Neutrophils in respiratory syncytial virus disease

Untangling the NET

Cortjens, B.

Link to publication

Creative Commons License (see https://creativecommons.org/use-remix/cc-licenses):
Other

Citation for published version (APA):
Cortjens, B. (2017). Neutrophils in respiratory syncytial virus disease: Untangling the NET.
Chapter 1

General Introduction and Thesis Outline
General introduction

Respiratory syncytial virus (RSV) is one of the most important causes of respiratory infections in young children worldwide.\textsuperscript{1,2} Approximately 80\% of all children have been infected at least once during the first two years of life.\textsuperscript{3} Globally, a total of thirty-three million children are infected with RSV each year, and mortality due to RSV infection is estimated between 66,000-199,000 children per year, primarily in the developing world.\textsuperscript{2} In developed countries with access to highly specialized paediatric intensive care units (PICU), the mortality rates are much lower.\textsuperscript{4} Mortality in the Netherlands is estimated at 2.8 per 100,000 children younger than 12 months.\textsuperscript{3} Nevertheless, 8-10\% of infants infected with RSV develop severe lower respiratory tract disease (RSV-LRTD) warranting hospital admission and even mechanical ventilation.\textsuperscript{4} This poses a great burden on both hospital capacity, as well as the costs associated with these admissions.\textsuperscript{6,7} The estimated annual costs associated with RSV disease are 600 million USD in the US and 18 million CAD in Canada.\textsuperscript{8,9} In the Netherlands, RSV associated costs are estimated at 8 million euros per year.\textsuperscript{5} Treatment of severe RSV-LRTD is hampered by the lack of effective treatments, and supportive care is the only available treatment to date.

Originally, RSV was discovered during a ‘common cold’ outbreak in chimpanzees in 1956 \textsuperscript{10}, but the virus proved to be able to infect humans as well. In retrospect, the oldest RSV-positive sample was found in lung-tissue of a child who died from ‘atypical pneumonia’ in 1931.\textsuperscript{11} Since then, our insights into the mechanisms of infection and the pathogenesis of RSV disease have greatly improved, but unfortunately not enough to be able to develop sufficiently successful treatments.

Respiratory syncytial virus

RSV is a member of the pneumoviridae, genus Orthopneumovirus. There are several closely related species, including bovine (b)RSV and pneumonia virus of mice (PVM, fig. 1).\textsuperscript{12} All pneumoviruses are negative-sense enveloped RNA viruses. The RSV genome spans 15,000 nucleotides and encodes for ten proteins. Three proteins are expressed as transmembrane surface proteins: fusion (F), attachment (G), and small hydrophobic (SH) protein of which the F and G protein are the most immunogenic.\textsuperscript{13,14} Five structural proteins (L, N, P, M and M2-1) and two non-structural proteins (NS1 and NS2) complete the genome and serve in various functions, including encapsulation, RNA replication, assembly and transcription.\textsuperscript{15}

RSV infects ciliated lung epithelial cells in the bronchus, bronchioli and nasal area (Fig. 2), but also type 1 and type 2 pneumocytes in the alveoli.\textsuperscript{11,16} After contact of the virus with these cells, the host can detect infection through binding of viral peptides or DNA to toll-like receptors (TLRs). For example, the RSV F protein can bind to TLR4 and viral dsRNA can be detected by TLR3.\textsuperscript{17,18} After infection, the lung epithelial cells start producing a range of im-
General Introduction

Figure 1: Phylogenetic tree of pneumoviridae
Dendrogram showing the protein distance of the HRSV, PVM and BRSV F proteins tested. The amino acid distance scale is indicated with a value of 10% distance. Adapted from Corti et al.90

Figure 2: RSV infection of primary human airway epithelial cells in vitro.
RSV-GFP infected cells (green) are ciliated cells (red, anti-beta-tubulin) and are located at the apical surface. Non-infected cells, detected with the nuclear staining (blue, Hoechst) are located at the basal side of the infected cells and do not express cilia.
mune-regulating cytokines, including IL-8, type 1 interferons and MMP-9. These secreted chemo- and cytokines orchestrate a complex host response to RSV infection. Some chemokines activate and recruit immune cells (e.g. IL-8). Others activate lung cells, for example, IL-9 promotes mucus production by goblet cells. This increased mucus production is a characteristic feature of severe RSV-LRTD and results in airway obstruction. Eventually, the adaptive immune system is activated and starts to contribute to viral clearance. Together these pathways determine how the body responds to RSV infection and, in combination with direct viral-induced damage, dictate the clinical presentation.

