Histol Histopathol (1999) 14: 185-201

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Invited Review

Cytokines and pulmonary inflammatory and immune diseases

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Summary. Cytokines are important soluble signalling molecules that dictate and coordinate inflammatory and immune responses. Further understanding the role of cytokines in the pathobiologic mechanisms of pulmonary inflammatory and immune diseases holds the key to the development of effective prophylactic and therapeutic strategies. In the last several years, the use of models of human pulmonary diseases established either in normal adult animals, mice deficient for a given immune cell type or cytokine, or mice engineered to overexpress a given cytokine, has remarkably facilitated our understanding of the mechanisms operating in human disease. Cytokines that are involved in pulmonary inflammatory and immune conditions may be generally divided into groups of pro-inflammatory, anti-inflammatory and growth-stimulatory cytokines. While pro-inflammatory cytokines can be detrimental under such severe conditions as endotoxemia and fibrosis, they are required in host resistance against infectious agents. Anti-inflammatory cytokines play an important role in controlling the extent of tissue inflammatory/immune responses. Overexpression of growth-stimulatory cytokines are often directly associated with tissue fibrotic responses. In this review, the findings attained from experimental models by us and others were discussed with emphasis on cellular and histopathologic alterations, cytokine-mediated molecular mechanisms and the prospects of cytokine-based therapeutic strategies. Due to the restricted space, we chose to focus only on models for endotoxic lung, endotoxemia, acute pulmonary infections by extracellular Gram-negative bacteria, chronic pulmonary infections by intracellular myco-bacteria, allergic airways inflammation and pulmonary fibrosis.

Key words: Lung, Cytokines, Infections, Asthma, Fibrosis

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Introduction

Biomedical research aims to understand ourselves in order to fulfill two goals: to improve the quality of life and to better prevent and treat illnesses. Understandably, many experiments are either unfeasible or ethically unallowable to be carried out in humans (Botting and Morrison, 1997). Animal models, particularly, mouse models of human pulmonary diseases have been instrumental for us not only to understand the disease mechanisms but also to evaluate the effectiveness of intervention strategies. Cytokines are of paramount importance in mediating the cross-communication between cells that carry out essential inflammatory and immune activities. Most cytokines can be released by multiple cell types except a few that are restricted primarily to lymphocytes, particularly T cells. During inflammatory responses elicited by non-immune stressors, many cytokines may be released from multiple cellular sources other than lymphocytes. In contrast, during host responses elicited by immunogenic antigens, antigen-specific T lymphocytes are a prominent source of selective cytokines, dependent upon the nature of antigens and the genetic background of host. These cytokines may be grouped into Th1 and Th2 cytokines with the former including IL-2 and IFNγ and the latter IL-4 and IL-5 (Mosmann and Sad, 1996). The Th1 and Th2 cytokines usually dictate a different pattern of tissue immune-inflammatory responses. Certain cytokines that are not exclusively released by lymphocytes but are involved in Th cell differentiation or Th1 or Th2 tissue responses, may also be categorized into Th1 or Th2-type cytokines. The use of animal models has remarkably helped dissect the role of cytokines in pulmonary inflammatory or immune diseases. One important advantage of using the mouse rather than other species is the unique accessibility to many immunologic reagents including gene knock-out mouse strains. However, it is also important to bear in mind that there are certain differences in respiratory anatomy and histology between ourselves and rodents. For instance, the left lung of rodents consists of only one lobe and the right

lung is divided into the apical, middle, posterior and a small accessory lobe (Boorman and Eustis, 1990). Histologically, rodent airways do not have well-developed respiratory bronchioles and, from the lower portion of trachea there is no submucosal glands. Of prominence, in contrast to those in humans, the conducting airways lack the submucosal layer, comprising only the epithelial and fibrous subepithelial connective tissue layers. While examinations of cytology, morphology, gene expression and protein levels in models established in normal adult rodents still remain important and useful, the "loss of function" or "gain of function" approaches by genetic engineering have revolutionized the way by which we study the function of cells or cytokines of interest. In this regard, many pulmonary diseases can now be studied in a mouse strain defective in a given immune cell type or cytokine, or in a mouse strain overexpressing a cytokine (Ho, 1994). Discoveries from such studies have remarkably helped us unravel the previously unknown cause of certain human ailments. For instance, mice deficient in IFN-γ were found to succumb to systemic or pulmonary tuberculous infection (Cooper et al., 1993), and this experimental finding has recently led to the finding that a certain number of children who developed disseminated tuberculous infection following vaccination with *Mycobacterium bovis* BCG or who were susceptible to infections caused by opportunistic mycobacteria, carried a genetic defect in the ligand binding subunit of IFN-γ receptor (Jouanguy et al., 1996; Newport et al., 1996). Mice deficient in GM-CSF suffered a pulmonary disease called pulmonary alveolar proteinosis (Dranoff et al., 1994) and this finding has recently led to identification of a mutation in the gene coding for the common ß chain of receptors for GM-CSF/IL-3/IL-5, in patients with pulmonary alveolar proteinosis (Dirksen et al., 1997). Findings of such nature will undoubtedly foster the determination of relative genetic propensity to certain diseases and the development of new therapeutic strategies.

Cytokines in experimental models of endotoxic lung

The alveolar macrophage resides both in the alveolar and interstitial spaces, serving as part of the innate defense mechanism in the lung. Normally, this cell type outnumbers the lymphocyte in the lung with a ratio 5-10 to 1. In situations in which the number of invading microorganisms exceeds the capacity of primary defense mechanisms, neutrophils (PMN) are called in in response to microbial-derived products, complement, leukotrienes and chemokines, thus operating as a powerful back-up defense mechanism during acute pulmonary infection (Stockley, 1995). Such neutrophilic accumulation in the lung is a pathologic hallmark of acute lung inflammation. Since the Gram-negative bacterium represents a frequent pathogen causing pneumonia through either a direct local or systemic route, Gram-negative bacterium-derived lipopolysaccharide (LPS) is commonly used to elicit an acute neutrophilic inflammatory response in the lung to study the molecular mechanisms (Friedland and Griffin, 1992; Manthey and Vogel, 1992). Routinely, LPS is intratracheally (i.t.) instilled into rat or mouse lung. We have also developed a mouse model of acute lung inflammation by delivering aerosolized LPS in a Plexiglas chamber (Xing et al., 1998). This technique simplifies the procedure of delivery and avoids potential homeostatic alterations induced by surgical trauma during i.t. procedure. LPS triggers a quick neutrophil response in the lung which can be appreciated microscopically within the pulmonary vasculature and in the insterstitium at as early as 0.5-1h post-challenge. Significant accumulation of PMN in bronchoalveolar lavage fluid (BAL) was noticed by 3h, peaked at 12 to 24h and declined to background levels by 72h (Xing et al., 1993, 1998). There was also an increase in the number of alveolar macrophages (Xing et al., 1993; Shellito et al., 1995). These kinetics of PMN trafficking mimic the course of acute pneumonia in humans, thus providing an opportunity to study the role of cytokines in the pathogenesis of acute pneumonia. Expression in lung tissue of mRNAs for both TNFα and MIP-2 was quickly turned on by 1h, followed by IL-1ß and IL-6 (Xing et al., 1994). It is quite likely that the onset of TNFα expression preceded MIP-2 if such examination was carried out at earlier time points. At cytokine protein levels, the release of TNFα, MIP-2 and IL-6 peaked at about 4h and markedly declined by 72h. Thus, the cascade of cytokines TNFα, MIP-2 and IL-6 is part of important mechanisms orchestrating the host response to such nonimmune stressors as LPS. In addition to direct induction of MIP-2 and IL-6 by LPS, TNFα may amplify such induction (Fig. 1).

