An important role for intestinally derived T cells in respiratory defence

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Margaret Dunkley, Reinhard Pabst and Allan Cripps discuss the role of intestinally derived T cells in protecting the lung against Gram-negative bacterial infection. They describe the factors directing T-cell migration from gut-associated lymphoid tissue to lung, and focus on the role of T cells and T-cell-derived cytokines in bacterial clearance from the lung.

Many of the early studies of respiratory defence addressed the role of mucosa-derived antibody, and it has only recently become apparent that T cells are also responsible for many protective effects in the lung. Not only are T cells necessary to provide help for most antibody responses, but they also interact directly with some pathogens, as well as recruit and activate other effector cells. Furthermore, it has been shown that intestinal immunization with killed bacteria protects the lung against bacterial infection and that T cells are involved in this process. This article discusses the migration of T cells from immunized gut to lung, and analyses their role in protecting this organ. Much of the information on protection and migration has been obtained from animal models, whereas the role of factors such as cytokines has been determined principally using *in vitro* systems.

Intestinal immunization and protection against respiratory infection

Immune mechanisms at the various mucosal sites in the body interact through the common mucosal immune system[1], with the gut playing a central role by disseminating effector cells to the lung and other mucosal sites (reviewed in Refs 2,3). Consequently, oral vaccines for protection against lung infection are being developed. Indeed, studies using acute respiratory infection models of *Haemophilus influenzae* and *Pseudomonas aeruginosa* in the rat have demonstrated significant clearance of bacteria, both from the airways and the lung tissue of intestinally immunized animals compared with unimmunized animals[4–6]. Although oral, intra-Peyer's patch (IPP), systemic and intratracheal (IT) immunization all protect the lung against acute *P. aeruginosa* infection[6], the effectiveness of oral delivery is of particular interest because of the ease and safety of the vaccination route. However, the use of IPP as an intestinal immunization route for experimental purposes does have the advantage of stimulating a more vigorous immune response than oral immunization[6,7]. Whereas unimmunized animals die of pneumonia and septicaemia within 12–15 h of challenge with a lethal dose of *P. aeruginosa*, animals immunized IPP with killed bacteria survive and recover[6]. One week after challenge, all bacteria are cleared from the airways of IPP-immunized animals and only minimal numbers remain in the lung tissue. Animals immunized six months previously still exhibit this enhanced bacterial clearance upon challenge, suggesting a definite role for acquired immunity in this process. Although oral immunization with killed bacteria also provides significant protection, the level of protection is lower, with less bacterial clearance and fewer long-term survivors than for IPP-immunized animals[8].

Migration of gut-derived lymphocytes to the lung

To understand why intestinal immunization protects against lung infection, the factors controlling lymphocyte migration must be considered. The current consensus of opinion concerning lymphocyte homing is that naive lymphocytes recirculate through lymph nodes and other organized lymphoid tissue such as Peyer's patches (secondary lymphoid tissue), where priming may take place. Memory cells and effector cells then home to tissue related to the site of their initial activation (reviewed in Refs 9,10), where they accumulate and/or proliferate upon local antigenic stimulation. Thus, oral immunization of pigs with *Actinobacillus (Haemophilus) pleuropneumoniae* has been shown to result in an increase in the number of lymphocytes in bronchoalveolar lavage (BAL) fluid, indicating an increased migration of gut-derived lymphocytes to the lung[11]. Similarly, following intestinal immunization, IgA-containing B cells migrate from Peyer's patches to mucosal sites, including the lung[3,12]. The T cells that arise following intestinal IPP immunization with keyhole limpet hemacyanin (KLH) provide help for antibody production and have a mucosally restricted distribution[7], as do alloantigen-specific T cells arising after oral immunization of rats with allogeneic lymphocytes[13]. $CD4^+$ T-cell blasts, obtained by *in vitro* restimulation of thoracic duct lymphocytes from animals immunized IPP with KLH, traffic selectively to mucosal lamina propria. By contrast, small resting $CD4^+$ T cells (predominantly comprising naive cells) traffic to organized lymphoid tissue such as Peyer's patches, spleen, mesenteric lymph nodes and peripheral lymph nodes[14]. Similarly, when compared with small lymphocytes, a larger proportion of lymphoblasts from the lung migrates to non-organized lymphoid tissue[15].