Clinical RSV disease

Sixty-eight percent of all children become infected with RSV during the first 12 months of life, and as such it is one of the most common infections during early childhood. Primary RSV infection usually results in mild upper respiratory tract disease characterised by rhinorrhoea, nasal congestion and sub-febrile temperature. Often these children recover within 1-2 weeks. However, 2% of RSV infected children develop lower respiratory tract disease, which can present as either bronchiolitis, (broncho-) pneumonia or both. These patients usually exhibit tachypnea, coughing and laboured breathing. Also, wheezing can be heard upon auscultation of the lungs. Furthermore, very young infants (< 2 months) can present with apnea’s as the first and only sign of bronchiolitis. If children present with severe RSV-LRTD with the risk of respiratory failure, hospital admission is warranted. As mentioned above, the only available treatment is supportive in nature, and consists of supplemental oxygen and feeding through a naso-gastric tube. In approximately 10% of hospital admitted children these supportive measures are insufficient, and these children progress to respiratory failure. This becomes apparent by the inability to ventilate carbon dioxide (resulting in hypercapnia) and/or reduced oxygen uptake (resulting in hypoxaemia). These patients will need intubation and mechanical ventilation. A majority of patients with severe RSV-LRTD who need mechanical ventilation fulfil the current clinical criteria of (paediatric) acute respiratory distress syndrome (ARDS), a syndrome characterized by extensive inflammatory damage to the lungs with pulmonary oedema and severe hypoxemia. However, there are still little data about the exact inflammatory responses in the lungs of these severely ill patients. The few available pathology samples of fatal RSV cases show RSV infection of bronchiolar and alveolar epithelial cells with sloughing of necrotic cells and intra-luminal infiltration by macrophages, lymphocytes and, most notably, neutrophils. The combination of sloughed epithelial cells and immune cell infiltration result in the, for RSV infection, characteristic airway obstruction.
Treatment of RSV

Options to treat RSV infection are scarce. Anti-viral medications are only partially effective and are not recommended as standard care.\textsuperscript{33-34} Although prevention by vaccination against RSV would be the most desirable option, 60 years of vaccine research has not yet resulted in an effective vaccine. Vaccine development is hampered by a series of obstacles, including the prematurity of the immune system at this young age, suppression of the immune system by maternal antibodies, the narrow balance between low virulence and enough immunogenicity, and the absence of characterized neutralizing epitopes on viral proteins.\textsuperscript{35} The latter was painfully demonstrated in the formalin-inactivated vaccine trials in the 60’s, in which vaccinated children developed more severe disease compared to the control group upon consecutive natural infection.\textsuperscript{36,37} Prevention of severe RSV infection is currently possible with the use of a monoclonal anti-RSV antibody – palivizumab. However, passive immunization is expensive and currently only indicated in high risk patients (e.g. premature babies, patients with congenital heart abnormalities and immunocompromised patients). Although these patients form risk groups for developing severe RSV disease, the group of healthy term born children are responsible for the majority of hospital and PICU admissions.\textsuperscript{38,39} Furthermore, passive immunization is not fully efficient, as only 55% of the treated children are protected against severe RSV disease.\textsuperscript{40} Taken together, the global impact of RSV disease on child health remains significant. It is interesting that, despite years of research, we do not completely understand the pathophysiology of RSV infection. Gaining insight into the mechanistic and pathological processes influencing RSV disease severity is essential to understand this disease and to develop new therapeutic strategies.