TNFα is a well-known pro-inflammatory cytokine capable of multiple functional activities including inducing cytokine release from many cell types and enhancing the adhesion of PMN to the endothelium by upregulating cell surface molecules intercellular adhesion molecule 1 (ICAM-1) and CD11a/CD18 on the endothelium and PMN, respectively. MIP-2, like KC, is a member of the C-X-C chemokine family with potent chemotactic activity for rodent PMN both in vitro and in vivo (Huang et al., 1992; Strieter et al., 1993; Gupta et al., 1996; Foley et al., 1996). An anti-MIP-2 antibody reduced, but not completely inhibited, lung neutrophilia by LPS, suggesting an important role for this chemokine and the participation of additional chemotactic factors in the full-blown lung neutrophilia (Schmal et al., 1996). The issue regarding the role of IL-6 during acute neutrophilic inflammation in the lung is of fundamental importance. IL-6 is clearly a critical cytokine which together with TNFα and IL-1, induces acute phase reactions comprising fever, hepatic release of acute phase proteins and adrenal corticosteroid release, during almost any alarm responses including acute pulmonary infections (Baumann and Gauldie, 1994). In this regard, IL-6 is expressed at the tissue site of inflammation and

released into the circulation whereby IL-6 operates as an exocrine hormone-like molecule (Gauldie et al., 1990). The functional role of IL-6 in the lung during acute inflammation had remained an unsolved issue until our recent studies by using mice deficient in IL-6 (Xing et al., 1998). We demonstrate that endogenous IL-6 in the lung is an antiinflammatory cytokine functioning to control the level of proinflammatory cytokines TNFα and MIP-2 and subsequently the level of lung neutrophilia and that such functions of IL-6 cannot be compensated for by other members of the IL-6 cytokine family (Kishimoto et al., 1992). Of interest, IL-6 appears to have little effect on the duration of neutrophilic inflammation since like in the wild-type mice, lung neutrophilia largely subsided by 72h in IL-6-deficient mice (Xing et al., 1998). This finding does not support a role of IL-6 in enhancing PMN apoptosis as suggested from a previous in vitro observation (Afford et al., 1992). Exogenously administered recombinant IL-6 has been shown to attenuate LPS-induced lung neutrophilia (Ulich et al., 1991a). Among other antiinflammatory cytokines that are likely involved in controlling acute lung inflammation are IL-10 and IL-1 receptor antagonist protein (IRAP). IL-10 is a well recognized antiinflammatory cytokine (de Vries, 1995). Interestingly, in contrast to marked induction by LPS of IL-6 locally in the lung, we found that a low constitutive level of IL-10 was not enhanced by LPS (Xing et al., 1998), suggesting that this cytokine plays a relatively less important role in controlling acute lung inflammation. However, whether this is also the case in humans remains to be determined. Local exposure to LPS also induced IRAP expression and i.t. administration of recombinant IRAP attenuated LPS-induced neutrophilia (Ulich et al., 1991b, 1992).

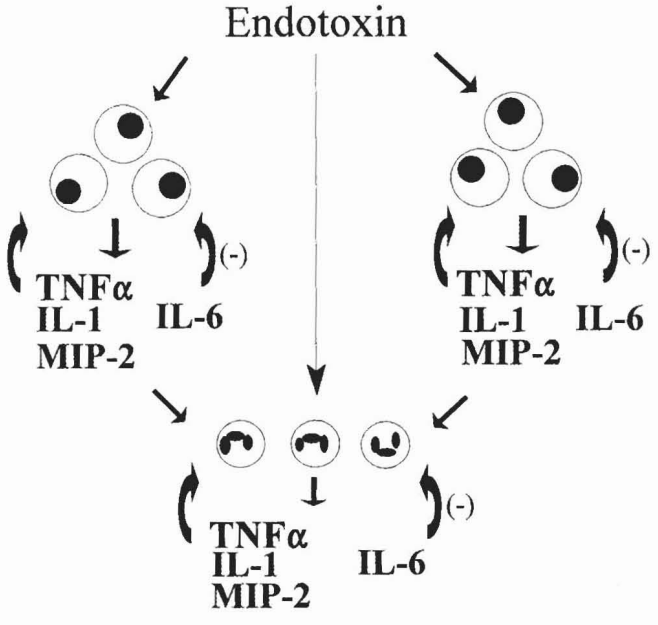

Fig. 1. Overview of cytokine actions and cellular sources during endotoxin-induced acute pulmonary inflammation. Endotoxin activates alveolar macrophages to release cytokines including TNFα, MIP-2, IL-1 and IL-6. Neutrophils are then recruited into the lung in response to both endotoxin and macrophage-derived cytokines and become an active source of these cytokines themselves at the time when expression level in macrophages wanes. All of these cytokines act in an autocrine and paracrine fashion. In contrast to TNFα, MIP-2 and IL-1, IL-6 operates as an antiinflammatory cytokine during acute pulmonary inflammation.

The cellular sources of cytokines during acute lung inflammation have been an interesting topic of research. In a rat model of LPS-induced acute lung inflammation, we dissected the cellular sources of cytokines by using Northern hybridization with total cellular RNA from purified alveolar macrophages (AM) and PMN, immunohistochemistry, and in situ hybridization. We found that AM represented an early source of pro-inflammatory cytokines which may not be of total surprise since these cells are the first line of defense in the deep airways. These cells expressed remarkable messages for TNFα, MIP-2, IL-1ß and IL-6 by 1h post-LPS challenge which declined markedly between 6 and 12h (Xing et al., 1994). Interestingly, at these later times when cytokine expression waned in AM and neutrophil infiltration started to be prominent, PMN became a striking source for all of these cytokines expression of which peaked at 6h and remained still significant by 12h. These findings by Northern hybridization were verified in the lung by immunohistochemistry and in situ hybridization (Xing et al., 1993, 1994). These studies have provided the first experimental evidence that AM and PMN account for temporal waves of cytokine expression during the course of acute lung inflammation, and that cytokines from AM may be important in triggering PMN trafficking and activation whereas those from PMN contribute to the amplification of responses and the autoactivation of PMN (Fig. 1).

Whether rodent models of endotoxic lung represent ideal models of acute lung injury remains a contentious issue. Such models are not models of adult respiratory distress syndrome (ARDS). Apparently, pathologies seen in LPS models lack the two essential components characteristic of ARDS: severe alveolar epithelial damage and fibrotic reaction (Bachofen and Weibel, 1977). Unfortunately, a real straightforward model of ARDS is not yet in immediate reach, which restricts investigations into the mechanisms by which the disease progresses into the stage of fibrosis. During LPS-induced acute inflammation, the lack of severe lung injury boils down to the issue regarding whether PMN are fully activated. From cytokine expression point of view, LPS, perhap together with AM-derived cytokines, indeed activates PMN to express a wide array of cytokines. However, whether these granulocytes undergo degranulation to release proteases such as elastase is unclear. It appears that it is difficult to demonstrate elastase activities in BAL fluids from rodent lungs (Delclaux et al., 1997) while increased elastase activities have been demonstrated in BAL from guinea pig lungs post-LPS challenge (Sakamaki et al., 1996). Never-

theless, these drawbacks should not detract these models from studies aiming to dissect the role of cytokines and other soluble mediators and adhesion molecules in the development and resolution of acute lung inflammation.

Indeed, the rodent models of LPS-induced acute lung inflammation with a self-limiting nature have proven useful for studies looking into the mechanisms underpinning the resolution of inflammation. It has been generally believed, primarily based upon in vitro observations that one major mechanism by which acute neutrophilic inflammation undergoes an effective clearance is apoptosis and subsequent engulfment by macrophages of apoptotic PMN (Savill et al., 1993). We have demonstrated a close correlation between the resolution of neutrophilic accumulation and the appearance and the subsequent macrophage engulfment, of apoptotic PMN during the course of acute lung inflammation (Cox et al., 1995). These latter two events were found slightly lagged behind the peak neutrophilic response to i.t. challenge with LPS, suggesting that such mechanisms play a critical role and are amazingly efficient in clearing massive neutrophilic accumulation in the lung.

