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Energy required for processes that sustain life is derived from degradation of carbohydrates, lipids and proteins. This degradation process is associated with the release of CO$_2$ as a by-product. CO$_2$ is therefore released in all live cells and tissues in living organisms. Since CO$_2$ cannot be allowed to accumulate in tissue, it is rapidly transported to lungs where it is exchanged with O$_2$. Haemoglobin molecules packed in red blood cells (RBC) are responsible for binding and transporting CO$_2$ in blood. Sustenance of the living state is dependent upon efficient gaseous exchange through lung membranes. This membrane comprises epithelial cells that line the lung alveoli and must therefore be kept clean and free of pathogens. These lung membranes however face constant onslaught of airborne microbes, polluting gaseous and fine particulates suspended in ambient air. During evolution several biological mechanisms have evolved to keep the lung membranes clean and healthy so that the gaseous exchange process can go on efficiently. Special immune mechanisms have evolved to eliminate pathogens that may reach lung alveoli through inspired air. These mechanisms may essentially be classified under the heads of natural and adaptive immunity.

Natural immunity within lungs has several components. One of the most important components of natural immunity present in lung alveoli is the population of alveolar macrophages (AMs). AMs can phagocytose the intruding microbes and kill them. In addition AMs can also be activated by PAMPs (pathogen associated molecular patterns) and release mediators including chemotactic agents that in turn initiate localized inflammatory responses with an overall aim of eliminating the intruding pathogens. Recognition of pathogen derived PAMPs by AMs is through special Toll-like receptor (TLR). TLRs are conserved during evolutionary process and are a ubiquitous part of the pathogens sensing mechanisms in a variety of living organisms.

Besides AMs, the other cell type in alveoli that come in intimate contact with pathogens is the epithelial cells that line the alveoli and airways within the lungs. In the present thesis we have focussed our investigations to determine whether lung epithelial cells have any significant role in lung immunity. This role could either be in sensing or recognition of PAMPs and may extend even to a role in processing and presentation of antigens to T helper (Th) cells.
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Recent studies have shown that the expressions of various TLRs are not entirely restricted to cells of immune system. A variety of cell types other than the immune cells may also express TLRs and may have a role in recognition of pathogen associated molecules (Bals and Hiemstra, 2004; Fan et al, 2003; Immler and Hoffmann, 2001; Xiang and Fan, 2010). In an organ like lung where the possibility of encountering of pathogens is rather high, it appears logical that lung epithelial cells too express TLRs, participate in the recognition of pathogens and contribute in some manner to the process of natural immunity in lungs. There are indeed some reports that point to the expression of TLRs on lung epithelial cells and cell lines derived from lung epithelial cell lines (Armstrong et al, 2004; Bals and Hiemstra, 2004; Doremann et al, 2003). We started our investigation by comparing expression of TLR2, TLR4 and TLR9 molecules on lung epithelial cells and alveolar macrophages.

During the first stage of the work, the study remained confined to certain established cell lines of lung epithelial cells as well as alveolar macrophages. We found that there is little or no expression of TLRs molecules on the cells surface of epithelial cells whereas a significant expression of these molecules could be demonstrated on alveolar macrophages. Interestingly while there was no surface expression of TLRs on lung epithelial cells, a significant expression of TLR4 receptors could be demonstrated intracellularly on epithelial cells. Treatment with cytokines (TNFα and IFNγ) resulted in substantially higher surface expression of all TLR receptors. On LA4 epithelial cell line, LPS and BCG could also induce a marginal increase in TLR2 and TLR4 expression. In these experiments LPS has been used as a prototype PAMP molecule of Gram negative bacteria that activates cells by binding to TLR4 receptors. BCG has been used as a prototype Gram positive pathogen. BCG lung infection in mice has been used as a model of tuberculosis infection in humans (Saxena et al, 2002a; Saxena et al, 2002b). Unlike the epithelial cells, BCG and LPS could substantially boost the expression of TLR2 receptor on MH-S macrophage cell line, though the expression of TLR4 molecule was not significantly altered on these cells. Basal as well as BCG induced surface expression of TLR2 and TLR4 molecules could be significantly inhibited by micro-fine crystalline silica particles. Silica particles have been used in this study as a prototype of air borne particulates that have been shown to cause a variety of health effects. Results of expression of TLR molecules on lung epithelial cell lines and MH-S
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macrophage cell line were further confirmed by using primary epithelial and macrophages cells.