Neutrophils

The innate immune response forms the first line of defence against invading pathogens. One of the main effector cells of the innate immune system is the neutrophilic granulocyte or neutrophil. Neutrophils are, together with dendritic cells and macrophages, responsible for antigen presentation, which in turn triggers the adaptive immune system to elicit a specific immune response. In last 50 years, RSV research has mainly focussed on local responses (i.e. epithelial responses, alveolar macrophages), adaptive immune responses (i.e. cytotoxic CD8$^+$ lymphocytes and antibody responses) and for the largest part: vaccine research. Despite the diversity of RSV research, one immune cell type is often overlooked: the neutrophil. Yet, one of the hallmarks of severe RSV disease is extensive neutrophilic infiltration of the airways and lungs. Almost 85% of all immune cells present in the lung lumen during RSV disease are neutrophils.\textsuperscript{41-43} These cells are known to possess a large arsenal of defensive strategies, which can not only attack microbes but also elicit injury to host tissue.\textsuperscript{44} Detailed knowledge regarding their role during severe RSV disease is lacking.
Main effector functions of neutrophils include the classical mechanisms of degranulation with secretion of antimicrobial proteins, reactive oxygen species (ROS) and phagocytosis of pathogens. Neutrophils can also produce neutrophil extracellular traps (NETs) after the process of NETosis, a distinct form of cell death by which the neutrophil spills its DNA covered with granule proteins into the extracellular space. These NETs, consisting of extracellular DNA packed with nuclear (e.g., histones) and granule (e.g., MPO, elastase) proteins, can trap and/or neutralize pathogens.

**Neutrophil biology in the lungs**

In human blood approximately 50-70% of white blood cells are neutrophils. They are derived from the bone marrow under influence of granulocyte colony stimulating factor (G-CSF) and are produced at a rate of $10^{11}$ cells per day under homeostatic conditions. When tissue injury and release of pro-inflammatory mediators, the extravasation and sequestration of neutrophils result in a transient neutropenia. This is subsequently compensated through the release of young neutrophils from the bone marrow, which can speed up production to $10^{12}$ cells per day. Under normal physiological conditions most neutrophils patrol the body for 24-48 hours. However, once recruited to sites of injury or infection, neutrophils receive survival-signals from macrophages and mesenchymal stem cells to delay their regulated cell death by the process of apoptosis that prolongs their lifespan for up to several days.

Neutrophils are quickly recruited to the lungs due to the presence of a natural neutrophil reservoir in the lung microvascular bed. Here, neutrophils lay margined against the vessel walls under regulation of CXCR4, ready to respond in case of damage or infection. Neutrophil recruitment into the lungs is regulated by chemokines (e.g. CXCL-8/IL-8, LTB4), which are produced locally by epithelial cells and macrophages. Through these mediators neutrophils are directed towards infected or damaged tissue. The epithelial cells release heat shock protein (HSP) 72, stimulating the toll like receptor (TLR) 4 receptors on neutrophils to...
induce NF-κB dependent production of IL-8 and TNF-α. This paracrine signal-transduction cascade amplifies their own recruitment and leads to massive neutrophil recruitment. This process has also been described for other respiratory viruses, for example during influenza infection.

Neutrophil extravasation in the lung is markedly different from other tissues. Neutrophils transmigrate into the lung via capillaries, instead of via the post-capillary venules, which is their normal place of exit from the blood compartment to the parenchyma of the organs. Under physiological conditions the flexible neutrophil (>7 µm) is forced through small capillaries (5 µm), but upon activation the neutrophil becomes more rigid and cannot deform to squeeze itself through the vascular lumen. This ensures very close contact with the endothelial wall which allows contact between adhesion receptors and facilitates extravasation. In addition, lung capillary endothelial cells have approximately 30 times higher expression of the integrin ligand ICAM-1 (CD54) compared to vessels in other tissues, increasing their binding potential for neutrophils. The combination of these two characteristics, that are unique to the lung, contribute to strong migration of neutrophils out of the capillary bed into the lungs. This is necessary to adequately cover the vast lung surface area (63m²) which has contact with the outer world.

**Neutrophil response to RSV**

Neutrophil recruitment and activation is also influenced by several viral factors. RSV infected cells shed soluble G-protein (sG) which represents about 15% of total produced G-protein. SG resembles CX3CL1 (or fractalkine), which, when attached to its CX3C-receptor, has a function in the recruitment of immune cells. The competitive blocking of this receptor leads to diminished immune cell recruitment, including neutrophils, as seen in a CX3C-receptor 1 knock-out mice infected with RSV. These mice display abrogated neutrophil recruitment to the lungs. Additionally, Caidi and colleagues confirmed a 79-90% reduction in neutrophils in the lungs when treating mice therapeutically with monoclonal antibodies against the CX3CL1 epitope. Thus, by targeting viral factors it is possible to modulate the immune response, including the response of neutrophils.