Cytokines in experimental models of endotoxemia

The acute neutrophilic inflammatory response is also often a local presentation in the lung during endotoxemia caused by Gram-negative infections that did not originate in the lung. In this case, the response results from exposure to endotoxin and cytokines in the bloodstream. Different from acute lung inflammation elicited by local endotoxin exposure during which there is primarily a compartmentalized cytokine response within the lung and an intraalveolar neutrophilic accumulation (Nelson et al., 1989), there is, during endotoxemia, an initial drop in the number of peripheral blood PMN and lymphocytes (Terebuh et al., 1992), and a large number of PMN becomes sequestered within the pulmonary vasculature with relatively few infiltrating the alveolar space (Nelson et al., 1989). Moreover, there is a swift cytokine surge in the bloodstream upon the onset of endotoxemia (Nelson et al., 1989; Manthey and Vogel, 1992). It was often observed that during endotoxemia, circulating TNFα level peaked earliest around 1h post-systemic LPS challenge (Manthey and Vogel, 1992). We recently observed that levels of MIP-2 and IL-10 also rapidly rose to a peak around the same time as TNFα during endotoxemia (Xing et al., 1998). These first waves of cytokines were followed by IL-6, GM-CSF and IFNγ which peaked at about 3h, 3h and 6h, respectively (Chensue et al., 1991; Tiegs et al., 1994; Xing et al., 1998). By examining mRNA expression for TNFα or IL-6, we investigated the potential contribution of various tissue sites to the circulating level of cytokines during endotoxemia. Among all organs analyzed, spleen/lung/heart and spleen/lung/heart/muscle are the major sites for TNFα and IL-6 expression, respectively (Xing et al., 1997a). It is clear that TNF-γ, MIP-2, GM-CSF and IFNα all contribute to the development of toxic syndromes and multiorgan injuries during endotoxemia (Tiegs et al., 1994; Standiford et al., 1995; Schmal et al., 1996; Bundschuh et al., 1997). The critical role for TNFα has been demonstrated not only by using an anti-TNFα antibody (Remick et al., 1990) but also by using a transgene-derived TNFα-antagonizing soluble TNFα receptor (Rogy et al., 1995). In addition, IL-1α but not IL-1ß may also be a critical player since mice deficient in both IL-1α and IL-1ß (ICE-deficient mice) but not mice only deficient in IL-1ß were protected from lethal challenge of LPS (Gutierrez-Ramos and Bluethmann, 1997). Of special interest, in contrast to little induction of IL-10 release in the lung post-LPS aerosol exposure (Xing et al., 1998), the circulatory level of IL-10 markedly increased during endotoxemia, suggesting an important role of this potent antiinflammatory cytokine. Indeed, neutralization of IL-10 increased lethality of mice in endotoxemia (Standiford et al., 1995) and administration of recombinant IL-10 or a plasmid DNA containing IL-10 transgene protected mice from lethal endotoxemia (Howard et al., 1993; Rogy et al., 1995). Recently, we have demonstrated inhibited cytokine production during endotoxemia by using adenoviral-mediated intramuscular IL-10 gene transfer (Xing et al., 1997a). The role of IL-6 in endotoxemia, in addition to its effect on the induction of acute phase reactions, has been a matter of debate, owing to conflicting experimental results obtained by using monoclonal antibodies against IL-6. Adequately addressing this issue is of fundamental importance. IL-6 is induced and released into circulation in almost all of alarm situations including trauma and acute infections and the circulating level of this cytokine is closely correlated with mortality (Zitnik and Elias, 1993). However, this correlation cannot provide a causal relationship since heightened IL-6 levels could be part of host attempt in trying to restore homeostasis, directly related to the extent of tissue injury. To address the role of endogenous IL-6, monoclonal antibodies against IL-6 were used in models of endotoxemia but the results are confusing, supportive of either a protective or detrimental role of IL-6 (Starnes et al., 1990; Heremans et al., 1992; Barton et al., 1993). Now it has become clear that anti-IL-6 monoclonal antibodies, instead of neutralizing IL-6 activities, chaperone circulating IL-6 by binding to this molecule to prolong its half-time and biologic activities (Heremans et al., 1992; May et al., 1993). To this end, we have investigated the cytokine profile and mortality in IL-6 gene knock-out ($IL\text{-}6^{-/-}$) mice during endotoxemia (Xing et al., 1998). We found that in the absence of IL-6, levels of proinflammatory cytokines including TNFα, MIP-2, GM-CSF and IFNγ were many times those in wild type mice while the level of antiinflammatory IL-10 remained similarly increased in both $IL\text{-}6^{-/-}$ and $IL\text{-}6^{+/+}$ mice. In addition, there was an increased mortality in $IL\text{-}6^{-/-}$ post-lethal i.p. challenge of LPS. These findings provide crucial evidence pointing to the antiinflammatory nature of IL-6 during endotoxemia by controlling the level of

proinflammatory, but not antiinflammatory, cytokines. In this respect, it is apparent that IL-10 per se and other members of the IL-6 cytokine family are not sufficient to compensate for the function of IL-6. Thus, the antiinflammatory activities of IL-6 in both local lung and systemic acute inflammatory responses are in keeping with other antiinflammatory aspects of this cytokine including induction of IRAP and TNFsR and protease inhibitors (Lotz and Guerne, 1991; Tilg et al., 1994). It is worth mentioning, however, that different from the role of IL-6 in acute inflammatory responses, IL-6 appears to enhance, instead of dampening, specific immune responses against chronic intracellular infections and tumor (Xing et al., 1998). In addition to IL-10 and IL-6, MCP-1 has recently been found to have anti-inflammatory properties in endotoxemia (Zisman et al., 1997a).

While the protective effect of anti-cytokine regimens may be attained in rodent models of endotoxemia, clinical trials using anti-TNFα or IRAP have not yielded consistent results (Bernard, 1995). The debate continues with regard to whether it is beneficial to dampen pro-inflammatory cytokines which are paradoxically important in fighting against the causative Gram-negative infectious agents. Recently, there has been emerging evidence supporting a protective role of certain pro-inflammatory cytokines in host resistance to systemic Gram-negative infections. Anti-IL-12 therapy was found to protect mice during lethal endotoxemia but impair bacterial clearance during *E. coli* induced peritoneal sepsis (Zisman et al., 1997b), and MCP-1 protected mice from systemic infection with *Pseudomonas aeruginosa* or *Salmonella typhimurium*. These suggest the relevance of using models of Gram-negative infections rather than models of LPS-induced endotoxemia to testing the efficacy of therapeutics such as recombinant or transgene-derived cytokine antagonists.

Cytokines in experimental models of acute pulmonary infections caused by Gram-negative bacteria

The morbidity and mortality of acute bacterial pneumonia have been increasing world-wide. Acute pneumonia is the leading cause of death of all nosocomial infections (Standiford and Huffnagle, 1997). Among the most frequent causative bacteria are Gram-negative *E. coli* and *Pseudomonas aeruginosa*. With increased mortality and emergence of drug-resistant strains of bacteria, there is an urgent need to understand host defense mechanisms in order to develop better prophylactic and therapeutic strategies. From this viewpoint, animal models of acute pulmonary infections are certainly superior to those elicited by LPS. Apparently, pathologic changes seen in acute lung inflammatory responses induced by LPS alone or by Gram-negative bacteria in rodent models are not identical. In a side-by-side comparison study, it was found that compared to pathologies induced by i.t. LPS, *Pseudomonas aeruginosa* induced a more pronounced lung injury marked by greater degrees of hemorrahage and proteinous exudation although the intensity of neutrophilia, albeit somewhat delayed, was similar to that by LPS (Delclaux et al., 1997). Such differences are likely accounted for by bacterial virulent factors in addition to LPS. Therefore, cautions should be taken when extrapolation is being made from the data obtained in models of endotoxic lung. It is generally believed that the innate immune components including alveolar macrophages, neutrophils and NK cells play a critical role in the primary pulmonary bacterial infection, whereas the acquired specific cellular and humoral immune responses play a critical role in host defense against the secondary infection. The role of T cells in host resistance to primary acute pulmonary bacterial infections remains unclear. Increased T cells were noticed at later stages of acute lung inflammation elicited by LPS but these cells were not involved in PMN clearance (Morris et al., 1997). And T cell accumulation and T cell-mediated host responses in primary, particularly chronic *Pseudomonas*-induced pneumonia have been reported (Stevenson et al., 1995; Wiebke et al., 1995). It should be noted that likely, there are no true primary infections in humans since most Gram-negative bacteria with pathogenic potentials colonize the upper respiratory, intestinal and lower urogenital tracts and quite some of us may have had subclinical local or systemic infections prior to the first clinical episode of pneumonia, and that host responses during chronic pulmonary pneumonia may differ from those involved in an acute episode. From this point of view, appropriate animal models of primary pulmonary infection must be accordingly developed to address the role of various cell types and cytokines involved in both innate and acquired immune-inflammatory responses. Mouse models of chronic pneumonia caused by *Pseudomonas aeruginosa* have been developed to study host responses against such chronic infections frequently seen in patients with cystic fibrosis. It is of interest that lung cellular responses and in vitro T cell responses appear different between various strains of mice with C57BL/6 and Balb/c mice being susceptible and resistant, respectively (Morissette et al., 1995; Stevenson et al., 1995).