For this purpose, epithelial cells were isolated from mouse lungs by using a commercially available kit. Essentially small pieces of mouse lung tissue were first digested with a cocktail of three enzymes (Collagenase, Dispase and DNase I) to disperse epithelial cells from lung tissue. Lung tissue as such contains large number interstitial lymphocytes and macrophages that are likely to contaminate the epithelial cell preparation derived from lung tissue digest. All cells of lymphoid and myeloid origin were removed by using anti-leukocyte common antigen coated magnetic bead based separation so that the final preparations of primary lung epithelial cells were essentially devoid of leukocytes. In general the recovery of PLE cells was about 3-5 million cells per mouse. These cells were maintained in culture wells in RPMI medium containing 10% FCS. The morphology of freshly isolated cells was rounded but the cells soon started to adhere to the culture wells and within 2-3 days acquired morphology very similar to that of LA4 lung epithelial cell line. Behaviour of cultured cells was examined by time lapse micro-cinematography where a significant number of cells were seen to divide. A cell cycle analysis further indicates that 8% of the cells were in G2/M phase and about 9% of cells were in DNA synthetic phase. These results clearly show that PLE cells may continue to grow in culture conditions for at least 3-4 days in culture.

Primary lung epithelial (PLE) cells, unlike LA4 cell line had a basal expression of TLR2 and TLR4 receptors that could be inhibited by silica particles. Exposure to BCG could significantly augment the expression of TLR2 molecules on both PLE cells as well as bronchoalveolar lavage (BAL) cells that essentially comprise alveolar macrophages.

Taken together our results indicate that lung epithelial cell line as well as primary lung epithelial cells may express TLR receptors, or may be induced to express these receptors by cytokines or pathogens / pathogen associated molecules. If these receptors are expressed on epithelial cells it appears likely that they may have some biological functions also. Activation of macrophages through TLR receptors is known to result in secretion of nitric oxide (Mizel et al, 2003). Our results however indicate that neither lung epithelial cell line (Figure 17, panel A) nor PLE cells
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(Results not shown) secrete significant amount of nitric oxide (NO) in response to BCG or LPS. It is likely that lung epithelial cells may not have the necessary enzymatic pathways responsible for NO production. In A549 human lung epithelial cells however, release of NO upon activation with cytokines (TNFα and IFNγ) has been shown (Roy et al, 2004). These results would indicate that lack of NO in response to BCG and LPS may not be due to lack of cellular machinery to make NO. Experiments to confirm the release of NO by cytokine activated PLE cells are currently underway. Interestingly our results also indicate that LPS and more potently BCG could induce the release of TNFα by PLE cells. Similarly the expression of the molecules associated with antigen presentation may also be boosted in epithelial cells by LPS and BCG (Figure 39-46, panel A). It is not unlikely that the release of TNFα and upregulation of antigen presentation molecules may be a consequence of signalling through TLRs. This proposition would however require further confirmation.

Since there is no basal expression of TLR molecules on cell surface of lung epithelial cells. It is possible that pathogens and PAMP molecules may be internalized by these cells and activation may thereafter be affected through intracellularly expressed TLRs in the epithelial cells. Interaction between BCG and lung epithelial cells as well as alveolar macrophages and a possible internalization of BCG by epithelial cells were examined. Addition of BCG as well as BCG sonicates resulted in decreased recovery of MH-S cells from cultures indicating that the BCG may be toxic to alveolar macrophages. Similar results were obtained by using peritoneal macrophages. Interestingly however lower doses of BCG improved the recovery of lung epithelial cells (both LA4 cell line as well as PLE cells) in the culture whereas higher dose of BCG has no significant effect on epithelial cell recovery. The reason for a toxic effect on macrophages but not on epithelial cells is not clear.