There are large gaps in knowledge about what neutrophils do when they have entered the airways, and if they contribute to viral clearance. Stokes et al. show that there is no difference in viral titers when comparing neutrophil depleted with non-depleted RSV-infected mice, indicating no significant role for neutrophils in viral clearance. However, they did see a reduction in mucus production during RSV infection in the neutrophil depleted mice. Mucus production by goblet cells is regulated via IL-9 produced by neutrophils, and a reduction in neutrophils might reduce the quantity of IL-9. This, in turn, could reduce the amount of airway obstruction during severe RSV-LRTD by mucus plugs.
**Neutrophil defence arsenal**

The neutrophil possesses an arsenal of defensive strategies (Fig. 3), including phagocytosis, production of reactive oxygen species (ROS) and release of immune defensive proteins (e.g. α-defensins, elastase) by degranulation.

The aim of phagocytosis is to remove cell debris and pathogens from sites of infection. The pathogens are engulfed into a vacuole which is called the phagosome. When the phagosome fuses with granules containing toxic antimicrobial proteins the environment turns hostile to pathogens. Besides the infused granule proteins, the NADPH oxidase is assembled on the phagosomal membrane, allowing ROS production inside the phagosome. Together these two mechanisms eliminate the pathogens within the phagosome.44 This internal antimicrobial process forms a safe way to dispose of pathogens. However, both ROS production and granule protein infusion can also take place at the outer cell membrane, leading to extracellular deposition of ROS and toxic proteins. Although still aimed to destroy pathogens, these ROS and proteins can now also damage host tissue, as has been shown in a number of diseases.61,62 The neutrophil and its phagocytic capabilities are classically associated with bacterial infections.44 However, the role of these and other defensive strategies of neutrophils in anti-viral immunity (e.g. limiting viral replication either by direct or indirect mechanisms) remain relatively unclarified. In theory, cellular cytotoxicity via production and excretion of oxygen radicals could kill virus-infected cells. It is also known that neutrophils can phagocytose virus(-infected cells) and consecutively destroy viruses.63-65 Calf neutrophils infected with bovine RSV contain non-replicating virus, indicating viral neutralization.66 A portion of human neutrophils phagocytose RSV particles, but it is not known whether these cells become infected or indeed neutralize the virus (unpublished data). RSV is found in both lung and blood human neutrophils.67 RSV RNA has also been found in heart tissue and cerebrospinal fluid. Indeed, extra-pulmonary complications during severe RSV disease (i.e. myocardial damage failure and apnoea’s) can occur in the heart and brain.68 It is unknown if RSV can replicate inside these neutrophils, and if virus-containing neutrophils cause viral dissemination.

**Neutrophil extracellular traps**

Besides phagocytosis, ROS-production and release of defensive proteins there is an additional defence mechanism of neutrophils; the formation of so-called ‘neutrophil extracellular traps’ (NETs). NETs are composed of extracellular networks of DNA-fibers studded with neutrophil-derived granule proteins and serve to capture and neutralize a broad spectrum of pathogens, including viruses.69,70 They were discovered in 2004 by Brinkmann et al., and are implicated in many other infectious and (auto-) immune diseases, including SLE and rheumatoid arthritis.71,72 The natural function of NETs is under heavy debate, and is currently unknown in RSV disease. NETs have both been implicated in anti-viral protection70, as well as in collateral
damage during viral infection. Funchal and colleagues found NET production upon stimulation of neutrophils with the RSV F protein in vitro, but little in vivo data exists for RSV. Dissecting their role during RSV infection is important in order to establish if, and how NETs contribute to disease severity.

**Neutrophil subsets**

Although neutrophils are often considered a single group of immune cells acting alike in a seemingly undirected and non-specific manner, this view seems too simplistic. Besides different neutrophil effector functions, there is also evidence for different ‘subsets’ of neutrophils, which could differentially influence disease outcome. Indeed, over the last few years many reports identified new subsets of neutrophils with unique functions. These subsets have distinct phenotypes (e.g. anti-microbial activity or immune suppressive activity). Especially immune suppression is an interesting function for neutrophils, as neutrophils typically appear early during disease and could potentially impact subsequent immune activation. For example, a study in which lipopolysaccharide (LPS), a component present in the membrane of gram-negative bacteria, was administrated in healthy adult volunteers showed the development of three distinct neutrophil subsets in blood, including one subset with T-cell inhibitory functions. However, little is known about subset formation during viral infections. Inhibitory neutrophils or the lack of inhibitory neutrophils could have a major impact on viral infections with predominant neutrophilic inflammation, like RSV.