Although certain cytokines have been analyzed at mRNA levels in these models to address the potential mechanisms underlying such strain differences and TNFα seems expressed at a higher level in the lung of resistant Balb/c mice (Gosselin et al., 1995), it remains to be determined whether immune-modulatory cytokines are involved at protein levels. By using a mouse model of acute bacterial pneumonia elicited by i.t. inoculation of virulent *Klebsiella pneumoniae,* it was found that accumulation of PMN and AM peaked around 48 h post-infection (Standiford et al., 1996). These peak cellular responses appeared correlated with peak production in lung homogenates of pro-inflammatory cytokines IL-12, TNFα, MIP-2 and MIP-1α and antiinflammatory

cytokine IL-10 (Greenberger et al., 1995, 1996; Standiford et al., 1996). Such cytokine kinetics are apparently much delayed compared to those elicited by i.t. LPS challenge (Xing et al., 1994), perhaps due to the fact that small inocula of bacteria required a certain period of time to replicate within the lung. IL-12, TNFα and MIP-2 have each been shown to be required for host resistance to *Klebsiella* infection (Standiford et al., 1995; Greenberger et al., 1996; Laichalk et al., 1996) or *Pseudomonas aeruginosa* infection in the lung (Kolls et al., 1995). Local lung induction of IL-10 by bacterial infection is in contrast to our recent finding that little IL-10 was induced during acute lung inflammation by LPS (Xing et al., 1998). Interestingly, neutralization of this endogenous IL-10 decreased the mortality of mice which was accompanied with increased levels of TNFα, MIP-2 and MIP-1α, and decreased bacterial counts in the lung and peripheral blood (Greenberger et al., 1996), suggesting a critical role of these cytokines in host defense. The relative contribution of these cytokines and their cellular sources remain to be elucidated. TNFα, IL-1α and IL-6 were also induced in the lung during acute pulmonary infection by Gram-positive *Streptococcus pneumoniae* (Bergeron et al., 1998). Little is known about the role of IFNγ and GM-CSF during acute pulmonary infections by extracellular Gram-negative bacteria. The critical role of IFNγ in host defense against acute lung infection by Gram-positive *Streptococcus pneumoniae* has recently been demonstrated in IFNγ $^{-/-}$ mice but interestingly, this cytokine was induced only in the peripheral blood but not in the lung of wild type mice (Rubins and Pomeroy, 1997). GM-CSF has been shown to be induced during LPS-induced lung neutrophilia (Huffman Reed et al., 1997). While chemokines MIP-2 and MIP-1α have been shown to increase the bactericidal activities of PMN and AM *in vitro* (Standiford et al., 1996), it remains to be fully understood how these proinflammatory cytokines contribute to host responses in the lung.

Thus, these findings support an emerging notion that proinflammatory cytokines are required for effective host defense mechanisms against acute pulmonary bacterial infection. This notion has prompted a number of preclinical and clinical trials in which a selected proinflammatory cytokine is overexpressed locally or systemically by transgene approaches or administering recombinant proteins to boost innate immune responses (Standiford and Huffnagle, 1997). The local i.t. administration of a replication-deficient adenoviral vector expression IL-12 decreased the mortality of mice with *Klebsiella pneumonia* (Greenberger et al., 1996). By using similar techniques, IFNγ expression was found to aid in the clearance from the lung of *Pseudomonas aeruginosa* in mice (Kolls et al., 1997). Currently, we are investigating the role of cytokines and the potential application of adenoviral vectors expressing pro-inflammatory cytokines in models of pulmonary infections by *Pseudomonas aeruginosa* or *E. coli*. It is important to realize that these experimentations are still in their infancy. Further understanding is required about the relative contribution of each cytokine involved in order to decide which cytokine should be used for immunotherapy as an adjuvant to chemotherapy, and this should be explored in the context of a given pulmonary bacterial infection since the protective immune-inflammatory response to each infection may not involve identical immune-modulatory molecules. Furthermore, different techniques and routes of administration must be explored to deal with the confounding issue that it is the circulating proinflammatory cytokines that cause endotoxic syndromes often seen in patients with Gram-negative infection-associated endotoxemia.

Cytokines in experimental models of chronic pulmonary infections caused by intracellular bacteria

The most common intracellular bacteria that cause chronic pulmonary infections are mycobacteria, particularly *Mycobacterium tuberculosis* (MT). The mycobacterium is an obligate intracellular pathogen of macrophages. Pulmonary tuberculous infection (TB) accounts for increasing morbidity and morbility across the world. Approximately eight millions of new TB cases emerge and three millions of people die of this disease every year. The socioeconomic changes, the ineffectiveness of BCG vaccine and the spread of drug resistant strains of MT, particularly in patients with HIV infection have all contributed to increased incidence and mortality (Cooper and Flynn, 1995). Ironically, relatively little is known about the nature of protective immune-inflammatory responses. The Th1 cytokines including IL-12, IFNγ and TNFα are believed to play a determining role in the development of immune-inflammatory responses against pulmonary TB (Munk and Emoto, 1995). Animal models, particularly those established in mice, are instrumental to helping understand the mechanisms of disease including the role of cytokines. The majority of studies have been carried out by i.v. administration of virulent MT or attenuated live *M. bovis* BCG. Since TB is primarily a respiratory disease and recent evidence indicates that the pattern of responses in the lung may differ from that at other tissue sites (Medina and North, 1996), increasing efforts are being made to establish mouse models of pulmonary TB elicited by airway inoculation of mycobacteria. Indeed, if the same cfu of MT that lodged in the lung following i.v. infection were aerosolized into the lung, the pathological sequela were different. Airway inoculation elicited a faster and greater development of lung pathology characterized by mononuclear cell accumulation, alveolar injuries and interstitial fibrotic reactions (North, 1995). And these mice succumbed to such infection much sooner than mice infected via a systemic route. In addition, compared to other organs, lung is the only organ that allows progressive replication of MT regardless of the route of inoculation (North, 1995), suggesting differential immune-inflammatory mecha-

nisms involved in controlling mycobacterial infection in the lung. Mouse models of pulmonary tuberculous infection may be ideally set up by aerosolization of virulent MT, a process mimicking aerobic mycobacterial infection in humans (North, 1995). The experimental duration is lengthy with such models and the majority of immune-competent mice do not succumb to infection until about 120 days post-infection. Alternatively, i.t. administration may be carried out to deliver larger doses of mycobacteria and this approach seems to shorten the duration of experiments and elicits pathologic processes similar to those by aerosol infection (Hernández-Pando et al., 1996). The models elicited by virulent strains of mycobacteria are more relevant to human diseases but the requirement of higher levels of biohazard containment facility poses restrictions to carrying out detailed dissections of cellular and molecular events. On the other hand, while the models established with attenuated strains allow such studies, they are not ideal models for studying certain aspects of TB as well as the efficacy of therapeutic agents.

