-induced and spontaneous apoptosis in human eosinophils.

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RESPIRATORY RESEARCH

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Nitric oxide-induced eosinophil apoptosis is dependent on mitochondrial permeability transition (mPT), JNK and oxidative stress: apoptosis is preceded but not mediated by early mPT-dependent JNK activation

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Abstract

Background: Eosinophils are critically involved in the pathogenesis of asthma. Nitric oxide (NO) is produced in high amounts in asthmatic lungs and has an important role as a regulator of lung inflammation. NO was previously shown to induce eosinophil apoptosis mediated via c-jun N-terminal kinase (JNK) and caspases. Our aim was to clarify the cascade of events leading to NO-induced apoptosis in granulocyte macrophage-colony stimulating factor (GM-CSF)-treated human eosinophils concentrating on the role of mitochondria, reactive oxygen species (ROS) and JNK.

Methods: Apoptosis was determined by flow cytometric analysis of relative DNA content, by Annexin-V labelling and/or morphological analysis. Immunoblotting was used to study phospho-JNK (pJNK) expression. Mitochondrial membrane potential was assessed by JC-1-staining and mitochondrial permeability transition (mPT) by loading cells with calcein acetoxymethyl ester (AM) and $CoCl_2$ after which flow cytometric analysis was conducted. Statistical significance was calculated by repeated measures analysis of variance (ANOVA) or paired t-test.

Results: NO-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) induced late apoptosis in GM-CSF-treated eosinophils. SNAP-induced apoptosis was suppressed by inhibitor of mPT bongkrekic acid (BA), inhibitor of JNK SP600125 and superoxide dismutase-mimetic AEOL 10150. Treatment with SNAP led to late loss of mitochondrial membrane potential. Additionally, we found that SNAP induces early partial mPT (1 h) that was followed by a strong increase in pJNK levels (2 h). Both events were prevented by BA. However, these events were not related to apoptosis because SNAP-induced apoptosis was prevented as efficiently when BA was added 16 h after SNAP. In addition to the early and strong rise, pJNK levels were less prominently increased at 20–30 h.

Conclusions: Here we demonstrated that NO-induced eosinophil apoptosis is mediated via ROS, JNK and late mPT. Additionally, our results suggest that NO induces early transient mPT (flickerings) that leads to JNK activation but is not significant for apoptosis. Thereby, we showed some interesting early events in NO-stimulated eosinophils that may take place even if the threshold for irreversible mPT and apoptosis is not crossed. This study also revealed a previously unknown physiological function for transient mPT by showing that it may function as initiator of non-apoptotic JNK signalling.

Keywords: Eosinophils, Apoptosis, Nitric oxide, Mitochondrial permeability transition, JNK, Reactive oxygen species

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Introduction

Eosinophils play a crucial role in the pathogenesis of asthma. By releasing toxic granule proteins, lipid mediators and other proinflammatory components, eosinophils contribute especially to exacerbations of asthma [1]. Additionally, eosinophils have gained increasing attention as antigen-presenting cells and as important regulators of T-helper (Th) 2 cytokine production and airway remodelling [2,3]. Eosinophils typically exist in low numbers in human peripheral blood complicating studies on their functions. They undergo spontaneous apoptosis in few days in the absence of any survival-prolonging cytokines. Blood eosinophils obtained from patients with asthma show delayed apoptosis when compared to eosinophils from healthy controls [4] and elevated levels of eosinophil survival-prolonging cytokines interleukin (IL)-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) have been found from the bronchoalveolar lavage fluid of asthmatics [5]. GM-CSF has been demonstrated as the main eosinophil-survival prolonging cytokine in asthmatic airways [6]. Apoptosis is an efficient way to discard eosinophils from the airways by avoiding inflammation. It is characterized by cell shrinkage, chromatin condensation, nuclear coalescence, DNA fragmentation, mitochondrial changes and transfer of phosphatidyl serine residues from the inner to the outer leaflet of the cell membrane.

Nitric oxide (NO) is a gaseous molecule possessing both physiological and pathophysiological role in human tissues [7]. During inflammation, inducible nitric oxide synthase (iNOS) is rapidly activated by bacterial endotoxin and inflammatory cytokines IL-1, tumor necrosis factor (TNF)-α and IFN-γ resulting in high production of NO. In inflamed airways, NO is produced by iNOS in alveolar macrophages and bronchial epithelial cells. Patients with asthma show elevated levels of NO in the exhaled air, which correlates to clinical symptoms of asthma, sputum eosinophilia and eosinophil activation markers [8,9]. However, in a rat-model of asthma NO-releasing glucocorticoid was shown to be more potent than glucocorticoid alone. In addition, NO-releasing compound diethylenetriamine (DETA)-NONOate was shown to be as potent as a glucocorticoid in inhibiting eosinophilic inflammation [10]. NO may have both pro- and anti-inflammatory properties in asthmatic inflammation.

Interestingly, we have previously shown that exogenous NO induces human eosinophil apoptosis *in vitro* in the absence and presence of IL-5 and GM-CSF, which may act as a counter regulatory mechanism to limit eosinophilia in inflamed lungs [11,12]. Apoptotic rate of sputum eosinophils was found to positively correlate with exhaled NO in children [13] indicating that induction of eosinophil apoptosis by NO may have clinical relevance. NO was shown to possess its pro-apoptotic effect via c-Jun-N-terminal kinase (JNK) [11] and caspases 6 and 3 [12]. In previous studies with other cell types and cell-free systems treatment with NO has been found to lead to formation of reactive oxygen species (ROS), stimulation of mitochondrial permeability transition (mPT) and disruption of mitochondrial function [14,15]. Mitochondrial permeability transition pore is a Ca^{2+}- and voltage-dependent channel in mitochondrial inner membrane for molecules up to 1.5 kDa. Ca^{2+}-overload induces mPT pore to open resulting in equilibration of small molecules across the inner membrane, loss of mitochondrial membrane potential ($\Delta\Psi_m$), mitochondrial swelling and finally rupture of the outer mitochondrial membrane which releases cytochrome c and other pro-apoptotic factors to cytosol to initiate apoptosis [16]. Only scarce information exists of the function of mPT in eosinophils [17]. JNK is a stress-regulated kinase that has been previously shown to mediate apoptosis by increasing transcription of several pro-apoptotic molecules and by phosphorylating B-cell lymphoma (Bcl) 2 family members thereby participating in mitochondrial apoptotic pathway [18]. This study was conducted to find out the cascade of events and signalling mechanisms leading to NO-induced eosinophil apoptosis in the presence of survival-prolonging cytokine GM-CSF, especially concentrating on the role of ROS, JNK and mitochondria.