In vivo however, this relative resistance of the epithelial cells to BCG may be advantageous. As later results indicated, epithelial cells could present antigens to Th cells. In view of the resistance of epithelial cells to toxic effect of BCG, antigen presentation may go on even if macrophages are compromised. Our studies on the uptake of fluorescence coupled (Dil C18) BCG by macrophages and epithelial cell lines clearly indicate both cell types could take up BCG in a time dependent manner,
though the uptake is substantially greater for MH-S macrophages compared to epithelial cell lines. It is possible that the toxic effect of BCG on macrophages may be related to higher internalization of bacteria by these cells.

Silica and Acid functionalized single walled carbon nano-tube (AF-SWCNTs) preparations were used to study the effect of particulates on various functional parameters of PLE cells. Silica particles were toxic to PLE cells at 50 & 100 \( \mu \text{g/ml} \) concentrations. Similarly AF-SWCNTs were also found to be toxic to PLE cells at 10 & 50 \( \mu \text{g/ml} \) concentrations. It is not clear whether the inhibitory effect of silica and AF-SWCNTs were purely mechanical effects that would be induced by any particle preparation, or was more specific effect related to their structure. It is interesting to note that both crystalline silica as well as AF-SWCNTs have negative charge. It is possible that the biological effects of Silica and AF-SWCNTs may be related to their negative charge. There have been reports in literature that the silica particles could structurally mimic some of PAMPs (Pernis, 2005). Further work would be required to elucidate the mechanism of effect of these particles on lung epithelial cells.

Since we have evidence for the internalization of BCG by lung epithelial cells, we further investigated the proposition of lung epithelial cells being capable of processing and presenting antigens to T helper (Th) cells. Presentation of antigens requires the expression of several crucial molecules on the antigen presenting cells (APCs). These include the MHC class II molecules that physically bind antigenic peptides derived from the degradation of bacterial antigens. Presentation of peptides derived from virus that infect epithelial cells would require the expression of MHC I molecules on epithelial cells. Besides class I and II MHC molecules, expression of accessory molecules like B7.1 and ICAM-1 is also necessary for antigen presentation of peptide antigens (Bachmann et al, 1997; Sedwick et al, 1999). MHC II expression was virtually undetectable on LA4 epithelial cells and could only be boosted minimally (though significantly) by BCG and LPS. Macrophages constitute efficient APCs for bacterial antigens but MH-S macrophage cell line also expressed very low levels of MHC class II molecules that too could not be boosted significantly by LPS and BCG. A mixture of cytokines (TNF\( \alpha \) and IFN\( \gamma \)) could however significantly augment the expression of MHC class II molecules on LA4 epithelial cells.
As expected, very high proportion of LA4 epithelial cells and MH-S macrophages express MHC I molecules. Quantitative expression of MHC I on per cell basis could significantly be boosted by treatment with LPS and BCG. Expression of B7.1 was higher on LA4 cells as compared to MH-S cells and in both cases a further augmentation of expression of B7.1 molecules could be observed in response to LPS and BCG. Almost 50% of the MH-S cells express ICAM-1 molecules but this molecule was not expressed on LA4 epithelial cells. BCG and LPS could marginally upregulate the expression of ICAM-1 on LA4 cells. Treatments with cytokine mixture (TNFα and IFNγ) in general augmented substantially the expression of antigen presentation markers on LA4 cells.