We have developed a mouse model of pulmonary tuberculous infection by i.t. administration of attenuated live *M. bovis* BCG. In immune-competent C57BL/6 mice, BCG elicited an early granuloma formation in the peribronchial and perivascular areas between days 7 and 14 and such granulomatous responses became culminant by day 27, involving large areas of lung parenchyma and consisting of macrophages, epithelioid cells, lymphocytes and neutrophils as well as hyperplastic type II epithelial cells (Wakeham et al., 1998). Different from pathologies in humans, there was a lack of giant multinuclear cells. The degree of lung structural damage was less than that by virulent mycobacteria. Of note, such pulmonary inflammatory responses were self-limiting in nature in immune-competent mice which by days 57 and 71 started to decline leaving only mild degrees of foamy macrophage accumulation. The resolution of responses was in accordance with decreasing numbers of bacilli detected in lung tissue at later time points. The effective control of mycobacterial infection in the lung was mirrored by minimal systemic dissemination of bacilli to the spleen. We have analyzed in detail the cytokines that are involved in immune-inflammatory responses in the lung. We found that the protein levels of Th1 cytokines IL-12, IFNγ and TNFα were not markedly induced in the lung until day 14, became maximal by day 27, markedly declined by day 57, suggesting that specific cellular immune responses were required for such optimal cytokine responses and these cytokines contributed to vigorious tissue inflammatory responses. By FACS analysis, in addition to increased pulmonary macrophages and NK cells, the majority of T cells were found to be CD4 T cells, pointing to the importance of this T cell subset. Of interest, there was little induction of a Th2 cytokine IL-4 throughout the entire course of our study, 71 days, suggesting a little role of Th2 cytokines during pulmonary mycobacterial infection. The lack of IL-4 response in the lung was also confirmed by in vitro antigen stimulation assay using total lung mononuclear cells (Wakeham et al., 1998). We found a lack of Th2 cytokines in both C57BL/6 and Balb/c strains of mice, which does not support the previous in vitro observations that IL-4 may play a role at later stages of immune responses against mycobacterial infection (Huygen et al., 1992; Orme et al., 1993). Neither was a Th2 cytokine response found in human tuberculosis (Lin et al., 1996). Instead, a weakened Th1 cytokine response caused by a genetic defect or immune suppressive infections may be the etiology of increased susceptibility of certain populations to tuberculous infections (Jouanguy et al., 1996; Newport et al., 1996). To address the relative role of IL-12 in the development of protective host responses during pulmonary mycobacterial infection, we carried out studies using mice deficient in IL-12. In the absence of functional IL-12, mice demonstrated a defect in mounting granulomatous responses in the lung with minimal formation of atypical granulomas comprising primarily lymphocytes throughout 71 days. BAL cellular analysis revealed a decrease not only in the number of macrophages but lymphocytes and neutrophils. These led to uncontrolled replication of bacilli in the lung and systemic dissemination. On FACS analysis, there was a lack of CD4 and CD8 T cell responses. Not only was there a defect in such primary immune-inflammatory responses in the lung of IL-12$^{-/-}$ mice, but also a defect in memory immune responses as shown by a lack of IFNγ recall response in antigen-stimulated purified lung mononuclear cells. The underlying molecular mechanisms were found to be a complete lack of IFNγ and a severely impaired TNFα response both in the lung and peripheral blood (Wakeham et al., 1998). And to our surprise, such a lack of Th1 cytokines did not favor a shift to a Th2 cytokine response since the level of IL-4 protein remained similar to that in immune-competent mice. Our findings lend support to a recent report that IL-4 was not induced in the liver following i.v. infection with MT in IL-12$^{-/-}$ mice (Cooper et al., 1997). Together, these findings do not seem to support the theory of reciprocal inhibition of expression between Th1 and Th2 cytokines, at least in the context of mycobacterial infection (Mosmann and Sad, 1996). It is possible that the in vivo T cell differentiation process is regulated in a far more complicated fashion and that certain soluble signals required for Th2 cell differentiation may be missing in the absence of IL-12 and other down-stream cytokines IFNγ and TNFα. However, in a model of intracellular parasitic infection, leishmaniasis, the lack of IL-12 polarized a Th2 cytokine response (Mattner et al., 1996). Furthermore, it is well known that the type of Th responses depends on the MHC genetic background during leishmaniasis with a Th1 or a Th2 phenotype developed in C57BL/6 and Balb/c mice, respectively. But an antigen ovalbumin elicited a typical Th2 airways response in both C57BL/6 and Balb/c mice (Stæmpfli et al., 1998). These findings thus suggest that the initial

cytokine microenvironment determined by the nature of eliciting agents upon the interaction with antigen-presenting cells and NK cells, and the differential expansion of T cell progenitors determined by antigenic epitopes presented specific to each MHC haplotype, may all dictate the type of Th responses.

IL-12$^{-/-}$ mice have also recently been employed to examine the role of IL-12 in host defense during systemic mycobacterial infection elicited by MT (Cooper et al., 1997). The lack of this cytokine led to much delayed mRNA expression of IFNγ and TNFα in the liver and uncontrolled mycobacterial replication in various organs. The rebounded expression of IFNγ and TNFα in the liver at later stages of infection is of particular interest which is in contrast to our findings that there is a lack of these cytokines throughout the entire course of pulmonary mycobacterial infection, suggesting the lack of potential compensatory mechanisms in the lung. One mechanism could be linked to IL-18 which has recently been shown to have certain redundant functions and synergistic activities with IL-12 (Lamont and Adorini, 1996; Kohno et al., 1997). Indeed, in the study by Cooper et al., high levels of IL-18 mRNA were found in the liver but it remains unclear whether there was a correlation between IL-18 induction and the rebound of IFNγ and TNFα expression. Examining IL-18 at the protein level and using specific IL-18 abrogating antibodies shall help clarify the role of IL-18 in this model. It would be of interest to also examine IL-18 expression at various tissue sites during pulmonary mycobacterial infection. Apparently, identification in molecular terms of the difference in immune-inflammatory responses between the lung and liver/spleen will help understand why the lung but not other tissues allows the progressive growth of MT (North, 1995). From all of these findings, it is plausible to consider IL-12 as an upstream molecule required for the elicitation of effective cellular immune-inflammatory responses during pulmonary mycobacterial infection. The downstream events include the differentiation and expansion of Th1 cells and CD8 T cells, the release of IFNγ from T and NK cells and the activation of pulmonary macrophages (Fig. 2). IL-12 appears required for a full TNFα response as well but whether an impaired TNFα response that we observed was a result of the lack of IL-12 or IFNγ remains to be fully understood. It is possible that in our model TNFα is primarily released from pulmonary macrophages but not lymphocytes. While IFNγ may activate macrophages to release TNFα, recent evidence has also suggested that IL-12 may induce a TNFα response independent of IFNγ (Taylor and Murray, 1997), and that IL-12 can directly act upon macrophages (Puddu et al., 1997). Thus, IL-12 may play a much broader role than IFNγ in immune-inflammatory responses during pulmonary mycobacterial infection, despite that mice deficient for IFNγ or TNFα or TNFα receptor are more susceptible to systemic or local lung infection by MT or *M. bovis* BCG (Kindler et al., 1989; Flynn et al., 1993, 1995). In consistence with the role of IL-6 in the amplification of specific immune responses, mice deficient in IL-6 were found to succumb to inoculation of MT at doses that were sublethal to w.t. mice (Ladel et al., 1997).

Understanding the cellular and molecular events and the nature of key cytokines involved in protective host responses will certainly help design better vaccines including recombinant cDNA vaccines (Huygen et al., 1996) and foster the notion of immunotherapy for TB patients, particularly those with drug-resistant TB. Recombinant viral or cDNA vaccines can be potentially engineered to co-express immunogenic antigens and immunomodulatory cytokines. These vaccines may enhance immunization efficacy in hosts who fail to mount adequate immune memory responses to conventional TB vaccines due to different MHC genetic background. Aerosol delivery of recombinant IFNγ has been tested on TB patients and some efficacy noticed (Condos et al., 1997). Cytokine gene therapy represents an economical and efficient way to boost immune responses and has the potential to be used to complement the current chemotherapy. However, what to express, which type of gene transfer vectors to use and how to deliver the transgene will entail rigorous experimental explorations ahead. Since tuberculous infection primarily originates in the lung and it assumes

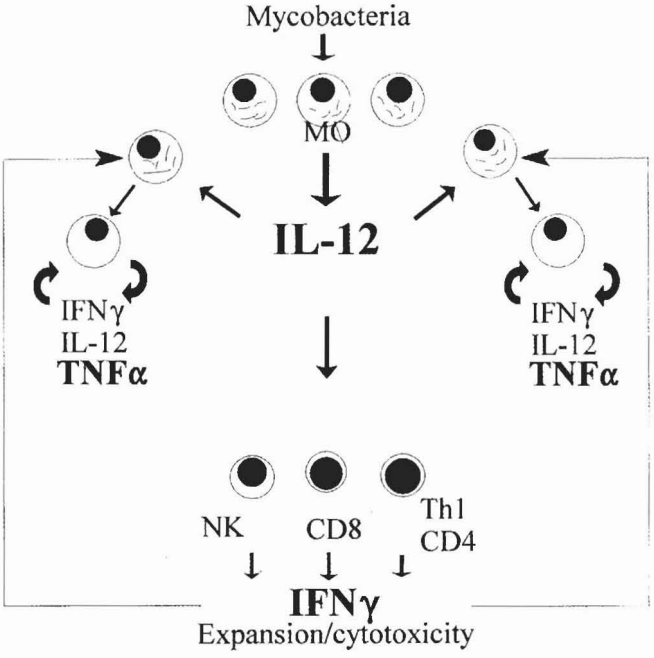

Fig. 2. Putative role of Th1 cytokines in host defense against pulmonary mycobacterial infection. Interaction of mycobacteria with macrophages releases IL-12 which plays an important role in induction of IFNγ by NK and T lymphocytes and Th1 phenotype differentiation. Activated antigen-specific T cells undergo proliferation and become cytotoxic. NK/T cell-derived IFNγ and perhaps macrophage-derived IL-12 both are required for full activation of macrophages, leading to enhanced release of TNFα, IFNγ and IL-12 by macrophages and increased mycobactericidal activities.

a chronic course, a prolonged cytokine gene expression locally in the lung will be highly desirable in addition to systemic delivery. Beside direct application of gene transfer vector, ex vivo cytokine gene transfer to peripheral blood monocytes also holds promise. Since macrophages within granulomas have a fast turn-over rate and the local replenishment of these cells may depend upon a continuing influx of peripheral blood monocytes, transfusion of monocytes transduced with cytokine genes may target the site of infection.