Methods

Materials

AEOL 10150 was a kind gift from Prof. James Crapo (University of Colorado, Denver, USA). Materials were purchased as previously described [12] or as follows: SP600125, negative control for SP600125, JNK inhibitor VIII, bongkrekic acid, apocynin (Merck, Darmstadt, Germany), diphenyleneiodonium chloride (DPI) (Sigma-Aldrich Co., St. Louis, MO, USA), JC-1 mitochondrial membrane potential detection kit (Biotium Inc., Hayward, CA, USA), MitoProbe transition pore assay kit (Molecular Probes Inc., Eugene, OR, USA), phospho-JNK (pJNK) antibody (Thr183/Tyr185) (Cell Signaling Technology Inc., Danvers, MA, USA), JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Human eosinophil purification and culture

The blood samples (100 ml) were taken from healthy, allergic or asthmatic individuals. All donors gave written informed consent to a study protocol approved by the ethical committee of Tampere University Hospital. Eosinophils were isolated to >99% purity and cultured under sterile conditions as previously described [12].

Apoptosis assays and western blotting

Relative DNA fragmentation assay of propidium iodide (PI)-stained cells, morphological analysis, Annexin-V

binding assay, eosinophil lysis and western blotting were performed as previously described [12].

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by staining eosinophils with cationic mitochondrial dye JC-1 followed by flow cytometric analysis [19]. In cells with intact $\Delta\Psi_m$ JC-1 accumulates into mitochondrial matrix. When the critical concentration is achieved, JC-1 aggregates releasing red fluorescence. In cells with collapsed $\Delta\Psi_m$ JC-1 remains in a monomeric form in the cytoplasm emitting green fluorescence. Cells (2.5-5 x 10^5) were stained for 15 min with JC-1 at 37°C, washed twice in medium and re-suspended in PBS, after which flow cytometric analysis was conducted. K^+ ionophore valinomycin is a drug able to collapse $\Delta\Psi_m$ and was used as a positive control [19]. The gates defining cells with intact and lost $\Delta\Psi_m$ were based on cells treated with GM-CSF (10 pM) and valinomycin (1 μM), respectively, in each experiment individually.

Determination of mitochondrial permeability transition

Mitochondrial permeability transition was determined by MitoProbe transition pore assay kit (Molecular Probes Inc., Eugene, OR, USA), a technique based on calcein acetoxymethyl ester (AM) and $CoCl_2$ [20]. Calcein AM is a non-fluorescent molecule which accumulates into cytosol and mitochondria. Cleavage of calcein AM by intracellular esterases liberates fluorescent calcein dye that does not cross plasma or mitochondrial membrane. In normal cells, addition of $CoCl_2$ quenches the fluorescence of cytosolic but not mitochondrial calcein. In cells with mPT, $CoCl_2$ enters also mitochondria to quench the mitochondrial calcein fluorescence. Calcein AM/$CoCl_2$-method requires activity of intracellular esterases that are functional only in vital cells restricting use of this method. Cells (5 x 10^5) were labeled with 10 nM calcein AM, 400 μM $CoCl_2$ and/or 0.5 μM ionomycin for 15 min at 37°C. Cells were washed with HBSS, resuspended in PBS and analyzed by flow cytometry. Ionomycin, known to induce complete mPT, always produced minimal fluorescence of 2–4 mean fluorescence intensity (MFI) in the presence or absence of GM-CSF, penicillamine or SNAP indicative of complete mPT. Calcein fluorescence is expressed as percentage of its initial value ((mean of calcein fluorescence of cells treated with calcein AM and $CoCl_2$ / mean of calcein fluorescence of cells treated with calcein AM) * 100). This data was finally normalized against control cells (cells treated with GM-CSF and penicillamine).

Statistics

Results are shown as mean ± SEM. Statistical significance was calculated by repeated measures analysis of variance with Dunnett's post-test or by paired t-test by using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

Results

SNAP induces apoptosis in GM-CSF-treated eosinophils

NO-donor SNAP but not the negative control N-acetyl-D,L-penicillamine induced apoptosis in GM-CSF-treated eosinophils as previously described and as indicated in Table 1 [12]. Time-course of SNAP-induced apoptosis revealed late initiation of cell death. SNAP induced only a minor increase in DNA fragmentation at 16 and 24 h time-points and a significant increase was observed only after 40 h of incubation (Figure 1).

SNAP-induced apoptosis is dependent on late mPT but preceded by early partial mPT

Mitochondrial membrane permeabilization including mPT and loss of $\Delta\Psi_m$ are critical steps in mitochondrial apoptotic pathway [16]. We used calcein AM/$CoCl_2$-method to assess whether SNAP induces mPT in GM-CSF-treated eosinophils. We found that SNAP reduced calcein fluorescence by 34.6 ± 7.7% when compared to penicillamine-treated cells at 1 h indicating that mPT occurs and $CoCl_2$ enters mitochondria to quench calcein fluorescence (Figures 2A-D). This effect was mostly prevented by bongkrekic acid, an inhibitor of mPT (Figures 2A-D). However, when compared to ionomycin-induced complete mPT as indicated by total quench of calcein fluorescence (data not shown), mPT raised by SNAP was partial.