There is no evidence that the antigen specificity of circulating lymphocytes influences their extravasation at particular sites of antigen deposition (reviewed in Ref. 16), and antigen alone is insufficient to direct

localization of adoptively transferred IgA^+ B cells[17] or $CD4^+$ T cells[14]. However, the presence of antigen does enhance the retention/accumulation of antigen-specific lymphocytes after extravasation.

The proportion of T cells migrating to the lung from sites of immunization, as opposed to those arising locally or in the regional lymph nodes, is not known. Some T cells may be activated locally by migratory antigen-presenting cells (APCs), such as dendritic cells, which themselves have migrated from the site of immunization. However, as shown by *in vitro* proliferation assay, analysis of the distribution of T cells specific for *P. aeruginosa*, following intestinal immunization of rats with killed *P. aeruginosa*, has revealed that these cells appear in Peyer's patches, mesenteric lymph nodes and blood, and can be detected in lung tissue following challenge with live bacteria[18]. Thus, bacteria-specific lymphocytes do migrate from the gut to the lung, where they reside until re-stimulated by antigen.

Mechanisms of extravasation

The process by which leukocytes enter tissues, and the lung in particular, is not well understood. However, the differential specificity of leukocyte homing appears to be due, at least in part, to the expression of leukocyte receptors for tissue-specific addressins on the venules of different tissues. Several tissue-specific addressins have been identified; for example, mice express a peripheral lymph node addressin and a mucosal addressin[19].

Naive cells express homing receptors for mucosal and peripheral secondary lymphoid tissue[20]. This explains the ability of populations of normal lymphocytes (predominantly comprising naive cells) to recirculate more or less uniformly through the different lymph nodes, irrespective of the lymphoid organ from which the cells originate[14].

Effector/memory cells show a differential expression of homing receptors. For instance, a cutaneous lymphocyte receptor is expressed on memory/effector cells homing to skin, whereas a mucosal lymphocyte receptor is expressed on cells homing to mucosal tissue. Homing to peripheral lymph nodes is at least partially due to lymphocyte expression of L-selectin, which binds to the peripheral lymph node addressin; this ligand is also involved in nonspecific homing during inflammation (as discussed below). However, unlike the gut or skin, the lung appears to have a mixed homing specificity that is intermediate between peripheral and mucosal specificities, and is comparable in specificity to mesenteric lymph nodes. This has been demonstrated *in vitro* by binding peripheral lymph node and Peyer's patch cells to cryostat sections of bronchus-associated lymphoid tissue (BALT)[21]. Thus, mucosally primed and peripherally primed cells would both be predicted to migrate to lung and, therefore, systemic and mucosal immunization should be equally successful in preventing lung infection, as is the case[6]. Thus, homing of intestinally derived lymphocytes to the lung may be understood in terms of mucosa-specific homing.

Distribution of lung T cells

Lymphocytes in the lung reside in four major compartments[22]: the pulmonary intravascular pool; the interstitial pool, containing transient lymphoid emigrants; the alveolar pool (sampled by BAL fluid); and the BALT (Ref. 23), which is not a constitutive structure in normal human lung, but can be formed under the influence of, as yet undefined, microbial factors[22]. The interstitial lymphocyte pool is of considerable size[24], comprising approximately 40% T cells. The role of the large intravascular pool of lymphocytes, which is retained by a physiological process, is unclear, but it may be of some importance in immune reactions of the respiratory tract[25]. Presumably, intestinally derived effector T cells reside in these compartments, where they may be reactivated upon exposure to antigen; however, the migration of cells to, and between, these compartments has yet to be fully elucidated.