Cytokines in experimental models of antigen-induced allergic airways inflammation

Asthma is an antigen-mediated chronic airways inflammatory disease. The prevalence of this disease in the Western world has been rising in the last twenty years. Although not always life-threatening, most cases of asthma are incurable. Both the direct and indirect costs from asthma-related health operations are all but enormous (Seaton, 1994). The failure to effectively control this disease owes primarily to the lack of our understanding of critical pathogenic events. In the last several years, progress has been made in understanding the role of cytokines in the pathogenesis of asthma by using mouse models of asthmatic inflammation. Although these models may not completely duplicate the pattern of airways physiologic changes and the pathologic course of human asthma (there has been a lack of mouse models of chronic asthma), they allow to dissect the cellular and molecular events underpinning airways eosinophilic inflammation, a characteristic feature of asthmatic airways (Seminario and Gleich, 1994). Although the protocol used to set up mouse models varies slightly between laboratories, the basic scheme is similar. We have established a mouse model of antigen-induced allergic airways inflammation by two intraperitoneal sensitizations with ovalbumin (OVA), 5 days apart, and OVA aerosol challenge 12 days later twice on the same day. There was marked margination of eosinophils and mononuclear cells within the pulmonary vasculature as early as 3h post-OVA challenge, perivascular accumulation of these cells by 12h and marked eosinophil infiltration around bronchi and bronchioles (Ohkawara et al., 1997). The peak of such cellular responses was around 3 days. While there was a lack of evidence of overt epithelial damages such as discontinued epithelia and naked basement membrane, epithelial goblet cell hyperplasia was evident. Such histologic changes faded markedly by day 21, suggesting a self-limiting nature of responses elicited by the current protocol. A chronic inflammatory process including certain degrees of airways fibrotic reactions have been observed in a model elicited by using a protocol that involves repeated OVA challenges (Blyth et al., 1996). It should be pointed out that eosinophils are not the only cell type infiltrating the airways and the other cell types also increased including lymphocytes, macrophages and neutrophils (Ohkawara et al., 1997). This feature has also been noticed in human cases (Howarth et al., 1994). Since eosinophils represent a very small fraction of total peripheral blood leukocytes in both human and rodents, the increased number of eosinophils in the bone marrow and peripheral blood may represent an important event preceding eosinophil infiltration in the lung. Indeed, we observed a twin-peak of peripheral blood eosinophilia, one at 7 days post-second sensitization and one at 3 and 9 days post-OVA challenge. The kinetics of bone marrow eosinophilia were very similar to those in the peripheral blood. These findings suggest the importance of events in the peripheral compartments in the development of airways eosinophilia.

The development of airways eosinophilia in asthma is an immunologically mediated event. It is generally believed that lymphocytes, both B and Th2 cells, play a critical role in the initiation of airways inflammation. The mechanisms driving the initial Th2 differentiation of T progenitor cells are still unclear but may involve IL-4 released from $CD4^+/NK1.1^+$ cells which appear to be selected through the interaction with CD1 molecules on antigen presenting cells (Mosmann and Sad, 1996; Spinozzi et al., 1998). Upon reexposure to the antigen, antigen-presenting cells (APC) present immunogenic epitopes to specific T memory cells at the airway mucosal site and regional lymph nodes. Activation of T cells likely requires not only the antigen presentation by APC but also the cognate interaction between T cells and APC via co-stimulatory molecules CD28/B7, and gp39/CD40. It is believed that TNFα, IL-4, IL-5 and GM-CSF as well as chemokines released from activated Th2 cells play important roles in the development of airways eosinophilic inflammation in asthma (Robinson et al., 1993; Seminario and Gleich, 1994; Lukacs et al., 1996). To dissect the role of cytokines during antigen-induced allergic airways eosinophilia, we examined cytokine profiles both in the lung and peripheral blood. Right after second i.p. sensitization, the circulatory level of IL-5 was markedly elevated, thus likely accounting for the first wave of peripheral blood eosinophilia detected 4 days after second sensitization (Ohkawara et al., 1997). This raised IL-5 declined to the baseline prior to aerosol challenge. Upon aerosol challenge, the level in BAL of TNFα quickly reached a peak in the first several hours, perhaps reflecting a contribution from mast cells. This was followed by an increase in IL-4 and IL-5 which peaked and subsequently declined at days 1 and 5, respectively, post-challenge. The kinetics of these cytokines correlated well with the development of airways eosinophilia which became maximal at about day 3. Of interest, the circulating levels of IL-4 and IL-5 also increased to levels similar to those in the lung. However, two differences were noticed: these cytokines peaked earlier than those in the lung; the raised levels fell much quicker. These suggest that the circulating IL-4 and IL-5 may have derived, at least in part, from lymphoid tissues outside the lung parenchyma. The level of GM-CSF, either in the circulation or in the lung, was increased to a much lower degree, than IL-4 or IL-5.