To determine whether mPT is important for apoptosis in the presence or absence of SNAP and GM-CSF, we treated the cells with bongkrekic acid. Indeed, bongkrekic acid significantly reversed the effect of SNAP on DNA fragmentation (Figure 2E), cell morphology (Figure 2F) and Annexin-V labeling (Figure 2G). Only a

Table 1 Effect of SNAP on GM-CSF-induced eosinophil survival as previously described [12]

	Apoptotic cells (%)
Untreated	60.8 ± 4.1
GM-CSF 10 pM	11.1 ± 1.6
+SNAP 10 μM	10.5 ± 1.8
+SNAP 100 μM	28.4 ± 10.9
+SNAP 1000 μM	44.6 ± 8.6 **
GM-CSF 10 pM	9.3 ± 1.0
+Penicillamine 100 μM	8.6 ± 1.2
+Penicillamine 1000 μM	8.6 ± 2.1

Apoptosis was determined after 40 h of incubation by DNA fragmentation assay. Shown are mean ± SEM, n = 7. ** indicates $p < 0.01$ as compared with GM-CSF-treated cells.

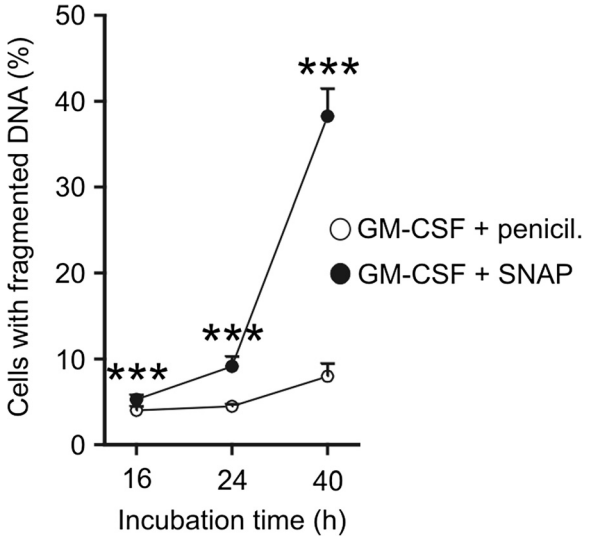

Figure 1 SNAP induces late apoptosis in GM-CSF-treated eosinophils. Eosinophils were incubated in the presence or absence of 1 mM SNAP, 1 mM penicillamine and/or 10 pM GM-CSF for the indicated times. Apoptosis was measured by DNA fragmentation assay. *** indicates $p < 0.001$ as compared with cells treated with GM-CSF and penicillamine at the corresponding time-point. Mean ± SEM is shown from six independent experiments.

small portion of spontaneous apoptosis was prevented by treatment with bongkrekic acid (Figures 2E-G). To examine whether the early mPT at 1 h time-point is critical for SNAP-induced apoptosis, we added bongkrekic acid to eosinophils at later, arbitrary time-point, 16 h after SNAP and GM-CSF. Surprisingly, SNAP-induced apoptosis was prevented even when bongkrekic acid was added to the cells 16 h after SNAP and GM-CSF (Figure 2H) indicating that early partial mPT is not significant for apoptosis. Thus, the threshold for apoptosis-inducing complete mPT is achieved 16–40 h after addition of SNAP.

SNAP induces late loss of mitochondrial membrane potential

Two forms of mPT have been previously identified. Sustained opening of the mPT pore leads to loss of ΔΨm resulting in cell death. However, transient or flickering openings of the mPT pore may only lead to mitochondrial membrane depolarization spikes, but not to permanent loss of $\Delta\Psi_m$ and cell death [21,22]. Next our aim was to determine the effect of SNAP on $\Delta\Psi_m$ at different time-points to get further evidence that only mPT occurring at late stage results in loss of $\Delta\Psi_m$ and is critical for apoptosis. As expected, we found that SNAP did not significantly increase the proportion of cells with lost $\Delta\Psi_m$ after 20 h of incubation (Figure 3A). At 40 h time-point, loss of $\Delta\Psi_m$ occurred in most of SNAP-treated but not penicillamine-treated cells (Figures 3A-C). This result supports the conclusion that SNAP induces permanent mPT leading to loss of $\Delta\Psi_m$ and apoptosis at late stage.

JNK mediates SNAP-induced apoptosis but is also induced by early non-apoptotic mPT

We have previously shown that SNAP-induced apoptosis is dependent on JNK and demonstrated early activation of JNK by SNAP [11]. To more carefully explore the pattern of JNK activation we studied pJNK levels during longer time-scale. SNAP induced a strong JNK phosphorylation at 2 h time-point and smaller increases in pJNK levels at 1 h, 20 h and 30 h time-points (Figure 4A). As expected, untreated eosinophils showed stable pJNK levels (Figure 4A). To examine if early activation of JNK is a consequence of early mPT, we used bongkrekic acid to inhibit mPT. Treatment with bongkrekic acid completely prevented SNAP-induced increase in pJNK levels at 2 h time-point (Figure 4B).

In previous studies it has been demonstrated that rapid, strong and transient JNK activation is a stress response resulting in cell survival signalling while delayed and sustained JNK activation is related to apoptosis [23,24]. To clarify whether the early (2 h) and/or late (20–30 h) JNK activation is important for SNAP-induced eosinophil apoptosis, we used JNK inhibitor SP600125 added before and/or 16 h after SNAP. We found that if SP600125 was added only at 16 h time-point it had no effect on SNAP-induced apoptosis (Figure 4C). Surprisingly, SP600125 added only before SNAP was not effective either (Figure 4C). Nevertheless, a clear inhibition of SNAP-induced apoptosis was seen when SP600125 was added at both of these time-points (Figure 4C). A chemically different inhibitor of JNK, JNK inhibitor VIII (1 μM), partly prevented SNAP-induced eosinophil apoptosis when added once 30 min before SNAP ($P < 0.05$, $n = 7$, data not shown). The results suggest that the later JNK activation is involved in mediating apoptosis but the later phase may begin earlier than 16 h and persist at least up to 30 h after addition of SNAP.