T cells and production of protective and nonprotective antibodies

Elevated, bacteria-specific antibody levels correlate with poor lung function in patients with cystic fibrosis[26], and several studies have demonstrated that at least some pathogen-specific antibodies may contribute to lung damage rather than to protection in chronic respiratory infection elicited by *P. aeruginosa*[27]. However, there is evidence that certain antibodies may have an important role in elimination of Gram-negative bacteria. An association between opsonizing antibody specific for mucoid exopolysaccharide and a lack of detectable *P. aeruginosa* colonization in some older, relatively healthy, patients with cystic fibrosis indicates a protective role for this antibody[28]. Protection of guinea pigs and rodents against *P. aeruginosa* infection following the administration of monoclonal antibodies against lipopolysaccharide (LPS) and opsonizing anti-alginate antibodies, respectively, has also been described[29,30]. These protective antibodies are IgG and IgM, and their production is almost certainly T-cell dependent.

The mucosal IgA response is regulated by T cells[31], which are required to provide an initial signal to the B cell *via* cell contact, and by another signal provided by transforming growth factor β (TGF-β), which switches B cells from expression of surface IgM to surface IgA (Ref. 32). The T-cell cytokines interleukin 5 (IL-5) and IL-6 are necessary for the final differentiation of B cells to IgA-secreting cells[33]. IgA enhances IgG-mediated phagocytosis by neutrophils[34], and Fcα receptors for IgA are induced on neutrophils by chemoattractants[35]. IgA-dependent monocyte-mediated antibacterial activity may also be important in bacterial clearance[36].

Following intestinal immunization with *P. aeruginosa*, specific antibodies are found both in serum and in BAL fluid. However, the proportion of BAL antibodies derived from serum as opposed to those produced locally in the lung is not yet known. Nonetheless, examination of total immunoglobulins in the sheep respiratory tract demonstrated that approximately 81% of IgA was produced locally, whereas IgM and IgG were wholly derived from plasma[37]. Whether T cells that provide help for antibody production following intestinal immunization are acting only in the gut, where large amounts of antibody are formed, or also in the lung, has yet to be clarified.

Non-antibody-mediated protection by T cells

Examination of bacteria-specific antibody levels in serum, saliva and BAL fluid of intestinally immunized animals has demonstrated that protection does not always correlate with antibody levels. Indeed, in some cases, protection was observed in the absence of detectable antibody, suggesting that other mechanisms of protection also operate. Results of cell transfer studies indicate that this protection is mediated by T cells[8,38]. In this instance, the mechanism of $CD4^+$ T-cell-mediated protection does not appear to be *via* help for antibody production, as bacteria-specific antibody was not detectable in recipients of purified T cells. Furthermore, the protection is also antigen specific, since $CD4^+$ cells from donors immunized with non-crossreacting bacteria do not confer protection.

Similarly, others have demonstrated the presence of a protective T-cell response in the absence of detectable antibody in mice that have been given low intraperitoneal doses of live *P. aeruginosa*[39]. Protection was transferred to nonimmune mice by $CD8^+$ T cells[40], which were demonstrated to lyse live *P. aeruginosa* by interaction with macrophages, resulting in secretion of a T-cell-derived cytokine. The development of cell-mediated immunity in the lungs of mice that had been infected by aerosol with live, virulent *Bordetella pertussis* has been shown to parallel the clearance of the infection from the lungs, whereas the antibody response only developed after the infection had cleared[41]. These findings are consistent with studies undertaken in patients with chronic bronchitis, which suggest that specific antibody does not correlate with immune status to non-typable *H. influenzae*[42].

T cells, macrophages and neutrophils – a crucial role in the elimination of Gram-negative bacteria from the lung?