This does not appear to agree with the findings from human studies (Robinson et al., 1993). IL-5 seems to play a much more prominent role than GM-CSF in the development of allergic airways inflammation. Indeed, the important role of IL-5 in the development of eosinophilia has been suggested by evidence from studies using monoclonal antibodies to IL-5 or IL-5 deficient mice. Systemic administration of anti-IL-5 reduced OVA-induced airways eosinophilia (Kung et al., 1995). IL-$5^{-/-}$ mice failed to develop airways eosinophilia elicited by OVA sensitization and challenge unless IL-5 transgene was delivered into the lung (Foster et al., 1996). Local overexpression in the lung of IL-5, in contrast to systemic overexpression of this cytokine (Dent et al., 1990), induced profound airways eosinophilia and hypersensitivity in transgenic mice (Lee et al., 1997). Of note, as a consequence of local overproduction by airway epithelial cells in this model, there were markedly elevated circulating levels of IL-5 and increased eosinopoiesis in the bone marrow. Systemic overexpression of GM-CSF only induced mild peripheral blood eosinophilia with striking macrophage accumulation and activation (Lang et al., 1987; Gearing et al., 1989), whereas local overexpression in the lung of GM-CSF induced only type II alveolar epithelial hyperplasia but not airways eosinophilia (Huffman Reed et al., 1997). It should not be surprising that IL-5 has a relatively specific effect on eosinophils whereas GM-CSF has a much more broad spectrum of biologic effects on various leukocytes due to differential receptor distribution (Xing et al., 1996a). One reason why local lung GM-CSF did not induce any airways eosinophilia could be that the circulatory level of GM-CSF was not adequate enough to mobilize eosinophil progenitors in the bone marrow. Indeed, compared to IL-5 content in the lung (Lee et al., 1997), the level of GM-CSF was about 500 times lower (Huffman Reed et al., 1997). This notion is supported by our recent findings that overexpression of GM-CSF in rat airways epithelial cells by a transient transgene approach led to not only macrophage accumulation but prominent airways eosinophilia (Xing et al., 1996b). It was noted that in this model circulating transgene product GM-CSF that leaked from the lung caused a significant peripheral blood eosinophilia. Similar approaches also induced airways eosinophilia in mouse lung but in a delayed fashion (Wang et al., 1998a). Thus, in these transgene models, it appears that both local and systemic signals are required for the onset of airways eosinophilia with local IL-5 or GM-CSF serving as a chemotactic force to peripheral blood eosinophils (Resnick and Weller, 1993) and systemic IL-5 or GM-CSF as an eosinopoietic factor acting on the bone marrow. It should be pointed out that the local level of a given transgene product in the lung may be much higher than that detected during the course of allergic airways inflammation. For instance, the maximal level of IL-5 in BAL induced by OVA challenge is mostly in pg/ml ranges. Compared to chemokines, IL-5 or GM-CSF is chemotactic to eosinophils in vitro only at higher concentrations (Resnick and Weller, 1993). It is therefore likely that the levels in the lung of IL-5 or GM-CSF detected during allergic inflammation are not by themselves sufficient to be chemotactic to eosinophils. The question is then what is the function of these locally present hematopoietic cytokines during antigen-induced airways inflammation. We have recently obtained evidence that GM-CSF transgene expression in mouse lung induces macrophage activation and dendritic cell differentiation as indicated by increased surface expression of MHC II, B7 and CD11c, thus implicating GM-CSF in enhancing antigen presentation and antigen-specific immune responses in the lung (Wang et al., 1998a). The requirement of GM-CSF in antigen-mediated T cell immune responses has been observed in GM-$CSF^{-/-}$ mice (Wada et al., 1997). Regarding the role of local IL-5 in the lung during allergic airways inflammation, we have recently observed that there is a lack of airways eosinophilia induced by OVA in IL-$5^{-/-}$ mice but airways eosinophilia can be reconstituted by intramuscular IL-5 gene transfer (Wang et al., 1998b). Such reconstitution was always accompanied by a bone marrow and peripheral blood eosinophilia. On the contrary, intrapulmonary IL-5 transgene expression, when compartmentalized to the lung, failed to do so. Of importance, the levels in the lung of IL-4 and chemokines eotaxin and MIP-1α were similar in both IL-$5^{+/+}$ and IL-$5^{-/-}$ mice. So was the extent of neutrophilia and lymphocytic responses. These findings suggest that systemic but not local, IL-5, together with eosinophil chemokines play an important role in the initiation of airways eosinophilia (Fig. 3). The findings from these studies shall provide important mechanistic insights into key events in allergen-induced airways eosinophilic inflammation. We have recently found that in addition to its critical role in IgE isotype switch, IL-4 together with TNFα plays a role in upregulating VCAM- 1 expression by pulmonary endothelium which is required for the initiation of OVA-induced airways eosinophilia as shown in CD40 ligand knock-out mice (Lei et al., 1998). In addition, IL-4, when overexpressed in mouse lung, also induces profound airways eosinophilia and this effect is likely through its direct chemotactic activities (Dubois et al., 1994) and induction of chemokines. IL-4 induced the release of MIP-1α and MCP-1, suggesting that IL-4 contributes not only to the development of airways eosinophilia but mononuclear cell infiltration seen in the lung during asthmatic inflammation (Waldhauser et al., 1998).

All of the currently available therapies for asthma can only control symptoms. It remains to be seen whether a better-than-steroid therapeutic modality will be developed. With increasing understanding of the pathogenesis of asthma, targeted therapeutic strategies may be developed to intervene selected key events or molecules ranging from antigen presentation, T cell activation, Th2 cytokines, eosinophil differentiation in the bone marrow, to the development of airways

inflammatory responses. In this regard, we have recently examined the effect of IL-10 by using a transgene approach. We found that IL-10 could inhibit the release of Th2 cytokines and the extent of airways eosinophilia (Ohkawara et al., 1996). IL-10 exerted its inhibitory effect perhaps through inhibition of the function of antigen-presenting cells and other inflammatory cell types.

Cytokines in experimental models of pulmonary fibrosis

Pulmonary fibrosis is a pathologic sequela to certain insults and represents an excessive form of tissue repair by the host who is unable to restore the normal tissue architecture that has been severely destructed. Despite of heterogeneous etiologies, established pulmonary fibrosis

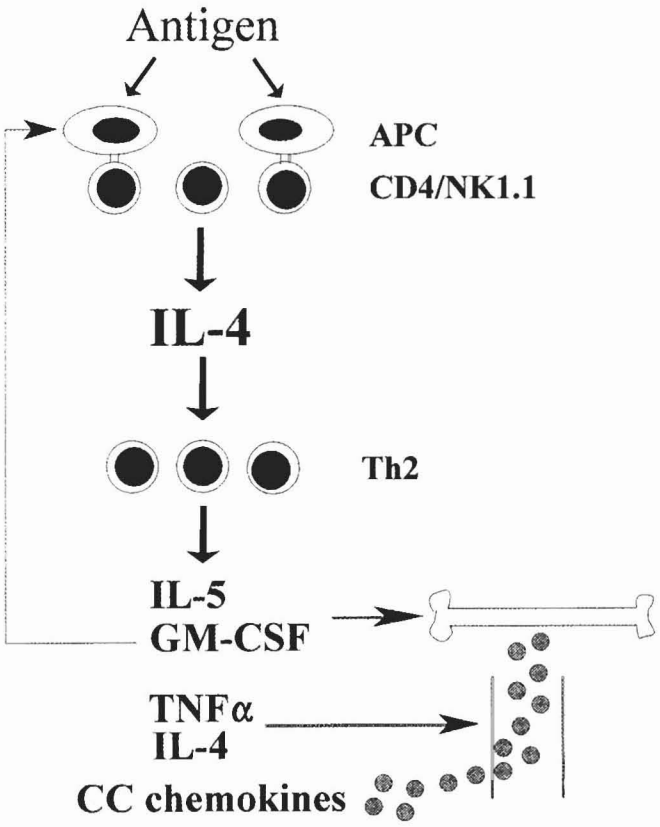

Fig. 3. Role of Th2 cytokines and C-C chemokines in the development of antigen-induced allergic airways eosinophilic inflammation. Upon interaction of antigen with CD4/NK1.1 T cells, the latter release IL-4 which is a key cytokine in driving Th2 differentiation. After clonal expansion, activated antigen-specific Th2 lymphocytes release IL-5, GM-CSF, IL-4, TNFα and CC chemokines. GM-CSF in the lung may amplify the immune response by further activating antigen-presenting cells (APC). Circulatory IL-5 and GM-CSF act on eosinophil and/or other myeloid progenitor cells in the bone marrow, releasing increased numbers of eosinophils and neutrophils into peripheral blood. Increased levels in the lung of TNFα and IL-4 enhance vascular VCAM-1 expression to promote eosinophil margination. Local CC chemokines such as eotaxin serve to attract eosinophils into the airway.

is characterized by abnormal fibroblast accumulation and connective tissue matrix deposition (Gauldie et al., 1993; Phan, 1995). Since the end-stage pulmonary fibrosis is irreversible, understanding the nature of earlier cascade events holds the key to the development of intervention strategies. A group of *Streptomyces verticillatus*-derived glycopeptides, bleomycin, that is used in chemotherapy for cancers, has been widely used to establish experimental models of pulmonary fibrosis to study pathogenic events (Piguet, 1994). We have observed significant accumulation of PMN, lymphocytes, monocytes and eosinophils at day 3 following i.t. instillation of bleomycin. The number of PMN steadily declined thereafter while the number of other cell types remained markedly increased in the following weeks. Damage to lung parenchyma, including the airways epithelium and alveolar septum was evident even in the early phase of inflammation, which was contrary to a minimum of tissue destruction seen in endotoxic lung. Patches of fibroblastic accumulation and matrix deposition were seen from 7 days, progressing into diffuse severe fibrotic pathologies in the following weeks. Cytokines and growth factors, such as TNFα and TGF-ß, are believed to play an essential role in the development of such overzealous type of tissue repair. However, a central issue has remained elusive regarding what triggers this fibrogenic cytokine cascade which is apparently missing in acute pulmonary inflammatory diseases (Jordana et al., 1993; Xing et al., 1994). In the latter, many pro-inflammatory cytokines but not fibrogenic growth factors are turned on in a limiting fashion (Xing et al., 1994). We believe that the nature of causative agents dictates the type of tissue responses and that severe tissue injury occurs as a result of toxicity of fibrogenic agents to the tissue or of the destructive type of inflammation elicited by these agents or both. The sustained severe tissue injury determines, by as yet unknown mechanisms, the duration of expression of proinflammatory cytokines such as TNFα and the type of growth factor responses. It has been demonstrated that expression of TNFα mRNA increases over a period of at least 14 days with a peak seen between 4 to 7 days post-bleomycin administration (Phan and Kunkel, 1992). This sustained TNFα expression contrasts the transient nature of TNFα expression during acute lung inflammation by LPS or Gram-negative bacteria (Xing et al., 1993, 1998; Standiford et al., 1996). Expression of TGF-ß was induced and peaked, almost concurrent with TNFα (Raghow et al., 1989; Phan and Kunkel, 1992). The critical role of TNFα in triggering fibrotic events is suggested from studies using anti-TNFα antibodies (Piguet et al., 1989, 1990; Zhang et al., 1997). However, TNFα is unlikely the only upstream signal required for switching on growth factors. Interestingly, while macrophages from bleomycin-treated lungs were found to release increased amounts of TNFα in vitro, to the best of our knowledge the protein level of this cytokine in BAL has not been reported and we found no detectable or only marginally increased levels of TNFα