Activation of JNK has been previously shown to mediate cell death by participating in the induction of mPT and loss of $\Delta\Psi_m$ [25,26]. We did not find, however, any role for JNK in stimulating these mitochondrial changes because inhibition of JNK by SP600125 or JNK inhibitor VIII did not reverse SNAP-induced loss of $\Delta\Psi_m$ at 40 h time-point ($p > 0.05$, $n = 4$-5, data not shown).

Significant role of early ROS production in SNAP-induced apoptosis

Cellular stress often results in increased production of ROS such as superoxide (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) by mitochondrial

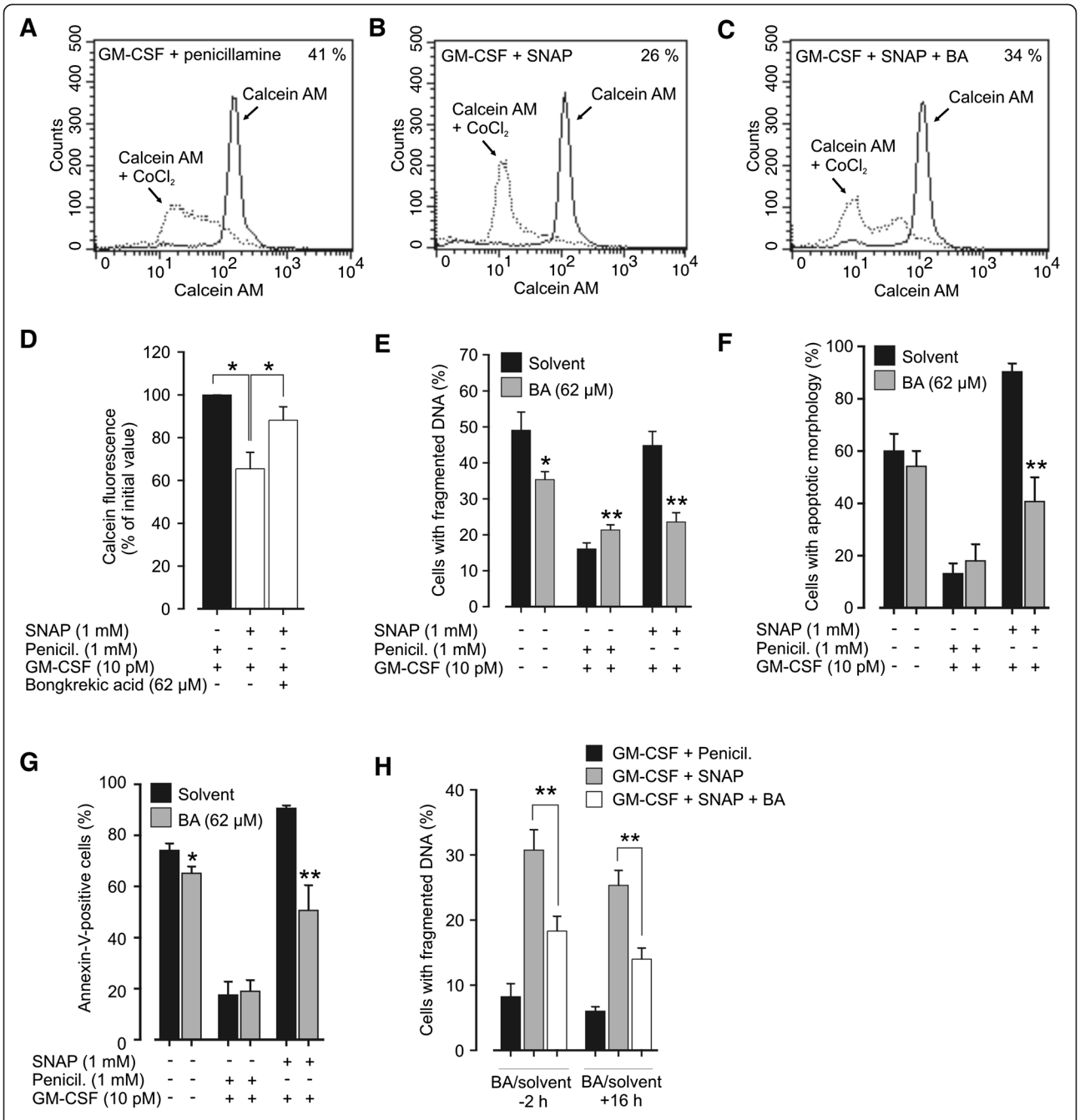

Figure 2 SNAP-induced apoptosis is dependent on late mPT but preceded by early partial mPT. Bongkrekic acid (62 µM) or solvent was added either 2 h before (**A-H**) or 16 h (**H**) after 10 pM GM-CSF, 1 mM SNAP and/or 1 mM penicillamine. In A-D Eosinophils were incubated for 1 h after which mPT was determined by calcein AM/$CoCl_2$-method as described in materials and methods. In A-C shown are representative graphs from analysis of mPT and in the top right corner percentage of calcein fluorescence of its initial value describing the extent of mPT. In E-H total incubation time of eosinophils was 40 h after which apoptosis was determined by DNA fragmentation assay (**E**, **H**), morphological analysis (**F**) or Annexin-V FITC staining (**G**). Values are mean ± SEM from five to six independent experiments, * indicates $p < 0.05$ and ** $p < 0.01$ as compared with the respective control.

respiratory chain, NADPH oxidase or other enzymes such as cyclooxygenase (COX). These radicals participate in the generation of several toxic metabolites. One of the most toxic metabolites formed in the presence of superoxide and nitric oxide is peroxynitrite, which induces DNA damage, lipid peroxidation and inhibits several cytoplasmic and mitochondrial enzymes [27]. To determine whether SNAP-induced apoptosis in the presence of GM-CSF is dependent on superoxide and/or peroxynitrite formation, we used a small-molecule