**Neutrophils in immuno-pathogenesis**

Neutrophils are important immune cells, as illustrated in patients with chronic granulomatous disease. Neutrophils from these patients are deficient in their capacity to produce oxygen radicals which results in impaired function, including impaired formation of NETs, leading to an increased risk of bacterial infections. On the other side of the spectrum are the destructive capabilities of neutrophils as seen during sepsis and ARDS. Here, neutrophils contribute to widespread alveolar endothelial and epithelial damage. They do so by spilling toxic granule proteins in the extracellular space, producing ROS, or by forming NETs. Increased activation and delayed clearance could also (additionally) influence tissue damage. After exerting its function the neutrophil usually undergoes apoptosis (regulated cell death) and is cleared by macrophages. If apoptosis is inhibited this could lead to longer exposure of the tissue to activated neutrophils. The examples above illustrate that, although our body needs neutrophils, it must maintain their destructive capabilities within limits.

It has been hypothesized that neutrophils also contribute to tissue damage during RSV infection. This would be particularly true for the cases in which RSV-LRTD leads to ARDS. In vitro studies show augmented RSV infected epithelial cell detachment when co-incubated
with neutrophils. If neutrophils indeed exert epithelial cellular toxicity during RSV infection, this could lead to epithelial sloughing and obstruction of the bronchioles, but no in vivo studies addressing these questions exists. The question remains whether the extensive influx of neutrophils during disease is cause or consequence of disease severity. Attracting neutrophils to the site of inflammation is key in the resolution of disease, but what if the body overshoots this target? In vitro co-incubation of human neutrophils with RSV induces anti-apoptotic pathways, but these results could not be confirmed in neutrophils in nasopharyngeal aspirates and blood of RSV infected children that showed increased apoptosis. Possibly, the micro-environment in the lung creates an overall pro-apoptotic signal. Yet, neutrophils seem to be capable of more than just phagocytosis. It is not likely that a single effector cell, like the neutrophil, could be solely responsible for all the immunopathology associated with viral infections. However, how big is their contribution?

**RSV animal models**

Ideally, in order to investigate the abovementioned questions all research related to RSV is studied in humans, and in particular in susceptible young children. To identify pathogenic pathways and assess efficacy of new drugs many invasive procedures are necessary, which is obviously unethical in these severely ill young children. Thus, to study RSV related research questions we make use of different models, which allow us to mimic certain disease characteristics (e.g. anti-viral host response or viral induced pathology). Specific in vitro models allow us to study single effector mechanisms (e.g. NET formation) using (usually) one or two specific cell types (e.g. neutrophils and epithelial cells). However, in the body countless different cells contribute to the hosts reaction upon infection. Findings from in vitro experiments thus need to be tested in (preferentially) relevant host-specific animal models to establish their role in vivo. The more the animal model mimics disease as seen in humans, the better are the chances that the results acquired from these models can be extrapolated to humans. It is therefore imperative to choose the most suitable animal model to assess the topic of interest.

**Small RSV animal models**

Many different RSV models exist to date, ranging from rodent models (e.g. mice, rats, cotton rats) to animal models utilizing larger animals like sheep or calves. RSV research has traditionally been relying on the smaller, i.e. mouse and cotton rat, models, due to the costs, the large availability of (genetically) modified mice and the broad range of species specific reagents. However, mice and cotton rats are semi-permissive to infection with human RSV and do not develop the same severe symptoms as children, and large inoculation doses are necessary to establish productive infection. The lack of susceptibility is explained by the non-natural host-pathogen relation, as RSV is not a mouse adapted virus and does not replicate efficiently in mouse cells. Alternatively, mice do become infected with the RSV related
pneumovirus: pneumonia virus of mice (PVM). This virus is related to RSV (Fig. 1) but is rodent specific and produces profound infection upon a small inoculum (as low as 30 PFU).\textsuperscript{86} Infection with PVM results in severe respiratory disease, eventually leading to respiratory failure and death. There are differences in susceptibility to PVM between mouse species, e.g. BALBc mice are more susceptible than C57BL6 mice, in which a slight delayed response is seen.\textsuperscript{87} Comparable to RSV disease in humans, PVM infection in mice is accompanied by potent neutrophilic inflammation, although not reaching the extent as seen in human disease.\textsuperscript{86} Utilizing a host-pathogen (PVM) specific mouse model with strong neutrophilic recruitment appears to offer a very relevant small rodent model to study neutrophil related research questions during RSV disease.