Although the mechanism(s) by which T cells protect the lung is still under debate, it could be proposed that one mode of action is *via* activation of the phagocytic response. Four hours after IT challenge with live bacteria, a time when bacterial clearance in immunized rats is significantly enhanced, the BAL fluid of intestinally immunized rats contains a significantly greater number of activated neutrophils compared with unimmunized rats[4,43]. The phagocytosis of *P. aeruginosa* by these cells can account for bacterial clearance in immunized animals[43]. Similarly, others have shown that the prompt appearance of neutrophils in lungs is necessary for the early clearance of non-typable *H. influenzae* in mice[44]. This implies that neutrophils mediate an important role in acute bacterial infections, and that immunization enhances this activity.

Alveolar macrophages have also been shown to be important in protection in the lower portions of the lung[45]. Protective immunity occurring early after intrapulmonary challenge with live *P. aeruginosa* is associated with significant macrophage activation in the bronchoalveolar space of immune animals[46]. This is only apparent in the early phase of infection and, by four hours, this increased macrophage activity has diminished. The enhancement of macrophage activation, as well as neutrophil recruitment and activation, in immunized animals suggests that a trigger or amplifying effect is provided by immune lymphocytes. Together with reports that T cells can recruit and activate neutrophils *in vitro*[47], these observations have led to the hypothesis that immune T cells may enhance elimination of bacteria from the lung *via* recruitment and activation of macrophages and neutrophils. This effect is likely to be mediated by cytokines released by T cells, according to a sequence of events predicted largely from *in vitro* evidence, as detailed below. A proposed mechanism for protection in the immune lung is depicted in Fig. 1.

Stage 1: activation of alveolar macrophages and tissue T cells

Bacterial LPS activates monocytes and macrophages, and induces expression of tumour necrosis factor α (TNF-α) and IL-1 (Ref. 48). The role of IL-1 and TNF-α in response to infection and inflammation has been summarized in recent reviews[49,50]. IL-1 activates T cells to secrete IL-2, which acts to increase TNF-α production by macrophages. TNF-α amplifies its own release and also induces IL-1 production. Interferon γ (IFN-γ) is secreted by T helper 1 (Th1) cells, and possibly by $CD8^+$ T cells and natural killer (NK) cells, and also amplifies LPS-mediated TNF-α release from macrophages[50]. Ultimately, TNF-α primes macrophages to produce superoxide anion and, thus, enhances killing of opsonized *P. aeruginosa*[51]. This sequence is consistent with observations of increased recruitment and activation of macrophages in the early stages after introduction of bacteria to the lung[46].

Stage 2: recruitment of leukocytes (predominantly neutrophils)

Although leukocyte homing occurs under normal circumstances, inflammation triggered by certain antigens or live bacteria can cause increased leukocyte infiltration into tissues. Several molecules are involved in leukocyte–endothelial adhesion (reviewed in Refs 10, 52–54; summarized in Table 1), and many studies have examined this interaction. A number of adhesion molecules essential to the transmigration of leukocytes are expressed or upregulated on activated endothelium. For instance, an L-selectin ligand induced on stimulated endothelium may be different to the ligand present on peripheral tissues and is involved in recruitment of leukocytes[54]. However, it is not yet clear which molecules play a role in leukocyte accumulation in the lung.

Bacterial factors alone can also cause cell recruitment, since it is known that LPS upregulates the L-selectin ligand, and other ligands, on endothelium[55]. Although little information is available regarding the detailed mechanisms occurring in the lung, several *in vitro* studies have shed some light on the interactions between leukocytes, endothelial cells and cytokines (reviewed in Refs 54,56). In particular, IL-1 and TNF-α play an important role in cell recruitment. TNF-α increases intrapulmonary expression of endothelial intercellular adhesion molecule 1 (ICAM-1) (within one hour) and E-selectin (within hours)[57], and has been shown to increase expression of complement receptor 3 (CR3; Mac-1, CD11b/CD18) and CR4 (p150.95, CD11c,d/CD18), but not lymphocyte function-associated