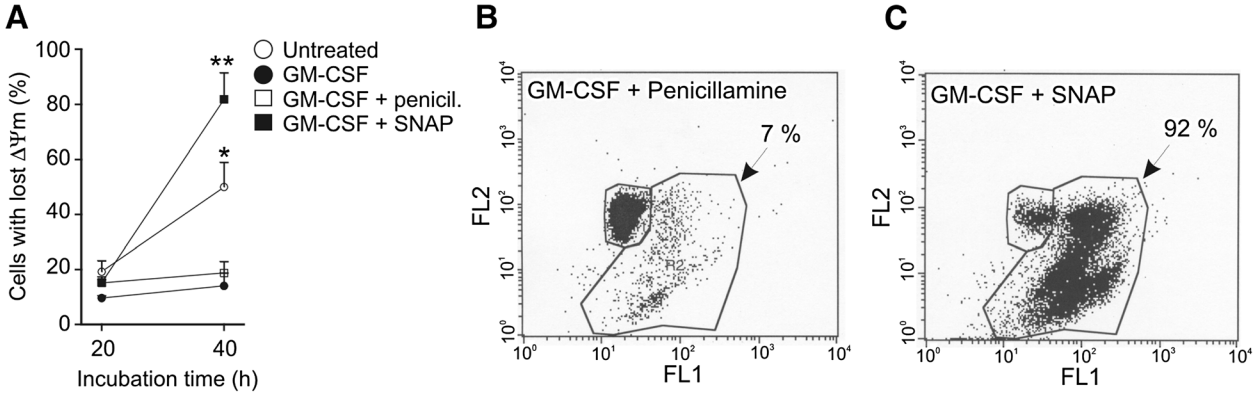

Figure 3 SNAP induces late loss of $\Delta\Psi_m$. Eosinophils were incubated with solvent, SNAP (1 mM), penicillamine (1 mM) and/or GM-CSF (10 pM) for indicated times (**A**) or for 40 hours (**B**-**C**) after which JC-1-staining and analysis of mitochondrial membrane potential by flow cytometer was conducted as described in materials and methods. In A shown are mean ± SEM from five independent experiments. * indicates $p < 0.05$ and ** $p < 0.01$ when compared to cells treated with GM-CSF or GM-CSF and penicillamine, respectively. In B-C shown are representative graphs from analysis of $\Delta\Psi_m$ with percentages of cells with lost $\Delta\Psi_m$.

antioxidant AEOL 10150 with a structure analogous to the catalytic site of SOD [27]. AEOL partly prevented the pro-apoptotic effect of SNAP but not spontaneous apoptosis indicating that treatment with SNAP specifically increases cellular ROS production (Figure 5A). When AEOL was added 16 hours after SNAP and GM-CSF, the treatment had no effect any more on SNAP-induced apoptosis (Figure 5B) indicating importance of early production of ROS. Inhibitors of NADPH oxidase, DPI and apocynin, had no significant effect on SNAP-induced apoptosis suggesting that NADPH oxidase is not the source of superoxide (Figures 5C-D). As a conclusion, early superoxide and/or peroxynitrite production is an important step in SNAP-induced apoptosis but superoxide does not originate from NADPH oxidase.

Discussion

Nitric oxide is produced in high amounts in asthmatic lungs and has an important role as a regulator of lung inflammation. In this study, we found that NO prevents the survival-prolonging effect of GM-CSF in eosinophils by inducing apoptosis consistently with our previous reports [11,12]. We focused here to the cascade of events leading to NO-induced eosinophil apoptosis particularly concentrating on the role of mitochondria, ROS and JNK. We showed that NO-induced eosinophil apoptosis is dependent on early ROS production, JNK and late mPT. In addition, we found that NO induced an early partial mPT and mPT-dependent JNK activation but those events seemed not mediate NO-induced apoptosis detected at later time points.

Mitochondrial permeability transition pore is a channel in the inner mitochondrial membrane that is composed of several proteins in a complex manner. The molecular structure of the channel has not yet been resolved despite of numerous attempts and it has been postulated that the structure may vary depending on the cell type and/or the trigger. Inhibitor of mPT, bongkrekic acid, acts as a ligand to adenine nucleotide translocator (ANT), which is either a component or an important regulator of mPT [28,29]. ANT has been shown to act as a critical target in mitochondrial membrane permeabilization induced by NO and peroxynitrite [30], giving ground for usage of bongkrekic acid as an inhibitor of mPT in this study. Here, we also showed that bongkrekic acid inhibits mPT in eosinophils.

Our results demonstrate that NO has a marked and varied effect on mPT at long time-scale. We found that NO induces partial mPT at 1 h in eosinophils that does not lead to early permanent loss of $\Delta\Psi_m$. This strongly suggests that early NO-induced mPT is transient or flickering. Previously, flickering mPT has been demonstrated in healthy intact cells [21,31] and it has been postulated to act as a fast calcium release mechanism [32,33] participating thereby in diverse range of Ca^{2+} -mediated cellular activities. Flickering mPT may also be involved in cell protection during minor stress [34] or it may act as an early signal for oxidative stress-induced apoptosis [31]. In accordance with our results, NO was shown to induce and modulate mPT in a reversible manner in isolated mitochondria [35]. We found that the early mPT is not significant for initiation of NO-induced apoptosis because addition of mPT inhibitor bongkrekic acid at 16 h after SNAP and GM-CSF still efficiently prevented NO-induced apoptosis. This seems to be in contrast to the findings of Ma et al. [31] who showed that early flickering mPT and associated superoxide flashes induced by selenite are early signals of apoptosis. They showed that manipulation of selenite-