**Large RSV animal models**

In addition to small animal RSV models, several large animal RSV models have been developed (e.g. chimpanzees, cattle, sheep). Although more difficult to perform due to size, costs and low availability of reagents, these animals are generally more closely related to humans in terms of respiratory tract anatomy and physiology, and produce very similar disease to RSV infection in humans.\textsuperscript{85} Another advantage of these models is that longitudinal data (e.g. sequential blood gasses, bronchoscopies) can be more easily collected. As a large animal model, calves experimentally infected with bovine (b)RSV are of particular interest. In both humans and calves, the young infants and young calves are at the highest risk of developing severe disease. Furthermore, bovine RSV epidemics occur with the same seasonal incidence as human RSV.\textsuperscript{88,89} Bovine (b)RSV is member of the same pneumoviridae family and is closely related to human (h)RSV (Fig. 1). It is accompanied by profound neutrophilic inflammation in the lungs, with comparable histopathology as hRSV.\textsuperscript{85} The clinical symptoms following infection can be severe, including wheezing, tachypnoea and eventually respiratory failure, again similar to severe hRSV infection in children. As such, the bovine RSV model in calves serves as an ideal pre-clinical model to study research questions regarding RSV.

In conclusion, the precise role of the neutrophil is far from clear. Better insight in the beneficial and detrimental effects of this important immune cell during severe RSV disease may therefore offer anchor points for new therapeutic strategies. This thesis aims to contribute to the bridging of this knowledge gap by:

- Assessing several RSV animal models for their use in neutrophil related RSV research.
- Evaluating the phenotype plasticity of neutrophils during viral respiratory infections.
- Assessing the role of neutrophil effector functions during severe RSV-LRTD, and investigate if interventions can prevent neutrophil related injury.
- Describing the effect natural occurring antibodies can have on RSV infection.
Thesis outline

Part I. Modelling Respiratory Syncytial Virus Disease

Animal models provide an essential bridge to investigate the many research questions regarding the pathophysiology of RSV disease, including the role of neutrophils. In part I we describe two host-specific RSV animal models. In chapter 2 we describe the bovine calf model as a suitable host-specific pre-clinical animal model for RSV. We determine clinical outcome, histopathologic damage and immunological responses in both bRSV and human-RSV infected young calves. In chapter 3 we explore another RSV animal model; the pneumonia virus of mice (PVM) model in mice. In this study we aim to unravel the role neutrophils play during acute PVM infection in C57BL6 and BALBc mice, by utilizing an antibody-mediated depletion method.

Part II. Neutrophils and the Pathogenesis of Respiratory Syncytial Virus Infection

In part II of this thesis we investigate the occurrence of neutrophil subsets and one of the effector functions of neutrophils; neutrophil extracellular traps (NETs) during RSV infection. In chapter 4 we investigate if viral respiratory infections are accompanied by different neutrophil subsets, potentially resulting in diverse functional phenotypes. In chapter 5 we summarise what is known about NET formation during (paediatric) respiratory diseases. We expand on this question in chapter 6. Here, we first investigate whether RSV was able to induce NET formation by human neutrophils in vitro, and if these NETs could trap viral particles and aid in the anti-viral response to RSV. We next explore the natural occurrence of NETs during severe RSV infection in humans and calves.

Part III. Therapeutic Interventions

In part III of this thesis we describe potential intervention strategies, including modulating neutrophils responses, to treat severe RSV infection. Chapter 7 describes the effect of targeting NETs by local dornase alfa treatment in bovine RSV infected calves. Chapter 8 is focusing on therapeutic monoclonal antibodies targeting the RSV G protein as a potential immune-modulatory target.
General Introduction

References

34. Venter K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. The Cochrane database of systematic reviews. 2007;CD000181
General Introduction
General Introduction