in BAL collected at a number of time points (Unpublished data). Lung cell proliferation and collagen/proteoglycan production were found closely associated with induction of TGFß1 mRNA (Raghow et al., 1989; Westergren-Thorsson et al., 1993). There are three isoforms of TGF-ß, TGF-ß1/2/3, active in mammals (Kelly, 1993). However, most studies have focused on the expression of TGF-ß1 and the relative contribution of each of these three isoforms remains unclear in bleomycin models. One recent study suggests a unique role of TGF-ß1 but not TGFß2 or 3 (Coker et al., 1997) whereas the other suggests the involvement of all three isoforms (Santana et al., 1995). The former finding is in line with observations made in human lung tissues, suggesting the importance of TGFß1 in the pathogenesis of fibrosis and a physiologic role of TGF-ß2/3 in maintaining lung homeostasis (Khalil et al., 1996a). The concurrent administration of anti-TGFß with bleomycin into mouse lung reduced pulmonary collagen deposition, thus providing the direct evidence to support the role of TGFß in pulmonary fibrosis (Giri et al., 1993). Of note, there has been no report on the protein level of TGF-ß1 in BAL. Since there are both inactive and active forms of TGF-ß molecules (Sime et al., 1997a,b), ideal immunodetection assays should differentiate these two forms. By using an ELISA detecting total or only active TGF-ß1, we found that the majority of increased measurable TGF-ß1 in the BAL samples from bleomycin-treated mouse lung was inactive, indicating the difficulty in assessing the regulation of activation of TGF-ß molecules in models of pulmonary fibrosis. It is likely that the active form of TGF-ß1 binds to the extracellular matrix, a way by which the host controls its bioactivity. By using ex vivo approaches, alveolar macrophages isolated from rat fibrotic lungs were found to spontaneously release active TGF-ß1 which involves the cleavage of cell-associated latent TGF-ß1 by plasmin secreted by these cells (Khalil et al., 1996b). It appears that different cell types can express antigenic TGF-ß1, depending on the stages of pulmonary fibrotic reactions (Zhang et al., 1995). In the early stage, epithelial and mononuclear cells and eosinophils expressed TGF-ß1 whereas at later stages eosinophils and fibroblasts/myofibroblasts became the major sources as shown by in situ hybridization techniques. Unfortunately, current in situ techniques including immunohistochemistry cannot differentiate the two forms of TGF-ß1. It is of particular interest that eosinophils may play a prominent role in pulmonary fibrosis. The presence of eosinophils in bleomycin-induced fibrotic lung and in human clinical situations with prominent fibrotic features including ARDS and IPF is a consistent histologic feature and recently IL-5 has been implicated in eosinophil chemotaxis and activation (Gharaee-Kermani and Phan, 1997). However, what regulates the expression of this Th2-type cytokine during pulmonary fibrotic responses is unclear. IL-5 is primarily produced by T cells and this study has not yet pinned down the precise nature of mononuclear cells that were found localizing IL-5. It would be of interest to find out whether GM-CSF and IL-4 and eosinophil-specific chemokines are also induced in this model. In addition to the aforementioned cytokines, there is recent evidence implicating the other growth factors PDGF and IGF, and chemokines MIP-1α and MCP-1, in the pathogenesis of bleomycin-induced pulmonary fibrosis (Smith et al., 1994; Zhang et al., 1994; Maeda et al., 1996). The role of lymphocytes in the development of bleomycin-induced fibrosis remains to be clarified. It was found that the majority of infiltrating lymphocytes were $CD4^+$ T cells with the rest being $CD8^+$ and γ/δ T cells (Braun et al., 1996). Elimination of CD4 or CD8 T cells did not seem to affect the final fibrotic outcome. In addition to macrophages, PMN, lymphocytes and eosinophils, the number of mast cells was also increased. These cells have been shown to be part of the fibrogenic constellation in a mast cell-deficient model (O'Brien-Ladner et al., 1993).

To examine the role of cytokines in the pathogenesis of pulmonary fibrosis, we have established models of cytokine transgene expression directed to the respiratory epithelium by using adenoviral -mediated gene transfer technologies (Xing et al., 1996a; Sime et al., 1997a). Different from lung specific transgenic models (Korflhagen et al., 1994; Miyazaki et al., 1995), adenoviral-mediated lung transgene expression is achieved in adult animals in a sustained but relatively transient fashion, a situation that mimics events occurring during inflammatory responses in humans. The limitation of these models is that the biologic events induced by transgene product are taking place in the lung over a viral infection background. By using these models, we have demonstrated the fibrogenic effect of TNFα in the lung (Sime et al., 1998). These effects appears to correlate with a mild degree of induction of TGF-ß1 release in the lung elicited by prolonged TNFα expression. We have also provided the first evidence that the active, but not the latent, form of TGF-ß1 has potent fibrogenic effects in the lung (Sime et al., 1997b). Overexpression of active TGF-ß1 in rat lung led to the formation of an aggressive type of pulmonary fibrosis with massive fibroblastic and myofibroblastic accumulation and connective tissue matrix protein deposition. These models will be invaluable for us to understand the mechanisms by which the latent TGF-ß is activated in the lung and to test intervention strategies targeting TGF-ß-mediated connective tissue metabolism. Overexpression of GM-CSF, used to be deemed as a hematopoietic growth factor, caused macrophage and eosinophil accumulation followed by granulation tissue formation and appearance of fibrotic patches in the lung (Xing et al., 1996b). The fibrotic sequela appeared to involve a cascade of events including macrophage and eosinophil activation, lung injury, TGF-ß1 upregulation, myofibroblast accumulation and granulation tissue formation (Xing et al., 1997b). Of interest, TNFα was not involved in these processes in a significant manner. Intradermal GM-CSF trans-gene expression also elicited subepidermal fibrosis characterized by fibroblast

hyperplasia (Xing et al., 1997c). Fibrogenic activities of GM-CSF were also demonstrated in the subcutaneous tissue following delivery of recombinant GM-CSF by a minipump (Rubbia-Brandt et al., 1991). Thus, GM-CSF may serve as a macrophage activator to release TGF-ß1, downstream of TNFα response. Together, these findings provide important experimental evidence to support a critical role of TGF-ß1 and myofibroblasts in the pathogenesis of pulmonary fibrosis.

Pulmonary fibrosis in humans is a chronic inflammatory disease and, in most cases, when clinically manifested, the disease is already in its later stages. Most patients with pulmonary fibrosis respond poorly to the current antifibrotic agents (Hunninghake and Kalica, 1995). The challenging issue is whether we can develop therapeutic strategies that are able to modulate the later fibrotic events, particularly TGF-ß-mediated events. Development of anti-growth factor agents may hold great promise in this regard. Recently, transgene expression of decorin, a TGF-ß1 inhibitor, has been shown to prevent renal fibrosis in rats (Isaka et al., 1996). We are currently exploring the usefulness of adenoviral gene transfer vectors for decorin or LAP (TGF-ß latency associated peptide) in ameliorating pulmonary fibrosis in experimental models.

Acknowledgments. We thank Sara DeSilvio for her secretarial assistance. The work presented in this article was supported by grants from The Medical Research Council of Canada, The Ontario Thoracic Society and Astra Draco AB, Sweden. ZX is a scholar of the Medical Research Council of Canada.

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Accepted May 19, 1998