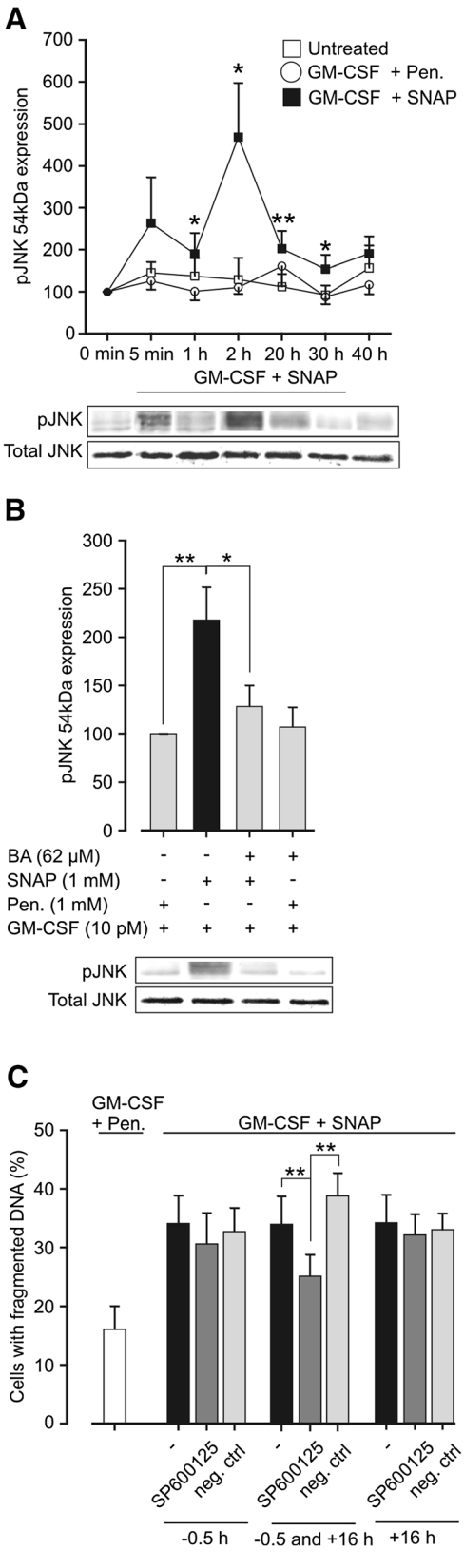

Figure 4 JNK mediates SNAP-induced apoptosis but is also strongly induced by early non-apoptotic mPT. In **A** eosinophils were incubated in the presence or absence of 1 mM SNAP, 1 mM penicillamine and/or 10 pM GM-CSF for indicated times and lysed for western blotting. pJNK and total JNK levels were normalized against 0 min GM-CSF (10 pM) sample. Total JNK was used as loading control and shown are 54 kDa pJNK and total JNK. * indicates $p < 0.05$ and ** $p < 0.01$ as compared with eosinophils stimulated with GM-CSF and penicillamine for the corresponding time, n = 7. Untreated cells were compared to 0 min GM-CSF sample, n = 5. In **B**, eosinophils were preincubated with bongkrekic acid for 2 h before adding SNAP, penicillamine and/or GM-CSF. Incubation was continued for 2 h before cell lysis and western blotting. Shown are mean ± SEM of 6 individual experiments. In **C**, time-points shown below the columns refer to the addition times of solvent, SP600125 (10 µM) or its negative control (10 µM). Time zero represents the time of adding GM-CSF (10 pM) and SNAP (1 mM) or penicillamine (1 mM). Total incubation time was 40 h and DNA fragmentation assay was used for determination of apoptosis. Values are mean ± SEM of 5 individual experiments. * indicates $p < 0.05$ and ** $p < 0.01$ versus respective control.

induced flickering mPT and associated superoxide flashes by knockdown or overexpression of mPT component cyclophilin D decreased or increased selenite-induced apoptosis, respectively [31]. However, this manipulation also affects the irreversible mPT and there is no conclusive evidence that flickering mPT and the associated superoxide flashes are necessary for apoptosis. Nevertheless, because treatment with NO ends up in mPT-mediated apoptosis it is likely that flickering mPT is an important point where anti-apoptotic and pro-apoptotic signals converge and fate of the cell is determined. Most probably, if a certain threshold is achieved flickering mPT is turned into permanent mPT and cell undergoes apoptosis or necrosis.

We showed that the early partial mPT led to strong activation of JNK at 2 h. A smaller activation of JNK was observed at later time-points (20–30 h). Consistently, a previous study showed that apoptosis-inducing N-methyl-4-phenylpyridinium (MPP+) induced early and late phases of JNK activation in a mammalian cell line and the early phase was preventable by bongkrekic acid [36]. They also found that the late JNK activation was independent of mPT, which remains unsolved in our study. However, in contrast to our study, Casarino et al. did not show whether the early mPT-dependent JNK activation or late phase of JNK activation are relevant for MPP+ –induced apoptosis. To our knowledge, this is the first study to demonstrate mPT-mediated JNK activation not related to apoptosis. According to our study, one physiological function of flickering mPT may, therefore, be initiation of JNK signalling in response to oxidative stress probably aiming to cell rescue. Plenty of evidence from studies conducted by others supports the conclusion that the early mPT and the following JNK activation are a