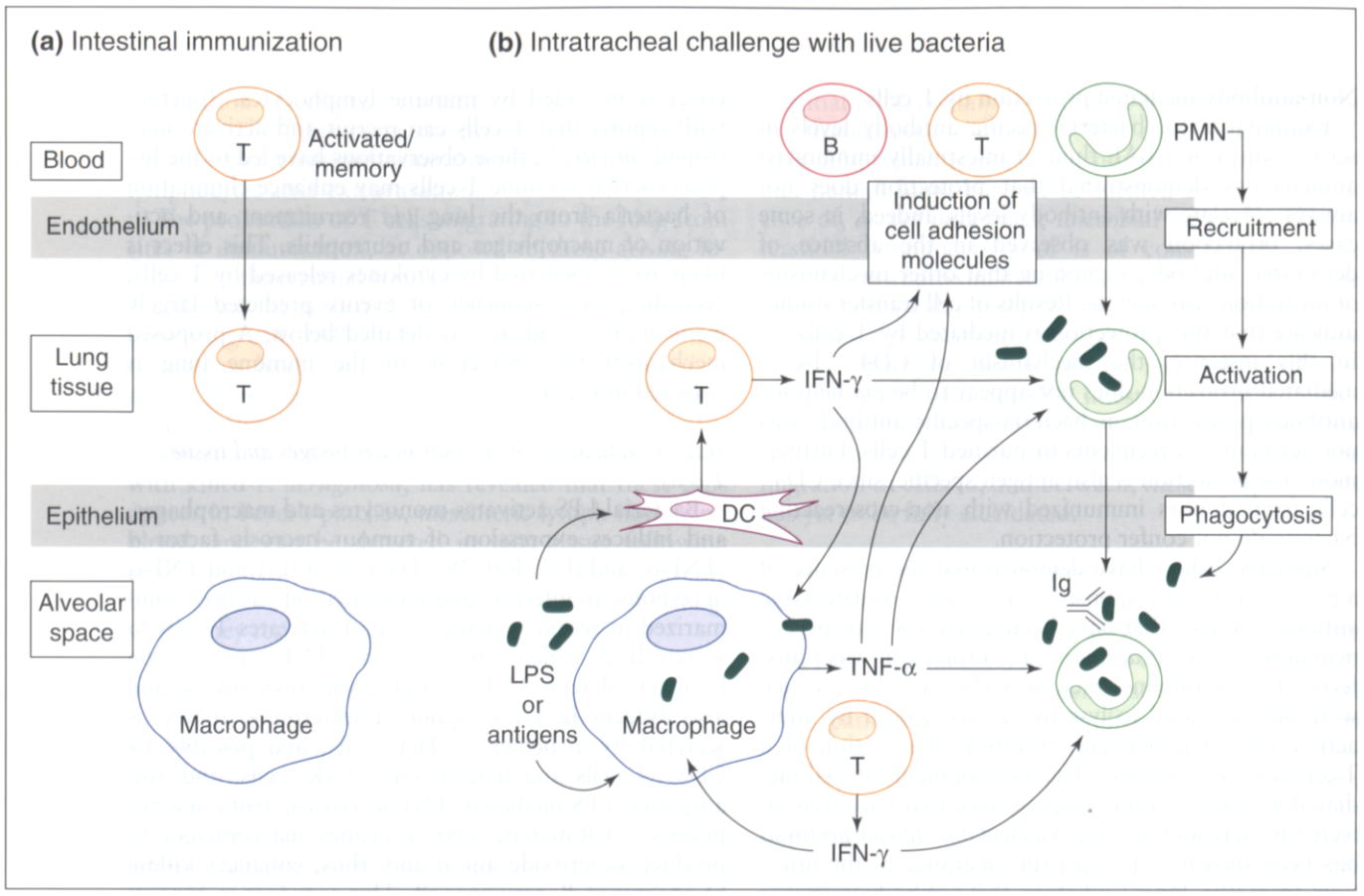

Fig. 1. *Proposed scheme for the role of intestinally derived T cells in the recruitment and activation of neutrophils. (a) Migration of T cells from the gut to the lung after intestinal immunization with killed bacteria. (b) Cell recruitment and activation after pulmonary challenge with live bacteria. Abbreviations: T, T cell; B, B cell; PMN, polymorphonuclear leukocyte (neutrophil); IFN-γ, interferon γ; DC, dendritic cell; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor α; Ig, immunoglobulin.*

molecule 1 (LFA-1), on neutrophils. IL-1 and TNF-α both stimulate lung fibroblasts and epithelial cells to release IL-8, which is probably the most potent neutrophil chemotactic factor in the lung[58]. Endothelium activated by TNF-α secretes a neutrophil chemotactic factor[59], binds neutrophils[60] and recruits leukocytes[61]. T cells are also implicated in the recruitment process, as IFN-γ causes some upregulation of endothelial L-selectin ligand[55], and induces ICAM-1 on endothelium[62].

Stage 3: activation of recruited neutrophils

TNF-α is released by macrophages, as described in stage 1, and activates neutrophils[63]. IFN-γ derived from activated T cells stimulates macrophage synthesis of reactive nitrogen intermediates[64], enhances macrophage killing of opsonized *P. aeruginosa*, and activates neutrophil oxygen metabolism, lysosomal enzyme release and antibody-dependent cell-mediated cytotoxicity. TNF-β (lymphotoxin), another Th1-derived cytokine, inhibits locomotion, and stimulates respiratory burst and degranulation of neutrophils[65]. Granulocyte–macrophage colony-stimulating factor (GM-CSF), which may be produced either by Th1 or Th2 cells, enhances the neutrophil respiratory burst[66]. TNF-α, TNF-β and GM-CSF have a rapid, short-lived effect on neutrophils *in vitro*, whereas IFN-γ has a slower, longer-lasting effect[65].