A comparison of expression of antigen presentation marker on epithelial and macrophage cell lines and freshly isolated PLE cells and macrophages cells indicated some interesting differences. While almost all LA4 and MH-S cells express MHC I molecules only one third of PLE cells express MHC I molecules. 80% of the BAL macrophages also express MHC I molecules. A central dogma in immunology states that all nucleated cells express MHC I molecules. Functionally it makes sense because MHC class I molecules are involved in the presentation of viral antigens and Cytotoxic T lymphocytes (CTLs) generated in response to the antigens are expected to kill the infected cells harbouring the infection. There are however exception of this dogma. A large proportion of brain neurons do not express MHC I molecules (Joly and Oldstone, 1992). The rationale of absence on certain cells of expression of MHC I molecules could be that these cells may be spared from killing by CTLs even if they are infected with virus. In such cases, such cells may not be killed even if they are infected with virus because of the crucial biological function they perform. Non-expression of MHC I molecules on two third of PLE cells may be based on such a rationale. We also considered the possibility that lack of detectable MHC I molecules on large proportion of lung epithelial cells could be an artefact resulting from digestion of the lung tissue with degradative enzymes. This however appears unlikely because, even after allowing the cells to recuperate for 3 days in culture, MHC I expression was not restored on these cells. A large proportion of PLE as well as primary macrophages express MHC II molecules as compared to cell lines. Almost 60% of the PLE cells expressed ICAM-1 whereas less than 1% of LA4 cells express these molecules. In contrast, one third of LA4 cells express B7.1 whereas less than
3% of PLE cells express these molecules. On the whole it can be said that the set of molecules associated with antigen presentation are either expressed on lung epithelial cells or their expression may be boosted by activating agents like pathogens and cytokines. Interestingly exposure to silica particles did not interfere with the expression of antigen presentation molecules on either PLE cells or primary macrophages. Figure 53 shows that under certain conditions silica may even up-regulate the expression of antigen presentation molecules.

Finally in this thesis attempts were made to get direct evidence for the ability of PLE cells to process and present antigens to Th cells. Due to MHC restriction phenomenon, it is necessary that the T cells and antigen presenting cells must share the same MHC haplotype for the T cells activation to take place. Experiments were therefore designed to use antigen sensitized Th cells as well as the antigen presenting cells from the same mouse strain. Sensitized Th cells were derived from the spleens of BCG sensitized mice and were purified by using a positive selection process with anti CD4 coated magnetic beads. Whereas the proportion of Th cells from mice spleen ranged from 25-30%, purified sensitized Th cells preparation had above 95% CD4+T cells. PLE cells, and as control peritoneal macrophages were used as APCs in an antigen presentation assays. APCs were allowed to interact with BCG for a period of 24 hrs following which the cells were washed and fixed by using gluteraldehyde. Antigen pulsed and fixed APCs have extensively been used for antigen presentation (Vallejo et al, 1992). Our results indicated that PLE cells were able to present BCG antigen to sensitized Th cells as indicated by the release of IL-2 and IFNγ by Th cells. Interestingly cytokine response resulting from T cells activation was comparable when either PLE cells or macrophages were used as APCs. These results suggest that epithelial cells may atleast under the experimental condition we used, would be as efficient in antigen presentation as macrophages are.

There are several reports in literature where cells other than professional APCs (dendritic cells, macrophages, and B cells) have been shown to have the ability to present antigens to Th cells (Akuthota et al, 2010; Marelli-Berg and Jarmin, 2004; Rescigno, 2010; Smyth et al, 2006). Recently Debbabi et al (2006) have shown that the primary lung alveolar epithelial cells can present microbial antigens to antigen sensitized T helper cells. Bulk of their results were obtained by using T7 lung alveolar
cell line that could present M. tuberculosis antigens to a homologous T cell hybridoma. In addition, they demonstrated an IFNγ response by M. tuberculosis sensitized Th cells by using primary lung epithelial cells pulsed with M. tuberculosis sonicate antigens. Our results confirm the results of Debbadi et al. and further show that BCG infected lung epithelial cells can be efficient APCs for Th cells. Further, we have compared the antigen presentation efficacy of the PLE cells and macrophages and found that the two cell types may have comparable antigen processing and presentation activity. In lungs the ability of the epithelial cells to present antigens may be highly advantageous for the host. This is because this ability of epithelial cells would markedly enhance the capacity and effectiveness of antigen presentation of antigens in lungs by increasing the sheer number of potential APCs. Moreover, pathogens like BCG that could be toxic to macrophages may still be efficiently presented by epithelial cells.

In conclusion, results presented in this thesis show that the lung epithelial cells besides providing the membranous surface where the crucial life sustaining process of gaseous exchange takes place, may also play an important role as participants in natural as well as the adaptive immune responses against pathogens in the lung. Further, particles like crystalline silica and poly-dispersed carbon nano-tubes may adversely affect this function by being toxic to the lung epithelial cells.