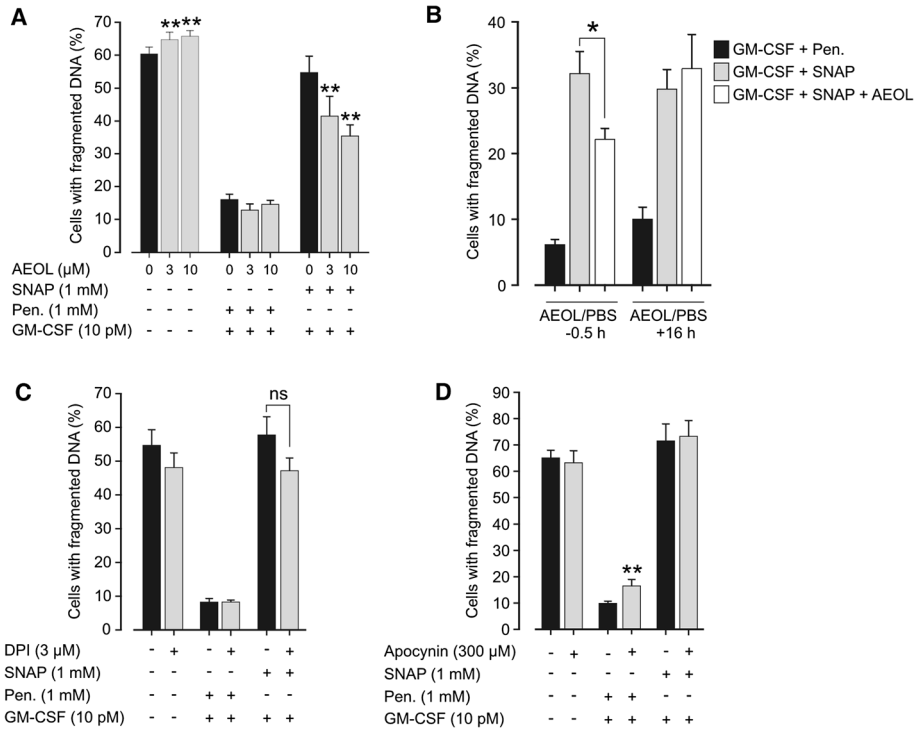

Figure 5 Early ROS-production is involved in SNAP-induced apoptosis. Eosinophils were incubated with AEOL for 15 min (**A**), DPI (**C**) or apocynin (**D**) for 20 min prior to stimulation with SNAP, penicillamine and/or GM-CSF for 40 hours. In **B**, AEOL was added 30 min prior to or 16 h after SNAP and GM-CSF. Apoptosis was measured by DNA fragmentation assay. * indicates $p < 0.05$ and ** $p < 0.01$ versus respective control. Shown are results from five to seven independent experiments.

protective response. First, several groups have demonstrated that rapid, strong and transient JNK activation is a stress response resulting in cell survival signalling while delayed and sustained JNK activation is related to apoptosis [23,24]. Second, flickering mPT was shown to be involved in cell protection during minor stress [34]. Additionally, Beltran et al. have demonstrated that long exposure to NO by DETA-NONOate initially stimulates a protective response by inhibiting complex IV in the mitochondrial respiratory chain. This led to maintenance of $\Delta\Psi_m$ by hydrolysis of glycolytic ATP instead of the respiratory chain and increased cell viability. The protective response induced by NO turned into apoptotic response by an unknown mechanism that was speculated to involve accumulation of oxidative damage [37]. In eosinophils $\Delta\Psi_m$ is maintained exceptionally by hydrolysis of glycolytic ATP rather than respiratory chain in contrast to most eukaryotic cells [38] indicating that this mechanism is already functional in eosinophils. Evidence of the survival-increasing potential of NO in eosinophils has been shown by several groups [39-41]. Hebestreit and co-workers showed that NO stimulates eosinophil survival in the presence of apoptosis-inducing Fas at 24 h time-point [39]. Further time-points were not studied to see whether the survival signalling would have turned into apoptotic signalling. In our experiments GM-CSF produced maximal survival of eosinophils at 10 pM concentration making it impossible to show that NO activates survival machinery in eosinophils at early time-points. This evidence supports the conclusion that NO-induced early mPT and the following JNK activation are a protective response of the cell.

ROS/RNS are known inducers of JNK in eosinophils [42], which suggests that ROS formation may be involved in the early mPT-dependent activation of JNK. Studies by Zorov et al. [43] have shown a relationship between ROS and mPT which raises an interesting possibility for the mechanism of mPT-induced JNK activation. They showed that mitochondrial ROS accumulation leads to mPT, which was followed by mitochondrial ROS burst that can be released to the cytosol [43]. Also Ma et al. demonstrated mPT-dependent superoxide flashes in response to oxidative stress [31]. However, whether this mechanism explains mPT-mediated early JNK activation in NO-treated eosinophils remains to be clarified.

Stimulation of GM-CSF-treated eosinophils with NO resulted in permanent loss of $\Delta\Psi$m and apoptosis at 40 h. Lost $\Delta\Psi_m$ is often, but not always, a consequence of permanent mPT [44]. Prevention of NO-mediated

apoptosis by late addition of bongkrekic acid, however, gives further evidence that the threshold for permanent mPT is crossed at time-point beyond 16 h. Previously, NO was shown to induce permanent mPT in isolated mitochondria and thymocytes [15]. In eosinophils, mPT mediated dexamethasone-induced apoptosis [17].

JNK has been previously reported to mediate spontaneous apoptosis and apoptosis induced by several drugs and nitric oxide in eosinophils [11,42,45]. In concordance with our previous results, we found here that NO activates JNK and JNK has a role in NO-induced apoptosis [11]. Similarly to the results with JNK peptide inhibitor 1 (L-JNKI1) in the previous study [11], two additions of JNK inhibitor SP600125 (at 30 min before and 16 h after SNAP) was required to suppress the pro-apoptotic effect of NO. Either addition alone had no statistically significant effect on SNAP-induced apoptosis. The result implies that the later phase of JNK activation is required for apoptosis but the later phase may initiate before 16 h. This is possible because we did not study pJNK levels in time-points between 2 h and 20 h. With the limited amounts of cells available for these studies and the sensitivity of the current assays, it was not possible to determine the exact time, when the later phase of JNK activation was initiated. The result also suggests that the initial 10 μM concentration of SP600125 was not adequate to inhibit JNK during long time-scale of activation in contrast to 1 μM concentration of JNK inhibitor VIII that slightly but statistically significantly suppressed the pro-apoptotic effect of SNAP. The mechanism by which JNK participates in NO-induced eosinophil apoptosis remains unclear. In previous studies with other cell types and stimulants JNK has promoted apoptosis by inducing mPT [25,26]. However, this seems not to be the mechanism in NO-induced apoptosis in eosinophils since inhibition of JNK had no effect on the SNAP-induced loss of $\Delta\Psi_m$. It is also possible that JNK has a pro-apoptotic mechanism that is independent on mPT. For example, JNK-mediated transcription has been shown to enhance expression of Fas-ligand [46] indicating that JNK activation may stimulate extrinsic Fas pathway of apoptosis in parallel to intrinsic mitochondrial pathway. Alternatively, JNK-dependent mechanisms may mediate the pathway from mPT to DNA fragmentation.