Thus, after initial triggering of macrophages and immune T cells by bacterial factors, an amplifying cycle would lead to increased macrophage activation, increased cell recruitment (particularly neutrophils) and enhanced activation of recruited neutrophils. The predominantly macrophage-derived cytokines TNF-α and IL-1, and the Th1-type cytokines such as IFN-γ, would be predicted to be important to this process in the lung. However, *in vivo* experiments suggest that TNF-α is involved in protection in immunized rats, whereas IL-1 is not. Nonimmune rats show increasing levels of TNF-α and IL-1 in BAL fluid after acute respiratory infection with a lethal dose of *P. aeruginosa*[46]. Interestingly, by contrast, intestinal immunization causes an early, higher, transient release of TNF-α in the bronchoalveolar space, and prevents the production of IL-1 that is seen in unimmunized animals[46], thereby preventing the damage that is ultimately caused by high IL-1 levels. Overall, the regulation of TNF-α and IL-1 production may well be under the control of T-cell cytokines.

Studies assessing the *in vivo* role of cytokines in the process of bacterial elimination show that administration of IFN-γ after bacterial challenge can protect against one strain of *P. aeruginosa* but not another[67], owing to the inactivation of IFN-γ by proteases produced by the latter strain. IFN-γ alone is incapable of inducing protection when administered IT, either simultaneously or a short time after challenge in a rat model of acute respiratory infection induced by *P. aeruginosa* (M. Dunkley, A. Buret, R. Clancy and A. Cripps, unpublished). These data suggest either that the IFN-γ is being destroyed, or that synergy with other cytokines is required for a full effect. However, intestinally

immunized rats, which exhibit enhanced bacterial clearance compared with unimmunized rats, have higher levels of IFN-γ in the BAL fluid than do unimmunized controls, suggesting a role for IFN-γ in bacterial elimination (M. Dunkley, A. Buret, R. Clancy and A. Cripps, unpublished).