Under normal physiological condition, few per cent of the oxygen consumed by mitochondria is converted to superoxide. Cellular stress often leads to further increase in superoxide production. Inhibition of the components of the mitochondrial respiratory chain has been demonstrated as one mechanism by which NO increases superoxide production [47]. Reaction between nitric oxide and superoxide leads to formation of peroxynitrite. By using SOD mimetic AEOL 10150, we showed that the pro-apoptotic effect of SNAP on eosinophils in the presence of GM-CSF is partly dependent on superoxide and/or peroxynitrite production. However, addition of SOD mimetic at 16 h time-point was no longer effective in reversing the pro-apoptotic effect of SNAP suggesting that early formation of superoxide and/or peroxynitrite is critical for SNAP-induced apoptosis. Early NO-induced ROS-production is in concordance with the results of other studies [48,49]. Previously in other cell

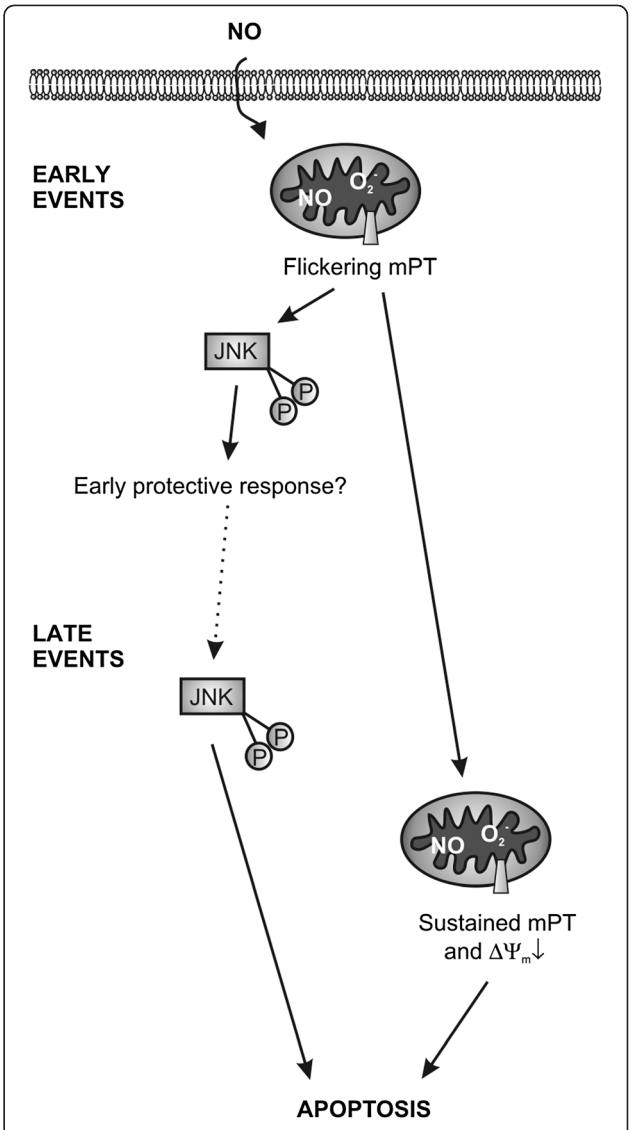

Figure 6 A proposed model of the action of NO in GM-CSF-treated eosinophils. As early events (1–2 hours), NO induces flickering mPT that leads to JNK activation but these events are not related to apoptosis and may act as an early protective response. Later, the flickering mPT turns into sustained mPT, mitochondrial membrane potential ($\Delta\Psi_m$) collapses and cell undergoes apoptosis. A new phase of JNK activation occurs before late loss of mitochondrial function and is involved in mediating apoptosis but not via inducing disruption of mitochondrial function.

types the peak of ROS production by NO has been demonstrated to occur before caspase activation and the following apoptosis [49,50]. However, the importance of this early ROS peak for apoptosis has been unclear. Our study shows that early increase of ROS is a critical event mediating NO-induced eosinophil apoptosis. NO-induced apoptosis seems to be initiated relatively late suggesting importance of accumulation of oxidative damage. Interestingly, because both apoptosis-related formation of ROS and activation of JNK seem to initiate before 16 h and ROS/RNS are known inducers of JNK [42], ROS may also participate in activating the later apoptosis-related JNK. Inhibition of apoptosis by AEOL was only partial suggesting that NO-induced formation of ROS is not the only key event for initiation of apoptosis. NO may also have direct apoptosis-stimulating effects on eosinophils. Another possibility that may explain the result is inefficiency of the used SOD-mimetic in dismutating all superoxide and peroxynitrite production. According to our results with inhibitors of NADPH oxidase, this enzyme is not the major source of superoxide in NO-treated eosinophils. This leaves mitochondrial electron transport chain as the most likely source of superoxide.

Nitric oxide is abundant in the lungs of asthmatics and thereby most likely affects eosinophil functions in a physiological situation. In this study, in addition to the mechanism of NO-induced apoptosis, we showed some interesting early events in NO-stimulated eosinophils that may take place even if the threshold for irreversible mPT and apoptosis is not crossed. In fact, levels of NO in the exhaled air has been shown to correlate to eosinophilic inflammation [8,9] which makes it tempting to speculate that NO might only induce flickering mPT and JNK activation ending up in a protective response but not apoptosis in a physiological situation.

Conclusions

We showed here that NO induces apoptosis of GM-CSF-treated human eosinophils by a mechanism that involves early oxidative stress, JNK activation and late mPT. However, before irreversible activation of apoptotic machinery NO induces early flickering mPT that leads to JNK activation but is not related to apoptosis (Figure 6). Thereby, we showed some interesting early events in NO-stimulated eosinophils that may take place even if the threshold for irreversible mPT and apoptosis is not crossed. This study also revealed a previously unknown physiological function for transient mPT by showing that it may act as an initiator of non-apoptotic cell signal transduction by activating JNK.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PI-S participated in the study design, carried out experiments, performed data analysis and wrote the manuscript. EM participated in conceiving of the study and the study design and revised the manuscript critically for important intellectual content. VLK revised the manuscript critically for important intellectual content. HK conceived the study, participated in the study design, data analysis and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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