A downregulatory role for Th2-type cytokines in the lung?

Th2-type cytokines inhibit many of the activating properties of IFN-γ and, therefore, may be involved in regulation of the inflammatory response in the lung. IL-4 and IL-10 synergize to inhibit cell-mediated immunity, and both cytokines inhibit IFN-γ production[68]. Although IL-4 causes some upregulation of L-selectin ligand[55], it has also been shown to inhibit macrophage production of IL-1α, IL-6 and TNF-α (Ref. 69), and to inhibit cytokine synthesis by Th1 cells[70]. IL-10 inhibits parasite killing and nitric oxide production by IFN-γ-activated macrophages[71], and inhibits cytokine production by activated macrophages[70]. Human IL-10 is also an inhibitor of IL-8-induced $CD4^+$ T-cell migration[72]. Thus, the effects of Th2-derived cytokines would be expected to downregulate elimination of Gram-negative bacterial infection in the lung. However, these cytokines may positively aid the elimination of bacteria as a result of their contribution to antibody production. IL-5 enhances IgA production, and hence may contribute to neutrophil phagocytosis, and IL-6 is important in the final differentiation of B cells. IL-10 has stimulatory effects upon B-cell activation and immunoglobulin secretion[73]. Thus, at the sites of antibody production (which, following intestinal immunization, would be predominantly in the gut mucosa and, to a lesser extent, in the bronchial mucosa), Th2-derived cytokines may play an enhancing role in bacterial elimination.

Many other cytokines produced by T cells, and by other cells such as fibroblasts and epithelial cells, may also be implicated in the protective process in the lung. For example, TGF-β may play a role in the immune response to acute *P. aeruginosa* infection. This cytokine is produced by numerous cell types, including T cells, macrophages, B cells and platelets, and has both pro- and anti-inflammatory effects[74]. It has been suggested that TGF-β may play a key role in cell recruitment and activation during the early phases of an inflammatory response, and that it may subsequently downregulate such responses once they are initiated, while promoting tissue repair and establishing a state of memory that allows for induction of secondary responses upon antigenic rechallenge[75].

Concluding remarks

Determination of the precise contribution of cytokines and T-cell subsets to immune protection in the lung awaits examination of *in vivo* levels of individual cytokines at various stages of the response against acute and chronic infection. The *in vivo* use of neutralizing antibodies against various cytokines should also shed light on their respective roles in promoting or inhibiting bacterial elimination. Thus, although a role for T cells in respiratory defence is becoming more clearly defined, there is much to be elucidated with regard to defining which compartments contain the activated T cells, the route of migration to the lung compartments and the precise mechanisms by which these cells exert their protective effects.

Table 1. Adhesion molecules involved in interactions between leukocytes and endothelial cells during inflammation

Leukocyte	Adhesion molecule on leukocyte	Ligand on endothelium
T, B, Mo, M, N, NK	LFA-1 (CD11a/CD18)	ICAM-1, ICAM-2, ICAM-3
Mo, M, N, E, NK	CR3 (Mac-1, CD11b/CD18)	ICAM-1
Mo, N	sialyl-Lewis x	E-selectin (ELAM-1), P-selectin (GMP-140)
Mo, M, N, NK	CR4 (p150.95, CD11c,d/CD18)	VCAM-1
L, Mo, E	VLA-4 (CD49d/CD29)	VCAM-1
L, Mo, N, E	L-selectin (LECAM-1)	L-selectin ligand

Abbreviations: T, T cell; B, B cell; Mo, monocyte; M, macrophage; N, neutrophil; NK, natural killer cell; L, lymphocyte; E, eosinophil; LFA-1, lymphocyte function-associated molecule 1; CR3, complement receptor 3; VLA-4, very late antigen 4; LECAM-1, leukocyte cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; ELAM-1, endothelial–leukocyte adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1.

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